

# UDP-Xylose-Stimulated Glucuronyltransferase Activity in Wheat Microsomal Membranes: Characterization and Role in Glucurono(arabino)xylan Biosynthesis<sup>1[C]</sup>

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Microsomal membranes from etiolated wheat (*Triticum aestivum*) seedlings cooperatively incorporated xylose (Xyl), arabinose, and glucuronic acid residues from their corresponding uridine 5'-diphosphosugars into an ethanol-insoluble glucurono(arabino)xylan (GAX)-like product. A glucuronyltransferase activity that is enhanced by the presence of UDP-Xyl was also identified in these microsomes. Wheat glucuronyltransferase activity was optimal at pH 7 and required manganese ions, and several lines of evidence suggest its involvement in GAX-like biosynthesis. The GAX characteristics of the <sup>14</sup>C-product were confirmed by digestion with a purified endo-xylanase from *Aspergillus awamori* (endo-xylanase III) and by total acid hydrolysis, resulting in a Xyl:arabinose:glucuronic acid molar ratio of approximately 105:34:1. Endo-xylanase III released only three types of oligosaccharides in addition to free Xyl. No radiolabel was released as xylobiose, xylotriose, or xylotetraose, indicating the absence of long stretches of unbranched Xyl residues in the nascent GAX-like product. High-pH anion exchange chromatography analysis of the resulting oligosaccharides along with known arabinoxylan oligosaccharide standards suggests that a portion of the nascent GAX-like product has a relatively regular structure. The other portion of the [<sup>14</sup>C]GAX-like polymer was resistant to proteinase K, endo-polygalacturonase, and endo-xylanase III (GH11 family) but was degraded by Driselase, supporting the hypothesis that the xylan backbone in this portion of the product is most likely highly substituted. Size exclusion chromatography indicated that the nascent GAX-like polymer had an apparent molecular mass of approximately 10 to 15 kD; however, mature GAXs from wheat cell walls had larger apparent molecular masses (>66 kD).

Xylans represent a large family of hemicellulosic polysaccharides with considerable importance to many industries and to the integrity of plant cell walls. Xylans are a complex group of molecules identified in primary as well as secondary cell walls. They come in a variety of structural variants that include simple as well as more complex structures, such as glucuronoxylans (GXs) in the wood, glucurono(arabino)xylans (GAXs) in vegetative tissues of grasses, and neutral arabinoxylans (AXs) found in the endosperm of cereal grains. All of these xylans have a  $\beta(1,4)$ -D-xylan backbone in common that is substituted at the C-2 and/or C-3 position, mainly with  $\alpha$ -Araf residues or, to a lesser extent, with  $\alpha$ -GlcA or O-methyl- $\alpha$ -GlcA (MeGlcA) residues at the C-2 position. The degree and variation in the substitution, and the content of Ara, depend on the species and tissues. For example, in cell walls of grasses, GAXs make up 35% of cell walls

(Bacic and Stone, 1981; Carpita, 1983, 1996; Carpita and Whittern, 1986; Vietor et al., 1994; Ebringerova and Heinze, 2000), while endosperm cell walls in cereals have up to 70% of AX that is lacking GlcA residues with a high content of Araf side chains (Bacic and Stone, 1981; Fincher and Stone, 1986; Vietor et al., 1994). In addition, the secondary walls of dicots contain up to 35% GXs that are substituted with little or no Araf and have approximately 10% to 20% of the Xyl residues substituted with MeGlcA residues (Aspinall, 1980; Ebringerova and Heinze, 2000). In contrast to many hemicelluloses such as xyloglucan (XyG), galactomanan, and  $\beta(1,3;1,4)$ -D-glucan (called also mixed-linked glucan [MLG]), for which the biosynthetic pathways have been elaborated and genes cloned, the biochemistry of the biosynthesis of xylans has proven to be more difficult to investigate and therefore has received relatively little attention.

Only recently has research on the *irregular xylem (irx)* mutants from *Arabidopsis* (*Arabidopsis thaliana*) revealed candidate genes that are most likely to be involved in GX biosynthesis in secondary cell walls (Brown et al., 2005, 2007; Zhong et al., 2005; Pena et al., 2007; Persson et al., 2007). Mitchell et al. (2007) used a bioinformatics approach to identify additional candidate genes that might be involved in the synthesis and feruloylation of AX. No direct biochemical function, however, was obtained for any of these candidate genes. The characterization of the five

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Arabidopsis mutants *irx8*, *fra8*, *irx9*, *irx14*, and *parvus* indicated that GX content is affected in these mutant plants and that the main difference resides in the abundance of an oligosaccharide sequence located at the reducing end of GX, namely,  $\beta$ -D-Xyl-(1,4)- $\beta$ -D-Xyl-(1,3)- $\alpha$ -L-Rha-(1,2)- $\alpha$ -D-GalA-(1,4)-D-Xyl, and in the length of the xylan chains (Brown et al., 2007; Pena et al., 2007; Persson et al., 2007). Consequently, it was concluded that GX biosynthesis in secondary cell walls uses this oligosaccharide to either initiate (prime) or terminate the synthesis process. The mechanism of this initiation/termination step is still unknown. It is speculated that IRX7, IRX8, and PARVUS proteins are needed for the synthesis of this oligosaccharide at the reducing end to terminate the synthesis of GX, whereas IRX9 and IRX14 proteins would be involved in the elongation of the GX backbone during GX synthesis (Brown et al., 2007; Lee et al., 2007). However, the exact and direct biochemical functions of these proteins are unknown and their biochemical mechanisms are not understood; both require experimental confirmation.

On the other hand, although several studies have demonstrated that xylan-like polymers were synthesized from UDP-Xyl microsomal fractions from various species, such as pea (*Pisum sativum*) epicotyls (Waldron and Brett, 1983; Baydoun et al., 1989; Waldron et al., 1989), sycamore (*Platanus* spp.) and poplar (*Populus* spp.) trees (Dalessandro and Northcote, 1981), wheat (*Triticum aestivum*; Porchia and Scheller, 2000), maize (*Zea mays*; Bailey and Hassid, 1966), and *Zinnia* (Suzuki et al., 1991), in no case have the individual proteins involved in the biosynthesis been clearly identified. Earlier enzymological studies on GX biosynthesis in the pea system indicated that a cooperative action between xylosyltransferase (XylIT) and glucuronyltransferase (GlcAT) activities is required for the synthesis of the GX polymer; however, the role of the GlcAT activity in the process and the structural details of the product formed were not provided (Waldron and Brett, 1983; Baydoun et al., 1989). All of these biochemical studies generated xylan-like polymers without the addition of exogenous xylo-oligosaccharide acceptors, and it is not known whether an oligosaccharide primer (as for *irx8* and *irx9* mutants) is required to initiate the synthesis in these tissues. Several works, however, reported the presence of XylIT activities that would require the addition of exogenous xylo-oligosaccharides as acceptors (Kuroyama and Tsumuraya, 2001; Urahara et al., 2004; Brown et al., 2007; Lee et al., 2007). Furthermore, Lee et al. (2007) demonstrated that microsomes from Arabidopsis stems exhibited both XylIT and GlcAT activities when supplied with exogenous xylo-oligosaccharides (degree of polymerization [DP] 2–6), but no cooperative action was observed between these two activities. Clearly, there is still a considerable lack of knowledge about the enzymological aspects of xylan biosynthesis and a lack of detailed product characterization. For example, it is unknown whether all xylan types (GAXs,

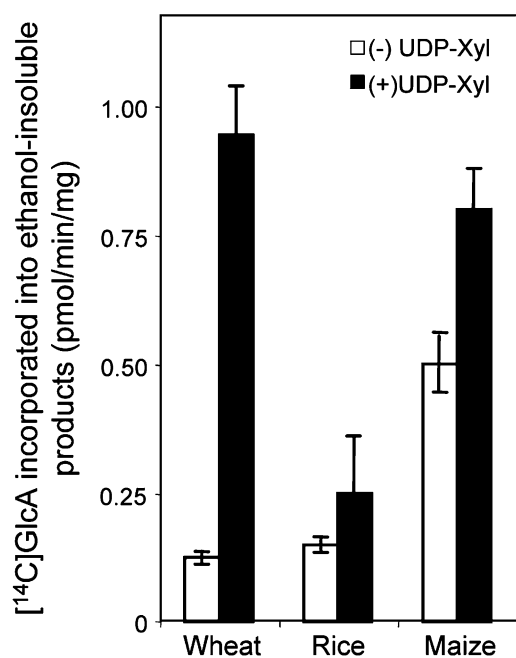
AXs, and GXs) are synthesized by similar mechanisms/enzymes and how xylan synthase (XylS), XylIT, arabinosyltransferase, and GlcAT cooperate to generate the polymers. Thus, it is unknown whether glucuronation and arabinosylation of the xylan backbone occur randomly or in a regular pattern.

This study describes the identification of a GlcAT activity in wheat Golgi-enriched microsomes that is enhanced by the presence of UDP-Xyl in the reaction medium and its participation in GAX biosynthesis. We showed that wheat microsomes produced a GAX-like polymer that is partly degradable by a purified endo-xylanase from *Aspergillus awamori*, and the total acid hydrolysis of the product released  $^{14}\text{C}$ -radiolabel solely as Xyl, Ara, and GlcA residues in a molar ratio of approximately 105:34:1, respectively. The combination of endo-xylanase III treatment with high pH anion exchange chromatography (HPAEC) analysis revealed that the in vitro-synthesized GAX-like polymer has a relatively regular structure. To our knowledge, this is the first report that presents evidence for a cooperative biosynthetic mechanism for GAX biosynthesis in grasses; this evidence is based upon our structural analysis of the nascent GAX-like products.

## RESULTS

### Identification and Characterization of GlcAT Activity That Is Stimulated by the Addition of UDP-Xyl

In previous work on GX biosynthesis in the pea system, it was demonstrated that the capacity of pea microsomes to transfer GlcA from UDP-GlcA into ethanol-insoluble products was enhanced in the presence of UDP-Xyl in the reaction medium (Waldron and Brett, 1983). The analysis of the products supported the conclusion that the pea GlcAT is involved in the synthesis of GX polymer (Waldron and Brett, 1983; Baydoun et al., 1989). Because cell walls of etiolated pea contain low amounts of GX, we hypothesized that microsomes from plants containing higher amounts of GAX in their cell walls (i.e. wheat, rice [*Oryza sativa*], or maize) may possess high GlcAT activity and could be an excellent model in which to investigate GAX biosynthesis and the role of GlcAT in the process. Therefore, Golgi-enriched microsomal fractions were prepared from etiolated wheat, rice, and maize seedlings and tested for their ability to transfer [ $^{14}\text{C}$ ]GlcA from UDP-[ $^{14}\text{C}$ ]GlcA to ethanol-insoluble material in the presence or absence of UDP-Xyl. Figure 1 shows that rice microsomes did not present a significant GlcA transfer activity regardless of the presence or absence of UDP-Xyl in the reaction mixture, whereas wheat and maize microsomes did show substantial [ $^{14}\text{C}$ ]GlcA incorporation into ethanol-insoluble products in the presence of UDP-Xyl. The low activity in rice microsomes may indicate the need for a better optimization of the reaction conditions. Since maize microsomes



**Figure 1.** [<sup>14</sup>C]GlcA transfer from UDP-[<sup>14</sup>C]GlcA into ethanol-insoluble products formed by Golgi-enriched microsomal preparations (approximately 0.2 mg of protein) from wheat, rice, and maize etiolated seedlings. [<sup>14</sup>C]GlcA incorporation was measured in the presence (black bars) or absence (white bars) of 0.5 mM UDP-Xyl for 90-min reactions. The assays were conducted in triplicate.

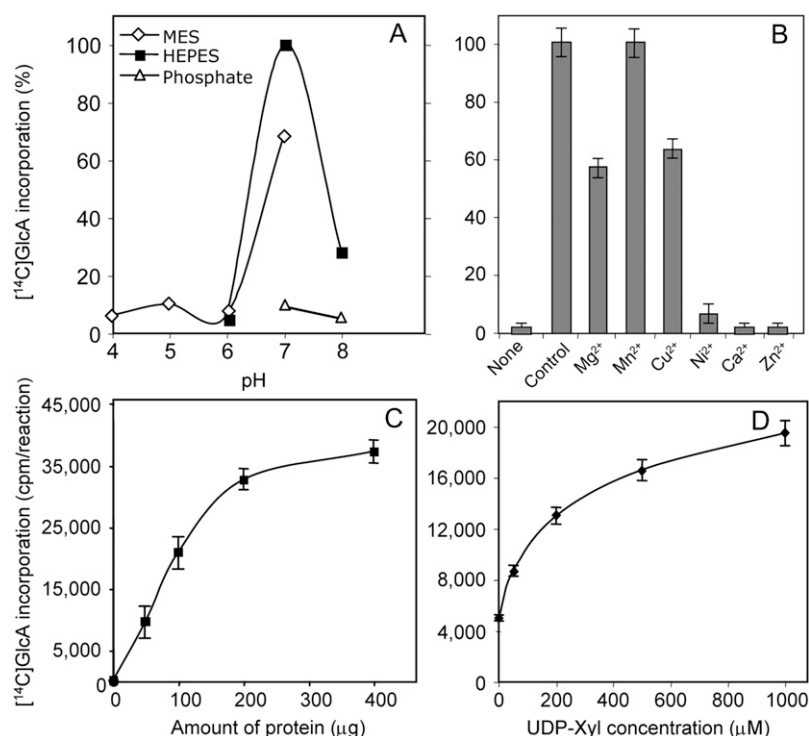
had a higher radioactivity background in the control (minus UDP-Xyl), we decided to focus on etiolated wheat seedlings, as their microsomes gave the highest [<sup>14</sup>C]GlcA transfer activity and the lowest background. Therefore, Golgi-enriched microsomes from etiolated wheat seedlings were used for the biochemical characterization of the enzyme and the generation of GAX-like polymer.

As a first step, biochemical characterization of the wheat GlcAT activity was carried out, and the results are summarized in Figure 2. As indicated, wheat enzyme works optimally at pH 7, and its activity decreases dramatically at and below pH 6 and at and above pH 8. HEPES buffer, in which standard reactions were performed, was found to be the best buffer. Potassium phosphate buffer, on the other hand, had a strong inhibitory effect (90%) on the activity (Fig. 2A). Regarding the divalent cation requirement for the optimal activity, wheat GlcAT activity required the presence of Mn<sup>2+</sup> ions (5 mM) in the reaction medium (Fig. 2B). The inclusion of 5 mM Mg<sup>2+</sup> or Cu<sup>2+</sup> into the reaction medium produced approximately 60% of the activity compared with the control (Fig. 2B). Thus, in standard reactions, both Mn<sup>2+</sup> and Mg<sup>2+</sup> were included at 5 mM each. As expected from an enzymatic reaction, [<sup>14</sup>C]GlcA incorporation into ethanol-insoluble products showed a linear increase as a function of protein amount used up to 0.2 mg per reaction (Fig. 2C). Boiled microsomes did not show any transfer

activity (data not shown). The enhancing effect of UDP-Xyl was also tested for higher concentrations, and as expected, wheat GlcAT activity increased as a function of the increasing concentration of UDP-Xyl, with a plateau reached at 0.5 mM UDP-Xyl in 90-min incubation reactions (Fig. 2D). This result confirms further the stimulatory effect of UDP-Xyl on GlcAT activity. Thus, in the remaining experiments, 0.5 mM UDP-Xyl was used for the optimal stimulation of GlcAT activity. We noticed also that wheat GlcAT activity was stable at -80°C for at least 6 months without any loss of activity. The use of Suc at concentrations greater than 0.8 M produced a strong inhibitory effect (90%) on the [<sup>14</sup>C]GlcA transfer activity (data not shown).

Five nucleotide sugar precursors (UDP-Glc, UDP-Gal, UDP-GalNAc, GDP-Man, and GDP-Fuc) were tested for their stimulatory effect on the [<sup>14</sup>C]GlcA transfer reaction under the standard conditions used for UDP-Xyl. Interestingly, UDP-Glc and GDP-Fuc were found to induce wheat GlcAT activity by 50% and 44%, respectively, compared with the control (stimulation by UDP-Xyl; Table I). UDP-Gal, GDP-Man, and UDP-GalNAc, however, had lower but reproducible stimulatory effect (approximately 30% of the control; Table I). The stimulatory effect of UDP-Ara was not tested because wheat microsomes contained UDP-Xyl-4-epimerase activity that would produce UDP-Xyl, which in turn would affect the reaction. The physiological significance of GlcAT stimulation by UDP sugars other than UDP-Xyl is still unknown.

To investigate the physiological role of wheat GlcAT activity with respect to plant growth and development, the activity was monitored during the growth of etiolated wheat seedlings. Etiolated wheat seedlings represent not only a good experimental model for biochemical studies but also an excellent system in which to investigate the importance of cell wall constituents (and the enzyme activities that produce these constituents) in cell growth and elongation, as the mesocotyl of the young etiolated wheat seedlings have rapid growth and division rates. Figure 3 represents the growth stages of an etiolated wheat seedling along with the corresponding GlcAT activity (in the presence or absence of UDP-Xyl) in the Golgi-enriched microsomal membranes from coleoptile and mesocotyl regions of the seedlings. As expected, microsomes from young developing seedlings have the highest [<sup>14</sup>C]GlcA transfer rate in comparison with older seedlings, with the maximum GlcAT activity observed in 4-d-old seedlings (Fig. 3). However, substantial [<sup>14</sup>C]GlcA incorporation still occurs in older seedlings. When the GlcAT activity was measured in the 5-cm upper region (top of the coleoptile with the emerging primary leaves), the 5-cm middle region (base of the coleoptile and the top of the mesocotyl), and the 5-cm lower region (base of the mesocotyl) of an 8-d-old seedling, the base of the mesocotyl, which corresponds to the cell elongation and division region, was found to have the highest activity rate (Fig. 3). This supports the



**Figure 2.** Biochemical characteristics of the UDP-Xyl-enhanced GlcAT activity in Golgi-enriched microsomes from wheat etiolated seedlings. Microsomal membranes (approximately 0.1 mg of protein unless indicated otherwise) of 6-d-old wheat seedlings were incubated with UDP- $^{14}\text{C}$ GlcA in the presence of 0.5 mM UDP-Xyl (unless indicated otherwise) for 90 min, and the  $^{14}\text{C}$ GlcA incorporation is expressed either as a percentage of the total activity in the control in a standard assay or as cpm. A and B, Effects of pH (A) and divalent cations (B) on  $^{14}\text{C}$ GlcA transfer as a percentage of the optimal activity. C and D, Effects of increasing amounts of enzyme (up to 0.4 mg of protein; C) and increasing amounts of UDP-Xyl (up to 1 mM; D) on wheat GlcAT activity. The cations were used at a final concentration of 5 mM.

hypothesis that the product of the GlcAT activity may be involved in cell growth and elongation. For practical reasons (enough tissue and easy handling), most of the experiments were conducted using microsomal membranes from 6-d-old wheat seedlings.

#### Wheat Microsomes Cooperatively Incorporate $^{14}\text{C}$ -Radiolabel from UDP- $^{14}\text{C}$ Xyl, UDP- $^{14}\text{C}$ Arabinopyranose, and UDP- $^{14}\text{C}$ GlcA into a GAX-Like Product

The three common monosaccharides found in GAXs from primary cell walls in grasses (commonly called type II walls) are Araf, Xyl, and GlcA (Carpita, 1996). In this work, we sought to investigate GAX synthesis in wheat Golgi-enriched microsomal membranes and whether the GlcAT activity incorporates GlcA into this GAX polymer. Like pea UDP-Xyl-enhanced GlcAT activity (Waldron and Brett, 1983; Baydoun et al., 1989), wheat GlcAT was also expected to be involved in GAX biosynthesis in this system.

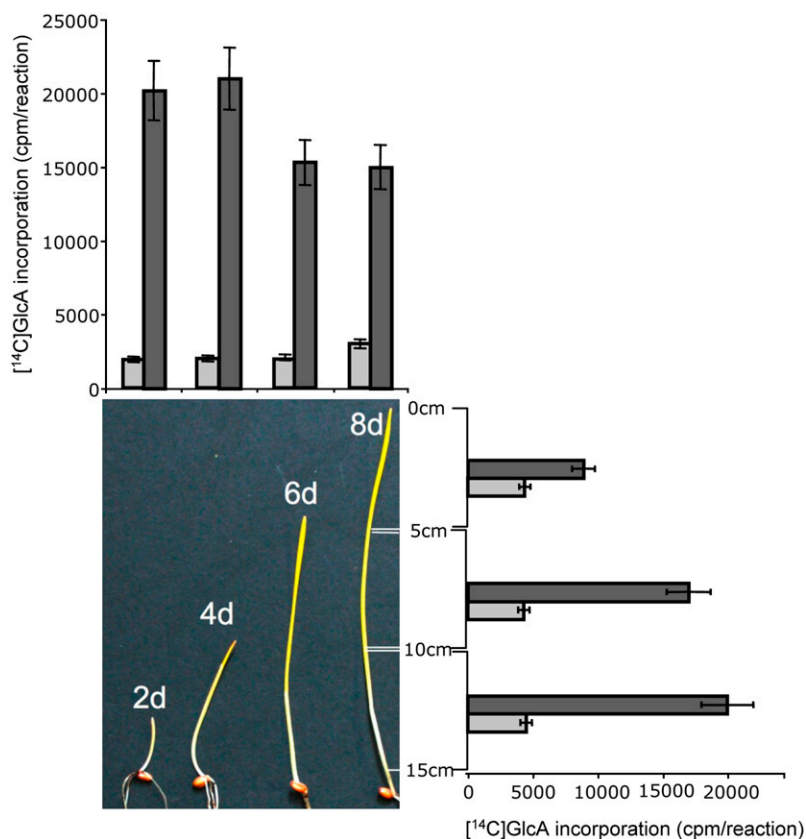
In a first set of experiments, wheat microsomal membranes were supplied with individual UDP- $^{14}\text{C}$ -sugar precursors and  $^{14}\text{C}$ -radiolabel incorporation into ethanol-insoluble products was monitored. The data indicated that  $^{14}\text{C}$ -products were formed but could not be digested (less than 10%) with purified endo-xylanases (data not shown), indicating that no substantial  $^{14}\text{C}$ GAX was produced. Therefore, it was hypothesized that the synthesis of GAX polymers in wheat may require the simultaneous addition of multiple precursors in the reaction mixture, as was shown for XyG biosynthesis, which requires the presence of

both UDP-Glc and UDP-Xyl (Ray, 1980; Hayashi and Matsuda, 1981; Faik et al., 2002). Thus, it is expected that the presence of UDP-Xyl, UDP-Araf, and UDP-GlcA together in reaction medium would produce a stimulatory effect on the incorporation of these sugars into the GAX products. To test this hypothesis, a set of experiments were conducted in which the effect of increasing concentrations of UDP-Xyl on the incorporation of  $^{14}\text{C}$ GlcA and  $^{14}\text{C}$ Ara into products was monitored. Already the data in Figure 2D indicated that  $^{14}\text{C}$ GlcA incorporation was greatly enhanced by increasing amounts of UDP-Xyl, suggesting a cooperative action between GlcAT and XylIT activities. Figure 4A, however, shows that  $^{14}\text{C}$ Araf incorporation was stimulated with concentrations up to 0.2 mM UDP-Xyl but that higher UDP-Xyl concentrations (>0.3 mM) appeared to cause partial inhibition of

**Table 1.** Effects of nucleotide sugars (0.5 mM) on  $^{14}\text{C}$ GlcA transfer from UDP- $^{14}\text{C}$ GlcA ( $2\ \mu\text{M}$ ,  $90,000\ \text{cpm}\ \text{reaction}^{-1}$ ) into ethanol-insoluble products

Nucleotide Sugar Added	GlcA Transfer into Ethanol-Insoluble Product	
	$\text{pmol}\ \text{reaction}^{-1}$	% of control
None	$1.1 \pm 0.06$	12
UDP-Xyl (control)	$8.9 \pm 0.18$	100
UDP-Glc	$4.5 \pm 0.15$	50
GDP-Man	$3.4 \pm 0.15$	38
UDP-Gal	$3.6 \pm 0.18$	40
UDP-GalNAc	$2.1 \pm 0.12$	23
GDP-Fuc	$3.9 \pm 0.15$	44

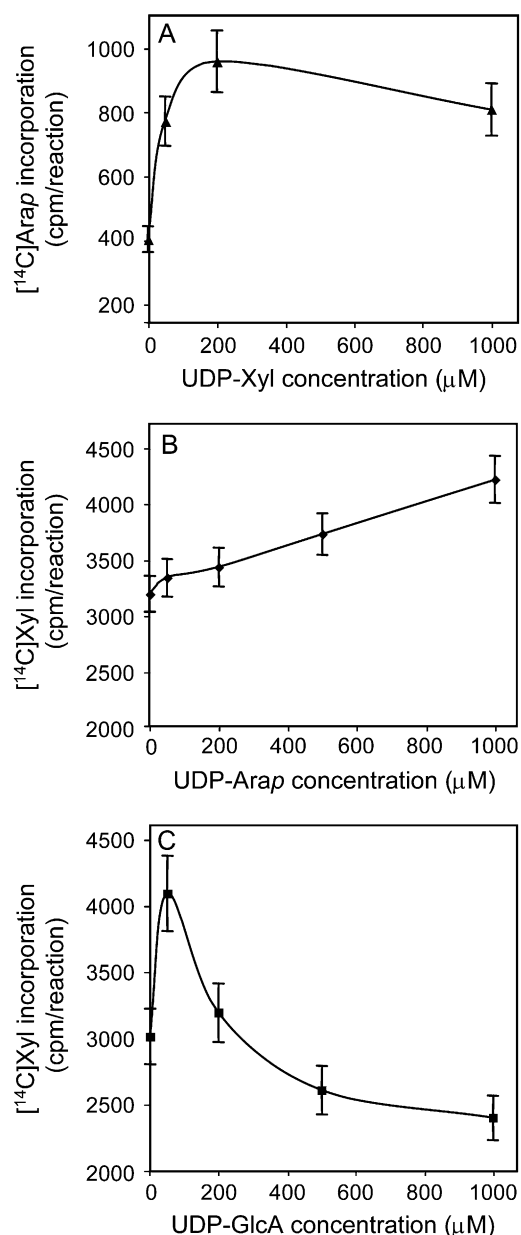
**Figure 3.** Monitoring GlcAT activity in growing etiolated wheat seedlings (up to 8 d after germination) and in different growth zones (coleoptile and mesocotyl) of 8-d-old seedlings. The epicotyls of 2-d-old (2d), 4-d-old (4d), 6-d-old (6d), and 8-d-old (8d) seedlings and various regions (coleoptile and mesocotyl) of 8-d-old seedlings were harvested separately for Golgi-enriched membrane preparations as described in "Materials and Methods." The GlcA transfer activity was tested in a standard GlcAT assay as described for product 4 in Table II.



$[^{14}\text{C}]\text{Ara}$  incorporation. This apparent inhibition can be explained by the presence of UDP-Xyl-4-epimerase activity, which would convert UDP-Xyl to UDP-Arap, thereby diluting the UDP- $[^{14}\text{C}]\text{Arap}$  pool with nonradioactive UDP-Arap, which would result in a lower apparent  $[^{14}\text{C}]\text{Ara}$  incorporation into products. When an increasing concentration of UDP-Arap was added to the reactions in the presence of a constant concentration of UDP- $[^{14}\text{C}]\text{Xyl}$ , only a slight stimulatory effect on the  $[^{14}\text{C}]\text{Xyl}$  incorporation into products was observed (Fig. 4B). This low stimulatory effect may be explained again by the presence of the epimerase activity, which produces an equilibrium between UDP-Xyl and UDP-Arap and dilutes the  $^{14}\text{C}$ -radiolabel over both UDP sugars. On the other hand, increasing concentrations of UDP-GlcA showed a stimulatory effect on  $[^{14}\text{C}]\text{Xyl}$  incorporation only for UDP-GlcA concentrations lower than 0.1 mM; in contrast, higher UDP-GlcA amounts had inhibitory effects (Fig. 4C). This inhibition could be the result of a competitive inhibition of XylS activity by UDP-GlcA, as the only difference between GlcA and Xyl residues is the carboxy group of the GlcA residues.

To demonstrate that  $^{14}\text{C}$ -radiolabel is solely associated with Ara, Xyl, and GlcA, four products were prepared and analyzed for their monosaccharide composition by total acid hydrolysis and HPAEC fractionation. Products 1 and 2 were obtained by incubation of microsomes with 0.5 mM UDP- $[^{14}\text{C}]\text{Xyl}$  in the absence

or presence of 0.2 mM UDP- $[^{14}\text{C}]\text{Arap}$  (specific radioactivity, approximately 3.5 cpm pmol $^{-1}$ ), respectively. Product 3 was the result of the incubation of microsomes in the presence of 0.5 mM UDP- $[^{14}\text{C}]\text{Xyl}$  and 2  $\mu\text{M}$  UDP- $[^{14}\text{C}]\text{GlcA}$  (specific radioactivity, approximately 900 cpm pmol $^{-1}$ ). Product 4 resulted from incubation in the presence of 0.5 mM UDP- $[^{14}\text{C}]\text{Xyl}$ , 0.2 mM UDP- $[^{14}\text{C}]\text{Arap}$ , and 2  $\mu\text{M}$  UDP- $[^{14}\text{C}]\text{GlcA}$ . The resulting products were precipitated with 70% ethanol and washed extensively to remove any unincorporated UDP- $^{14}\text{C}$ -sugars. Ethanol-insoluble materials were resuspended in an alkali solution, which solubilized approximately 95% of the  $^{14}\text{C}$ -radiolabel. The alkali-soluble products were neutralized and centrifuged to remove insoluble material. This step yielded a loss of an additional approximately 5% of the  $^{14}\text{C}$ -radiolabel as insoluble material after neutralization. The final soluble products contained approximately 90% of the initial  $^{14}\text{C}$ -radiolabel incorporated. Table II summarizes the data and indicates that 95% to 100% of the  $^{14}\text{C}$ -radiolabel was released as Ara, Xyl, or GlcA residues, depending on the product. In addition, the data indicate that, although UDP- $[^{14}\text{C}]\text{Arap}$  was not included in the reaction mixtures yielding products 1 and 3, substantial  $^{14}\text{C}$ -radiolabel (approximately 30%) was released as Ara, which confirmed the presence of UDP-Xyl-4-epimerase activity in the microsomal preparation. To determine whether Golgi-enriched microsomes contained UDP-GlcA-4-epimerase activity that



**Figure 4.** Effects of increasing concentrations (0–1 M) of UDP-Xyl, UDP-Arap, or UDP-GlcA on the incorporation of [ $^{14}\text{C}$ ]Xyl or [ $^{14}\text{C}$ ]Ara into ethanol-insoluble products by wheat Golgi-enriched membranes. A, Effect of increasing amounts of UDP-Xyl on [ $^{14}\text{C}$ ]Ara transfer from UDP-[ $^{14}\text{C}$ ]Ara (0.2 mM, 3.5 cpm pmol $^{-1}$ ). B, Effect of increasing amounts of UDP-Arap on [ $^{14}\text{C}$ ]Xyl transfer from UDP-[ $^{14}\text{C}$ ]Xyl (0.5 mM, 3.5 cpm pmol $^{-1}$ ). C, Effect of increasing amounts of UDP-GlcA on [ $^{14}\text{C}$ ]Xyl transfer from UDP-[ $^{14}\text{C}$ ]Xyl (0.5 mM, 3.5 cpm pmol $^{-1}$ ). In all reactions, the  $^{14}\text{C}$ -radiolabel incorporation was monitored as cpm per reaction, and the assays were carried out two times.

would convert UDP-[ $^{14}\text{C}$ ]GlcA to UDP-[ $^{14}\text{C}$ ]GalA (Gu and Bar-Peled, 2004) and UDP-GlcA decarboxylase activity that would produce UDP-[ $^{14}\text{C}$ ]Xyl from UDP-[ $^{14}\text{C}$ ]GlcA (Harper and Bar-Peled, 2002), two versions of  $^{14}\text{C}$ -labeled product 4 were prepared using either UDP-[ $^{14}\text{C}$ ]Xyl or UDP-[ $^{14}\text{C}$ ]GlcA as the source of the

$^{14}\text{C}$ -radiolabel. Total acid hydrolysis of the [ $^{14}\text{C}$ ]GlcA-labeled product 4 released radioactivity as free [ $^{14}\text{C}$ ]GlcA only (no [ $^{14}\text{C}$ ]GalA was detected), which indicates that wheat microsomal preparations are free of UDP-GlcA-4-epimerase activity; no radioactivity was released as [ $^{14}\text{C}$ ]Xyl, indicating that the microsomes were also free of UDP-GlcA decarboxylase activity (data not shown). Therefore, under our reaction conditions, there was no interference with UDP sugars interconverting activities (with the exception of UDP-Xyl-4-epimerase activity) and  $^{14}\text{C}$ -radiolabel in products was solely associated with GlcA, Xyl, and Ara. Taking into consideration the specific radioactivity of [ $^{14}\text{C}$ ]Xyl, [ $^{14}\text{C}$ ]Ara, and  $^{14}\text{C}$ -radiolabeled GlcA, the HPAEC analyses indicated that Ara:Xyl ratios in products 1 and 2 were approximately 1:2, and for products 3 and 4 the Xyl:Ara:GlcA ratios were approximately 105:34:1 (Table II). It is noteworthy that at higher UDP-GlcA concentrations (0.1 mM), [ $^{14}\text{C}$ ]GlcA incorporation into products increased by 10 to 15 times without a concurrent increase in Xyl incorporation (data not shown).

To further confirm that [ $^{14}\text{C}$ ]Xyl-labeled product 4 contained GlcA residues, which would make it acidic, Dowex resin was used to purify the product. The purified product was analyzed by HPAEC for its monosaccharide composition. Our data indicated that approximately 70% to 80% of the  $^{14}\text{C}$ -radiolabel was retained by the resin, and after washing, this  $^{14}\text{C}$ -radiolabel was released with 1 M NaCl. Total acid hydrolysis of the purified product yielded a [ $^{14}\text{C}$ ]Xyl:[ $^{14}\text{C}$ ]Ara ratio estimated at approximately 3:1 (data not shown), thus providing further support for the conclusion that GlcA, Xyl, and Ara were coinorporated into a single product. This result also indicates that the wheat UDP-Xyl-enhanced GlcAT activity is most likely involved in the transfer of GlcA into the newly synthesized GAX-like product. Moreover, when products 1, 2, and 4 (Table II) were fractionated on a Bio-gel P2 column, it was found that  $^{14}\text{C}$ -labeled product 4 eluted at the void volume ( $V_0$ ; Fig. 7, bottom), whereas products 1 and 2 eluted within the elution volume of the Bio-gel P2 column (Fig. 7, top and middle, respectively). This increase in size between these products due to the addition of UDP-Arap and/or UDP-GlcA is an additional indication that the three sugars are incorporated into the same product (nascent GAX-like polymer).

#### A Portion of the Nascent [ $^{14}\text{C}$ ]GAX-Like Polymer Is Susceptible to Endo-Xylanase III and May Have Relatively Regular Structure

One goal of this study was to determine whether the arabinosyl and glucuronyl acid residues were added to the xylan backbone randomly or in a regular pattern forming repeating basic subunits, like that observed in XyGs of dicots (Ray, 1980; Hayashi and Matsuda, 1981; Faik et al., 2002). Consequently, both [ $^{14}\text{C}$ ]GlcA- and [ $^{14}\text{C}$ ]Xyl-labeled product 4 were tested with respect to

**Table II.** Incorporation of  $^{14}\text{C}$ -radiolabel into ethanol-insoluble products by wheat Golgi-enriched membranes and its distribution among monosaccharides after total acid hydrolysis of the products and HPAEC analysis

Products 1 and 2 were obtained from the incubation of microsomes with 0.5 mM UDP- $^{14}\text{C}$ Xyl (approximately 3.5 cpm pmol $^{-1}$ ) in the absence or presence, respectively, of 0.2 mM UDP- $^{14}\text{C}$ Arap (approximately 3.5 cpm pmol $^{-1}$ ). Product 3 was prepared by incubation with 0.5 mM UDP- $^{14}\text{C}$ Xyl and UDP- $^{14}\text{C}$ GlcA (2  $\mu\text{M}$ , approximately 900 cpm pmol $^{-1}$ ), and product 4 was prepared by incubation with all three of these UDP- $^{14}\text{C}$ -labeled sugars. Incubation times were 90 min, and each reaction was conducted in triplicate. ND, Not detected.

Product	Total $^{14}\text{C}$ -Radiolabel Incorporated <i>cpm reaction<math>^{-1}</math></i>	Monosaccharide Incorporation Rate		
		Xyl	Ara	GlcA
		<i>pmol min<math>^{-1}</math> mg<math>^{-1}</math> protein</i>		
Product 1	2,500 $\pm$ 500	26.5 $\pm$ 0.1	12.0 $\pm$ 0.5	ND
Product 2	4,150 $\pm$ 600	44.5 $\pm$ 0.2	21.7 $\pm$ 0.6	ND
Product 3	15,000 $\pm$ 1,000	73.0 $\pm$ 0.8	19.3 $\pm$ 0.7	0.64 $\pm$ 0.01
Product 4	16,500 $\pm$ 1,500	65.7 $\pm$ 1.5	21.6 $\pm$ 0.8	0.70 $\pm$ 0.01

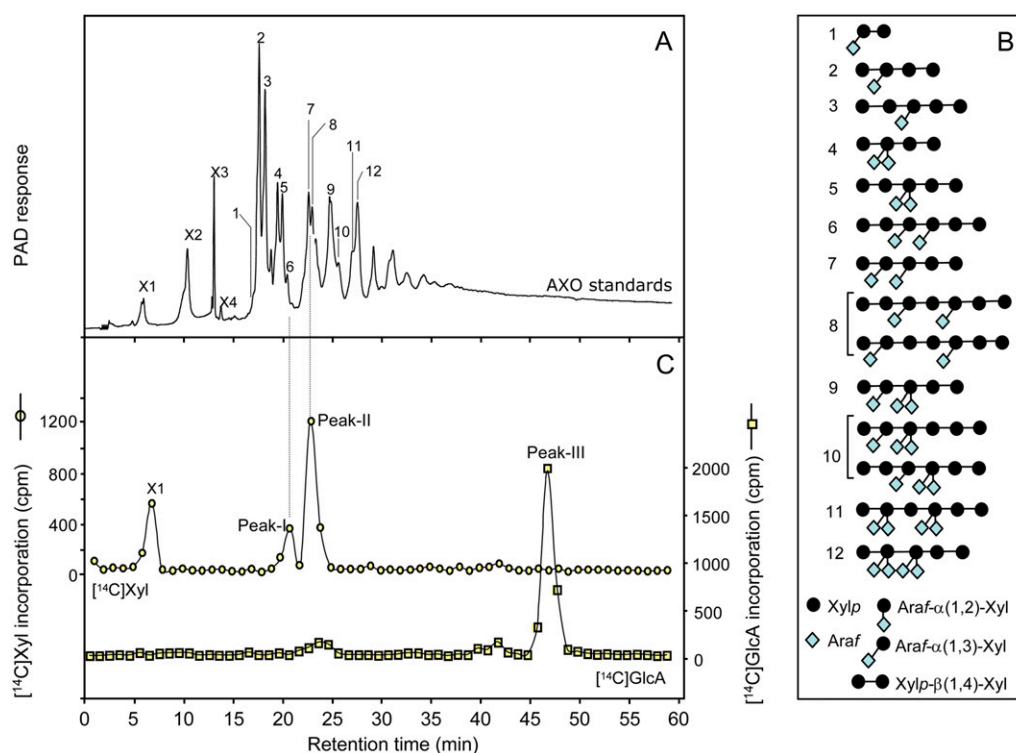
their susceptibility to endo-xylanase degradation using a purified and well-characterized endo-xylanase III from *A. awamori* (Kormelink et al., 1993b). The resulting oligosaccharides were compared with characterized AX oligosaccharide (AXO) standards prepared by the action of endo-xylanase III on purified wheat AX (Fig. 5A; Kormelink et al., 1993a). Endo-xylanase III is classified as a GH11 family member according to the CAZy database (<http://www.cazy.org/>); therefore, it does not tolerate substituted Xyl residues in the  $-2$ ,  $-1$ , and  $+1$  catalytic subsites (Kormelink et al., 1993b; Davies et al., 1997). This means that a minimum of three consecutive unbranched Xyl residues in the xylan backbone are required for the enzyme to cleave the backbone. As a result of the reaction mechanism, treatment of GAX or AX polymers with endo-xylanase III would generate predominantly oligosaccharides having two unbranched Xyl residues at the reducing end and one or two unbranched Xyl residues at the nonreducing end.

When  $^{14}\text{C}$ -labeled product 4 was treated with endo-xylanase III and fractionated on a Bio-gel P2 column, approximately 60% to 70% of the  $^{14}\text{C}$ -radiolabeled products were resistant to degradation and eluted at the Vo of the column ( $>DP$  12), and only approximately 30% to 40% of the radiolabel was released as oligosaccharides (DP 7–9) and monosaccharides (data not shown). The resulting degradation products were mixed with the characterized AXOs and the mixture was fractionated on a CarboPac PA-10 column (Dionex) as free  $^{14}\text{C}$ Xyl (retention time [RT] = approximately 6.5 min) and two broad  $^{14}\text{C}$ -labeled oligosaccharide peaks, peak I (RT = approximately 21 min) and peak II (RT = approximately 23 min; Fig. 5C). A typical elution profile of AXO standards is indicated in Figure 5A. Because of the breadth of the  $^{14}\text{C}$ -labeled oligosaccharide peaks, it was not possible to associate a specific AXO standard with the oligosaccharides. However, Figure 5C indicates that peak I eluted under AXO standards 4, 5, and 6, whereas peak II eluted under AXO standards 7 and 8. To confirm that peaks I and II indeed contained the

Xyl-to-Ara ratio predicted in these combined AXO standards, peaks I and II were combined (several runs were necessary to collect enough radioactive material for analysis) and subjected to total acid hydrolysis followed by HPAEC analysis. These data indicated that the radioactivity was associated with Xyl and Ara in a molar ratio of approximately 2.9:1, respectively (Table III), which fits the average from AXO standards 4 to 8 (Fig. 5B). Although our analyses may suggest that peaks I and II might correspond to standards 6 and 7, respectively, further detailed structural analysis is needed to elucidate the fine structure of these oligosaccharides. Unfortunately, because an unpurified mixture of enzymes was used to produce the  $^{14}\text{C}$ GAX-like polymer, performing detailed analyses such as matrix-assisted laser-desorption ionization time of flight or gas chromatography-mass spectrometry on the reaction product would not be accurate, as many other contaminant polymers would be present with our GAX-like product.

When  $^{14}\text{C}$ GlcA-containing product 4 was treated with the endo-xylanase III, the  $^{14}\text{C}$ -radiolabel was associated mainly (90%) with one broad  $^{14}\text{C}$ -labeled oligosaccharide peak (peak III) that eluted late in the gradient (RT = approximately 47 min; Fig. 5C). Since the composition of peak III could not be determined using xylo-oligosaccharide or aldo-uronic acid standards (RT = 25–27), it was subjected to total acid hydrolysis followed by HPAEC analysis. Table III indicates that most radioactivity from peak III was associated with free  $^{14}\text{C}$ GlcA. It is important to note that GlcA represented only a minor portion (approximately 1%) of the nascent GAX-like polymer. Thus, it was expected that  $^{14}\text{C}$ Xyl may not be detectable if only a few picomoles of GlcA were incorporated in the  $^{14}\text{C}$ -labeled product 4 (due to the difference in the specific radioactivity between  $^{14}\text{C}$ GlcA [approximately 900 cpm pmol $^{-1}$ ] and  $^{14}\text{C}$ Xyl [approximately 3.5 cpm pmol $^{-1}$ ]). More structural analysis of peak III is needed to elucidate its fine structure, as we did not have access to known larger GAX oligosaccharides.





**Figure 5.** HPAEC fractionation profiles of known AXO standards and  $^{14}\text{C}$ -radiolabeled oligosaccharides. A, AXO standards were prepared by digestion of wheat AXs with endo-xylanase III as described in “Materials and Methods.” B, Schematic representation of the structures of these AXO standards (● represents Xylp and ◇ represents Araf residues) as determined by Kormelink et al. (1993a). The AXO standards are numbered from 1 to 12 in A and B. C, Typical fractionation profiles of  $^{14}\text{C}$ -labeled oligosaccharides released from [ $^{14}\text{C}$ ]Xyl-labeled product 4 (○) and [ $^{14}\text{C}$ ]GlcA-labeled product 4 (□; see Table II) by treatment with endo-xylanase III. The  $^{14}\text{C}$ -radiolabel was monitored each minute at  $30^\circ\text{C}$  and eluted as three peaks around 21 min (peak I), around 23 min (peak II), and around 47 min (peak III). The CarboPac PA10 column was eluted at a rate of  $1\text{ mL min}^{-1}$  with  $0.1\text{ M}$  NaOH containing a gradient ( $0\text{--}0.5\text{ M}$ ) of sodium acetate for 60 min and then eluted isocratically for 5 min with  $1\text{ M}$  sodium acetate. RTs of Xyl, xylobiose, xylotriose, and xylo-tetraose are indicated as X1, X2, X3, and X4, respectively, in A. PAD, pulsed amperometric detection of carbohydrates using an electrochemical detector (ED50; Dionex). [See online article for color version of this figure.]

### A Portion of the Nascent [ $^{14}\text{C}$ ]GAX-Like Polymer Is Resistant to Endo-Xylanase III and Might Be Highly Substituted

As indicated above, approximately 60% to 70% of  $^{14}\text{C}$ -labeled product 4 was resistant to endo-xylanase III. This may be explained either by a higher degree of substitution of the xylan backbone of the product (lacking stretches of three consecutive unbranched Xyls needed for endo-xylanase III action) or by the production of  $^{14}\text{C}$ -radiolabeled polymers other than

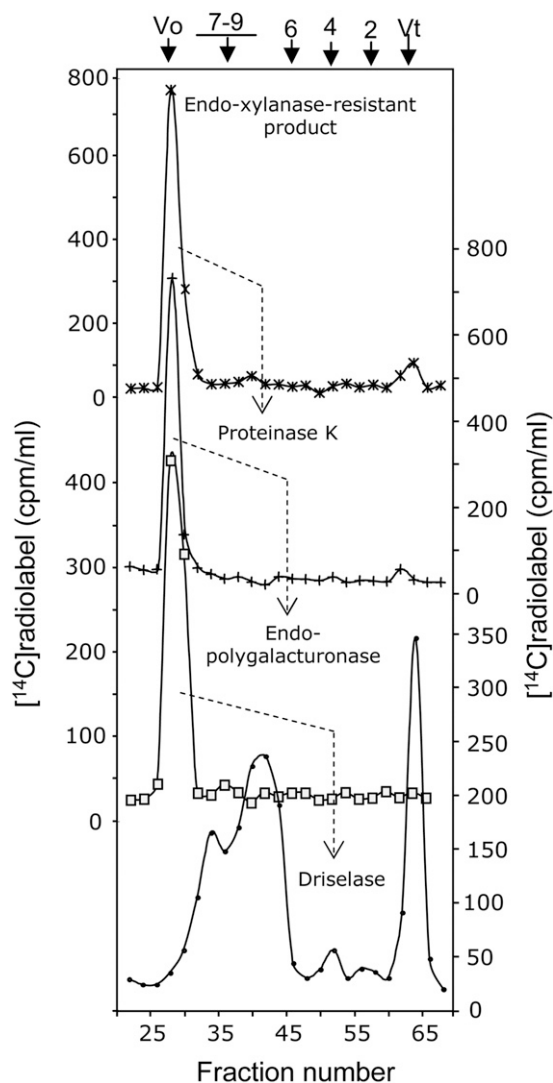
GAX (i.e. glycoproteins, pectins, or XyG). Thus, the endo-xylanase III-undigested radioactive material from [ $^{14}\text{C}$ ]Xyl-labeled product 4 was subjected to sequential enzymatic treatment with proteinase K and endo-polygalacturonase (*Aspergillus niger*) followed by fractionation on Bio-gel P2 columns. Figure 6 shows that proteinase K or endo-polygalacturonase treatment did not degrade the  $^{14}\text{C}$ -product (still eluting at Vo), which supports the hypothesis that [ $^{14}\text{C}$ ]Xyl was not incorporated into glycoproteins or pectins. On the other hand, if wheat Golgi-enriched micro-

**Table III.** Distribution of  $^{14}\text{C}$ -radiolabel recovered as monosaccharides released by total acid hydrolysis ( $2\text{ M}$  TFA) from peaks I and II and peak III (see Fig. 5)

Each analysis was carried out in duplicate using at least approximately 5,000 cpm. ND, Not detected.

Peak	$^{14}\text{C}$ -Radiolabel Recovery			Ratio Xyl:Ara
	Ara	Xyl	GlcA	
Peaks I and II	10	29	ND	2.9:1
Peak III	ND	ND	0.38	–





**Figure 6.** Bio-gel P2 fractionation of the endo-xylanase III-resistant portion of [ $^{14}\text{C}$ ]Xyl-labeled product 4 after sequential treatment with proteinase K, endo-polygalacturonase, and Driselase as described in "Materials and Methods." The column was eluted with degassed water, and the elution volumes of dextran (500 kD;  $V_0$ ) and DP 2, 4, 6, and 7 to 9 are indicated with arrows at the top.  $V_t$ , Total elution volume.

somes were producing [ $^{14}\text{C}$ ]GAX-like polymer with highly branched xylan backbone portions, the depolymerization of these portions would require a synergistic action of endo-xylanase and glycosidases such as  $\alpha$ -(1,2)/(1,3)-arabinofuranosidases and  $\alpha$ -(1,2)-glucuronosidase activities. To test this hypothesis, the product was subjected to Driselase treatment. Driselase is known to contain a mix of some of these activities and would be a good enzyme source to hydrolyze these portions of the polymer. Figure 6 shows that, as expected, Driselase converted this product to  $^{14}\text{C}$ -labeled oligosaccharides, with sizes in the range of DP 4 to 9, in addition to free  $^{14}\text{C}$ -labeled monosaccharides (eluted at the total elution volume).

Furthermore, Driselase lacks the  $\alpha$ -xylosidase activity that cleaves  $\alpha$ -(1,6) linkage between Xyl residues and the Glc of the glucan backbone of XyGs. As a result, Driselase degrades XyGs completely to isoprimeverose (Xyl  $\rightarrow$  Glc) and other monosaccharides. Isoprimeverose is usually used as diagnostic test for XyG synthesis (Faik et al., 2002). As indicated in Figure 6, no radioactivity was eluted as disaccharides (DP 2), which supports the hypothesis that wheat microsomes do not incorporate [ $^{14}\text{C}$ ]Xyl into XyG. Together, these results support the hypothesis that a significant portion of the xylan backbone of the nascent GAX-like polymer may be highly branched with Ara residues.

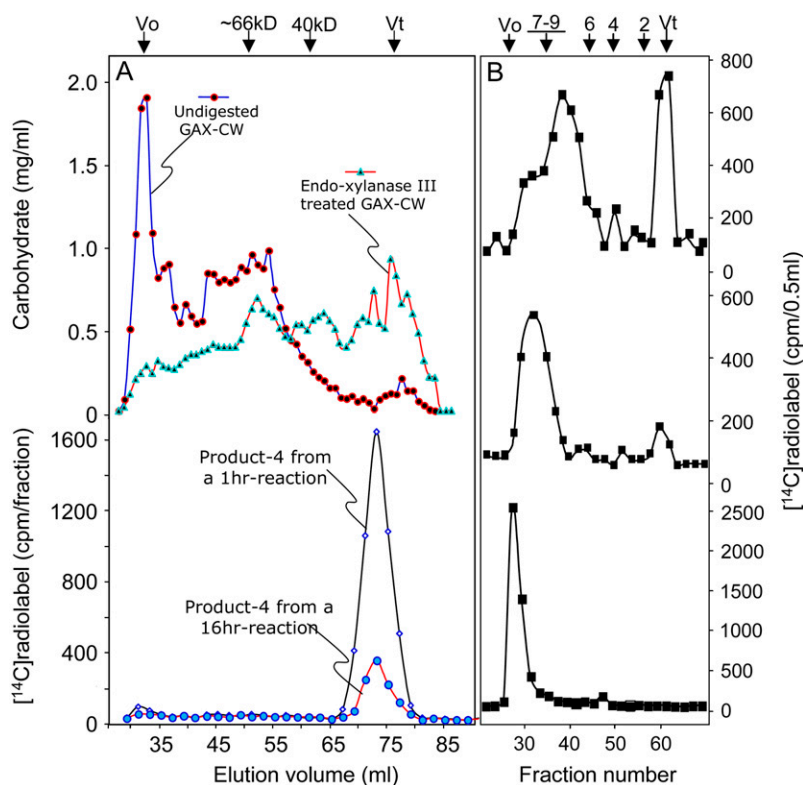
#### Estimation of the Molecular Mass of the [ $^{14}\text{C}$ ]GAX-Like Product Synthesized *In Vitro* and GAXs from Wheat Seedling Cell Walls

GAXs were extracted from wheat etiolated seedling cell walls using alkali solution and partially purified by enzymatic removal of starch and MLG polymers using  $\alpha$ -amylase and lichenase. Fractionation of these partially purified GAXs on Sepharose-CL-6B indicated the coexistence of two GAX populations with different sizes. One GAX population eluted as a broad peak at approximately 66 kD (referred to as 66-GAX) and the other with a high molecular mass (>500 kD) eluted as a sharp peak at the  $V_0$  of the column (referred to as HMW-GAX; Fig. 7A). 66-GAX and HMW-GAX represent approximately 70% and approximately 25% of the total alkali-extracted GAXs, respectively. While the HMW-GAX population was completely degraded with endo-xylanase III, the 66-GAX population was approximately 80% resistant to digestion, which suggests that this population of the mature GAX is highly branched (Fig. 7A). This 66-GAX population can be compared with the portion of the nascent GAX-like product that is resistant to endo-xylanase III.

When the nascent [ $^{14}\text{C}$ ]GlcA-labeled product 4 (Table II) was fractionated on Sepharose-CL-6B, the size of the product was much smaller than mature wall GAXs, with a molecular mass of approximately 10 to 15 kD (Fig. 7A), which would correspond to a DP of approximately 50 to 80. Longer incubation reaction times (up to 16 h of incubation) did not result in an increase in the size of the  $^{14}\text{C}$ -labeled product 4 (Fig. 7A). The maximum synthesis *in vitro* was reached after 90 min (data not shown). This result suggests that the polymer produced in our *in vitro* assay may not reflect the structure of the GAX polymers produced *in vivo*.

#### DISCUSSION

This work demonstrates that wheat Golgi-enriched microsomes synthesize a  $^{14}\text{C}$ -product that has GAX characteristics. Total acid hydrolysis of the product released  $^{14}\text{C}$ -radiolabel solely associated with Xyl, Ara, and GlcA residues in molar ratios comparable to those



**Figure 7.** Sepharose-CL-6B fractionation profiles (A) of the partially purified GAX from wheat seedling cell walls (before and after treatment with endo-xylanase III) along with the  $^{14}\text{C}$ GlcA-labeled product 4 (from 1- and 16-h incubation reactions), and Bio-gel P2 fractionation profiles (B) of  $^{14}\text{C}$ -radiolabeled product 1 (top), product 2 (middle), and product 4 (bottom) prepared in vitro by wheat Golgi-enriched membranes (see Table II). The Sepharose-CL-6B column was eluted with 0.1 mM NaOH containing 0.01%  $\text{NaBH}_4$ , and 1-mL fractions were collected. The elution volumes of dextrans (500 and 40 kD), BSA (66 kD), and Xyl are indicated with arrows at the top as  $V_0$ , ~66 kD, 40 kD, and total elution volume ( $V_t$ ), respectively. The Bio-gel P2 column was eluted as described in "Materials and Methods." [See online article for color version of this figure.]

of known GAXs from grasses (Aspinall, 1980; Vieter et al., 1994; Ebringerova and Heinze, 2000). This work demonstrates also that synthesis of the GAX-like polymer requires cooperative action between XylS, arabinosyltransferase, and GlcAT activities, as shown by the reciprocal stimulatory effects between of UDP-Xyl, UDP-GlcA, and UDP-Arap on the incorporation of  $^{14}\text{C}$ GlcA,  $^{14}\text{C}$ Ara, and  $^{14}\text{C}$ Xyl into ethanol-insoluble product. The wheat GlcAT activity was greatly enhanced in the presence of UDP-Xyl in a similar fashion as that described earlier from the pea system (Waldron and Brett, 1983). Besides the fact that wheat GlcAT was much more active than pea GlcAT, both enzymes appear to share many biochemical properties (i.e. UDP-Xyl stimulation,  $\text{Mn}^{2+}$  requirements, and pH). Several lines of evidence support the conclusion that wheat GlcAT activity is involved in GAX-like biosynthesis: (1) reaction conditions (buffer and ions) are similar for both GlcA transfer and GAX-like synthesizing activities (Fig. 2); (2) the increase in the size of products 1, 2, and 4 upon inclusion of UDP-GlcA and/or UDP-Arap in the reaction medium strongly suggests the incorporation of Xyl, Ara, and GlcA into the same product (Fig. 7); and (3) the nascent  $^{14}\text{C}$ Xyl-labeled product 4 (GAX-like polymer) also contained GlcA residues, as judged by its attachment to Dowex resin. Interestingly, despite the fact that the newly synthesized GAX-like polymer contains more Ara than GlcA side chains, its biosynthesis was dependent on the presence of UDP-GlcA in the reaction medium. In the absence of UDP-GlcA, microsomal membranes

make only smaller fragments that eluted within the volume of the Bio-gel P2 column. Thus, GlcAT seems to play a central role in controlling the elongation of the GAX backbone. A similar stimulatory effect of UDP-GlcA on xylan backbone elongation was also observed in earlier work on pea epicotyls that required a cooperative action between the XylT and GlcAT activities (Dalessandro and Northcote, 1981; Waldron and Brett, 1983; Baydoun et al., 1989). This cooperative mechanism between synthases and other glycosyltransferases is not unique to GAX and GX synthesis, for it was also observed in the synthesis of XyGs (Ray, 1980; Hayashi and Matsuda, 1981; Faik et al., 2002). In the case of GAXs and GXs, the distribution of GlcA residues along the  $\beta(1,4)$ -xylan backbone could serve as markers for the XylS to proceed or for endo-xylanases to cut at specific sites. In support of this hypothesis, Nishitani and Nevins (1991) and Hurlburt and Preston (2001) have reported the existence of bacterial endo-xylanases (both from the GH5 family) that require the presence of GlcA residues to recognize the cleavage sites on the xylan backbone.

This study sought to partially analyze the structure of the nascent GAX-like polymer using the purified and well-characterized endo-xylanase III from *A. awamori*, a member of the GH11 family (CAZy classification). Treatment of the nascent GAX-like polymer with this enzyme revealed that 60% to 70% of the polymer was resistant to digestion and only 30% to 40% was susceptible to endo-xylanase III, suggesting the existence of structural differences within the newly synthesized

polymer. Two lines of evidence suggest that the endo-xylanase III-susceptible portion of the polymer may have a relatively regular structure: (1) endo-xylanase III treatment released only free Xyl and no xylobiose, indicating that substituted  $\beta(1,4)$ -Xyl residues in the xylan backbone are separated by short stretches of unbranched  $\beta(1,4)$ -Xyl residues; and (2) endo-xylanase III treatment released only two major radioactive peaks (peaks I and II), as shown by HPAEC analysis. In addition, because endo-xylanase III belongs to the GH11 family, it requires at least three adjacent Xyl residues between branched Xyl residues to cut the xylan backbone (Kormelink et al., 1993a; Davies et al., 1997). Therefore, the degradation pattern observed here would be possible only if the structure of the polymer contained substituted  $\beta(1,4)$ -Xyl residues separated by stretches of no more than four unbranched  $\beta(1,4)$ -Xyls (xylotetraose). If the structure of the xylan backbone contained stretches of three unbranched Xyls, no free Xyl would be released. However, if the stretches consisted of more than four unbranched Xyl residues, endo-xylanase III would generate xylobiose among the final degradation products. On the other hand, stretches of less than three unbranched Xyls would make the product resistant to endo-xylanase III digestion. This latter possibility may explain why 60% to 70% of nascent GAX-like polymers are endo-xylanase III resistant. This result is in agreement with an earlier study by Vietor et al. (1994) showing that substantial proportions of the water-unextractable AXs from barley (*Hordeum vulgare*) and malt were resistant to digestion by endo-xylanase I from *A. awamori* (a member of the GH10 family). These AXs were highly branched polymers (55% of the Xyls were substituted). However, additional characterization of the fragments released by endo-xylanase III will be needed before its incorporation into a structural model for nascent GAX-like polymer produced in vitro. It is important to stress that this structure of the GAX-like polymer produced in our in vitro assay may not reflect the structure of the GAX polymers produced in vivo.

Cell walls from wheat seedlings appeared to contain two GAX populations with different molecular masses (66-GAX and HMW-GAX). This finding is consistent with earlier work on GAX from cell walls of dark-grown maize coleoptiles, in which three GAX polymers were observed with molecular masses of approximately 10 kD, approximately 70 kD, and greater than 500 kD (Carpita, 1983, 1984). On the other hand, wheat microsomes synthesized in vitro a GAX-like polymer that is approximately 10 to 15 kD, and longer incubation time of the reaction (up to 16 h) did not produce larger polymers. It is possible that our reaction conditions were not optimal for the microsomes to build larger polymers (i.e. lack of critical substrates/cofactors, inactivity of parts of the synthetic machinery, and nonfunctional regulation mechanisms). However, pulse-chase experiments in maize seedlings have suggested that the smaller and highly branched GAX population was the precursor for the

larger xylans (Carpita, 1984), which suggests that nascent GAX-like polymer synthesized as a highly branched and smaller polymer may be the precursor to the larger xylans. The mechanisms that would allow the generation of larger GAXs from the precursor in vivo are not known. Porchia and Scheller (2000) have identified a  $\beta(1,4)$ -XylT activity in etiolated wheat seedlings that produces in vitro a xylan-like polymer with a larger size (200 kD) than our product. Several observations indicate that this XylT activity is different from the XylS activity involved in our GAX-like polymer synthesis: (1) the XylT activity identified by Porchia and Scheller (2000) is active in potassium phosphate buffer, a buffer that inhibited approximately 90% of our GAX-like synthesizing activity (Fig. 2); (2) this activity did not require the presence of  $Mn^{2+}$  or  $Mg^{2+}$ , in sharp contrast with our activity; and (3) the addition of UDP-Arap did not stimulate Xyl incorporation into an ethanol-insoluble product, which appeared to be lowly substituted.

The other alternative mechanism to explain the formation of larger GAXs in vivo would be the involvement of a specific GAX endo-transglycosylating activity that could mediate the integration of small nascent GAX-like fragments into already existing GAXs in the cell wall. XyG endo-transglycosylase serves as a precedent for this mechanism (Faik et al., 1998; Hrmova et al., 2007). Yokoyama et al. (2004) have identified 29 XyG endo-transglycosylase (also called XTH) members in rice, a number that is comparable to the XTH genes found in Arabidopsis, despite the fact that rice cell walls have low amounts of XyG compared with Arabidopsis cell walls. It is possible that some of these rice XTH proteins might use GAX as a substrate. After integration into the cell wall, this highly substituted portion of the GAX would be subjected to rapid and extensive postmodifications that would include the removal of Ara and GlcA residues by arabinofuranosidase and glucuronosidase activities, respectively. As a result, long unbranched  $\beta(1,4)$ -Xyl stretches would be generated, which would result in tighter hydrogen bonding to cellulose or MLG molecules (Carpita, 1983, 1996; Kabel et al., 2007).

It is now accepted that GAXs and XyGs are the principal cross-linking polymers in walls of growing tissues (Carpita, 1983, 1996; Kabel et al., 2007), and earlier works have shown that walls from rapidly elongating regions of maize coleoptiles are rich in highly substituted GAXs (up to 20% of total hemicelluloses; Carpita, 1984; Carpita and Whittern, 1986). Therefore, we should expect higher GlcAT- and GAX-synthesizing activities in growing regions of wheat seedlings. This work is compatible with these earlier studies and provides direct evidence that Golgi-enriched microsomal preparations from rapidly elongating regions (mesocotyl) have higher GlcAT activity (Fig. 3) and are actively synthesizing GAX polymers.

In conclusion, this study reports significant progress with respect to the biochemistry of GAX biosynthesis

in wheat, with emphasis on the mechanism of synthesis and analysis of the product. The study also reports on the presence of a GlcAT that is enhanced by the addition of UDP-Xyl in the reaction medium and its role in GAX biosynthesis. However, the mechanism by which this GlcAT proceeds during GAX biosynthesis will require the purification and cloning of the genes that encode the enzyme, work that is now under way in our laboratory. Nevertheless, the picture that emerges here is that plant cells appear to use similar mechanisms (a cooperative mechanism with some variation) to synthesize at least three hemicellulosic polymers found in primary cell walls, namely GAXs, GXs, and XyGs.

## MATERIALS AND METHODS

### Plant Materials and Chemicals

Winter wheat (*Triticum aestivum*), rice (*Oryza sativa*), and maize (*Zea mays*) seeds were grown on soil in the dark at 24°C. UDP-[<sup>14</sup>C]GlcA (6.67–11.58 GBq mmol<sup>-1</sup>) was from MP Biomedicals, and UDP-[<sup>14</sup>C]Xyl (9.76 GBq mmol<sup>-1</sup>) was from Perkin-Elmer. UDP-Xyl and UDP-Arap were from CarboSource (Complex Carbohydrate Research Center, University of Georgia; [http://cell.ccr.uga.edu/~carbosource/CSS\\_items.html](http://cell.ccr.uga.edu/~carbosource/CSS_items.html)). A solution of purified endo-xylanase III from *Aspergillus awamori* (323 μg mL<sup>-1</sup>; specific activity = 18 units mg<sup>-1</sup> protein) and the authentic AXO standards were kindly provided by Drs. Henk Schols and Laurice Pouvreau (Laboratory of Food Chemistry, Wageningen University, The Netherlands). Known AXO standards were prepared by digesting 20 mg of wheat AX with endo-xylanase III (0.012 units) for 16 h (see the conditions below). Lichenase (*Bacillus subtilis*), endopolygalacturonase M2 (*Aspergillus aculeatus*), xylo-oligosaccharides, and aldouronic acids were from Megazyme. Proteinase K (*Tritirachium album*) was from Fisher. α-Amylase type VII-A (porcine pancreas), Dowex 1-X80 resin, Sepharose-CL-6B, and all chemicals were purchased from Sigma. The Bio-gel P2 column was from Bio-Rad. Driselase (*Irpex lacteus*) was from Kyowa-Hakko.

### Preparation of UDP-[<sup>14</sup>C]Arap

UDP-[<sup>14</sup>C]Arap was prepared from UDP-[<sup>14</sup>C]Xyl using recombinant *Arabidopsis thaliana* UDP-Xyl-4-epimerase gene (UGE3) expressed in bacteria (a gift from Dr. Wolf-Dieter Reiter, University of Connecticut; Burget et al., 2003). Purified epimerase (5 μg) was incubated with UDP-[<sup>14</sup>C]Xyl (5 μg in 200 μL of 50 mM HEPES buffer, pH 7) for 5 min at room temperature. UDP-[<sup>14</sup>C]Arap was purified by HPAEC using an isocratic concentration of 100 mM ammonium formate. Ammonium formate was removed by repeated freeze-dry cycles, and the purified UDP-[<sup>14</sup>C]Arap was resuspended in ethanol:water (7:1) solution and stored at -80°C until use.

### Preparation of Golgi-Enriched Membranes

Etiolated seedlings (wheat, rice, or maize) were used for microsomal preparation as described earlier (Faik et al., 2002) with additional modifications. All steps were carried out at 4°C. Briefly, approximately 25 g of etiolated seedling tissues was harvested and ground with mortar and pestle in 50 mL of extraction buffer (0.1 M HEPES-KOH, pH 7, 0.4 M Suc, 0.1% bovine serum albumin [BSA], 1 mM dithiothreitol, 5 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, 1 mM phenylmethylsulfonyl fluoride, and one tablet of Roche complete protease inhibitor cocktail). The suspension was filtered through two layers of Miracloth, and the filtrate was centrifuged at 3,000g for 20 min. The ground wheat tissue was kept at -20°C for cell wall GAX extraction (see below). The resulting supernatant was layered over 1.8 M Suc cushion buffer and centrifuged at 100,000g for 60 min. Crude microsomal membranes located at the top of the 1.8 M Suc cushion were further fractionated to obtain Golgi-enriched membranes by overlaying 1.1 M Suc solution on the top of the crude microsomal membranes, followed by another 0.25 M Suc solution. This discontinuous gradient was centrifuged for 60 min at 100,000g, and Golgi-

enriched microsomal membranes located at the 0.25/1.1 M Suc interface were collected and pelleted by another centrifugation at 100,000g, resuspended in 200 μL of extraction buffer, and stored at -80°C until use. This standard procedure usually yields membrane fractions with a protein concentration of approximately 5 μg μL<sup>-1</sup>. Protein content is estimated using Bradford reagent (Sigma) and various concentrations of BSA as standard.

### Standard GlcAT- and GAX-Synthesizing Activity Assay

The reaction mixture (approximately 60 μL) consisted of 40 μL of Golgi-enriched membranes (approximately 200 μg of proteins), approximately 2 μM UDP-[<sup>14</sup>C]GlcA (90,000 cpm, 900 cpm pmol<sup>-1</sup>), 0.5 mM UDP-Xyl, with or without UDP-Arap (0.2 mM). When UDP-[<sup>14</sup>C]Xyl or UDP-[<sup>14</sup>C]Arap was included in the reaction, it was supplied as approximately 3.5 cpm pmol<sup>-1</sup>. Reactions were incubated at room temperature for 90 min, terminated by adding 1 mL of cold 70% ethanol, and precipitated for at least 1 h at -20°C. Reaction products were collected by centrifugation at 10,000g for 10 min, and the pellets were washed (five times) with 1 mL of cold 70% ethanol to remove excess UDP-[<sup>14</sup>C]GlcA. The <sup>14</sup>C-radiolabel incorporation was estimated by resuspending the pellets in 300 μL of water and adding 3 mL of scintillation fluid (Fisher) before counting the cpm in a liquid scintillation counter (Beckman Coulter LS 6500).

### Effects of pH, Divalent Cations, and Nucleotide Sugars on the GlcA Transfer

The optimum pH for GlcAT activity was determined using Golgi-enriched membrane preparations from 6-d-old etiolated wheat seedlings and MES, HEPES, and potassium phosphate buffers at a final concentration of 100 mM. The activity was tested at pH 4, 5, 6, and 7 with MES-KOH buffer, pH 6, 7, and 8 with HEPES buffer, and pH 7 and 8 with potassium phosphate buffer. Incubation conditions, product washing, and <sup>14</sup>C-radiolabel quantification were carried out as described for the standard GAX-synthesizing assay.

To test the effect of divalent ions, Golgi-enriched membranes were prepared from 6-d-old seedlings as described above except that the ions were omitted from the extraction buffer. The membranes were used to test the GAX-synthesizing activity as described in the standard assay in the presence of 5 mM MnCl<sub>2</sub>, MgCl<sub>2</sub>, CaCl<sub>2</sub>, CuCl<sub>2</sub>, NiCl<sub>2</sub>, and ZnSO<sub>4</sub>. After stopping the reactions and washing, the radioactivity in pellets was quantified by scintillation counting as described above.

UDP-Glc, UDP-Gal, UDP-GalNAc, GDP-Man, and GDP-Fuc were tested for their stimulatory effect on the GlcA transfer into ethanol-insoluble product under the same conditions used to test UDP-Xyl. UDP sugars were included at 0.5 mM concentrations using 100 mM HEPES buffer, pH 7, at room temperature for 90-min reactions. UDP-Ara was not tested because wheat microsomes contained UDP-Xyl-4-epimerase activity that would produce UDP-Xyl, which in turn would affect the reaction.

### Extraction of GAX from Cell Walls of Etiolated Wheat Seedlings

Residual cell walls (from microsomal preparation procedures) were boiled for 15 min in water to denature hydrolases and eliminate soluble polymers and small molecules. After centrifugation (4,000g, 20 min, 4°C), the walls were extracted with chloroform:methanol (1:1) to remove lipids before extensive washes with acetone until the supernatant turned colorless. The residual walls were air dried before use for GAX extraction with 2 M KOH containing 0.01% NaBH<sub>4</sub> (v/g dry weight) for 16 h at room temperature. The extraction was repeated a second time, and both extracts were combined and neutralized with glacial acetic acid before dialysis for 24 h against water (with several water changes). The alkali extract was precipitated with 70% ethanol and resuspended in a smaller volume for lyophilization. This procedure usually produces approximately 1.2 g of material from approximately 5 g dry weight of cell walls.

To remove contaminant starch, the alkali extract material was treated with α-amylase type VII-A (100 units g<sup>-1</sup> material) in 20 mM sodium phosphate buffer, pH 6.9, containing 7 mM NaCl overnight at room temperature, and the reaction was terminated by boiling for 10 min. The amylase-treated product was dialyzed against water overnight, and the retained material was lyophilized. The removal of MLGs was carried out by treatment of the amylase-treated material with lichenase (100 units g<sup>-1</sup> material) in 25 mM Tris-HCl, pH

6.9, overnight at 50°C. After dialysis against water, the final retained material was lyophilized. Typically, approximately 5 g dry weight of starting cell walls would give approximately 0.6 g of final GAX-enriched material (approximately 50% of alkali-extracted material). This GAX-enriched fraction was used for further analyses on the Sepharose-CL-6B column and for endo-xylanase III treatment.

## General Analytical Methods Used

### <sup>14</sup>C-Product Preparation

Five to 20 standard GlcAT activity reactions (Table II) were used to generate enough <sup>14</sup>C-products for analyses. Ethanol-insoluble <sup>14</sup>C-products from these reactions were washed as described above and solubilized in 0.5 mL of 0.1 M NaOH before centrifugation at 10,000g for 5 min (room temperature) to remove alkali-insoluble materials. Usually, this step solubilizes approximately 95% of the <sup>14</sup>C-radiolabel. The resulting alkali-soluble <sup>14</sup>C-products were neutralized with acetic acid and centrifuged again as above to remove insoluble materials from the final soluble <sup>14</sup>C-products to be used for the subsequent analyses. Usually, the final soluble <sup>14</sup>C-products contain approximately 90% of the initial <sup>14</sup>C-radiolabel (Table II).

### Analysis of Monosaccharide Composition by Total Acid Hydrolysis and HPAEC

Samples were lyophilized and dissolved in 2 M trifluoroacetic acid (TFA). The mixture was autoclaved for 60 min at 121°C, and the excess TFA was removed by repeated evaporation in a Speed-Vac system. The identity of the monosaccharides (including acidic monosaccharides) was determined by HPAEC after fractionation on a CarboPac PA10 column (4 × 250 mm; Dionex) connected, in series with a CarboPac PA guard column (3 × 25 mm; Dionex), to a BioLC system using pulsed amperometric detection (ED50 electrochemical detector; Dionex). Known monosaccharides were treated under the same conditions and used as standards. The following conditions were used: isocratic elution at a flow rate of 1 mL min<sup>-1</sup> with 16 mM NaOH for 30 min, followed by 0.1 M NaOH containing 0.3 M sodium acetate for 10 min; the column was washed with double distilled water and calibrated again with 16 mM NaOH for the next run. One-milliliter fractions were collected.

### Fractionation of Oligosaccharides by HPAEC

Samples (<sup>14</sup>C-radiolabeled oligosaccharides or AXO standards) were fractionated on a CarboPac PA10 column by adapting the conditions used by Kormelink et al. (1993a). The column was eluted at a flow rate of 1 mL min<sup>-1</sup> with 0.1 M NaOH containing a gradient of sodium acetate from 0 to 0.5 M during 60 min, followed by isocratic elution for 5 min with 1 M sodium acetate in 0.1 M NaOH. In the final step, the column was eluted with 0.1 M NaOH for 15 min before use in the next cycle. <sup>14</sup>C-radiolabeled samples were injected in several repetitions to collect enough material for cpm counting and for other analyses. AXO standards were included with <sup>14</sup>C-radiolabeled samples as a control to ensure that their elution profiles did not change between injections.

### Endo-Xylanase III Treatment

Samples (<sup>14</sup>C-products or GAX from wheat seedling cell walls) were lyophilized and resuspended in 50 mM sodium acetate, pH 5, containing 0.012 unit of purified enzyme. Enzymatic digestion was conducted at 40°C for 16 h. The reactions were stopped by boiling for 10 min and centrifuging to remove insoluble materials before further analysis by gel filtration or HPAEC.

### Proteinase K Treatment

<sup>14</sup>C-products were concentrated to approximately 2 mL and adjusted to 50 mM Tris-HCl buffer, pH 7.6, before the addition of 5 μL of proteinase K (15 units). The reaction mixture was incubated at 37°C for 8 h before inactivation of the enzyme by boiling for 10 min.

### Endo-Polygalacturonase M2 Treatment

<sup>14</sup>C-products were concentrated to approximately 2 mL and adjusted to 0.1 M acetate buffer, pH 5, before the addition of 5 μL of endo-polygalacturonase

M2 (25 units). The reaction mixture was incubated at 40°C for 5 h before inactivation of the enzyme by boiling for 15 min.

### Driselase Treatment

Driselase powder (0.1 mg; *Irpex lacteus*) was resuspended in 0.25 mL of sodium acetate buffer (100 mM, pH 4.3), and the mixture was vortexed and incubated on ice for 10 min to solubilize the enzymes. After centrifugation at 10,000g for 5 min (4°C), the supernatant was mixed with the product that was lyophilized and resuspended in 0.5 mL of water. The reaction was conducted for 16 h at 40°C and stopped by boiling for 10 min. Insoluble material was removed by centrifugation, and the supernatant was used for fractionation by gel filtration on the Bio-gel P2 column.

### Gel Filtration on the Bio-gel P2 Column

<sup>14</sup>C-labeled oligosaccharides were fractionated on a 90 × 1.5-cm column and eluted under gravity with degassed water. Fractions of 2.3 mL were collected. Xyl, xylo-oligosaccharides (DP 2–6), and XyG-oligosaccharides (DP 7–9) were used as standards to calibrate the column.

### Gel Filtration on the Sepharose-CL-6B Column

Nascent <sup>14</sup>C-product 4 or GAX from cell walls was fractionated on a Sepharose-CL-6B column (60 × 1 cm) that was eluted with 0.1 M NaOH containing 0.01% NaBH<sub>4</sub>. Eluted products were collected in 1-mL fractions. Dextran (500 kD), dextran (40 kD), and BSA (66 kD) were used as standards to calibrate the column. Carbohydrate content was determined by the phenol-sulfuric acid method (Dubois et al., 1956).

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