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Identification of a disaccharide side chain 2-*O*-α-D-galactopyranosyl-α-D-glucuronic acid in *Arabidopsis* xylan

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Arabidopsis xylan consists of a linear chain of β-1,4-linked D-xylosyl residues, about 10% of which are substituted with single residues of α-D-glucuronic acid (GlcA) or 4-*O*-methyl-α-D-glucuronic acid (MeGlcA) at *O*-2. In addition, about 60% of xylosyl residues are acetylated at *O*-2 and/or *O*-3. Previous studies have identified a number of genes responsible for elongation of the xylan backbone, addition of the GlcA substituents, and methylation of the GlcA residues. Yuan et al. (2013) have recently reported that the 2-*O*- and 3-*O*-monoacetylation of xylosyl residues in *Arabidopsis* xylan requires a DUF231 domain-containing protein, ESKIMO1 (ESK1), and proposed that ESK1 and its homologs are putative acetyltransferases responsible for xylan acetylation. It was noticed that the ^{1}H nuclear magnetic resonance (NMR) spectra of the acetylated xylan from the *esk1* mutant and the wild-type *Arabidopsis* exhibited a prominent proton signal peak at 5.42 ppm in addition to resonances corresponding to known acetylated structural groups of xylan. Here, we performed detailed structural investigation of wild-type *Arabidopsis* acetylated xylan using 2-dimensional ^{1}H-^{1}H and ^{1}H-^{13}C NMR spectroscopy and found that the signal peak at 5.42 ppm in the ^{1}H NMR spectrum was attributed to GlcA residues substituted at *O*-2 with α-D-galactose (Gal), indicating the presence of Gal-GlcA disaccharide side chains in *Arabidopsis* xylan. This finding was further supported by analysis of endoxylanase-digested xylan using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. Our study demonstrates that *Arabidopsis* xylan contains Gal-GlcA disaccharide side chains in addition to GlcA, MeGlcA, and acetyl substitutions.

Xylan is the second most abundant polysaccharide in plant biomass. It is the predominant hemicellulose in secondary walls of xylem and fibers in angiosperms.[1] Xylan consists of a linear chain of β-1,4-linked xylosyl residues with a degree of polymerization up to 120.[2] The reducing end of the xylan backbone from gymnosperms and dicots also contains a distinct tetrasaccharide sequence, β-D-Xyl-(1→3)-α-L-Rha-(1→2)-α-D-GalA-(1→4)-D-Xyl.[3-6] Xylan from dicots is typically substituted with single residues of α-D-glucuronic acid (GlcA) and 4-*O*-methyl-α-D-glucuronic acid (MeGlcA) at *O*-2. Xylan from lignified tissues of grasses is substituted with α-L-arabinose (Ara) at *O*-3 in addition to the 2-*O*-linked GlcA and MeGlcA, and that from cereal grains is mainly substituted with Ara residues at *O*-2 and *O*-3.[1] Although xylan substituents are typically single sugar residues, those of grass xylan can be disaccharides composed of 2-*O*-Ara-Ara or 2-*O*-Xyl-Ara linked at *O*-3 to the xylan backbone, and the Ara substituents may be esterified by ferulic acid at *O*-5.[1] Xylan from wood of *Eucalyptus globulus* was found to contain disaccharide side chains composed of MeGlcA substituted at *O*-2 with α-D-galactose (Gal).[7] In addition to sugar substitutions, xylosyl residues in the xylan backbone may be acetylated at *O*-2 and/or *O*-3.[8] Since xylan is one of the factors contributing to the recalcitrance of cellulosic biomass to saccharification,[9] it is important to have a thorough understanding of xylan structure and of how genetic modification of xylan content and structure may alter lignocellulosic biomass recalcitrance in order to custom-design biomass composition tailored for biofuel production.

Arabidopsis has been used as a model to identify genes involved in xylan biosynthesis and to study how alterations of xylan content and structure impact secondary wall biosynthesis. As a typical dicot xylan, *Arabidopsis* xylan consists of the xylosyl backbone, the reducing end tetrasaccharide sequence, and substitutions of xylosyl residues with GlcA/MeGlcA residues and acetyl groups.[5,10] Genetic and biochemical studies of xylan biosynthesis in *Arabidopsis* have revealed that the elongation of the xylosyl backbone requires glycosyltransferases from both GT43 (IRX9/I9H and IRX14/I14H) and GT47 (IRX10/IRX10L) families,[5,11-16] the biosynthesis of the reducing end tetrasaccharide sequence involves glycosyltransferases from GT8 (IRX8 and PARVUS) and GT47 (FRA8/F8H) families,[5,6,11,17-19] the substitutions by GlcA residues is mediated by 3 GT8 glycosyltransferases (GUX1/2/3),[20,21] and the methylation of GlcA residues is catalyzed by 3 DUF579

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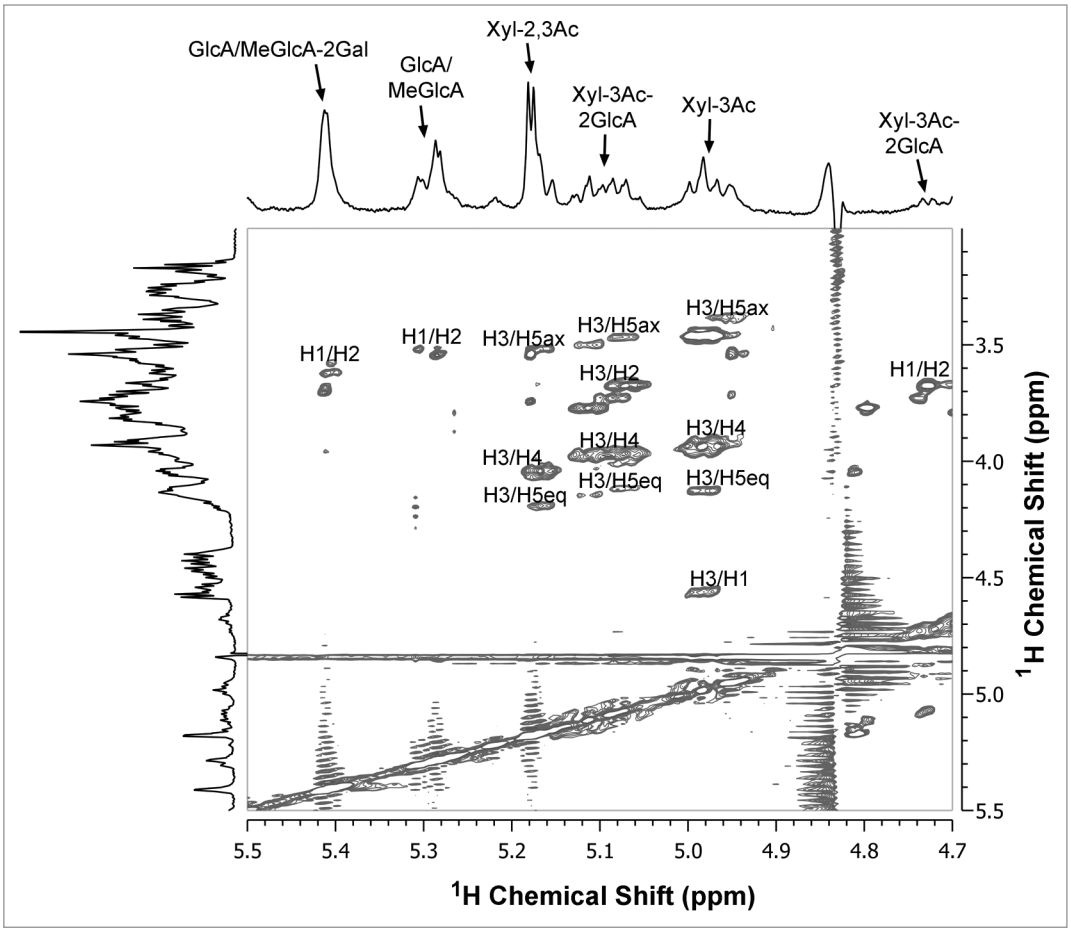

Figure 1. Two-dimensional ^{1}H-^{1}H TOCSY NMR spectrum of acetylated xylan from wild-type *Arabidopsis* stems. Acetylated xylan was extracted with DMSO[28] and digested with β-endoxylanase M6 (Megazyme) to generate xylooligosaccharides,[16] which were subsequently subject to structural analysis using NMR spectroscopy.[29] The spectra aligned at the top and the left of the figure are the 1D ^{1}H NMR spectra of *Arabidopsis* xylan. The identities of the resonance peaks in the top spectrum are marked. The following abbreviations are used: GlcA/MeGlcA-2Gal, GlcA/MeGlcA substituted at *O*-2 with galactose; Xyl-2,3Ac, 2,3-di-*O*-acetylated xylosyl residues; Xyl-3Ac-2GlcA, 3-*O*-acetylated xylosyl residues substituted with *O*-2 with GlcA/MeGlcA; Xyl-3Ac, 3-*O*-acetylated xylosyl residues.

domain-containing methyltransferases (GXM1/2/3).[22,23] Mutations of genes responsible for the biosynthesis of the xylan backbone and the reducing end tetrasaccharide sequence all lead to a reduction in xylan content and concomitantly a defective secondary wall thickening. Recently, Yuan et al.[24] have demonstrated that a DUF231 domain-containing protein, ESKIMO1 (ESK1), is required for the acetylation of xylan during secondary wall biosynthesis in *Arabidopsis*. The *esk1* mutation causes a specific reduction in the 2-*O*- and 3-*O*-monoacetylation of xylosyl residues in xylan and severe defects in secondary wall thickening and plant growth. It was hypothesized that ESK1 and its close homologs were putative acetyltransferases catalyzing *O*-acetylation of xylosyl residues in xylan.

It was noticed that the ^{1}H nuclear magnetic resonance (NMR) spectra of the acetylated xylan from the *esk1* mutant and the wild-type *Arabidopsis* exhibited a prominent proton resonance peak located at 5.42 ppm in addition to the resonances corresponding to 2-*O*- and 3-*O*-monacetylated xylosyl residues, 2,3-di-*O*-acetylated xylosyl residues, and 3-*O*-acetylated xylosyl residues substituted at *O*-2 with GlcA (**Fig. 1**).[24] A proton resonance at 5.42 ppm has been previously observed in the ^{1}H NMR spectrum of xylan from *E. globulus* and it was attributed to disaccharide side chains composed of MeGlcA substituted at *O*-2 with α-D-Gal.[7] About 10% xylosyl residues in *E. globulus* xylan are substituted with MeGlcA residues and one-third of the MeGlcA substituents are attached with Gal residues. The Gal-MeGlcA disaccharide substituents in xylan have thus far only been reported in *E. globulus*, and it is not known whether they are also present in xylans from other species. One study has found that xylan from maize bran contains Gal-Xyl-Ara trisaccharide substituents.[25]

To investigate what structure the observed 5.42-ppm proton resonance in *Arabidopsis* acetylated xylan is attributed to, we employed 2-dimensional (2D) total correlation NMR spectroscopy (TOCSY) to analyze the structural units of wild-type *Arabidopsis* xylan. It has previously been shown that in the 2D ^{1}H-^{1}H TOCSY spectrum of *E. globulus* xylan, the H-1 signal of the Gal-MeGlcA disaccharide substituents at 5.42 ppm has correlation cross peaks with H-2 proton at 3.77 ppm.[26] The H-1 signal of *Arabidopsis* xylan at 5.42 ppm also has correlation with H-2 proton at 3.7 ppm (**Fig. 1**), indicating that the cross peak corresponds

to the Gal-MeGlcA disaccharide substituents. Unlike *E. globulus* xylan that has only MeGlcA substituents,[26] *Arabidopsis* xylan contains both GlcA and MeGlcA substituents. Since the proton resonances for GlcA and MeGlcA are overlapped in the 2D NMR spectrum,[27] it is not discernable whether the cross peak at H-1 of 5.42 ppm and H-2 of 3.7 ppm in the 2D TOCSY spectrum of *Arabidopsis* xylan contains both Gal-GlcA and Gal-MeGlcA, and thus it is designated as Gal-GlcA/MeGlcA (**Fig. 1**). Other correlations in the 2D TOCSY spectrum of Arabidpsis xylan correspond to GlcA/MeGlcA (the H-1/H-2 cross peak at 5.3/3.55 ppm), 2,3-di-*O*-acetylated xylosyl residues (H-3/H-5ax at 5.17/3.53 ppm, H-3/H-4 at 5.17/4.05 ppm, and H-3/H-5eq at 5.17/4.2 ppm), 3-*O*-acetylated xylosyl residues substituted at *O*-2 with GlcA (H-3/H-5ax at 5.08/3.48 ppm, H-3/H-2 at 5.08/3.7 PM, H-3/H-4 at 5.08/3.98 ppm, H-3/H-5eq at 5.08/4.2 ppm, and H-1/H-2 at 4.73/3.69 ppm), and 3-*O*-monoacetylated xylosyl residues (H-3/H-5ax at 4.98/3.48 ppm, H-3/H-4 at 4.98/3.93 ppm, H-3/H-5eq at 4.98/4.13 ppm, and H-3/H-1 at 4.98/4.58 ppm). The spectral positions of these proton cross peaks are in good agreement with those reported for acetylated xylans from *E. globulus* and aspen,[8,26] thus validating the reliability of the 2D TOCSY spectrum of *Arabidopsis* xylan.

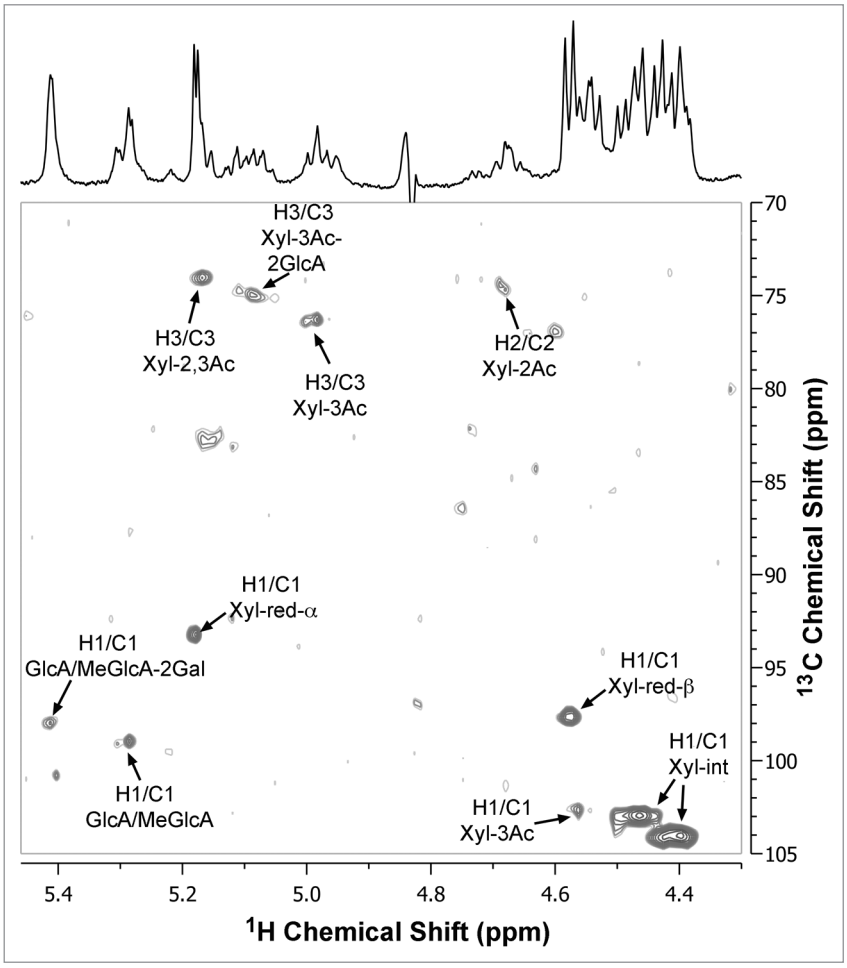

Figure 2. Two-dimensional ^{1}H-^{13}C HSQC NMR spectrum of acetylated xylan from wild-type *Arabidopsis* stems. Acetylated xylan was extracted with DMSO[28] and digested with β-endoxylanase M6 (Megazyme) to generate xylooligosaccharides,[16] which were subsequently subject to structural analysis using NMR spectroscopy.[29] The spectrum aligned at the top of the figure is the 1D ^{1}H NMR spectra of *Arabidopsis* xylan. See the abbreviations in **Figure 1**. Xyl-red-α, reducing end α-Xyl; Xyl-red-β, reducing end β-Xyl; Xyl-int, non-acetylated internal Xyl.

To confirm the identity of the 5.42-ppm proton resonance in *Arabidopsis* acetylated xylan as Gal-GlcA/MeGlcA disaccharide substituents, we next analyzed the structural units of *Arabidopsis* xylan using 2D heteronuclear single-quantum correlation NMR spectroscopy (HSQC). The 2D ^{1}H-^{13}C HSQC spectrum of *Arabidopsis* xylan showed a cross peak of H-1 and C-1 signals at 5.42 and 98 ppm (**Fig. 2**) corresponding to the resonances of Gal-MeGlcA disaccharide substituents, which is the same as that observed in *E. globulus* xylan.[26] Congruent with the 2D HSQC spectra of acetylated xylans from *E. globulus* and aspen,[8,26] other cross peaks of the ^{1}H and ^{13}C chemical shifts in the spectrum of *Arabidopsis* xylan correspond to GlcA/MeGlcA (the H-1/C-1 signals at 5.28/98.8 ppm), 2,3-di-*O*-acetylated xylosyl residues (H-3/C-3 at 5.17/74 ppm), 3-*O*-acetylated xylosyl residues substituted at *O*-2 with GlcA (H-3/C-3 at 5.08/75 ppm), 3-*O*-monoacetylated xylosyl residues (H-3/C-3 at 4.98/76.3 ppm and H-1/C-1 at 4.58/102.5 ppm), and non-acetylated internal xylosyl residues (H-1/C-1 at 4.42/104 and 4.48/103 ppm). Because xylooligomers released from xylanase digestion of acetylated xylan were used for NMR spectroscopy, cross peaks of H-1 and C-1 signals for reducing end α-xylose (H-1/C-1 at 5.18/93.2 ppm) and reducing end β-xylose (H-1/C-1 at 4.58/97.6 ppm) were prominent.

To further substantiate the existence of the disaccharide side chain Gal-GlcA/MeGlcA in *Arabidopsis* xylan, we next applied matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) to examine xylooligomers released from xylanase digestion of *Arabidopsis* xylan. The MALDI-TOF spectrum showed the expected prominent ion peaks $[M+Na]^{+}$ at mass-to-charge ratio (*m/z*) 745, 759, 877, and 891 that are attributed to GlcA-substituted Xyl_4, MeGlcA-substituted Xyl_4, GlcA-substituted Xyl_5, MeGlcA-substituted Xyl_5, respectively (**Fig. 3**).[17] Noticeably, the spectrum also had ion peaks at *m/z* 775 and 907, which correspond to the expected masses of (Gal-GlcA)-substituted Xyl_3 and (Gal-GlcA)-substituted Xyl_4, respectively. No ion peaks corresponding to the expected masses of (Gal-MeGlcA)-substituted Xyl_3 (*m/z* 789) and (Gal-MeGlcA)-substituted Xyl_4 (*m/z* 921) were detected. The MALDI-TOF-MS data showing the presence of ions corresponding to (Gal-GlcA)-substituted xylooligomers not only is consistent with the 2D NMR data but also demonstrate that the disaccharide side chain in *Arabidopsis* xylan is mainly composed

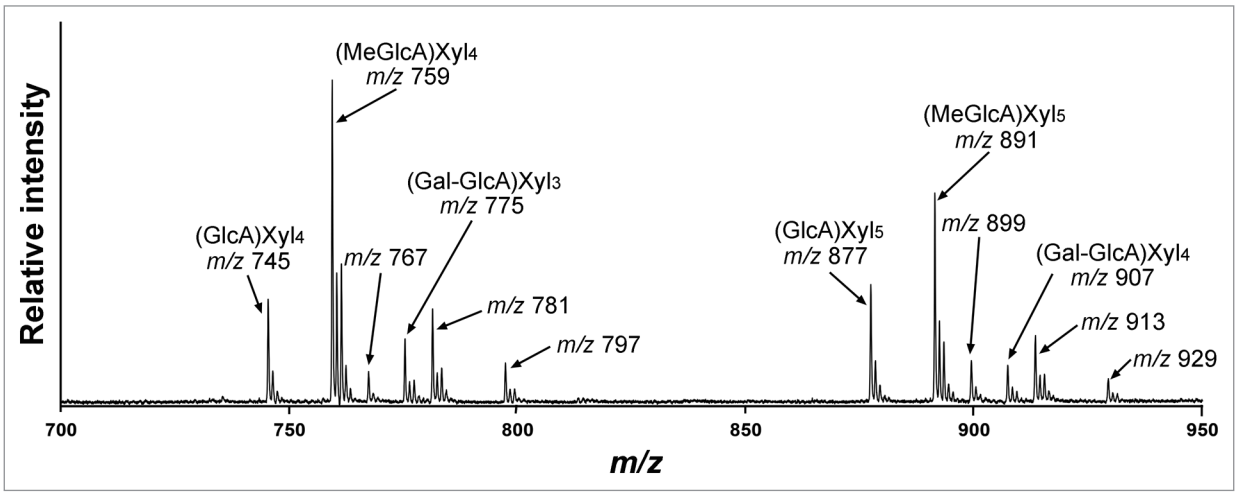

Figure 3. MALDI-TOF spectrum of xylooligomers generated by xylanase digestion of KOH-extracted *Arabidopsis* xylan. The ions $[M+Na]^+$ at *m/z* 745 and 759 are attributed to xylotetrasaccharides bearing a GlcA residue [$(GlcA)Xyl_4$] and a methylated GlcA residue [$(MeGlcA)Xyl_4$], respectively, and those at *m/z* 877 and 891 are attributed to xylopentasaccharides bearing a GlcA residue [$(GlcA)Xyl_5$] and a methylated GlcA residue [$(MeGlcA)Xyl_5$], respectively. Note the presence of ions at *m/z* 775 and 907 that correspond to the masses of xylotrisaccharides bearing Gal and GlcA residues [$(Gal-GlcA)Xyl_3$] and xylotetrasaccharides bearing Gal and GlcA residues [$(Gal-GlcA)Xyl_4$], respectively. The ions at *m/z* 767, 781, and 797 are attributed to the doubly sodiated species $[M+2Na]^+$ of $(GlcA)Xyl_4$, $(MeGlcA)Xyl_4$ and $(Gal-GlcA)Xyl_3$, respectively. The ions at *m/z* 899, 913, and 929 are attributed to the doubly sodiated species $[M+2Na]^+$ of $(GlcA)Xyl_5$, $(MeGlcA)Xyl_5$, and $(Gal-GlcA)Xyl_4$, respectively.

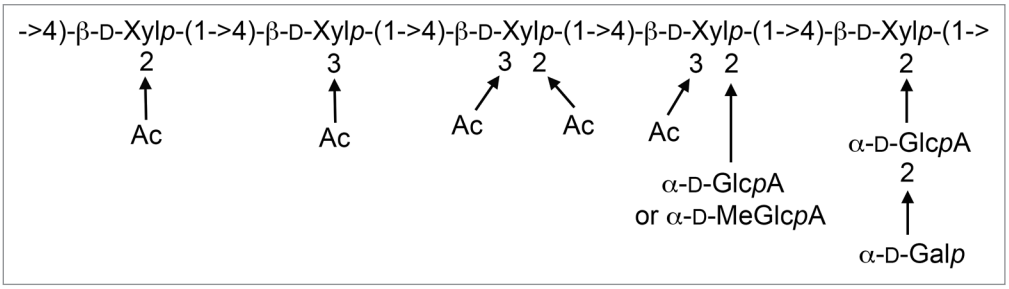

Figure 4. Diagram of *Arabidopsis* xylan structure with all known substituents. The reducing end tetrasaccharide sequence, β-D-Xyl-(1→3)-α-L-Rha-(1→2)-α-D-GalA-(1→4)-D-Xyl, is not shown.

of GlcA substituted with Gal and that MeGlcA substituted with Gal, if present, is a minor component.

In summary, our results from the 2D ^1H-^1H and ^1H-^{13}C NMR spectroscopy and the MALDI-TOF-MS provide unequivocal evidence demonstrating that *Arabidopsis* xylan contains Gal-GlcA disaccharide side chains in addition to substitutions with GlcA, MeGlcA and acetyl groups (**Fig. 4**). Since GlcA substituents are common structural units of xylans from different species, it will be interesting to investigate whether Gal-GlcA substitutions of xylan also occur commonly in various plant species. Further studies on the functional roles of the Gal-GlcA substitutions in xylan properties and identification of glycosyltransferases responsible for Gal-GlcA substitutions of xylan will enrich our understanding of the mechanisms controlling xylan biosynthesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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