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The *irregular xylem9* Mutant is Deficient in Xylan Xylosyltransferase Activity

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Xylan is the second most abundant polysaccharide in dicot wood, and thus elucidation of the xylan biosynthetic pathway is required to understand the mechanisms controlling wood formation. Genetic and chemical studies in *Arabidopsis* have implicated three genes, *FRAGILE FIBER8* (*FRA8*), *IRREGULAR XYLEM8* (*IRX8*) and *IRREGULAR XYLEM9* (*IRX9*), in the biosynthesis of glucuronoxylan (GX), but the biochemical functions of the encoded proteins are not known. In this study, we determined the effect of the *fra8*, *irx8* and *irx9* mutations on the activities of xylan xylosyltransferase (XylT) and glucuronyltransferase (GlcAT). We show that microsomes isolated from the stems of wild-type *Arabidopsis* exhibit XylT and GlcAT activities in the presence of exogenous 1,4-linked β-D-xylooligomers. Xylooligomers ranging in size from two to six can be used as acceptors by XylT to form xylooligosaccharides with up to 12 xylosyl residues. We provide evidence that the *irx9* mutation results in a substantial reduction in XylT activity but has no discernible effect on GlcAT activity. In contrast, neither XylT nor GlcAT activity is affected by *fra8* and *irx8* mutations. Our results provide biochemical evidence that the *irx9* mutation results in a deficiency in xylan XylT activity, thus leading to a defect in the elongation of the xylan backbone.

Keywords: *Arabidopsis* — *irregular xylem9* — Glucuronyltransferase — Xylan — Xylosyltransferase.

Abbreviations: AA, anthranilic acid; Csl, cellulose synthase-like; DTT, dithiothreitol; *fra8*, *fragile fiber8*; GlcA, glucuronic acid; GlcAT, glucuronyltransferase; GT, glycosyltransferase; GX, glucuronoxylan; *irx8*, *irregular xylem8*; *irx9*, *irregular xylem9*; MALDI-TOF MS, matrix-assisted laser desorption ionization-time-of-flight-mass spectrometry; MeGlcA, 4-O-methyl-glucuronic acid; Xyl, xylose; XylT, xylosyltransferase.

Introduction

Wood is of immense importance in human life as it is used for construction, pulping and paper-making, and has considerable value as a renewable source of biomass for biofuel production. Understanding the biosynthetic pathways that lead to the formation of wood will allow us to design strategies rationally to produce wood with improved properties by altering plant secondary cell wall composition.

Cellulose, xylan and lignin are the major components of dicot wood. Nevertheless, the biosynthetic pathway leading to the formation of xylan is poorly understood. Xylan is composed of a linear backbone of 1,4-linked β-D-xylosyl residues, of which about 10% are substituted with α-D-glucuronic acid (GlcA), 4-*O*-methyl-α-D-glucuronic acid (MeGlcA) and/or arabinose (Ebringerová and Heinze 2000). Xylans are classified based on the nature of the side chains: (methyl)glucuronoxylan (GX), arabinoxylan or glucuronoarabinoxylan. GX is the predominant xylan present in dicot wood, whereas arabinoxylan and glucuronoarabinoxylan are the most abundant hemicelluloses in grass cell walls (Ebringerová and Heinze 2000). GXs from birch, spruce and *Arabidopsis* have been shown to contain a distinct glycosyl sequence → 4)-β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-α-D-Gal*p*A-(1 → 4)-D-Xyl*p* at the reducing end (Shimizu et al. 1976, Johansson and Samuelson 1977, Anderson et al. 1983, Pena et al. 2007). Thus, GX biosynthesis is likely to involve glycosyltransferases responsible for the formation of the backbone and the reducing end sequence, as well as enzymes that add and modify side chains. The isolation and functional characterization of genes encoding xylan biosynthetic enzymes is a critical step toward increasing our understanding of xylan biosynthesis.

Numerous enzyme activities associated with xylan biosynthesis, including xylosyltransferase (XylT), glucuronosyltransferase (GlcAT), methyltransferase and arabinosyltransferase, have been described (Dalessandro and Northcote 1981, Baydoun et al. 1983, Baydoun et al. 1989, Suzuki et al. 1991, Porchia and Scheller 2000, Kuroyama and Tsumuraya 2001, Gregory et al. 2002, Porchia et al. 2002, Urahara et al. 2004). Microsomes isolated from wheat seedlings and barley endosperms contain XylT activity that adds up to five xylosyl residues to exogenous xylooligomer acceptors (Kuroyama and Tsumuraya 2001, Urahara et al. 2004). However, in other studies, the XylT was reported to transfer xylose to high

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molecular weight endogenous acceptors present in the microsomal preparations (Dalessandro and Northcote 1981, Baydoun et al. 1983, Suzuki et al. 1991, Porchia and Scheller 2000, Gregory et al. 2002). Gregory et al. (2002) partially purified a XylT activity-associated 50 kDa polypeptide from French bean. However, no secondary wall xylan biosynthetic enzymes have been purified to homogeneity and biochemically characterized, nor have their corresponding genes been identified.

Recent molecular and genetic studies have provided strong evidence that three *Arabidopsis* genes, *FRAGILE FIBER8* (*FRA8*), *IRREGULAR XYLEM8* (*IRX8*) and *IRX9* (Brown et al. 2005, Persson et al. 2005, Zhong et al. 2005, Bauer et al. 2006, Pena et al. 2007, Persson et al. 2007), as well as their respective poplar homologs, *PoGT47C*, *PoGT8D* and *PoGT43B* (Aspeborg et al. 2005, Zhou et al. 2006, Zhou et al. 2007), have roles in secondary cell wall GX biosynthesis. FRA8, IRX8 and IRX9 encode putative glycosyltransferases belonging to families GT47, GT8 and GT43, respectively. These genes are expressed in cells undergoing secondary wall thickening and their encoded proteins are localized in the Golgi (Zhong et al. 2005, Pena et al. 2007, Zhou et al. 2006, Zhou et al. 2007), the known site of GX biosynthesis. Mutations of the *FRA8*, *IRX8* and *IRX9* genes cause a substantial reduction in the GX content of secondary walls. Structural analysis of the GX in the *fra8* and *irx8* mutants revealed a decrease in the number of GX chains and a decrease in the amount of GlcA residues, together with the near absence of the GalA-containing glycosyl sequence at the GX reducing end. These results suggest that FRA8 and IRX8 are involved in the biosynthesis of the glycosyl sequence at the reducing end of GX or in the addition of GlcA side chains. In contrast, the *irx9* mutation leads to an increase in the number of GX chains, but a substantial reduction in GX chain length as well as in the number of GlcA side chains. These results have led to the suggestion that IRX9 is a xylan synthase (Pena et al. 2007). However, no XylT activity has been detected using recombinant IRX9 (Pena et al. 2007). Thus, the possibility cannot be discounted that the shortened GX chemotype in *irx9* may result from a defect in the addition of side chains, thereby resulting in reduced GX chain elongation (Pena et al. 2007). Indeed, previous studies have led to the suggestion that GX biosynthesis requires the cooperative actions of XylT and GlcAT (Baydoun et al. 1983).

In this report, we show that microsomes from wild-type *Arabidopsis* stems exhibit XylT and GlcAT activities in the presence of exogenous xylooligomers. We present biochemical data demonstrating that the *irx9* mutation results in a substantial reduction in XylT activity but has no discernible effect on GlcAT activity. The *fra8* and *irx8* mutations have no discernible effects on the activities of XylT and GlcAT. Our data are consistent with the notion that IRX9 is the XylT that is responsible for the normal elongation of the GX backbone. This is the first report showing a deficiency of a xylan biosynthetic enzyme in mutants defective in xylan biosynthesis.

Results

Biochemical properties of XylT activity in microsomes of wild-type stems

To investigate the effects of the *fra8*, *irx8* and *irx9* mutations on XylT and GlcAT activities, we first established conditions for the assay of these enzymes and for studying their biochemical properties. We found XylT activity in microsome preparations from wild-type *Arabidopsis* inflorescence stems in which a large amount of xylan is synthesized. XylT activity was dependent on the presence of exogenous Xyl_6. No XylT activity was detected without the addition of exogenous acceptors. The transfer of radiolabeled Xyl from UDP-[^{14}C]Xyl to Xyl_6 was dependent on the Xyl_6 concentration (Fig. 1A) with an apparent K_m value of 10.6 mM and V_{max} of 21.8 pmol min^{-1} mg^{-1} protein. The XylT activity was both time and protein concentration dependent (Fig. 1B, C). The optimal pH for the XylT activity was around 6.5 (Fig. 1D) and its optimal temperature about 10–20°C (Fig. 1E). UDP-GlcA stimulated the XylT activity up to 30% (Fig. 1F), probably because the addition of GlcA side chains by the GlcAT activity may increase the solubility of the elongated xylooligomers, which could further be used as acceptors for the XylT activity.

It has been shown that microsomes isolated from wheat and barley can use xylooligomers with different lengths as acceptors (Kuroyama and Tsumuraya 2001, Urahara et al. 2004). We next investigated the effectiveness of xylooligomers with different lengths as acceptors for the XylT activity (Fig. 2). The monomer Xyl_1- anthranilic acid (AA) was not an acceptor for Xyl transfer (Fig. 2B). A low level of Xyl transfer was obtained using Xyl_2-AA as an acceptor (Fig. 2C). The XylT activity readily transferred Xyl residues to acceptors ranging from Xyl_3 to Xyl_6 (Fig. 2D, F, G, H), resulting in the synthesis of xylooligosaccharides with a size up to Xyl_{12}.

To find out whether a longer reaction time can lead to the production of longer xylooligosaccharides by the XylTase activity, we performed a time course study using Xyl_4-AA as acceptors. Up to seven xylosyl residues were added to Xyl_4-AA with a 2 h incubation, leading to the production of a population of xylooligosaccharides ranging from Xyl_5 to Xyl_{11} (Fig. 3A). Prolonged incubation (5–8 h) did not result in the formation of xylooligosaccharides longer than Xyl_{12} but did cause a gradual increase in the proportions of Xyl_9–Xyl_{11} (Fig. 3A–C).

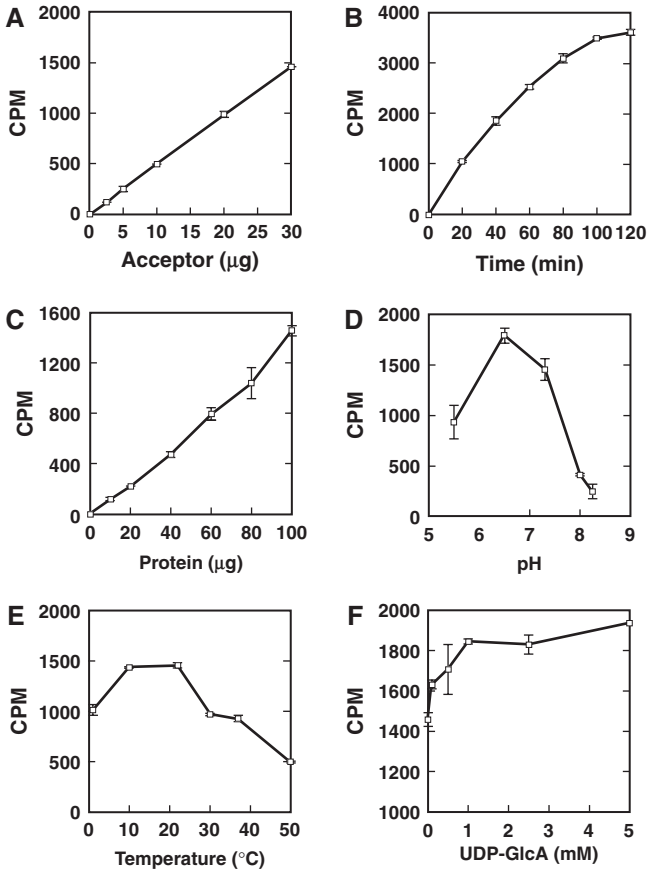

Fig. 1 Biochemical properties of the XylT activity in microsomes from *Arabidopsis* stems. Microsomes were incubated with UDP-[^{14}C]Xyl and Xyl_6 for 20 min unless otherwise indicated, and the XylT activity was measured by counting the radioactivity (c.p.m.) present in the reaction products. All assays were repeated twice and the data are means ± SE. (A) Dependence of the XylT activity on the concentration of Xyl_6 acceptor. (B) Time course of the transfer of radiolabeled Xyl onto the Xyl_6 acceptor by the XylT activity. Note that the XylT activity is linear in the first 40 min and reached a maximum after 100 min incubation under the assay conditions used. (C) The XylT activity is protein concentration-dependent. (D) Effect of pH on the XylT activity. (E) Effect of temperature on the XylT activity. (F) Effect of UDP-GlcA on the XylT activity.

We used enzymatic treatment and matrix-assisted laser desorption ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS) analyses to confirm that the reaction products formed by reacting Xyl_4-AA and UDP-Xyl with microsomes were 1,4-linked β-D-xylooligosacchrides. MALDI-TOF-MS gave masses (*m*/*z* 822, 954, 1,086, 1,218, 1,350, 1,482 and 1,614) for products that differed by 132 Da (Fig. 4). A series of oligosaccharides that differ by an incremental mass of 132 is consistent with the sequential addition of pentosyl residues to Xyl_4-AA (Fig. 4). Endo-β-(1,4)-xylanase and exo-β-(1,4)-xylosidase treatment of the XylT-generated products resulted in hydrolysis of the Xyl_n-AA oligomers to di- or tri-xylooligomer (Fig. 5). These results confirm that the XylT-generated products were composed of 1,4-linked β-D-xylosyl residues.

Biochemical properties of GlcAT activity in microsomes of wild-type stems

We found that microsomes isolated from *Arabidopsis* inflorescence stems also exhibited GlcAT activity in the presence of an exogenous 1,4-β-D-xylohexose (Xyl_6). The transfer of radiolabeled GlcA from UDP-[^{14}C]GlcA to Xyl_6 was shown to be dependent on the Xyl_6 concentration (Fig. 6A), with an apparent K_m value of 10.8 mM and V_{max} of 114.6 pmol min^{-1} mg^{-1} protein. The GlcATase activity was time and protein concentration dependent (Fig. 6B, C). The optimal pH for GlcAT activity was about 6.5 (Fig. 6D), with substantial activity over a temperature range of 10–37°C (Fig. 6E). Addition of UDP-Xyl in the reaction mixture did not stimulate GlcAT activity; instead high concentrations (0.5–5 mM) of UDP-Xyl inhibited its activity (Fig. 6F).

GlcAT and XylT activities in the fra8, irx8 and irx9 mutants

We next investigated the effects of the *fra8*, *irx8* and *irx9* mutations on XylT and GlcAT activities. Inflorescence stems were again used to determine the relative enzyme activities in wild-type and mutant plants as these tissues have the highest XylT and GlcAT activities (Fig. 7). Because FRA8, IRX8 and IRX9 proteins were previously shown to be targeted to the Golgi (Zhong et al. 2005, Pena et al. 2007) and the microsomes we prepared were enriched with the activity of inosine diphosphatase (Lait and Zwiazek 2001), a Golgi marker enzyme (data not shown), we reasoned that FRA8, IRX8 and IRX9 proteins were present in microsome preparations and therefore it was appropriate to use microsomal preparations to study the effects of the *fra8*, *irx8* and *irx9* mutations on XylT and GlcAT activities. GlcAT activity was somewhat elevated in the microsomal preparations of the three mutants compared with the wild type (Fig. 8A). XylT activity was also elevated in the *fra8* and *irx8* mutants. The elevation of the GlcAT and XylT activities could be due to a compensatory effect caused by the mutations. However, XylT activity was substantially reduced in the *irx9* mutant compared with the wild type (Fig. 8B). A time course analysis of the transfer of radiolabeled xylosyl residues from UDP-[^{14}C]Xyl onto the xylooligomer acceptor demonstrated that much less label was incorporated by *irx9* microsomes than by wild-type microsomes (Fig. 8C), a result that is consistent with IRX9 being involved with xylan synthesis. XylT activity in *irx9* plants was returned to near-normal levels by complementation of the mutant with the wild-type *IRX9* gene or its poplar homolog, the *PoGT47B* gene (Fig. 8D). Taken together, these results provide strong evidence that IRX9

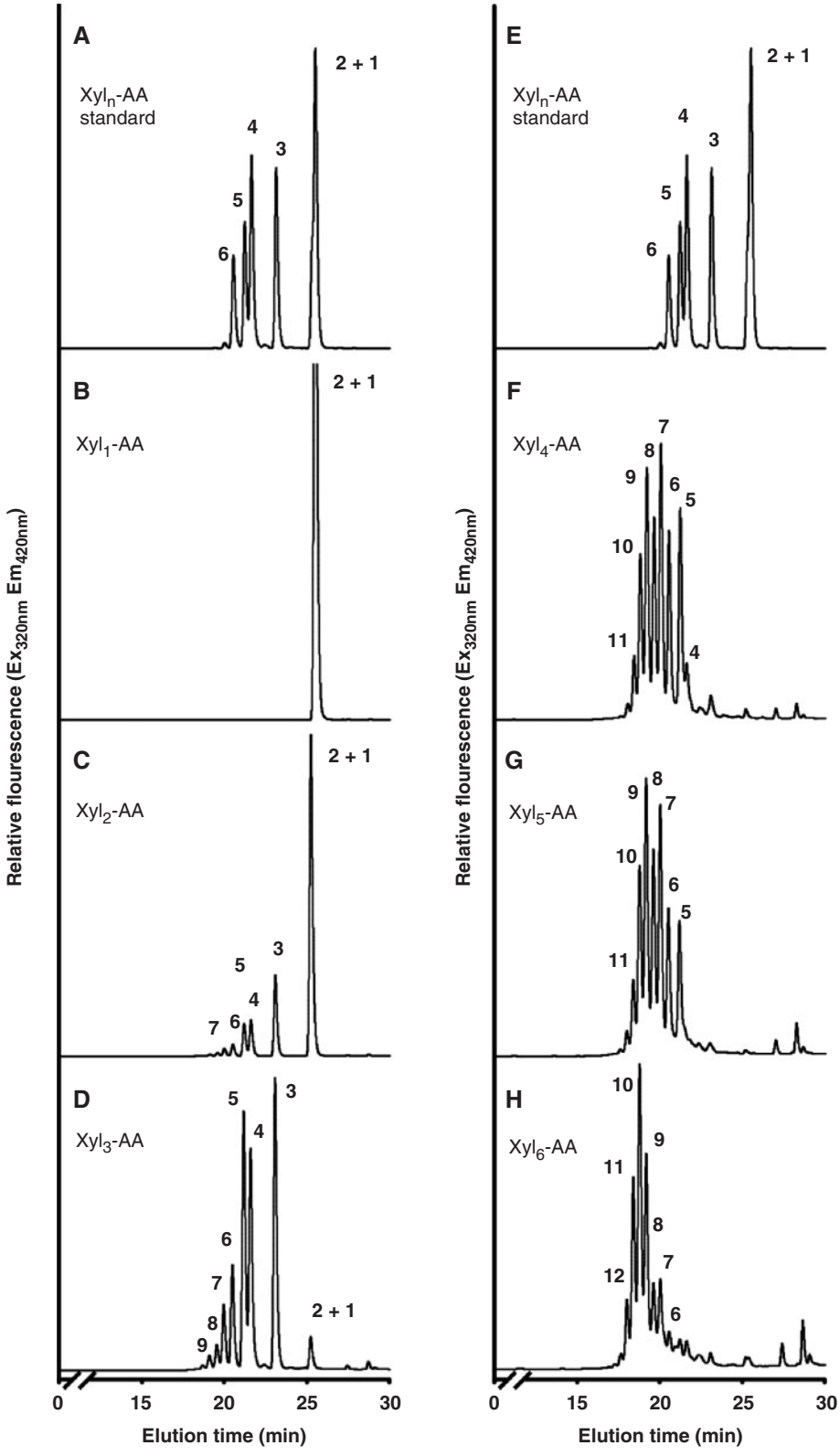

Fig. 2 Xylooligomers of different length as acceptors for the XylT activity. Microsomes were incubated with UDP-Xyl and the fluorescent acceptors Xyl$_1$-AA (B), Xyl$_2$-AA (C), Xyl$_3$-AA (D), Xyl$_4$-AA (F), Xyl$_5$-AA (G) or Xyl$_6$-AA (H) for 5 h, and the reaction products were analyzed by reverse-phase HPLC for separation and detection of the fluorescent xylooligosaccharides. A chromatogram of standard Xyl$_1$-AA to Xyl$_6$-AA is shown in (A) and (E) for reference. The XylT activity can transfer xylosyl residues onto xylooligomers as short as xylobiose.

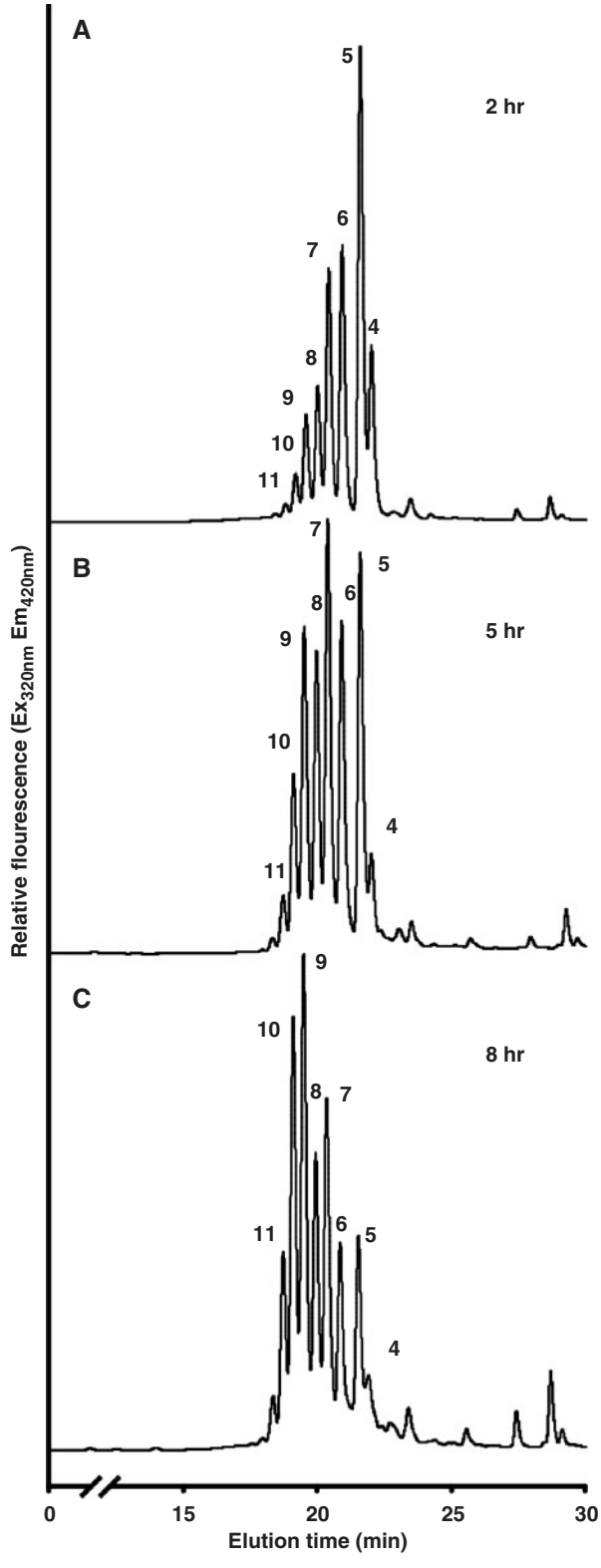

Fig. 3 Successive transfer of xylosyl residues onto the acceptors by the XylT activity. Microsomes were incubated with UDP-Xyl and the fluorescent Xyl_4-AA acceptor for 2 h (A), 5 h (B) and 8 h (C), and the reaction products were analyzed by reverse-phase HPLC. The XylT activity is able to elongate Xyl_4 up to Xyl_{12} successively.

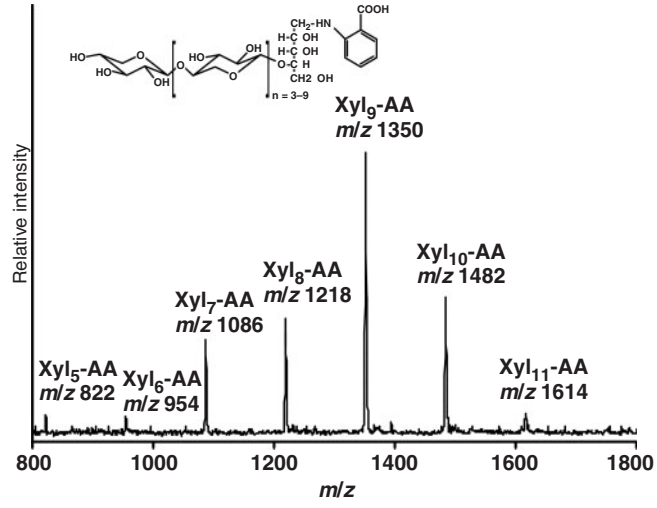

Fig. 4 MALDI TOF mass spectrum of the XylT-catalyzed reaction products. Microsomes were incubated with UDP-Xyl and the acceptor Xyl_4-AA, and the reaction products were purified and analyzed by MALDI-TOF-MS. The inset shows the chemical structure of Xyl_n-AA. A series of ions with a mass increment of 132 Da corresponding to one xylosyl residue was observed in the spectrum. These ions correspond to AA-labeled oligosaccharides $[Xyl_n\text{-AA} + Na]^+$ composed of 5–11 Xyl residues.

has a role in the synthesis of the xylan backbone of secondary wall GX.

To investigate in greater detail the effect of the *irx9* mutation on the elongation of xylooligosaccharides, we compared the number of xylosyl residues added onto the Xyl_6-AA acceptor by wild-type and *irx9* microsomes. With wild-type microsomes, Xyl_6-AA was elongated within 2 h to a mixture of xylooligosaccharides ranging from Xyl_7-AA to Xyl_{12}-AA, with Xyl_7-AA to Xyl_9-AA predominating (Fig. 9A). After 5 h of reaction time, the peaks of Xyl_{10}-AA to Xyl_{12}-AA were elevated, whereas those of Xyl_6-AA to Xyl_8-AA were diminished (Fig. 9B). In contrast, in the *irx9* mutant, Xyl_6-AA was only elongated to a mixture of Xyl_7-AA to Xyl_9-AA, and, even after 5 h of reaction time Xyl_7-AA remained the predominant peak (Fig. 9C, D). These results indicate that the *irx9* mutation reduces the XylT activity, thereby causing a substantial decrease in the transfer of xylosyl residues onto the xylooligomers.

Discussion

It was previously proposed that cellulose synthase-like (Csl) genes might encode enzymes for the biosynthesis of hemicelluloses (Richmond and Somerville 2001). Indeed, members of Csl gene families CslC, CslA and CslF have been shown to synthesize the backbones of xyloglucan (Cocuron et al. 2007), glucomannan (Dhugga et al. 2004, Liepman et al. 2005), and (1,3;1,4)-β-D-glucan (Burton et al. 2006), respectively. However, gene(s) involved in the

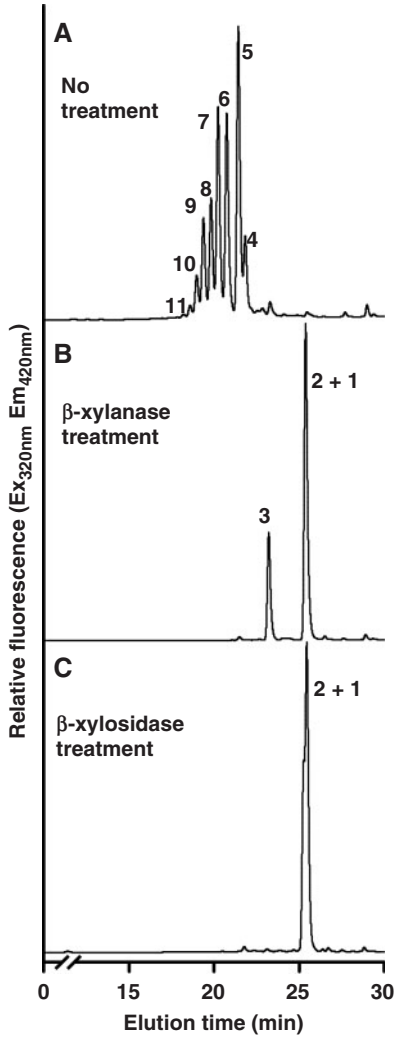

Fig. 5 Degradation of the XylT-catalyzed reaction products by endo-β-(1,4)-xylanase and exo-β-(1,4)-xylosidase. Microsomes were incubated with UDP-Xyl and the fluorescent Xyl_4-AA acceptor, and the reaction products were digested with endo-β-(1,4)-xylanase (B) or exo-β-(1,4)-xylosidase (C). The reaction products with (B, C) and without (A) enzymatic digestion were analyzed by reverse-phase HPLC. Treatment of the reaction products with β-(1,4)-xylanase and exo β-(1,4)-xylosidase results in their degradation into Xyl_1–Xyl_3, indicating that the reaction products have β-(1,4)-linkage.

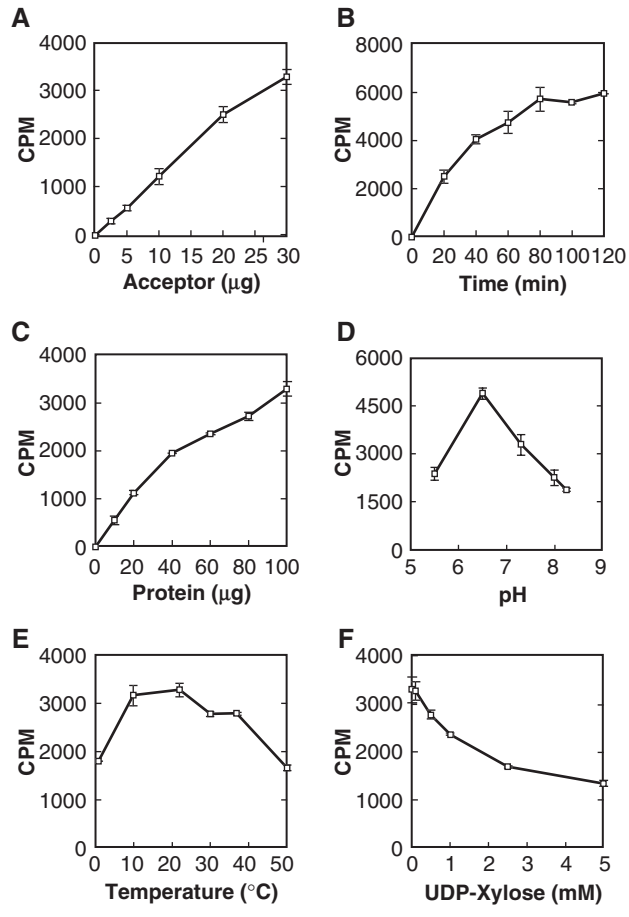

Fig. 6 Biochemical properties of the GlcAT activity in microsomes from *Arabidopsis* stems. Microsomes were incubated with UDP-[^{14}C]GlcA and Xyl_6 for 20 min unless otherwise indicated, and the GlcAT activity was measured by counting the radioactivity (c.p.m.) of the reaction products. All assays were repeated twice and the data are means ± SE. (A) Dependence of the GlcAT activity on the concentration of Xyl_6 acceptor. (B) Time course of the transfer of radiolabeled GlcA onto the Xyl_6 acceptor by the GlcAT activity. Note that the GlcAT activity is linear in the first 30 min and reached a maximum after 80 min incubation under the assay conditions used. (C) The GlcAT activity is protein concentration dependent. (D) Effect of pH on the GlcAT activity. (E) Effect of temperature on the GlcAT activity. (F) Effect of UDP-Xyl on the GlcAT activity.

biosynthesis of the xylan backbone have remained elusive (Lerouxel et al. 2006). No recombinant Csl proteins have been shown to have XylT activity (Liepman et al. 2005), nor has transcription profiling analysis revealed correlations between xylan biosynthesis and the expression of Csl genes, with the exception of CslA, during wood formation (Aspeborg et al. 2005). Our demonstration that IRX9 is required for normal XylT activity as well as normal elongation of xylan chains provides the first biochemical evidence that a glycosyltransferase other than a Csl protein is involved in the biosynthesis of the xylan backbone.

The irx9 mutation causes a deficiency in the transfer of xylosyl residues onto xylooligomer acceptors

The *irx9* mutation causes a number of changes in GX structure, including a decreased ratio of GlcA to MeGlcA residues, an increased number of GX chains and a substantial reduction in GX chain length (Pena et al. 2007). The findings that the GX chain number increases and its length decreases in the *irx9* mutant suggest that the primary effect of the *irx9* mutation may be on the elongation of GX chains rather than on the initiation of GX biosynthesis. However, the *irx9* mutation also decreases

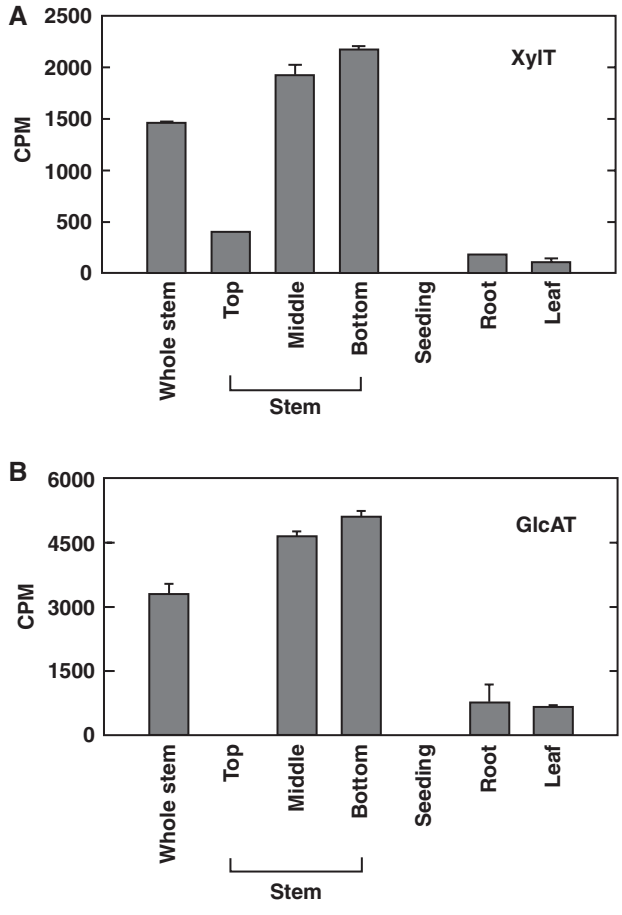

Fig. 7 The XylT and GlcAT activities in different *Arabidopsis* organs. Microsomes isolated from stems, seedlings, roots and leaves were incubated with Xyl_6 and UDP-[^{14}C]Xyl or UDP-[^{14}C]GlcA for 20 min, and the XylT and GlcAT activity was measured by counting the radioactivity (c.p.m.) of the reaction products. All assays were repeated twice and the data are means ± SE. The middle and bottom parts of stems exhibited the highest XylT and GlcAT activities.

the ratio of GlcA to MeGlcA residues on the GX. Thus, it is equally possible that IRX9 is involved in the transfer of GlcA residues on the GX, and that a defect in GlcA transfer may itself lead to an attenuation of GX chain elongation (Pena et al. 2007). Our biochemical data showed that the *irx9* mutation has no discernible effect on GlcAT activity, thereby limiting the possibility that IRX9 is required for GlcAT activity. Instead, our study demonstrated that the *irx9* mutation causes a reduction in XylT activity. The decreased XylT activity in the *irx9* mutant results in a reduced ability to transfer xylosyl residues onto xylooligomer acceptors, leading to the formation of much shorter xylooligosaccharides than wild-type plants (Fig. 9). This finding is consistent with the chemical analysis showing that the degree of polymerization of GX is reduced from 93 in the wild type to 28 in the *irx9* mutant (Pena et al. 2007). Thus, our study provides compelling evidence that the decreased GX chain length caused by the *irx9* mutation is due to a deficiency in XylT activity.

It is important to note that the *irx9* mutation does not completely eliminate the XylT activity. Because the T-DNA insertion (SALK_058238) in the *irx9* mutant disrupts an *IRX9* exon that encodes part of the putative catalytic domain, *irx9* is presumably a null mutant. Therefore, the low level of XylT activity remaining in the *irx9* mutant is probably contributed by its functional homolog(s), which may also result in a limited elongation of xylan backbone.

The fra8 and irx8 mutations do not affect GlcAT and XylT activities

A previous study demonstrated that *fra8* and *irx8* mutants synthesize GX that is nearly devoid of the reducing end glycosyl sequence → 4)-β-D-Xyl*p*-(1 → 4)-β-D-Xyl*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-α-D-Gal*p*A-(1 → 4)-D-Xyl*p*, indicating that FRA8 and IRX8 are involved in the biosynthesis of this sequence. However, the *fra8* and *irx8* mutations also cause a reduction in both the number of GX chains and the ratio of GlcA to MeGlcA residues in the GX (Zhong et al. 2005, Pena et al. 2007, Persson et al. 2007). Therefore, the results of genetic and chemical analyses do not exclude the possibility that FRA8 and IRX8 are involved in the transfer of GlcA residues onto the xylan backbone. Our biochemical data provide evidence that the *fra8* and *irx8* mutations do not result in a reduction in XylT or GlcAT activities, which suggests that FRA8 and IRX8 have no direct roles in the transfer of GlcA residues onto the xylan backbone or in the elongation of the xylan backbone by addition of xylosyl residues. The results of our study support the proposal that the FRA8 and IRX8 glycosyltransferases are involved in the biosynthesis of the glycosyl sequence at the GX reducing end (Pena et al., 2007).

The XylT and GlcAT activities in Arabidopsis microsomes require exogenous xylooligomers as acceptors

Xylan XylT activities have been identified in sycamore, poplar, pea, wheat, barley, zinnia and French bean (Dalessandro and Northcote 1981, Baydoun et al. 1983, Suzuki et al. 1991, Porchia and Scheller 2000, Kuroyama and Tsumuraya 2001, Gregory et al. 2002). In most cases, the XylT activity was detected without the addition of exogenous acceptors, suggesting that these XylTs use microsome-associated endogenous acceptors or that these enzymes do not require an acceptor. Indeed, a detergent-solubilized, and partially purified XylT fraction has been reported to have XylT activity in the absence of exogenous acceptors (Gregory et al. 2002). However, exogenous xylooligomer acceptors are required for XylT activities in the microsomes of asparagus (Feingold et al. 1959), wheat (Kuroyama and Tsumuraya 2001) and barley (Urahara et al. 2004). It is not known whether the discrepancy

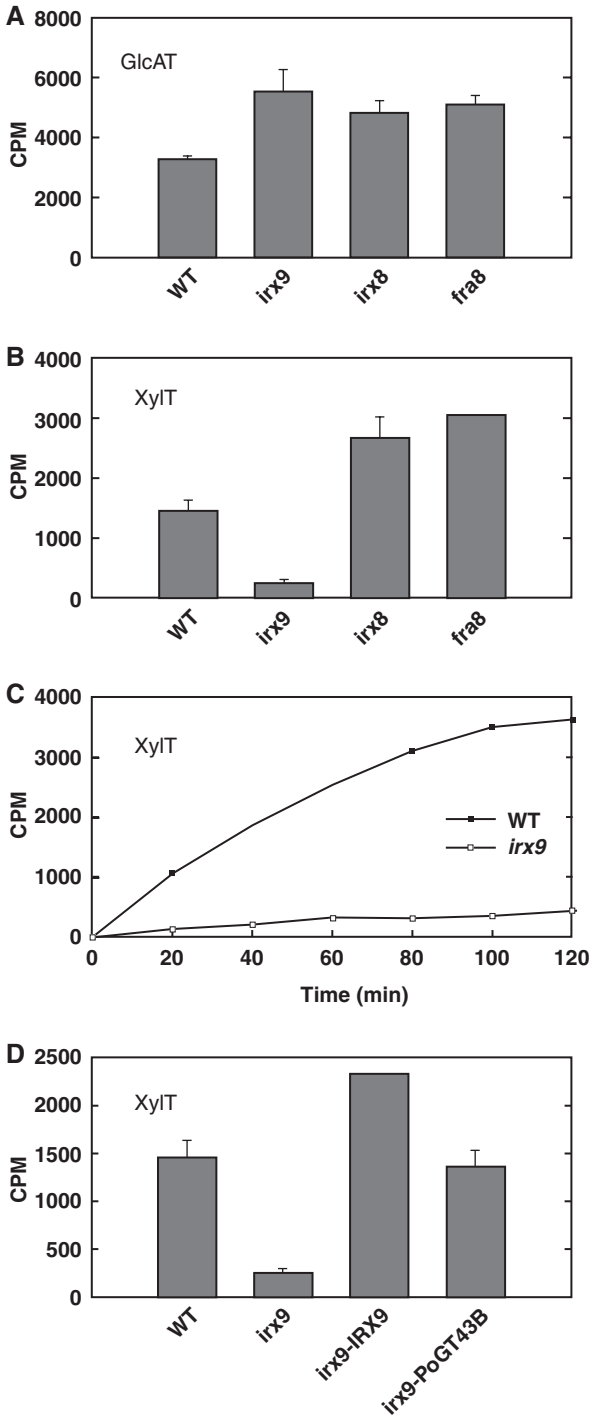

Fig. 8 The XylT and GlcAT activities in the stems of wild type, *irx9, irx8* and *fra8*. Microsomes isolated from the wild type and mutants were incubated with Xyl_6 and UDP-[^{14}C]GlcA or UDP-[^{14}C]Xyl for 20 min unless otherwise indicated, and the GlcAT and XylT activities were measured by counting the radioactivity (c.p.m.) of the reaction products. All assays were repeated twice and the data are means ± SE. (A) GlcAT activity in the *irx9, irx8* and *fra8* mutants compared with the wild type. (B) XylT activity in the *irx9, irx8* and *fra8* mutants compared with the wild type. Note that the XylT activity was drastically reduced in the *irx9* mutant but slightly increased in the *irx8* and *fra8* mutants. (C) Time course of the XylT activity in the wild type and the *irx9* mutant. The XylT activity in *irx9* remained low even after 2 h incubation. (D) Complementation of *irx9* with the *Arabidopsis* IRX9 or its poplar homolog PoGT43B restored the XylT activity.

regarding the requirement for exogenous acceptors is due to the difference in microsome preparations or the nature of XylTs from different species. In any case, it is clear that with *Arabidopsis* microsomes, exogenous xylooligomers are required for the XylT activity and that a xylooligomer as short as xylotriose or xylotetraose is sufficient to be an acceptor for the transfer of xylosyl residues.

The GlcAT activity in the *Arabidopsis* microsomes was also found to require exogenous xylooligomers as an acceptor, suggesting the presence of a GlcAT that transfers GlcA onto pre-formed xylooligomers. No GlcA was transferred in the absence of an exogenous xylooligomer acceptor, indicating that the microsome preparations contain little if any endogenous acceptors for GlcAT. The addition of exogenous UDP-Xyl to the reaction mixture did not increase the amount of GlcA incorporated. Because the Xyl_6 acceptor used is elongated to Xyl_{12} by the XylT activity, this indicates that no additional GlcA residues are transferred to the elongated xylooligomers by the GlcAT activity. This is consistent with the reported normal distribution of GlcA residues along the xylan backbone, which is typically about one GlcA residue for every 10 xylosyl residues (Ebringerová and Heinze 2000). Our finding that the GlcAT activity can transfer GlcA onto pre-formed xylooligomers is in contrast to the previous report showing that pea epicotyl microsomes can only add GlcA onto nascent xylan but not onto pre-formed xylan (Baydoun et al. 1989). This discrepancy may be due to our use of xylooligomers rather than newly synthesized xylan polysaccharides as acceptors.

In summary, our study demonstrates that IRX9 is required for the normal XylT activity. Previous attempts to demonstrate XylT activity using recombinant IRX9 protein have been unsuccessful (Pena et al. 2007). Thus, it is essential to design strategies to characterize IRX9 further biochemically. A complete description of the biochemical properties of IRX9 together with IRX8, FRA8 and other yet to be identified proteins involved in xylan biosynthesis will undoubtedly lead to a better understanding of the biosynthetic pathway of xylan, the second most abundant polysaccharide produced by woody plants.

Materials and Methods

Plant materials

Wild-type *Arabidopsis* (ecotype Columbia), *fra8* (Zhong et al., 2005), *irx8* (SALK_008642), *irx9* (SALK_058238), *irx9*-complemented with wild-type IRX9 (Pena et al. 2007) and

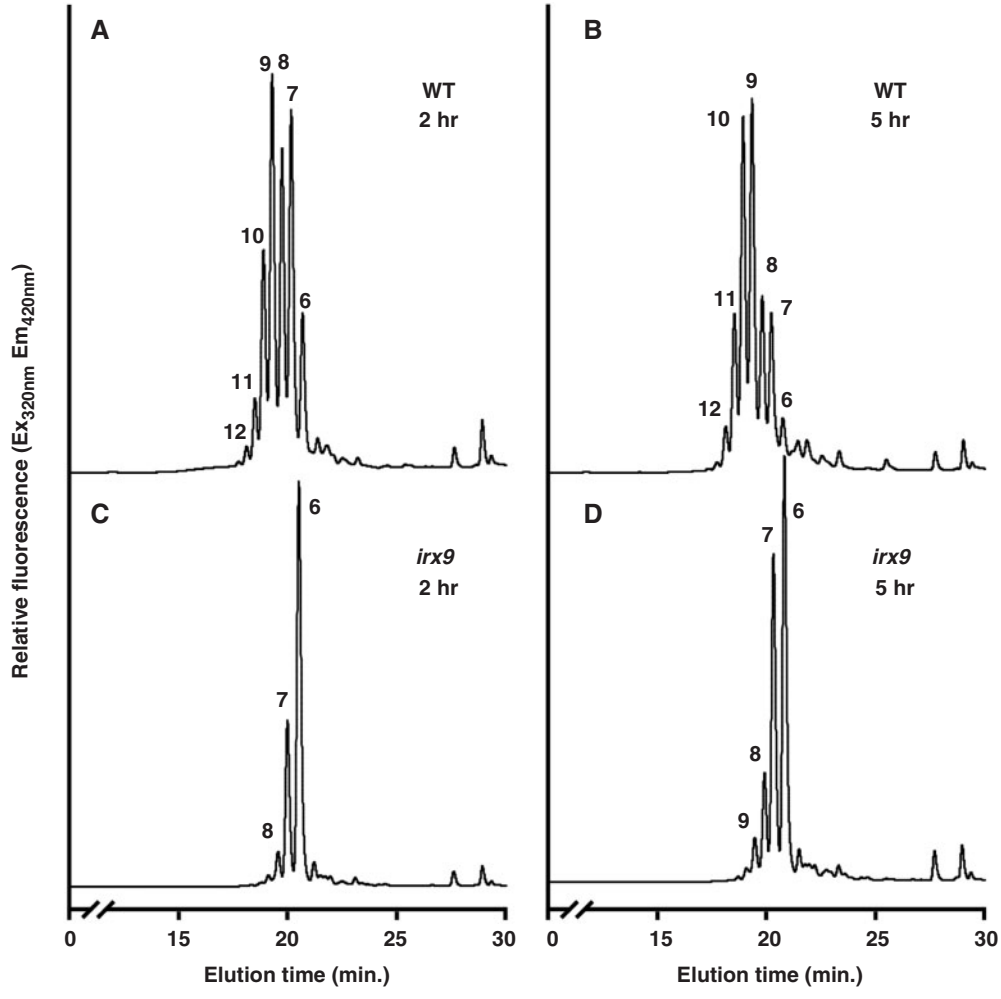

Fig. 9 Deficiency in the transfer of xylosyl residues onto xylooligomer acceptors in the *irx9* mutant. Microsomes from the wild type (A, B) and *irx9* mutant (C, D) were incubated with UDP-Xyl and the fluorescent Xyl_6-AA acceptor for 2 h (A, C) and 5 h (B, D), and the reaction products were analyzed by reverse-phase HPLC. Note that the XylT activity in the wild type transferred up to six xylosyl residues onto the acceptors, whereas only up to three xylosyl residues were transferred by the XylT activity in *irx9*.

irx9-compelemented with PoGT43B (Zhou et al. 2007) plants were grown in a greenhouse. Inflorescence stems, roots and leaves used for microsome isolation were collected from 6-week-old plants, and seedlings used for microsome isolation were 2 weeks old.

Enzymes and chemicals

Endo-β-xylanase M6 and xylooligomers (Xyl_2–Xyl_6) were purchased from Megazyme (Wicklow, Ireland). Xylose, UDP-GlcA, anthranilic acid and β-xylosidase were from Sigma (St Louis, MO, USA). UDP-Xyl was from CarboSource Service (Athens, GA, USA; supported in part by NSF-RCN grant #0090281). UDP-[^{14}C]Xyl (specific activity, 264 mCi mmol^{-1}) and UDP-[^{14}C]GlcA (specific activity, 300 mCi mmol^{-1}) were from American Radiolabeled Chemical (St Louis, MO, USA). Toyopearl HW-40 was from Tosoh Bioscience (Montgomeryville, PA, USA).

Anthranilic acid labeling of xylooligomers

Xylose and xylooligomers (Xyl_2–Xyl_6) were labeled at their reducing termini with AA according to Ishii et al. (2002). Briefly, oligosaccharides were reacted for 2 h at 65°C with 0.2 M AA in 20 mM sodium acetate, pH 5.5, containing 1 M $NaBH_3CN$. The reaction was terminated by the addition of 10 mM ammonium acetate (pH 7.0), and the AA-labeled oligosaccharides were separated from AA and $NaBH_3CN$ by chromatography on a column (1.5 × 100 cm) of Toyopearl HW-40F and eluted with 10 mM ammonium acetate (pH 7.0). The $A_{254\,nm}$ of the eluant was monitored and the UV-positive fractions containing the AA-labeled xylose, and xylooligomers were pooled and lyophilized.

Assay of GlcAT and XylT activities by radiolabeling

Microsomes were isolated and the GlcAT and XylT activities were determined following the procedures of Kuroyama and Tsumuraya (2001). Briefly, plant materials were homogenized with a mortar and pestle in grinding solution (50 mM HEPES-NaOH, pH 7.3, 0.4 M sucrose, 10 μg ml^{-1} aprotinin and 10 μg ml^{-1} leupeptin). After centrifugation at 3,000 × *g* for 15 min to remove debris, the supernatant was further centrifuged at 100,000 × *g* for 1 h. The pellets containing the microsomes were

re-suspended in the buffer containing 50 mM HEPES-NaOH (pH 7.3) and 0.4 M sucrose, and stored at −80°C. The protein concentration of the microsomal fraction was measured using the BioRad protein assay kit with bovine serum albumin (BSA) as the standard protein.

For assay of GlcAT activity, 100 μg of microsomes were incubated with the reaction mixture (a total volume of 30 μl) containing 50 mM HEPES-KOH, pH 6.8, 5 mM $MnCl_2$, 1 mM dithiothritol (DTT), 0.5% Triton X-100, 0.2 μg μl^{-1} Xyl_6 and UDP-[^{14}C]GlcA (0.1 μCi). After incubation at 21°C for various times, the reaction was stopped by addition of the termination solution (0.3 M acetic acid containing 20 mM EGTA).

For assay of XylT activity, 100 μg of microsomes were incubated with the reaction mixture (a total volume of 30 μl) containing 50 mM HEPES-KOH, pH 6.8, 5 mM $MnCl_2$, 1 mM DTT, 0.5% Triton X-100, 0.1 mM cold UDP-Xyl, 0.2 μg μl^{-1} Xyl_6 and UDP-[^{14}C]Xyl (0.1 μCi). After incubation at 21°C for various times, the reaction was stopped by addition of the termination solution.

The radiolabeled xylooligosaccharides were separated from UDP-[^{14}C]Xyl by paper chromatography according to Ishikawa et al. (2000). The terminated reaction mixture was spotted onto a strip of Whatmann 3 MM paper and developed with 95% ethanol : 1 M ammonium acetate (2 : 1, v/v) as the solvent. The radiolabeled xylooligosaccharides are retained at the original spot, whereas UDP-[^{14}C]Xyl moved along with the solvent. The spot containing the radiolabeled xylooligosaccharides was cut out, sonicated in 0.1 N NaOH, and the amount of radioactivity present was determined with a PerkinElmer scintillation counter (Waltham, MA, USA).

The activity of inosine diphosphatase in microsomes was determined according to Lait and Zwiazek (2001).

Assay of XylT activity using Xyl_n-AA as acceptors and HPLC analysis of Xyl_n-AA

Assay of XylT activity with Xyl_n-AA acceptors was determined in a reaction mixture (a total volume of 30 μl) containing 50 mM HEPES-KOH, pH 6.8, 5 mM $MnCl_2$, 1 mM DTT, 0.5% Triton X-100, 0.1 mM cold UDP-Xyl, 0.5 mM Xyl_n-AA and 200 μg of microsomes. After incubation at 21°C for various times, the reaction was terminated with 0.1 M acetic acid, and filtered with an Amicon Ultrafree-MC filter (0.22 μm). The products formed were analyzed by reversed-phase HPLC analysis. HPLC was performed with an Agilent 1100 series LC system and a Shimadzu RF-10A_{XL} fluorescence detector ($Ex_{320\,nm}$, $Em_{420\,nm}$). The incorporated Xyl_n-AA products were separated using a Luna C18 (5 μm) column (250 mm long, 4.6 mm i.d.). The column was eluted at 0.5 ml min^{-1} with aqueous 80% methanol containing 0.02% (v/v) trifluoroacetic acid (0–5 min) followed by a gradient to 100% methanol over 45 min.

Treatment of xylooligosaccharides with β-xylanase and β-xylosidase

The XylT activity reaction using Xyl_n-AA acceptors as described above was terminated by heating at 100°C for 3 min. The reaction mixture (a total volume of 30 μl) was centrifuged at 12,000 × *g* for 5 min and the supernatant was saved for treatment with β-xylanase and β-xylosidase. The supernatant (10 μl) was mixed with 50 mM sodium acetate, pH 6.0 (30 μl) containing β-xylanase (4.5 U) or β-xylosidase (0.005 U), and incubated for 6 h at 37°C. The reaction was terminated with 0.1 M acetic acid, and subjected to reverse-phase HPLC analysis.

MALDI-TOF-MS

The AA-labeled xylooligosaccharides from the XylT-catalyzed reactions were analyzed by MALDI-TOF-MS using a Hewlett-Packard LDI 1700 XP spectrometer operated in the positive-ion mode with an accelerating voltage of 30 kV, an extractor voltage of 9 kV and a source pressure of approximately 8×10^{-7} torr. The enzyme reaction mixture was mixed with an equal volume of water (200 μl) and then applied to a Strata C-18 solid phase extraction cartridge (3 ml, Phenomenex) that had been pre-washed with methanol and then with water. The cartridge was eluted with water (5 ml) and then with 60% methanol (5 ml) to elute the Xyl_n-AA oligomers. The solution was concentrated to dryness under a flow of air, and the residue dissolved in water (100 μl). A portion (1 μl) of the solution was mixed with the MALDI matrix (1 μl, 0.1 M 2,5-dihydroxbenzoic acid and 0.03 M 1-hydroxyisoquinoline in 50% acetonitrile) and dried on the stainless steel target plate. Spectra are the average of at least 100 laser shots.

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References

Andersson, S.-I., Samuelson, O., Ishihara, M. and Shimizu, K. (1983) Structure of the reducing end-groups in spruce xylan. *Carbohydr. Res.* 111: 283–288.

Aspeborg, H., Schrader, J., Coutinho, P.M., Stam, M., Kallas, A., et al. (2005) Carbohydrate-active enzymes involved in the secondary cell wall biogenesis in hybrid aspen. *Plant Physiol.* 137: 983–997.

Bauer, S., Vasu, P., Persson, S., Mort, A.J. and Somerville, C.R. (2006) Development and application of a suite of polysaccharide-degrading enzymes for analyzing plant cell walls. *Proc. Natl Acad. Sci. USA* 103: 11417–11422.

Baydoun, E.A.-H., Usta, J.A.-R., Waldron, K.W. and Brett, C.T. (1989) A methyltransferase involved in the biosynthesis of 4-*O*-methylglucuronoxylan in etiolated pea epicotyls. *J. Plant Physiol.* 135: 81–85.

Baydoun, E.A.-H., Waldron, K.W. and Brett, C.T. (1983) The interaction of xylosyltransferase and glucuronyltransferase involved in glucuronoxylan synthesis in pea (*Pisum sativum*) epicotyls. *Biochem. J.* 257: 853–858.

Brown, D.M., Zeef, L.A.H., Ellis, J., Goodacreb, R. and Turner, S.R. (2005) Identification of novel genes in *Arabidopsis* involved in secondary cell wall formation using expression profiling and reverse genetics. *Plant Cell* 17: 2281–2295.

Burton, R.A., Wilson, S.M., Hrmova, M., Harvey, A.J., Shirley, N.J., Medhurst, A., Stone, B.A., Newbigin, E.J., Bacic, A. and Fincher, G.B. (2006) Cellulose synthase-like CslF genes mediate the synthesis of cell wall (1,3;1,4)-β-d-glucans. *Science* 311: 1940–1942.

Cocuron, J.C., Lerouxel, O., Drakakaki, G., Alonso, A.P., Liepman, A.H., Keegstra, K., Raikhel, N. and Wilkerson, C.G. (2007) A gene from the cellulose synthase-like C family encodes a β-1,4 glucan synthase. *Proc. Natl Acad. Sci. USA* 104: 8550–8555.

Dalessandro, G. and Northcote, D.H. (1981) Increase of xylan synthetase activity during xylem differentiation of the vascular cambium of sycamore and poplar trees. *Planta* 151: 61–67.

Dhugga, K.S., Barreiro, R., Whitten, B., Stecca, K., Hazebroek, J., Randhawa, G.S., Dolan, M., Kinney, A.J., Tomes, D., Nichols, S. and Anderson, P. (2004) Guar seed β-mannan synthase is a member of the cellulose synthase super gene family. *Science* 303: 363–366.

Ebringerová, A. and Heinze, T. (2000) Xylan and xylan derivatives—biopolymers with valuable properties, 1. Naturally occurring xylans structures, isolation procedures and properties. *Macromol. Rapid Commun*. 21: 542–556.

Feingold, D.S., Neufeld, E.F. and Hassid, W.Z. (1957) Xylosol transfer catalyzed by an asparagus extract. *J. Biol. Chem*. 234: 488–489.

Gregory, A.C.E., Smith, C., Kerry, M.E., Wheatley, E.R. and Bolwell, G.P. (2002) Comparative subcellular immunolocation of polypeptides associated with xylan and callose synthases in French bean (*Phaseolus vulgaris*) during secondary wall formation. *Phytochemistry* 59: 249–259.

Ishii, T., Ichita, J., Matsue, H., Ono, H. and Maeda, I. (2002) Fluorescent labeling of pectic oligosaccharides with 2-aminobenzamide and enzyme assay for pectin. *Carbohydr. Res*. 337: 1023–1032.

Ishikawa, M., Kuroyama, H., Takeuchi, Y. and Tsumuraya, Y. (2000) Characterization of pectin methyltransferase from soybean hypocotyls. *Planta* 210: 782–791.

Johansson, M.H. and Samuelson, O. (1977) Reducing end groups in birch xylan and their alkaline degradation. *Wood Sci. Technol*. 11: 251–263.

Kuroyama, H. and Tsumuraya, Y. (2001) A xylosyltransferase that synthesizes β-(1 → 4)-xylans in wheat (*Triticum aestivum* L.) seedlings. *Planta* 213: 231–240.

Lait, C.G. and Zwiazek, J.J. (2001) A calcium-binding protein with similarity to serum albumin localized to the ER–Golgi network and cell walls of spinach (*Spinacia oleracea*). *Physiol. Plant* 112: 460–469.

Lerouxel, O., Cavalier, D.M., Liepman, A.H. and Keegstra, K. (2006) Biosynthesis of plant cell wall polysaccharides—a complex process. *Curr. Opin. Plant Biol*. 9: 621–630.

Liepman, A.H., Wilkerson, C.G. and Keegstra, K. (2005) Expression of cellulose synthase-like (*Csl*) genes in insect cells reveals that *CslA* family members encode mannan synthases. *Proc. Natl Acad. Sci. USA* 102: 2221–2226.

Pena, M.J., Zhong, R., Zhou, G.-K., Richardson, E.A., O'Neill, M.A., Darvill, A.G., York, W.S. and Ye, Z.-H. (2007) *Arabidopsis irregular xylem8* and *irregular xylem9*: implications for the complexity of glucuronoxylan biosynthesis. *Plant Cell* 19: 549–563.

Persson, S., Caffall, K.H., Freshour, G., Hilley, M.T., Bauer, S., Poindexter, P., Hahn, M.G., Mohnen, D. and Somerville, C. (2007) The *Arabidopsis irregular xylem8* mutant is deficient in glucuronoxylan and homogalacturonan, which are essential for secondary cell wall integrity. *Plant Cell* 19: 237–255.

Persson, S., Wei, H., Miline, J., Page, G.P. and Somerville, C.R. (2005) Identification of genes required for cellulose synthesis by repression analysis of public microarray data sets. *Proc. Natl Acad. Sci. USA* 102: 8633–8638.

Porchia, A.C. and Scheller, H.V. (2000) Arabinoxylan biosynthesis: identification and partial characterization of β-1,4-xylosyltransferase from wheat. *Physiol. Plant* 110: 350–356.

Porchia, A.C., Sorensen, S.O. and Scheller, H.V. (2002) Arabinoxylan biosynthesis in wheat. Characterization of arabinosyltransferase activity in Golgi membranes. *Plant Physiol*. 130: 432–441.

Richmond, T.A. and Somerville, C.R. (2001) Integrative approaches to determining Csl function. *Plant Mol. Biol*. 47: 131–143.

Shimizu, K., Ishihara, M. and Ishihara, T. (1976) Hemicellulases of brown rotting fungus, *Tyromyces palustris*. II. The oligosaccharides from the hydrolysate of a hardwood xylan by the intracellular xylanase. *Mokuzai Gaikkashi* 22: 618–625.

Suzuki, K., Ingold, E., Sugiyama, M. and Komamine, A. (1991) Xylan synthase activity in isolated mesophyll cells of *Zinnia elegans* during differentiation to tracheary elements. *Plant Cell Physiol*. 32: 303–306.

Urahara, T., Tsuchiya, K., Kotake, T., Tohno-oka, T., Komae, K., Kawada, N. and Tsumuraya, Y. (2004) A β-(1 → 4)-xylosyltransferase involved in the synthesis of arabinoxylans in developing barley endosperms. *Physiol. Plant* 122: 169–180.

Zhong, R., Pena, M.J., Zhou, G.-K., Nairn, C.J., Wood-Jones, A., Richardson, E.A., Morrison, W.H., Darvill, A.G., York, W.S. and Ye, Z.-H. (2005) *Arabidopsis Fragile Fiber8*, which encodes a putative glucuronyltransferase, is essential for normal secondary wall synthesis. *Plant Cell* 17: 3390–3408.

Zhou, G.K., Zhong, R., Richardson, E.A., Himmelsbach, D.S., McPhail, B.T. and Ye, Z.-H. (2007) . Molecular characterization of PoGT8D and PoGT43B, two secondary wall-associated glycosyltransferases in poplar. *Plant Cell Physiol*. 48: 689–699.

Zhou, G.K., Zhong, R., Richardson, E.A., Morrison, W.H.3rd, Nairn, C.J., Wood-Jones, A. and Ye, Z.-H. (2006) The poplar glycosyltransferase GT47C is functionally conserved with *Arabidopsis Fragile Fiber8*. *Plant Cell Physiol*. 47: 1229–1240.

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