PCP
PLANT & CELL PHYSIOLOGY

Arabidopsis GUX Proteins Are Glucuronyltransferases Responsible for the Addition of Glucuronic Acid Side Chains onto Xylan

Chanhui Lee[1], Quincy Teng[2], Ruiqin Zhong[1] and Zheng-Hua Ye[1,*]

[1]Department of Plant Biology, University of Georgia, Athens, GA 30602, USA
[2]National Exposure Research Laboratory, US Environmental Protection Agency, Athens, GA 30605, USA
*Corresponding author: E-mail, zhye@plantbio.uga.edu; Fax, +1-706-542-1805.
(Received February 3, 2012; Accepted April 18, 2012)

Regular Paper

Xylan, the second most abundant cell wall polysaccharide, is composed of a linear backbone of β-(1,4)-linked xylosyl residues that are often substituted with sugar side chains, such as glucuronic acid (GlcA) and methylglucuronic acid (MeGlcA). It has recently been shown that mutations of two Arabidopsis family GT8 genes, *GUX1* and *GUX2*, affect the addition of GlcA and MeGlcA to xylan, but it is not known whether they encode glucuronyltransferases (GlcATs) or indirectly regulate the GlcAT activity. In this study, we performed biochemical and genetic analyses of three Arabidopsis *GUX* genes to determine their roles in the GlcA substitution of xylan and secondary wall deposition. The *GUX1/2/3* genes were found to be expressed in interfascicular fibers and xylem cells, the two major types of secondary wall-containing cells that have abundant xylan. When expressed in tobacco BY2 cells, the GUX1/2/3 proteins exhibited an activity capable of transferring GlcA residues from the UDP-GlcA donor onto xylooligomer acceptors, demonstrating that these GUX proteins possess xylan GlcAT activity. Analyses of the single, double and triple *gux* mutants revealed that simultaneous mutations of all three *GUX* genes led to a complete loss of GlcA and MeGlcA side chains on xylan, indicating that all three GUX proteins are involved in the GlcA substitution of xylan. Furthermore, a complete loss of GlcA and MeGlcA side chains in the *gux1/2/3* triple mutant resulted in reduced secondary wall thickening, collapsed vessel morphology and reduced plant growth. Together, our results provide biochemical and genetic evidence that GUX1/2/3 are GlcATs responsible for the GlcA substitution of xylan, which is essential for normal secondary wall deposition and plant development.

Keywords: Arabidopsis • GlcA • Glucuronyltransferase • Secondary wall • Xylan • Xylan biosynthesis.

Abbreviations: *fra8*, *fragile fiber8*; GalA, galacturonic acid; GlcA, glucuronic acid; GlcAT, glucuronosyltransferase; GT, glycosyltransferase; GUS, β-glucuronidase; GUX, glucuronic acid substitution of xylan; *irx*, *irregular xylem*; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; MeGlcA, 4-*O*-methyl-glucuronic acid; NMR, nuclear magnetic resonance; RT–PCR, reverse transcription–PCR; Rha, rhamnose; RWA, reduced wall acetylation; SND1, secondary wall-associated NAC domain protein1; Xyl, xylose; Xyl_6, xylohexaose

Introduction

Xylan, a linear chain of β-(1,4)-linked xylosyl residues, is the second most abundant cell wall polysaccharide found in vascular plants. Although it is mainly deposited in the secondary walls of dicots and gymnosperms, in grass species it is found in both primary walls and secondary walls. Xylan is often substituted with side chains of α-(1,2)-linked glucuronic acid (GlcA) and/or methylglucuronic acid (MeGlcA), and may also be highly acetylated at C-2 and/or C-3 (Timell 1967). Xylan from grass species contains additional α-(1,2)- and α-(1,3)-linked arabinosyl side chains, which may be cross-linked with each other or with lignin by ferulate (Ishii 1997). Furthermore, the reducing end of xylan from dicots and gymnosperms has been found to include a unique tetrasaccharide sequence, β-D-Xyl*p*-(1→3)-α-L-Rha*p*-(1→2)-α-D-Gal*p*A-(1→4)-D-Xyl*p* (Shimizu et al. 1976, Johansson and Samuelson 1977, Andersson et al. 1983, Peña et al. 2007, Lee et al. 2009a). Study of xylan structure and biosynthesis has recently gained increasing interest due to the potential use of plant cellulosic biomass for biofuel production. The presence of xylan in cellulosic biomass has been shown to hinder the efficiency of conversion of biomass into bioethanol. For example, xylan, which is interwoven with cellulose, is believed to block the accessibility of cellulose to cellulase that is used to generate fermentable sugars (Himmel et al. 2007), and the acetyl groups released from xylan during biomass pre-treatment may inhibit microorganisms used to ferment sugars (Helle et al. 2003). Therefore, it is critical to identify and characterize all genes responsible for xylan biosynthesis in order to design strategies for genetic modification of biomass wall composition that is better tailored for biofuel production.

Plant Cell Physiol. 53(7): 1204–1216 (2012) doi:10.1093/pcp/pcs064, available FREE online at www.pcp.oxfordjournals.org

Editor-in-Chief's choice

Genetic analysis of Arabidopsis cell wall mutants has led to the identification of a number of genes involved in xylan biosynthesis. They include members of GT43, GT47 and DUF597 implicated in the biosynthesis of the xylan backbone (Brown et al. 2007, Lee et al. 2007a, Peña et al. 2007, Brown et al. 2009, Wu et al. 2009, Lee et al. 2010, Wu et al. 2010, Brown et al. 2011, Jensen et al. 2011); members of GT47 and GT8 required for the biosynthesis of the xylan reducing end sequence (Zhong et al. 2005, Brown et al. 2007, Lee et al. 2007b, Peña et al. 2007, Persson et al. 2007, Lee et al. 2009b); the *RWA* genes important for xylan acetylation (Lee et al. 2011); and two GT8 members essential for the GlcA substitution of xylan (Mortimer et al. 2010). Although these genes are implicated in xylan biosynthesis in genetic studies, biochemical assignment of their enzymatic activities is essential for our understanding of their functions. Two recent biochemical studies have established that GT43 members are xylosyltransferases responsible for the successive transfer of xylosyl residues during the elongation of the xylan backbone (Lee et al. 2012a, Lee et al. 2012b). However, biochemical proof of the enzymatic activities of other xylan biosynthetic genes is still lacking.

The substitution of xylan with GlcA and MeGlcA has been shown to be affected by mutations of a number of genes, including *IRX8*, *IRX9*, *IRX14*, *FRA8*, *PARVUS* and *GUX* genes (Brown et al. 2007, Lee et al. 2007b, Pena et al. 2007, Mortimer et al. 2010). While mutations of most of these genes only change the ratio of MeGlcA to GlcA, mutations of two *GUX* genes (*GUX1* and *GUX2*) result in a substantial loss of both GlcA and MeGlcA side chains (Mortimer et al. 2010), indicating that these GUX proteins are required for the addition of the GlcA side chains. It is unknown whether the GUX proteins possess glucuronyltransferase (GlcAT) activity since attempts to detect GlcAT activity using heterologously expressed GUX proteins were unsuccessful (Mortimer et al. 2010). Alternatively, they may act indirectly to regulate GlcAT activity (Mortimer et al. 2010).

In this study, we performed biochemical and genetic analyses of three Arabidopsis *GUX* genes. We showed that when heterologously expressed in tobacco BY2 cells, the GUX1, GUX2 and GUX3 proteins exhibited GlcAT activites that are able to add GlcA residues onto the xylooligomer acceptor. We provided further genetic evidence revealing that simultaneous T-DNA knockouts of GUX1/2/3 caused a complete loss of GlcA and MeGlcA side chains on xylan, reduced secondary wall thickening and altered plant growth. Our results demonstrated that GUX1/2/3 are GlcATs responsible for the GlcA substitution of xylan and are essential for normal secondary wall deposition and plant development.

Results

The *GUX1/2/3* genes are differentially expressed in secondary wall-forming cells

During the course of the study of SND1-regulated secondary wall biosynthetic genes in Arabidopsis (Zhong et al. 2010), we identified two family GT8 genes, *GUX1* and *GUX2*, whose expression was regulated by SND1, a secondary wall NAC master switch (**Fig. 1C, D**). These two genes were recently shown to be required for xylan glucuronylation (Mortimer et al. 2010). In addition to *GUX1/2*, three additional GUX homologs, *GUX3/4/5*, are present in the Arabidopsis genome (**Fig. 1A**). Quantitative PCR analysis of the expression of the *GUX* genes revealed that *GUX1/2* were predominantly expressed in stems, whereas *GUX3* was expressed at a similar level in all organs examined (**Fig. 1E**). *GUX4* and *GUX5* were found to be preferentially expressed in roots and in leaves and flowers, respectively. Expression analysis using laser-microdissected cell types from Arabidopsis stems demonstrated that the *GUX1/2* genes were expressed in both interfascicular fibers and xylem cells, whereas the expression of *GUX3* was only observed in xylem cells (**Fig. 1B**). This differential gene expression pattern was further confirmed by β-glucuronidase (GUS) reporter gene expression analysis showing that the *GUX1/2* expression was high in both interfascicular fibers and xylem cells (**Fig. 2A, B**), whereas *GUX3* expression was evident only in xylem cells (**Fig. 2C**). Since *GUX1/2/3* are expressed in secondary wall-forming cells in stems, we focused on them for further biochemical and genetic analyses.

GUX1/2/3 exhibit xylan GlcAT activity

GUX1/2 were recently shown to be required for the addition of GlcA side chains onto xylan, but it is not clear whether they are directly or indirectly involved in this process (Mortimer et al. 2010). In addition, it is not known whether GUX3 plays any role in xylan biosynthesis. To investigate the biochemical activity of the *GUX* genes, we heterologously expressed them in tobacco BY2 cells. Tobacco BY2 cells are parenchyma cells whose walls lack immunodetectable xylan (Lee et al. 2012a), which presents an ideal system for heterologous expression and activity detection of xylan biosynthetic enzymes. This system was recently employed to demonstrate that Arabidopsis GT43 members possess xylosyltransferase activities that are able to transfer xylosyl residues successively onto xylooligomer acceptors (Lee et al. 2012a). To assay the possible GlcAT activities of GUX proteins, we generated transgenic tobacco cell lines with expression of GUX1 alone, GUX2 alone, GUX3 alone and GUX1/2 together (**Fig. 3A**). Microsomes isolated from the transgenic cell lines were assayed for GlcAT activity using xylohexoase (Xyl_6) as an exogenous acceptor and UDP-[^{14}C]GlcA as a donor. As a control, microsomes isolated from BY2 cells transformed with an empty vector were used. At least 60 independent transgenic cell lines were assayed for the GlcAT activity, and representative data from five cell lines for each construct are shown in **Fig. 3**. It was evident that expression of GUX1, GUX2 or GUX3 resulted in a transfer of radiolabeled GlcA residues onto the Xyl_6 acceptor compared with the control (**Fig. 3B**), indicating that they exhibit GlcAT activities. Co-expression of GUX1 and GUX2 did not result in a further elevation in the GlcAT activity compared with GUX1 or GUX2 alone (**Fig. 3B**).

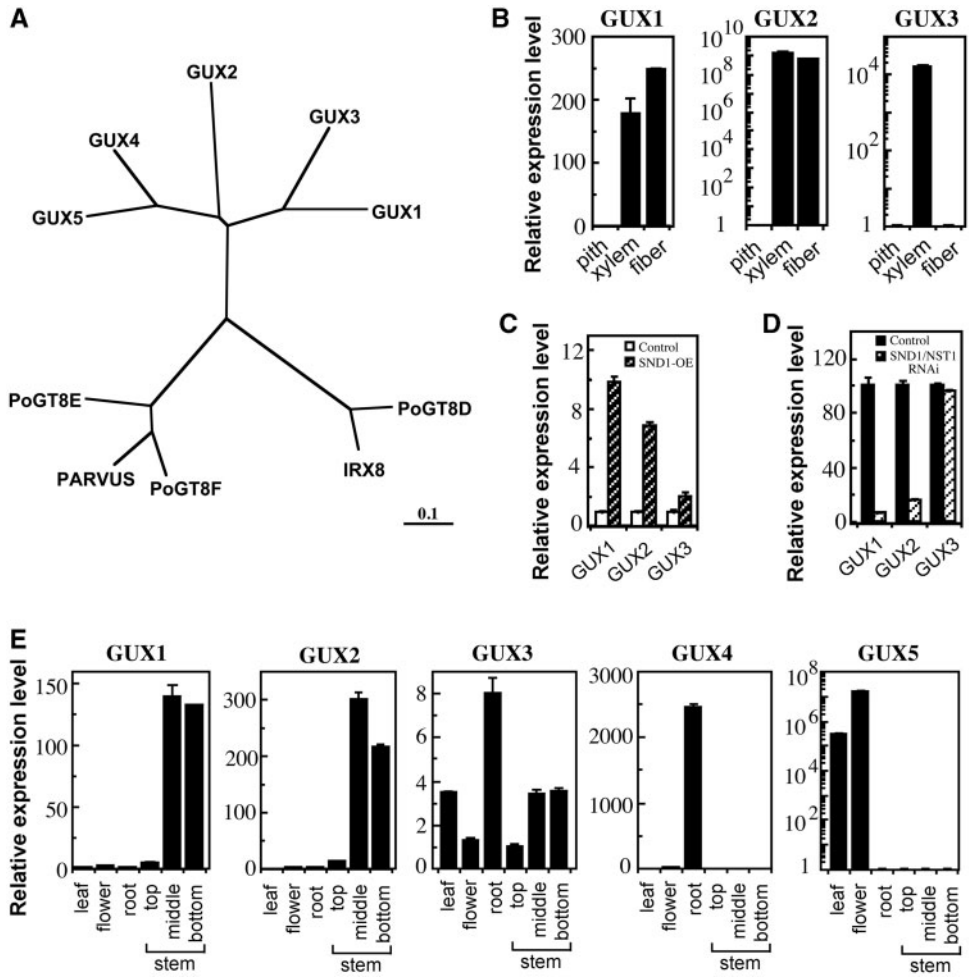

Fig. 1 Expression analysis of Arabidopsis *GUX* genes. (A) Phylogenetic relationship of family GT8 members that are known to be involved in xylan biosynthesis. The sequences of Arabidopsis GUXs, PARVUS and IRX8. and those of poplar PoGT8D, PoGT8E and PoGT8F were aligned using the ClustalW program (Thompson et al. 1994), and the phylogenetic tree was displayed using the TREEVIEW program (Page 1996). The 0.1 scale denotes 10% change. (B) Quantitative PCR analysis of the expression of *GUX* genes in pith, xylem and interfascicular fiber cells that were isolated from Arabidopsis stems. The expression level of each gene in pith cells was taken as 1. (C) and (D) Quantitative PCR analysis of the expression of *GUX* genes in the SND1 overexpressor (SND1-OE; C) and the SND1/NST1 RNA interference (RNAi) line (D). (E) Quantitative PCR analysis of the expression of *GUX* genes in different Arabidopsis organs. The lowest expression level of each gene among the organs analyzed was set to 1. Error bars in B–E denote the SE of three biological replicates.

The transgenic cell line that exhibited the highest GlcAT activity for each of the *GUX1/2/3* genes was chosen for further analysis. The GlcAT activities of microsomes from these lines were 40–50% of that of microsomes from Arabidopsis stems (**Fig. 3C**).

The GlcAT activity exhibited by GUX1/2/3 was further examined using different concentrations of the Xyl_6 acceptor, and was found to be dependent on Xyl_6 concentration (**Fig. 4**). The calculated K_m and V_{max} values are 4.7 mM and 35.8 pmol min^{-1} mg^{-1} protein, respectively, for GUX1 (**Fig. 4A**); 5.8 mM and 50.3 pmol min^{-1} mg^{-1} protein, respectively, for GUX2 (**Fig. 4B**); and 3.2 mM and 17.6 pmol min^{-1} mg^{-1} protein, respectively, for GUX3 (**Fig. 4C**). The K_m and V_{max} values exhibited by GUX1 and GUX2 are about half of the K_m (10.8 mM) and V_{max} (114.6 pmol min^{-1} mg^{-1} protein) values of the GlcAT activity displayed by wild-type Arabidopsis microsomes (Lee et al. 2007a).

To verify the transfer of GlcA residues onto the Xyl_6 acceptor in the reactions catalyzed by GUX1/2/3, we examined the reaction products using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS). It was found that in addition to the expected Xyl_6 peak (*m/z* 833), another signal peak (*m/z* 1009) was seen in the reaction products catalyzed by the microsomes of the transgenic cells expressing GUX1/2/3 (**Fig. 5**). This peak, which had an increase in the mass of 176 Da relative to Xyl_6, matches the expected mass of Xyl_6 substituted with one GlcA residue. As a positive control, we examined the reaction products catalyzed by microsomes from Arabidopsis stems. In addition to the expected peaks for the Xyl_6 acceptor and the GlcA-substituted

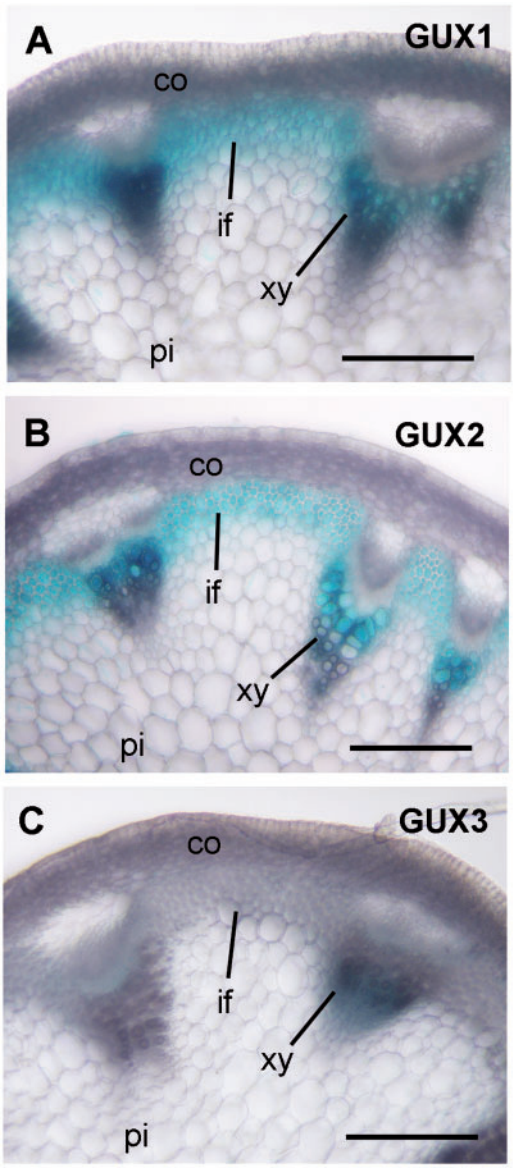

Fig. 2 Expression patterns of *GUX1*, *GUX2* and *GUX3* in Arabidopsis inflorescence stems. Inflorescence stems of transgenic Arabidopsis plants expressing the *GUX1* (A), *GUX2* (B) and *GUX3* (C) genes fused with the GUS reporter genes were sectioned and stained for GUS activity (shown as blue). co, cortex; if, interfascicular fiber; pi, pith; xy, xylem. Bars = 136 μm.

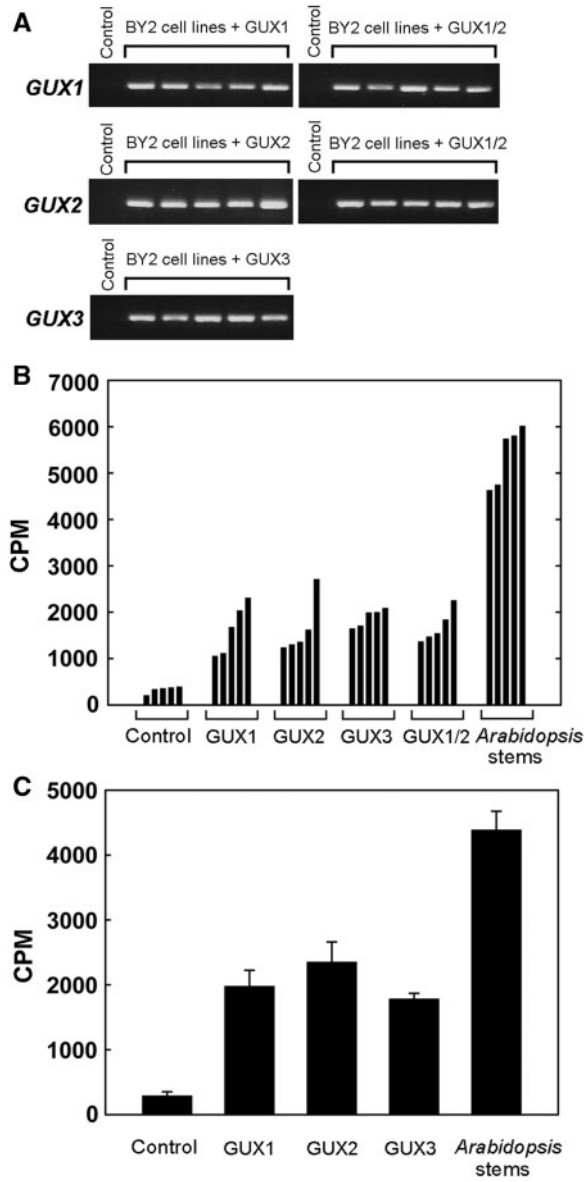

Fig. 3 Detection of GlcAT activity in transgenic tobacco BY2 cells expressing *GUX* genes. GlcAT activity was assayed by incubation of microsomes from GUX-expressing BY2 cells with UDP-[^{14}C]GlcA and Xyl_6, and subsequent detection of the incorporation of radiolabeled GlcA in the reaction products (CPM). (A) RT–PCR analysis showing the expression of Arabidopsis *GUX* genes in representative transgenic tobacco cell lines expressing *GUX1* alone, *GUX2* alone, *GUX3* alone or both *GUX1* and *GUX2* (*GUX1/2*). (B) GlcAT activity of representative transgenic BY2 cell lines expressing GUX1 alone, GUX2 alone, GUX3 alone or both GUX1 and GUX2 (GUX1/2). Note that microsomes from cell lines expressing GUXs exhibited a high level of GlcAT activity compared with those from cell lines transfected with the empty vector (control). The GlcAT activity from Arabidopsis stem microsomes is shown as a positive control. (C) Further confirmation of the GlcAT activity in the transgenic cell lines that exhibited the highest GlcAT activity for each *GUX* gene as shown in B. Error bars denote the SE of three independent assays.

Xyl_6, more signal peaks corresponding to Xyl_7 (*m*/*z* 965), Xyl_8 (*m*/*z* 1097) and the GlcA-substituted Xyl_7 (*m*/*z* 1141) were detected (**Fig. 5**). These additional oligosaccharide peaks are most probably attributed to the conversion of UDP-GlcA into UDP-Xyl by the endogenous UDP-GlcA decarboxylase (Harper and Bar-Peled 2002) and the subsequent addition of Xyl and GlcA residues onto the Xyl_6 acceptor. Taken together, these results provide direct biochemical evidence demonstrating that GUX1/2/3 are GlcATs that catalyze the transfer of GlcA residues onto the xylooligomer acceptor.

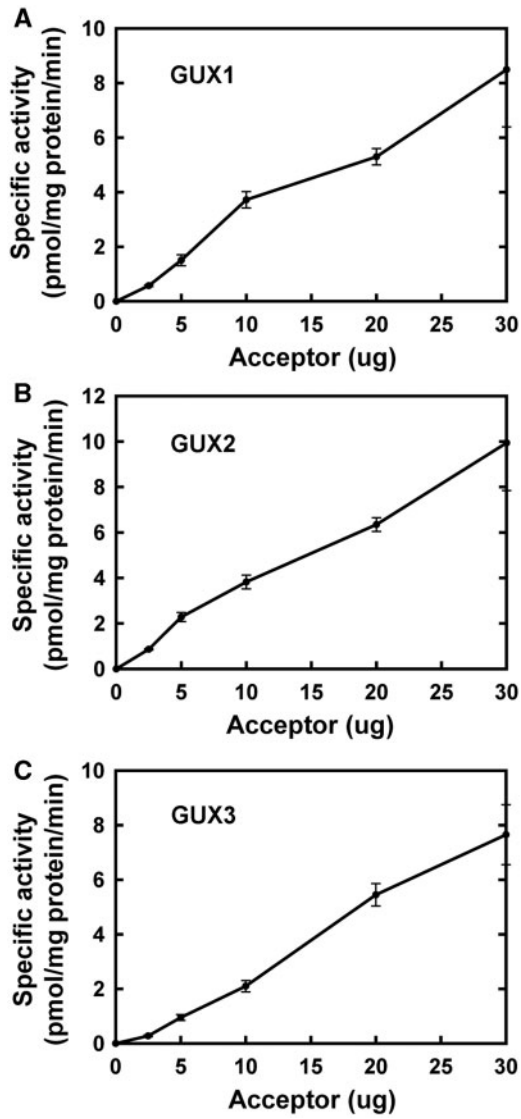

Fig. 4 Effect of the concentration of the Xyl_6 acceptor on the incorporation of radiolabeled GlcA catalyzed by microsomes of transgenic BY2 cells expressing GUX1 (A), GUX2 (B) and GUX3 (C).

Simultaneous mutations of *GUX1/2/3* lead to a complete loss of GlcA side chains on xylan

A previous report showed that simultaneous mutations of *GUX1/2* resulted in a partial loss of GlcA residues on xylan (Mortimer et al. 2010), indicating that either *GUX1/2* mutations are not null or additional GUX homologs are involved in the GlcA substitution of xylan. Because *GUX3* is expressed in the xylem and exhibits GlcAT activity, we hypothesized that in addition to GUX1/2, GUX3 is also involved in the addition of GlcA side chains on xylan. To test this hypothesis, we used the T-DNA insertion lines of *GUX1* (SALK_046841), *GUX2* (N469285) and *GUX3* (SALK_009971) to generate double and triple *gux* mutants. The inflorescence stems of the mutant plants were used for cell wall preparation and xylan extraction.

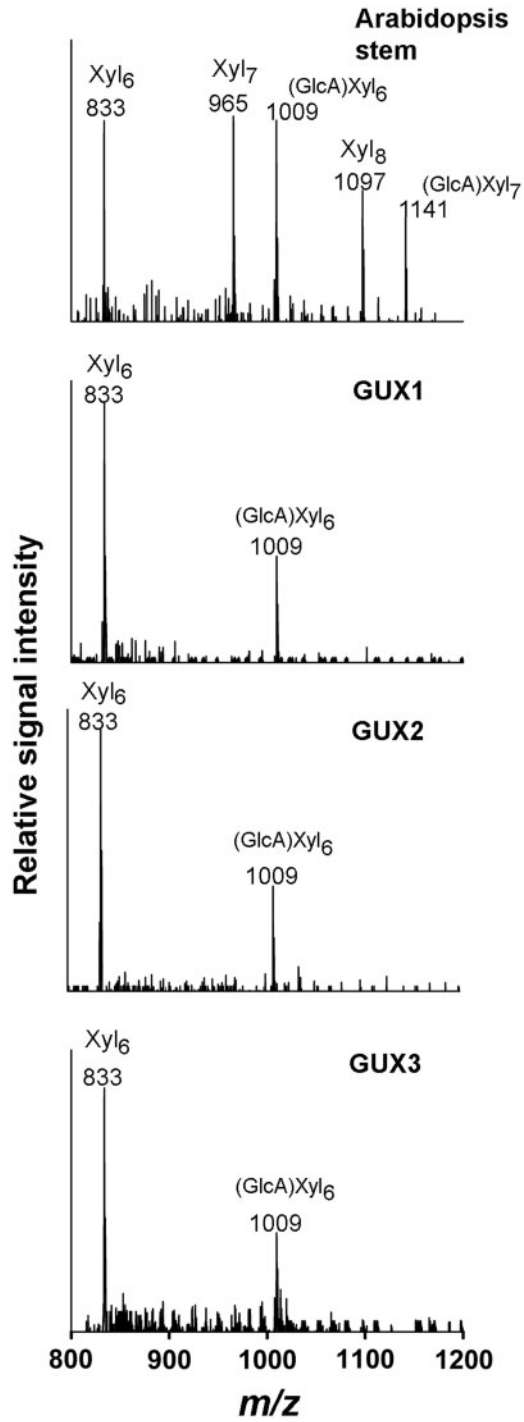

Fig. 5 MALDI-TOF mass spectra of the reaction products catalyzed by microsomes from Arabidopsis stems and tobacco BY2 cells expressing GUX1, GUX2 or GUX3. Microsomes were incubated with UDP-GlcA and the Xyl_6 acceptor, and the reaction products were analyzed by MALDI-TOF-MS. In addition to the ion corresponding to Xyl_6 (*m/z* 833), another ion (*m/z* 1009) with a mass increase of 176 Da corresponding to one GlcA residue was observed in the spectra of BY2 cells expressing *GUX* genes. In the reaction products catalyzed by Arabidopsis stem microsomes, three additional ions with *m/z* 965, 1,097 and 1,141, which correspond to Xyl_7, Xyl_8 and Xyl_7 substituted with one GlcA residue, respectively, were observed.

Quantitative measurement of GlcA and MeGlcA in total cell walls showed that the amount of GlcA was decreased further in the *gux1/2/3* triple mutant compared with that in the *gux1/2* and *gux1/3* double mutants (**Fig. 6A**). The MeGlcA content was undetectable in the *gux1/2/3* triple mutant and its content was drastically reduced in the *gux1/2* and *gux1/3* double mutants (**Fig. 6B**). Since the total cell walls used consist of other wall polysaccharides besides xylan that may also contain GlcA residues, we further isolated xylan for the analysis of GlcA side chains. The isolated xylan was digested with endoxylanase and the resulting xylooligosaccharides were subjected to MALDI-TOF-MS (**Fig. 7**). In the wild type, the signal peaks corresponding to the GlcA-substituted Xyl_4 (*m/z* 745) and the MeGlcA-substituted Xyl_4 (*m/z* 759) were evident. These GlcA side chain signal peaks were signficantly reduced in the *gux1/2* and *gux 1/3* double mutants and were absent in the *gux1/2/3* triple mutant. A signal peak corresponding to the expected mass of the xylan reducing end pentasaccharide (X-X-R-GA-X; *m/z* 761) was relatively elevated in the double and triple mutants due to the reduction in the signal peaks of GlcA side chains (**Fig. 7**). These results indicate that xylan isolated from the *gux1/2/3* triple mutant is devoid of GlcA side chains.

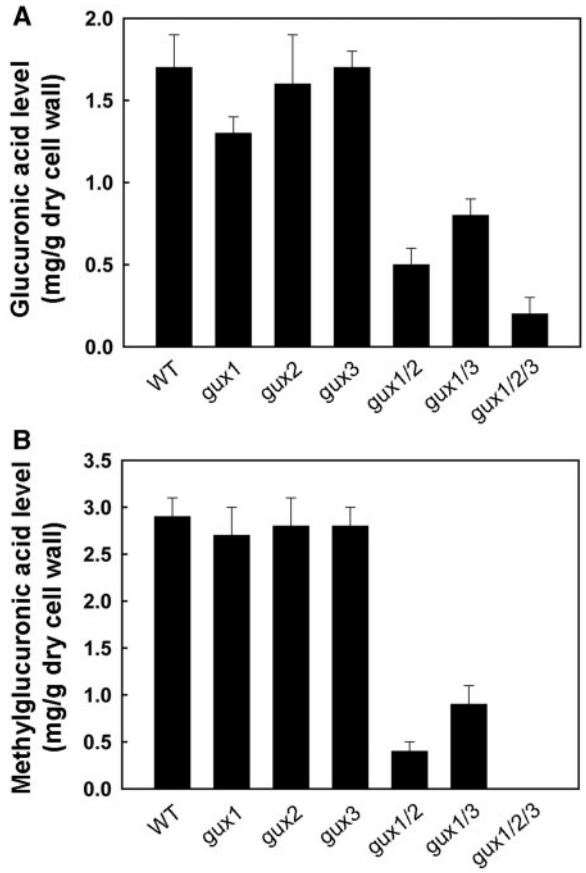

Fig. 6 Measurement of GlcA and MeGlcA amounts in cell wall residues isolated from the stems of wild-type Arabidopsis and various *gux* mutants. Error bars denote the SE of two independent assays.

To substantiate further the MALDI-TOF-MS data, the xylooligosaccharides generated by endoxylanase digestion were analyzed by nuclear magentic resonance (NMR) spectroscopy (**Fig. 8**). Xylan from the wild type displayed resonances characteristic of H1 of non-methylated and methylated GlcA side chains (at 5.29 and 5.28 p.p.m., respectively). In addition, it exhibited resonances attributed to the backbone xylosyl

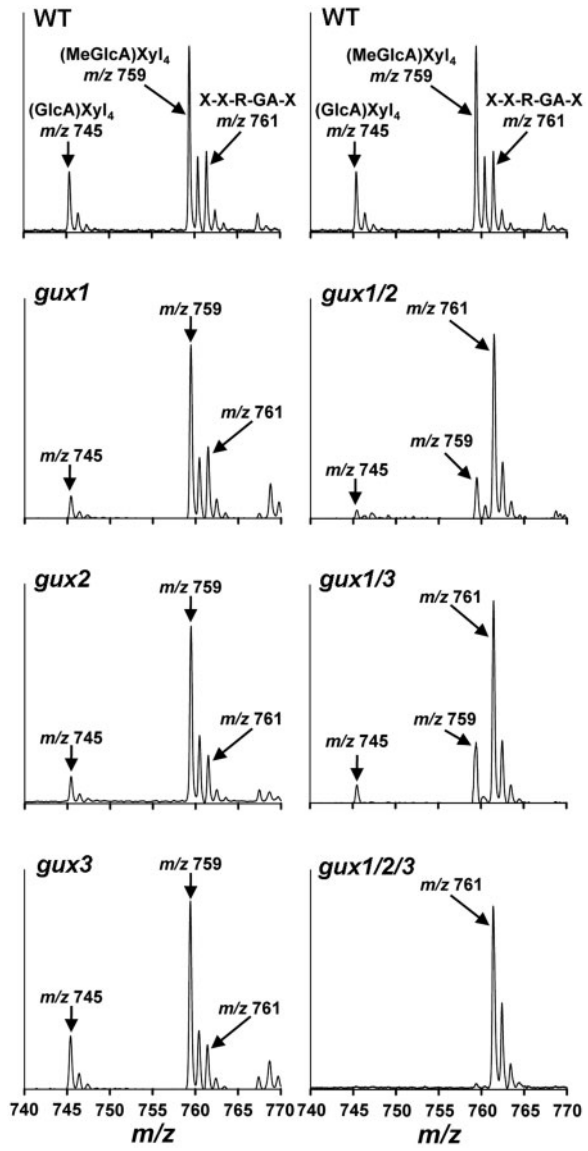

Fig. 7 MALDI-TOF mass spectra of acidic xylooligosaccharides generated by xylanase digestion of xylan from the stems of wild-type Arabidopsis and various *gux* mutants. The ions at *m/z* 745 and 759 correspond to xylotetrasaccharides bearing a GlcA residue [(GlcA)Xyl_4] and a methylated GlcA residue [(MeGlcA)Xyl_4], respectively. The ion at *m/z* 761 corresponds to the xylan reducing end pentasaccharide, β-D-Xyl-(1→4)-β-D-Xyl-(1→3)-α-L-Rha-(1→2)-α-D-GalA-(1→4)-D-Xyl (X-X-R-GA-X). Note the loss of ions at *m/z* 745 and 759 corresponding to (GlcA)Xyl_4 and (MeGlcA)Xyl_4, respectively, in the *gux1/2/3* triple mutant.

residues (H1 of branched and unbranched at 4.62 and 4.45 p.p.m., respectively) and the xylan reducing end tetrasaccharide sequence, β-D-Xyl-(1→3)-α-L-Rha-(1→2)-α-D-GalA-(1→4)-D-Xyl (H1 of 3-linked β-D-Xyl, H1 of α-L-Rha, H1 of α-D-GalA, H2 of α-L-Rha, and H4 of α-D-GalA) (Peña et al. 2007). Examination of xylan from the *gux* mutants showed the presence of a significant amount of GlcA and MeGlcA in the *gux1*, *gux2* or *gux3* single mutants and in the *gux1/3* double mutant. Only a small amount of GlcA and MeGlcA was detected in the xylan from the *gux1/2* double mutant, which is consistent with the previous report (Mortimer et al. 2010). Notably, xylan from the *gux1/2/3* triple mutant was completely devoid of GlcA and MeGlcA side chains (**Fig. 8**). Integration analysis of the NMR spectra revealed that in addition to the *gux1/2* and *gux1/2/3* mutants, the *gux1*, *gux2* and *gux3* single mutants and the *gux1/3* double mutant also had various degrees of reduction in the amount of GlcA and MeGlcA compared with the wild type (**Table 1**). Together, these results provide genetic evidence showing that all three *GUX* genes are involved in the addition of GlcA side chains onto xylan in Arabidopsis stems.

Simultaneous mutations of *GUX1/2/3* cause defects in secondary wall thickening and plant development

It was previously suggested that GlcA and MeGlcA substitutions of xylan are not required for the structural role of xylan in secondary walls since the *gux1/2* double mutant, with a partial loss of GlcA substitution of xylan, has no changes in cell wall morphology, vascular development or plant growth (Mortimer et al. 2010). We found that although the single and double *gux* mutants had no significant alterations in plant growth and stem mechanical strength, the *gux1/2/3* triple mutant showed a clear reduction in plant growth and stem mechanical strength compared with the wild type (**Fig. 9**). Since the mechanical strength of stems is largely conferred by interfascicular fibers

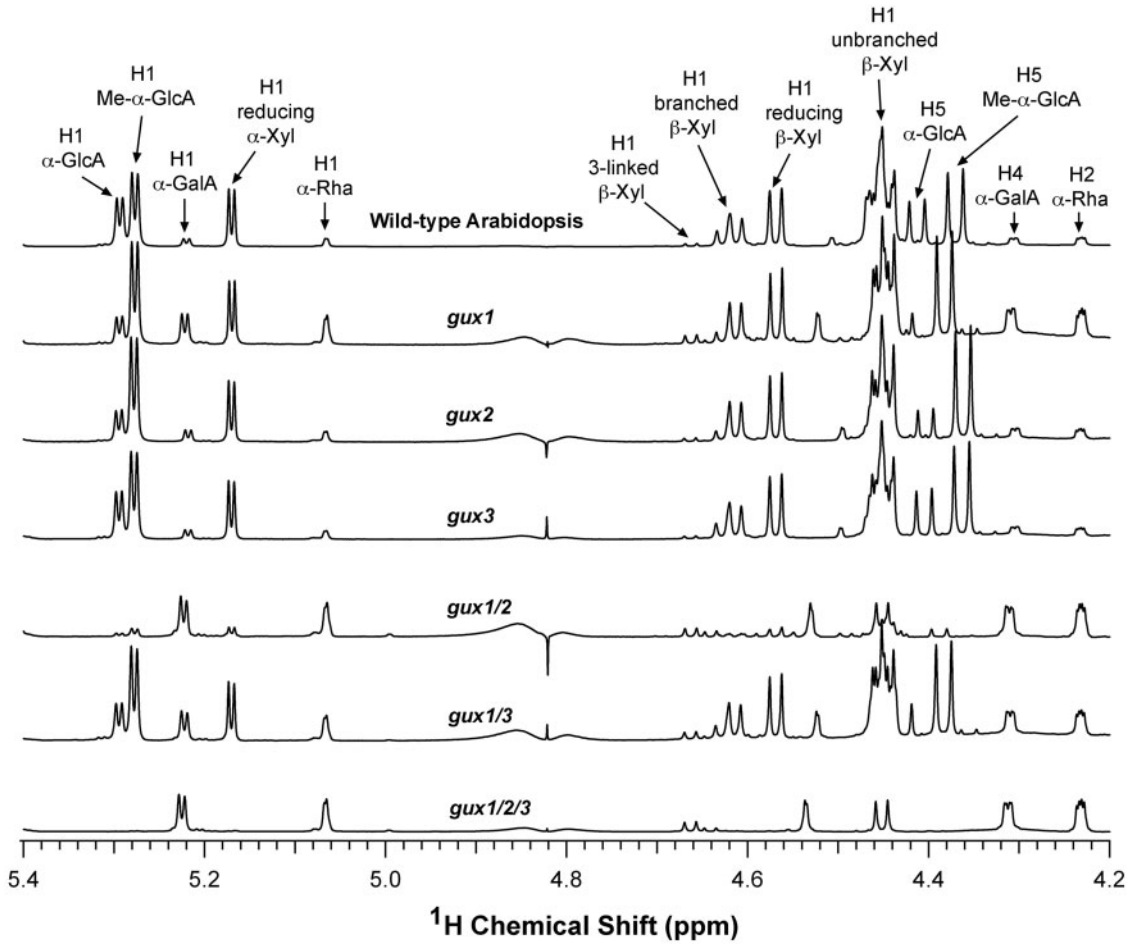

Fig. 8 Structural analysis of xylan from the wild type and the *gux* mutants by NMR spectroscopy. Xylooligosaccharides generated by β-endoxylanase digestion of alkaline-extracted xylan were subjected to ^{1}H-NMR analysis. Resonances are labeled with the position of the assigned proton and the identity of the residue containing that proton. The resonances of H1 of α-D-GalA, H1 of α-L-Rha, H1 of 3-linked β-D-Xyl, H4 of α-D-GalA and H2 of α-L-Rha are from the xylan reducing end tetrasaccharide sequence. Note the complete loss of resonances of α-GlcA and Me-α-GlcA in the *gux1/2/3* triple mutant compared with the wild type.

PCP PLANT & CELL PHYSIOLOGY

Table 1 Relative integrated values of GlcA and MeGlcA side chains in xylan from the stems of the wild type and the *gux* mutants

Sample	Reducing end sequence[a]	GlcA and MeGlcA side chains[b]	Relative amount of GlcA and MeGlcA side chains[c]
Wild type	1	2.76	100
gux1	1	0.70	25.3
gux2	1	1.93	69.9
gux3	1	2.12	76.8
gux1/2	1	0.08	2.9
gux1/3	1	0.81	22.4
gux1/2/3	1	ND[d]	ND[d]

[a] The integrated value of the xylan reducing end tetrasaccharide sequence was calculated from the NMR resonance of H1 of α-D-GalA, H1 of α-L-Rha, H1 of 3-linked β-D-Xyl, H4 of α-D-GalA and H2 of α-L-Rha (Fig. 8) and taken as 1.
[b] The integrated value of GlcA and MeGlcA side chains was determined from the ratio of the NMR resonance of GlcA and MeGlcA side chains to that of the reducing end sequence.
[c] The amount of GlcA and MeGlcA side chains in the wild-type xylan was taken as 100, and the relative value of GlcA and MeGlcA side chains in the *gux* mutants was calculated from the ratio of GlcA and MeGlcA side chains of the mutants over that of the wild type.
[d] ND, not detected.

and xylem, we examined the wall morphology of these cell types in the *gux* double and triple mutant. It was evident that the thickness of the secondary walls of interfascicular fibers and vessels was significantly reduced in the *gux1/2/3* triple mutant (Fig. 10D, H), which most probably contributes to the defective stem strength. In addition, a mild deformation of cell morphology was seen in some vessels of the *gux1/2/3* triple mutant (Fig. 10L, P). It was noted that the *gux1/2* double mutant also had a mild reduction in secondary wall thickness in interfascicular fibers (Fig. 10B, F). These results demonstrate that a complete loss of GlcA and MeGlcA side chains on xylan leads to a significant defect in secondary wall thickening and plant development.

The finding that the *gux1/2/3* triple mutant exhibited a defect in secondary wall thickening prompted us to investigate whether it had a decrease in xylan content as do most other xylan-defective mutants, such as *irx8*, *irx9*, *irx14*, *fra8* and *parvus* (Zhong et al. 2005, Brown et al. 2007, Lee et al. 2007b, Peña et al. 2007, Persson et al. 2007). It was unexpected to find that the xylose content in the inflorescence stems of the *gux1/2/3* triple mutant was significantly elevated compared with the wild type (Table 2). In addition, a slight increase in the xylose content was also seen in the *gux1/2* double mutant. Since the bulk of the xylose in the inflorescence stems is from xylan in the secondary wall-containing fibers, these results indicate that the content of xylan in the cell wall residues is relatively higher in the *gux1/2/3* mutant compared with the wild type. It was also noted that the density of cell wall residues significantly increased in the *gux1/2/3* triple mutant compared with the wild type and the *gux1/2* and *gux1/3* double mutants (Fig. 9C), suggesting that the *gux1/2/3* mutations caused an alteration in the physical property of cell walls.

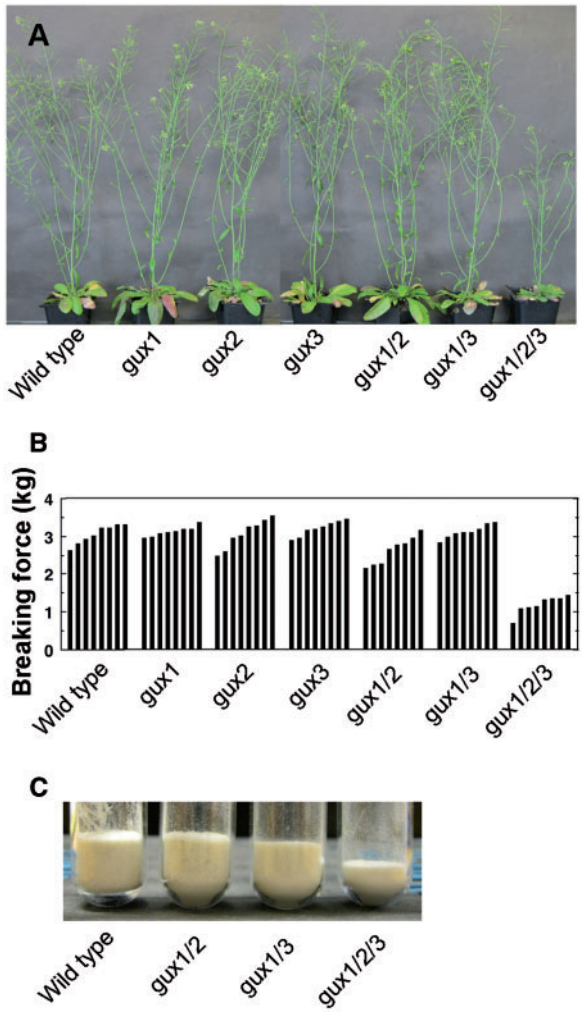

Fig. 9 The *gux1/2/3* triple mutations cause a reduction in plant growth and the mechanical strength of stems. (A) Ten-week-old wild-type Arabidopsis plant and single, double and triple *gux* mutant plants. (B) Breaking strength measurement showing that the mechanical strength of the stems of *gux1/2/3* is significantly reduced compared with the wild type. Each bar represents the breaking force of the inflorescence stem of individual plants. (C) Cell wall residues (120 mg) from the stems of the wild type, *gux1/2*, *gux1/3* and *gux1/2/3* showing a significant alteration in the density of *gux1/2/3* cell wall residues.

Loss of GlcA and MeGlcA side chains on xylan in the *gux* mutants has no effect on wall digestibility by cellulase

Because xylan is the major polysaccharide that interacts with cellulose in the secondary wall-rich cellulosic biomass and this interaction is believed to contribute to biomass recalcitrance (Himmel et al. 2007), we tested whether the altered cell wall property in the *gux1/2/3* mutant had any effect on the accessibility of cellulose to cellulase digestion. It was found that only a slight increase in the conversion of cellulose to glucose by cellulase and cellobiase was seen in the *gux1/2* double mutant

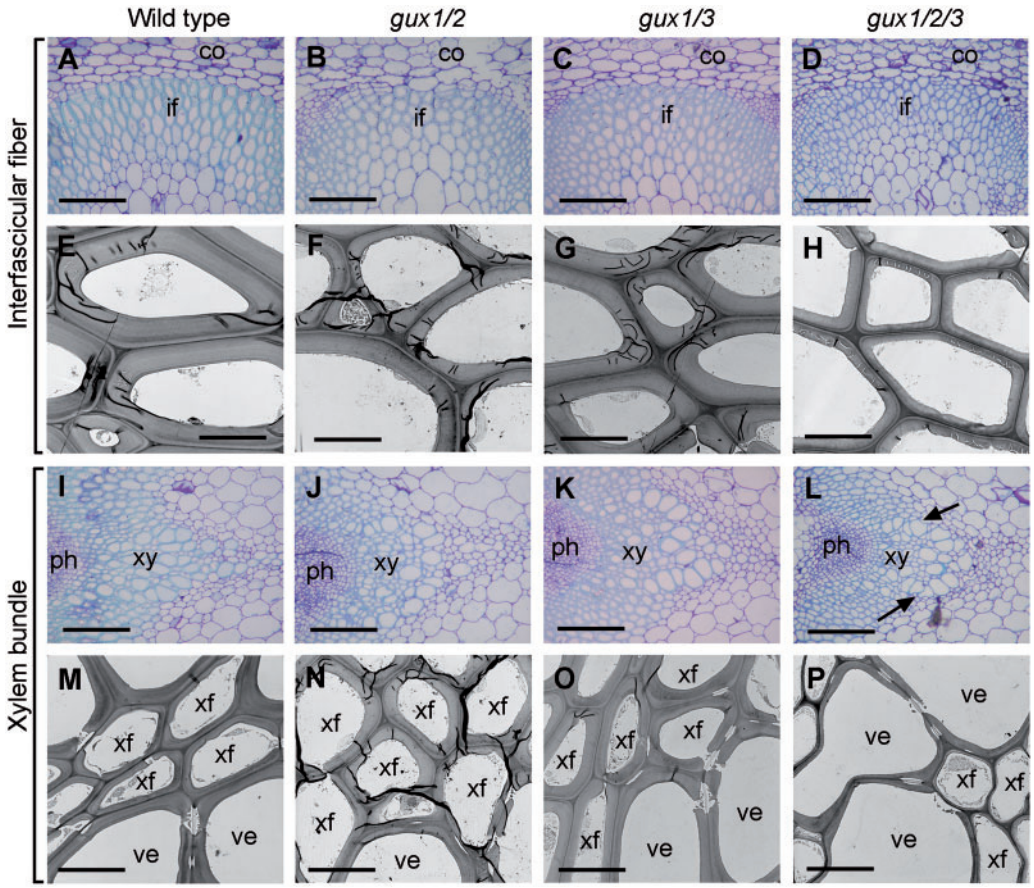

Fig. 10 Effects of *gux* mutations on secondary wall thickening and vessel morphology. Stems were sectioned and stained with toluidine blue for light microscopy (A–D and I–L) or sectioned for transmission electron microscopy (E–H and M–P). (A–H) Cross-sections of the interfascicular regions showing a mild (*gux1/2* and *gux1/3*) or severe (*gux1/2/3*) reduction in the secondary wall thickness compared with the wild type. (I–P) Cross-sections of xylem bundles showing a mild deformation of vessels only in *gux1/2/3* but not in *gux1/2* or *gux1/3* compared with the wild type. co, cortex; if, interfascicular fiber; ph, phloem; ve, vessel; xf, xylary fiber; xy, xylem. Bars = 86 μm in A–D and I–L and 7.2 μm in E–H and M–P.

and *gux1/2/3* triple mutant compared with the wild type (**Fig. 11**), indicating that removal of GlcA side chains of xylan has no major impact on the digestibility of cell walls by cellulase.

Discussion

GUX1/2/3 are glucuronyltransferases responsible for the addition of GlcA residues onto xylan

We have demonstrated that the GUX1/2/3 proteins possess GlcAT activities capable of catalyzing the addition of GlcA residues onto xylan. This biochemical proof of the GlcAT activities of GUX1/2/3 is consistent with their retaining catalytic mechanism of the GT8 family, to which GUX1/2/3 belong, as well as with the genetic evidence showing that simultaneous mutations of *GUX1/2/3* lead to a complete loss of GlcA and MeGlcA side chains on xylan. The findings that all three GUX proteins exhibit GlcAT activities and their genes are expressed in the same secondary wall-forming cell types indicate that they function redundantly in the GlcA substitution of xylan, which provides an explanation for why only simultaneous mutations of all three *GUX* genes result in a complete loss of GlcA and MeGlcA side chains on xylan. Mutations of a single or double *GUX* genes were also noticed to cause some degree of reduction in the level of GlcA side chains, indicating that expression of all three *GUX* genes is required for the normal rate of GlcA addition onto xylan.

A complete loss of GlcA and MeGlcA side chains of xylan has a drastic effect on secondary wall deposition and plant development

Our finding that the *gux1/2/3* triple mutant exhibits a reduction in secondary wall thickness and plant growth indicates that the GlcA substitution of xylan is essential for the normal function of xylan during secondary wall deposition. This is in contrast to the *gux1/2* double mutant in which no changes in wall morphology and plant growth were observed (Mortimer et al. 2010). Since small amounts of GlcA and MeGlcA side chains are still present in the xylan from the *gux1/2* double

Table 2 Neutral sugar composition of cell walls from the inflorescence stems of the wild type and the *gux* mutants

Sample	Xylose	Glucose	Mannose	Galactose	Arabinose	Rhamnose
Wild type	123.5 ± 5.6	426.2 ± 3.4	20.4 ± 0.3	17.1 ± 1.1	14.7 ± 1.1	13.6 ± 1.4
gux1	127.0 ± 4.0	411.5 ± 3.5	20.5 ± 0.5	18.7 ± 1.4	13.5 ± 0.5	14.6 ± 0.3
gux2	121.8 ± 6.5	444.7 ± 18.3	22.4 ± 1.4	21.4 ± 0.5	16.3 ± 1.2	13.2 ± 0.5
gux3	112.6 ± 8.5	434.4 ± 26.7	20.3 ± 3.2	21.2 ± 0.5	17.1 ± 0.5	13.4 ± 0.5
gux1/2	147.3 ± 3.5	406.2 ± 1.5	21.4 ± 1.5	21.2 ± 0.5	16.2 ± 1.11	4.2 ± 0.3
gux1/3	123.4 ± 2.5	439.2 ± 6.4	18.4 ± 4.2	18.1 ± 1.5	14.2 ± 1.5	11.8 ± 0.4
gux1/2/3	176.8 ± 3.8	402.7 ± 5.8	27.3 ± 0.5	21.7 ± 0.6	14.9 ± 0.7	15.3 ± 0.7

The wall residues for cell wall composition analysis were prepared from stems. The data are means (mg g^{-1} dry cell wall) ± SE of two independent assays.

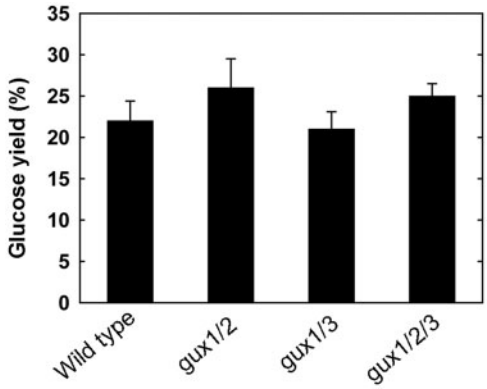

Fig. 11 Glucose release from the cell walls of the wild type and the *gux* mutants by cellulase and cellobiase. The enzymatic hydrolysis efficiency is shown as the percentage of glucose released by the enzymes over the total amount of cellulose in the walls. Error bars denote the SE of two independent assays.

mutant, it implies that removal of a majority of GlcA substitution has little effect on the normal function of xylan. However, it is evident from the analysis of the *gux1/2/3* triple mutant that a complete loss of GlcA substitution has a drastic effect on xylan properties, which is consistent with the previous observation that the degree of sugar substitutions of xylan has a large impact on the assembly of the xylan–cellulose network (Kabel et al. 2007). It has been shown that increasing the number of unsubstituted xylosyl residues in xylan induces formation of xylan–xylan interactions and, subsequently, favors the adsorption of xylan to cellulose (Kabel et al. 2007). Since the xylan in the *gux1/2/3* triple mutant is completely devoid of sugar substitutions, the unsubstituted xylan in the mutant most probably has an alteration in its interaction with cellulose and hence affects the overall secondary wall assembly, which leads to a defect in secondary wall thickening and a deformation of vessels. The reduced plant growth phenotype in the *gux1/2/3* triple mutant is most probably caused by the altered vessel morphology, which impedes normal solute transport, rather than by the defective secondary wall thickening in fibers. This hypothesis is consistent with the previous observations that a deformation in vessel morphology is often associated with a reduced plant growth phenotype (Zhong et al. 2005, Brown et al. 2007); on the other hand, a defect in the secondary wall thickening in fibers only does not affect the overall plant growth (Zhong et al. 2007). It should be noted that although no detectable *GUX3* expression was seen in the interfascicular fiber cells, the *gux1/2/3* triple mutant exhibited a more severe reduction in secondary wall thickness in interfascicular fibers than the *gux1/2* double mutant. One possible explanation for this observation is that although not detectable in the expression analysis, *GUX3* might be expressed at a very low level in the interfascuclar fiber cells, which plays a minor role in adding GlcA residues onto xylan in these cells.

In summary, our biochemical and genetic analyses of the Arabidopsis *GUX* genes demonstrate that GUX1/2/3 are xylan GlcATs responsible for the GlcA substitution of xylan and that a complete loss of GlcA side chains on xylan causes a significant impact on secondary wall deposition and plant development. Together with the recent biochemical study showing that GT43 members in Arabidopsis and poplar are xylosyltransferases responsible for the elongation of the xylan backbone (Lee et al. 2012a, Lee et al. 2012b), these findings mark an important advance in our understanding of the biochemical mechanisms underlying xylan biosynthesis.

Materials and Methods

Gene expression analysis

Total RNA from Arabidopsis tissues and tobacco BY2 cells was isolated with a Qiagen RNA isolation kit. RNA from different cell types (interfascicular fibers, xylem and pith cells) was isolated and amplified as described previously (Zhong et al. 2006). After treatment with DNase I, the total RNA was converted to first-strand cDNA, which was then used as a template for real-time quantitative PCR analysis with the QuantiTect SYBR Green PCR kit (Clontech). The PCR primers for *GUX1* are 5′-aattatgccgacggtcattgg-3′ and 5′-caagttatggcc gggaagtga-3′; those for *GUX2* are 5′-acgactccatggacgatgcat-3′ and 5′-aataacataagaacgacgtcg-3′; those for *GUX3* are 5′-atgata ccttcctcaagtcc-3′ and 5′-tagctacatagcgaggatcag-3′; those for *GUX4* are 5′-ccatcgccgcaagtcaacaaa-3′ and 5′-atctccggacaggta-gaagct-3′; and those for *GUX5* are 5′-ttgccggaaccggttaaacgg-3′

and 5′-ctaggaatgtgccacgtgtga-3′. The relative expression level was calculated by normalizing the PCR threshold cycle number of each gene with that of the *EF1α* reference gene. The data were the average of three biological replicates. Reverse transcription–PCR (RT–PCR) was applied to examine the transcript levels of *GUX* genes in transgenic tobacco BY2 cells.

GUS reporter gene analysis

For the GUS reporter gene analysis, the *GUX1*, *GUX2* or *GUX3* gene containing a 3 kb 5′ upstream sequence, the entire coding region, and a 2 kb 3′ downstream sequence was used. The GUS gene was inserted in-frame right before the stop codon of these genes, and then cloned into pBI101 (Clontech) to create the GUS reporter constructs. The constructs were transformed into wild-type Arabidopsis (ecotype Columbia) plants by *Agrobacterium*-mediated transformation (Bechtold and Bouchez 1994) to generate the GUS reporter transgenic plants. Inflorescence stems from 6-week-old transgenic plants were examined for the GUS activity described previously (Zhong et al. 2005).

Generation of transgenic BY2 cell lines

The full-length *GUX* cDNAs were ligated downstream of the *Cauliflower mosaic virus* (CaMV) 35 S promoter in a modified pBI121 to create the GUX overexpression constructs. Each GUX expression construct or an empty vector (as control) was introduced into tobacco BY2 cells by *Agrobacterium*-mediated transformation (An 1985). For co-expression of GUX1 and GUX2, agrobacteria containing the GUX1 and GUX2 expression constructs were used to co-transform tobacco cells. More than 60 independent transgenic cell lines were generated for each construct. They were confirmed for GUX expression by RT–PCR and used for subsequent GlcAT activity assay.

Assay of GlcAT activity

Microsomes were isolated from transgenic tobacco BY2 cells for the assay of the GlcAT activity following procedures by Kuroyama and Tsumuraya (2001). Microsomes (100 μg) 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 dithiothreitol (DTT), 0.5% Triton X-100, 0.2 μg μl^{-1} Xyl_6 (Megazyme), and UDP-[^{14}C]GlcA (0.1 μCi; American Radiolabeled Chemical). After incubation at 21°C for 30 min, the reaction was stopped by adding the termination solution (0.3 M acetic acid containing 20 mM EGTA). The radiolabeled xylooligosaccharides were separated from UDP-[^{14}C]GlcA by paper chromatography according to Ishikawa et al. (2000). The amount of radioactivity present in the xylooligosaccharides was determined with a PerkinElmer scintillation counter.

MALDI-TOF-MS

MALDI-TOF-MS was applied to analyze the GUX-catalyzed reaction products and the xylooligosaccharides generated by digestion of xylan with β-endoxylanase M6 (Megazyme) as described by Zhong et al. (2005). The spectrometer was 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 aqueous sample was mixed (1 : 1, v/v) with the MALDI matrix (0.2 M 2,5-dihydroxbenzoic acid and 0.06 M 1-hydroxyisoquinoline in 50% acetonitrile) and dried on a stainless steel target plate. Spectra are the average of 100 laser shots.

Histology

The basal parts of inflorescence stems of mature plants were fixed in 2% formaldehyde and embedded in low viscosity (Spurr's) resin (Electron Microscopy Sciences) (Burk et al. 2006). For light microscopy, 1 μm thick sections were cut with a microtome and stained with toluidine blue. For transmission electron microscopy, 85 nm thick sections were cut, post-stained with uranyl acetate and lead citrate, and observed using a Zeiss EM 902 A transmission electron microscope. Stems from at least eight plants for each *gux* mutant were examined, and representative data were shown.

The basal parts of inflorescence stems of mature plants were measured for their breaking force using a digital force/length tester (Larson System) (Zhong et al. 1997). Breaking force was calculated as the force needed to break apart a stem segment.

Cell wall sugar composition analysis

Inflorescence stems from mature plants were used for cell wall isolation according to Zhong et al. (2005). Neutral cell wall sugars (as alditol acetates) were determined following the procedure described by Hoebler et al. (1989). The alditol acetates of the hydrolyzed cell wall sugars were analyzed on a PerkinElmer Clarus 500 gas–liquid chromatograph instrument equipped with a 30 m × 0.25 mm (i.d.) silica capillary column DB 225 (Alltech Associates). GlcA and MeGlcA amounts in total cell walls were measured according to Doco et al. (2001).

Cell wall digestibility analysis

The digestibility of cell wall residues with cellulase enzymes was determined according to Chen and Dixon (2007). Briefly, the ethanol-insoluble cell wall residues (equivalent to 100 mg of glucose) were incubated with cellulase (52 U; Celluclast 1.5 L from Sigma) and cellobiase (16 U; Sigma) in 0.1 M sodium acetate buffer (pH 4.8) at 37°C for 72 h. After centrifugation, the supernatant was used to quantify neutral sugars released from cellulose using gas chromatography as described above. The enzyme mixture without the addition of cell wall residues was used as a blank. Under the same conditions, the enzyme mixture was able to digest 90% of Whatmann No. 1 filter paper cellulose into glucose. Two independent assays were performed on all samples.

^{1}H-NMR spectroscopy

Xylooligosaccharides were generated by digestion of 1 N KOH-solubilized wall preparations with β-endoxylanase M6

as described by Zhong et al. (2005). NMR spectra of the xylooligosaccharides from β-xylanase digestion were acquired at 20°C on a Varian Inova 600 MHz spectrometer (599.7 MHz, ^{1}H) using a 5 mm cryogenic triple resonance probe (Varian). All NMR samples were prepared with 100% D_2O in 3 mm standard NMR tubes. ^{1}H chemical shifts were referenced to DSS (2,2-dimethyl-2-silapentane-5-sulfonate sodium salt). For all experiments, 64 transients were collected using a spectral width of 6,000 Hz and an acquisition time of 5 s. The residual water resonance was suppressed by a 1 s pre-saturation pulse at a field strength of 40 Hz. 1D spectra were processed using MestReC (MestreC Research) with 0.2 Hz apodization followed by zero-filling to 128 k points. The ^{1}H-NMR assignments were done by comparing them with the NMR spectra data for xylan structure (Zhong et al. 2005, Peña et al. 2007).

Accession numbers

The Arabidopsis Genome Initiative locus identifiers for the Arabidopsis genes investigated in this study are *GUX1* (At3g18660), *GUX2* (At4g33330), *GUX3* (At1g77130), *GUX4* (At1g54940) and *GUX5* (At1g08990). The GenBank accession numbers for PoGT8E and PoGT8F are JQ905976 and JQ905977, respectively.

Funding

This work was supported by the Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences of the US. Department of Energy [Grant DE-FG02-03ER15415].

Acknowledgments

We thank the Arabidopsis Biological Resource Center and the European Arabidopsis Stock Center for the T-DNA knockout lines of GUXs, and the reviewers for their constructive comments. This paper has been reviewed in accordance with the US Environmental Protection Agency's peer and administrative review policies and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

References

An, G. (1985) High efficiency transformation of tobacco cells. *Plant Physiol.* 79: 568–570.

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.

Bechtold, N. and Bouchez, D. (1994) In planta *Agrobacterium*-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. *In* Gene Transfer to Plants. Edited by Potrykus, I. and Spangenberg, G. pp. 19–23. Springer-Verlag, Berlin.

Brown, D.M., Goubet, F., Wong, V.W., Goodacre, R., Stephens, E., Dupree, P. et al. (2007) Comparison of five xylan synthesis mutants reveals new insight into the mechanisms of xylan synthesis. *Plant J.* 52: 1154–1168.

Brown, D.M., Wightman, R., Zhang, Z., Gomez, L.D., Atanassov, I., Bukowski, J.P. et al. (2011) Arabidopsis genes *IRREGULAR XYLEM* (*IRX15*) and *IRX15L* encode DUF579-containing proteins that are essential for normal xylan deposition in the secondary cell wall. *Plant J.* 66: 401–413.

Brown, D.M., Zhang, Z., Stephens, E., Dupree, P. and Turner, S.R. (2009) Characterization of IRX10 and IRX10-like reveals an essential role in glucuronoxylan biosynthesis in Arabidopsis. *Plant J.* 57: 732–746.

Burk, D.H., Zhong, R., Morrison, W.H. III and Ye, Z.-H. (2006) Disruption of cortical microtubules by overexpression of green fluorescent protein-tagged α-tubulin 6 causes a marked reduction in cell wall synthesis. *J. Integr. Plant Biol.* 48: 85–98.

Chen, F. and Dixon, R.A. (2007) Lignin modification improves fermentable sugar yields for biofuel production. *Nat. Biotechnol.* 25: 759–761.

Doco, T., O'Neill, M.A. and Pellerin, P. (2001) Determination of the neutral and acidic glycosyl-residue compositions of plant polysaccharides by GC-EI-MS analysis of the trimethylsilyl methyl glycoside derivatives. *Carbohydr. Polymers* 46: 249–259.

Harper, A.D. and Bar-Peled, M. (2002) Biosynthesis of UDP-xylose. Cloning and characterization of a novel Arabidopsis gene family, UXS, encoding soluble and putative membrane-bound UDP-glucuronic acid decarboxylase isoforms. *Plant Physiol.* 130: 2188–2198.

Helle, S., Cameron, D., Lam, J., White, B. and Duff, S. (2003) Effect of inhibitory compounds found in biomass hydrolysates on growth and xylose fermentation by a genetically engineered strain of *S. cerevisiae*. *Enzyme Microb. Technol.* 33: 786–792.

Himmel, M.E., Ding, S.Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W. et al. (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science* 315: 804–807.

Hoebler, C., Barry, J.L., David, A. and Delort-Laval, J. (1989) Rapid acid-hydrolysis of plant cell wall polysaccharides and simplified quantitative determination of their neutral monosaccharides by gas–liquid chromatography. *J. Agric. Food Chem.* 37: 360–367.

Ishii, T. (1997) Structure and functions of feruloylated polysaccharides. *Plant Sci.* 127: 111–127.

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.

Jensen, J.K., Kim, H., Cocuron, J.C., Orler, R., Ralph, J. and Wilkerson, C.G. (2011) The DUF579 domain containing proteins IRX15 and IRX15-L affect xylan synthesis in Arabidopsis. *Plant J.* 66: 387–400.

Kabel, M.A., van den Borne, H., Vincken, J.-P., Voragen, A.G.J. and Schols, H.A. (2007) Structural differences of xylans affect their interaction with cellulose. *Carbohydr. Polym.* 69: 94–105.

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

Lee, C., O'Neill, M.A., Tsumuraya, Y., Darvill, A.G. and Ye, Z.-H. (2007a) The *irregular xylem9* mutant is deficient in xylan xylosyltransferase activity. *Plant Cell Physiol.* 48: 1624–1634.

Lee, C., Teng, Q., Huang, W., Zhong, R. and Ye, Z.-H. (2009a) Down-regulation of PoGT47C expression in poplar results in a

reduced glucuronoxylan content and an increased wood digestibility by cellulase. *Plant Cell Physiol.* 50: 1075–1089.

Lee, C., Teng, Q., Huang, W., Zhong, R. and Ye, Z.-H. (2009b) The F8H glycosyltransferase is a functional paralog of FRA8 involved in glucuronoxylan biosynthesis in *Arabidopsis*. *Plant Cell Physiol.* 50: 812–827.

Lee, C., Teng, Q., Huang, W., Zhong, R. and Ye, Z.-H. (2010) The Arabidopsis family GT43 glycosyltransferases form two functionally nonredundant groups essential for the elongation of glucuronoxylan backbone. *Plant Physiol.* 153: 526–541.

Lee, C., Teng, Q., Zhong, R. and Ye, Z.-H. (2011) The four Arabidopsis *REDUCED WALL ACETYLATION* genes are expressed in secondary wall-containing cells and required for the acetylation of xylan. *Plant Cell Physiol.* 52: 1289–1301.

Lee, C., Zhong, R., Richardson, E.A., Himmelsbach, D.S., McPhail, B.T. and Ye, Z.-H. (2007b) The *PARVUS* gene is expressed in cells undergoing secondary wall thickening and is essential for glucuronoxylan biosynthesis. *Plant Cell Physiol.* 48: 1659–1672.

Lee, C., Zhong, R. and Ye, Z.-H. (2012a) Arabidopsis family GT43 members are xylan xylosyltransferases required for the elongation of the xylan backbone. *Plant Cell Physiol.* 53: 135–143.

Lee, C., Zhong, R. and Ye, Z.-H. (2012b) Biochemical characterization of xylan xylosyltransferases involved in wood formation in poplar. *Plant Signal. Behav.* 7: 332–337.

Mortimer, J.C., Miles, G.P., Brown, D.M., Zhang, Z., Segura, M.P., Weimar, T. et al. (2010) Absence of branches from xylan in Arabidopsis *gux* mutants reveals potential for simplification of lignocellulosic biomass. *Proc. Natl Acad. Sci. USA* 107: 17409–17414.

Page, R.D.M. (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* 12: 357–358.

Peña, M.J., Zhong, R., Zhou, G.-K., Richardson, E.A., O'Neill, M.A., Darvill, A.G. et al. (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. et al. (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.

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.

Timell, T.E. (1967) Recent progress in the chemistry of wood hemicelluloses. *Wood Sci. Technol.* 1: 45–70.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673–4680.

Wu, A.M., Hörnblad, E., Voxeur, A., Gerber, L., Rihouey, C., Lerouge, P. et al. (2010) Analysis of the Arabidopsis IRX9/IRX9-L and IRX14/IRX14-L pairs of glycosyltransferase genes reveals critical contributions to biosynthesis of the hemicellulose glucuronoxylan. *Plant Physiol.* 153: 542–554.

Wu, A.M., Rihouey, C., Seveno, M., Hörnblad, E., Singh, S.K., Matsunaga, T. et al. (2009) The *Arabidopsis* IRX10 and IRX10-LIKE glycosyltransferases are critical for glucuronoxylan biosynthesis during secondary cell wall formation. *Plant J.* 57: 718–731.

Zhong, R., Demura, T. and Ye, Z.-H. (2006) SND1, a NAC domain transcription factor, is a key regulator of secondary wall synthesis in fibers of Arabidopsis. *Plant Cell* 18: 3158–3170.

Zhong, R., Lee, C. and Ye, Z.-H. (2010) Global analysis of direct targets of secondary wall NAC master switches in *Arabidopsis*. *Mol. Plant* 3: 1087–1103.

Zhong, R., Peña, M.J., Zhou, G.-K., Nairn, C.J., Wood-Jones, A., Richardson, E.A. et al. (2005) Arabidopsis *Fragile Fiber8*, which encodes a putative glucuronyltransferase, is essential for normal secondary wall synthesis. *Plant Cell* 17: 3390–3408.

Zhong, R., Richardson, E.A. and Ye, Z.-H. (2007) Two NAC domain transcription factors, SND1 and NST1, function redundantly in regulation of secondary wall synthesis in fibers of Arabidopsis. *Planta* 225: 1603–1611.

Zhong, R., Taylor, J.J. and Ye, Z.-H. (1997) Disruption of interfascicular fiber differentiation in an Arabidopsis mutant. *Plant Cell* 9: 2159–2170.