Absence of branches from xylan in Arabidopsis *gux* mutants reveals potential for simplification of lignocellulosic biomass

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As one of the most abundant polysaccharides on Earth, xylan will provide more than a third of the sugars for lignocellulosic biofuel production when using grass or hardwood feedstocks. Xylan is characterized by a linear β(1,4)-linked backbone of xylosyl residues substituted by glucuronic acid, 4-*O*-methylglucuronic acid or arabinose, depending on plant species and cell types. The biological role of these decorations is unclear, but they have a major influence on the properties of the polysaccharide. Despite the recent isolation of several mutants with reduced backbone, the mechanisms of xylan synthesis and substitution are unclear. We identified two Golgi-localized putative glycosyltransferases, GlucUronic acid substitution of Xylan (GUX)-1 and GUX2 that are required for the addition of both glucuronic acid and 4-*O*-methylglucuronic acid branches to xylan in Arabidopsis stem cell walls. The *gux1 gux2* double mutants show loss of xylan glucuronyltransferase activity and lack almost all detectable xylan substitution. Unexpectedly, they show no change in xylan backbone quantity, indicating that backbone synthesis and substitution can be uncoupled. Although the stems are weakened, the xylem vessels are not collapsed, and the plants grow to normal size. The xylan in these plants shows improved extractability from the cell wall, is composed of a single monosaccharide, and requires fewer enzymes for complete hydrolysis. These findings have implications for our understanding of the synthesis and function of xylan in plants. The results also demonstrate the potential for manipulating and simplifying the structure of xylan to improve the properties of lignocellulose for bioenergy and other uses.

bioenergy | glucuronoxylan | glycosyltransferase | plant cell wall | polysaccharide

The main component of the plant cell wall, cellulose, is assembled into crystalline microfibrils and embedded in a matrix containing pectins, lignin, proteins, and hemicelluloses such as glucomannan, xyloglucan, mixed linkage glucan, and xylan. The synthesis and function of the different polysaccharide components and their arrangement in the wall are not fully understood. Xylan, a polymer of β(1,4)-linked D-xylosyl (Xyl) residues, is quantitatively the major noncellulosic polysaccharide in angiosperms. The secondary cell walls of the woody tissues of eudicotyledonous plants, such as Arabidopsis and poplar, contain glucuronoxylan (GX), where the backbone is substituted with α(1,2)-linked D-glucuronyl (GlcA) or 4-*O*-methyl-GlcA (MeGlcA) residues. Xylan in the cell walls of grasses is, in addition, variably substituted with α(1,2)- and α(1,3)-linked L-arabinosyl (Ara) and other residues, such that in many tissues, a third or more of the Xyl residues can be decorated (1). These branches influence water solubility of the polysaccharide and its interaction with cellulose and lignin. For example, lignin is linked via feruloyl esters to the arabinosyl residues of grass xylan, and lignin may be esterified to the MeGlcA decoration of GX (1–3). The substitutions also influence the recognition of the polysaccharide by hydrolases, and can prevent enzymatic degradation to monosaccharides (4).

Xylan plays a key role in human and animal nutrition and in industrial uses of plant biomass. It is largely indigestible by humans and can improve passage of material through the gut, thereby helping to reduce the incidence of diseases such as colorectal cancer and type II diabetes (5). Conversely, highly substituted, indigestible xylan in animal feed leads to a loss of nutrition. During paper and pulp manufacture, xylan MeGlcA residues are converted to hexenuronosyl residues. This decreases the brightness of the final product and increases the requirement for chemicals, making the process expensive and more environmentally damaging (6).

Plant lignocellulosic biomass is being intensively researched as a potential renewable source of transport fuels, through enzymatic hydrolysis and fermentation of the released sugars (4). To achieve this, a reduction in the processing costs and improved fermentable sugar yield will be important. The recalcitrance of the cell wall polysaccharides to enzymatic hydrolysis necessitates extensive biomass pretreatment, such as the use of steam explosion, acids, or alkali. Xylan contributes to the recalcitrance, probably through direct interaction with cellulose fibrils and also through cross-links to lignin (3, 7). After pretreatment, relatively large quantities of several different enzymes are added to release most of the sugars as monosaccharides (saccharification). Organisms are being selected or engineered to ferment both hexoses and pentoses to improve the yield of fuel, as pentoses from xylan may provide more than one third of the sugar from many feedstocks. However, MeGlcA is not fermented by many organisms, and may therefore accumulate as an inhibitory compound in fermentation (4).

The many uses of xylan would benefit from the ability to alter the polysaccharide structure in plants. A number of genes have recently been identified as putative Golgi-localized glycosyltransferases (GTs) with a role in GX backbone synthesis. IRX9, IRX10, IRX10L, and IRX14 have been implicated in xylan backbone synthesis (8–13), whereas FRA8/IRX7, IRX8, and PARVUS are thought to synthesize a xylan chain primer or terminator oligosaccharide (8–10, 14). Mutations in all of these genes lead to reduced GX synthesis and collapsed xylem vessels. Severe dwarfing is seen in the more penetrant mutants, indicating that xylan is an important component for cell wall strength and plant growth.

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Based on a bioinformatic approach, candidate GTs have been proposed to catalyze α(1,2)- or α(1,3)-linked Ara substitution of grasses (15). Although xylan glucuronyltransferase activity (GuxT) has been detected in various plants (10, 11, 16, 17), no strong candidate GTs for addition of GlcA or MeGlcA, collectively named [Me]GlcA, to the xylan backbone have yet been identified. Here, we show that *gux* mutants in two Golgi-localized putative GTs have reduced GlcA and MeGlcA substitution on GX in Arabidopsis stems. The double *gux* mutants have unsubstituted xylan in their cell walls, yet appear normal in growth. These results have implications for our understanding of xylan synthesis, function, and industrial use.

Results

GUX1 and GUX2 Are Two Previously Uncharacterized Components of Xylan Synthesis Machinery. To discover unique GX biosynthetic enzymes, we searched for putative GTs that are coexpressed with known xylan synthesis proteins and that are colocalized with other polysaccharide synthesis enzymes in the Golgi apparatus. Approximately 450 characterized and putative GTs from Arabidopsis are categorized by homology into families in the CAZy database (18). We clustered all of the Arabidopsis predicted GTs according to their coexpression in different plant organs (Fig. 1*A* and Fig. S1. Several enzymes required for xylan synthesis were clustered with the secondary cell wall cellulose synthases (Fig. 1*B*). Only two additional putative GTs, At3g18660 and At4g33330, are coexpressed with these xylan synthesis genes. For reasons outlined below, we now refer to them as GUX1 (At3g18660) and GUX2 (At4g33330). These GTs have previously been shown to be coexpressed with secondary wall synthesis enzymes (19, 20). They are members of a subfamily of five enzymes belonging to CAZy family GT8 (18) Fig. S2*A*). Closely related genes, PttGT8A, PttGT8B, and PttGT8C are expressed during wood formation in poplar (21). However, GUX1 and GUX2 have previously been characterized as the starch initiation proteins PGSIP1 and PGSIP3 (22). The proteins were predicted to localize to the chloroplast, a finding inconsistent with a role in secondary cell wall synthesis. Nevertheless, in a quantitative proteomic approach to identify proteins in the Golgi apparatus of Arabidopsis cell cultures, we showed that the closely related protein At1g77130/PGSIP2/GUX3 is Golgi localized (23) (Fig. S3). This suggested that this family of five GT8 proteins might be Golgi localized and have a role in cell wall synthesis. Indeed, all five predicted proteins have a single N-terminal putative transmembrane domain, typical of Golgi-localized GTs (Fig. S2*B*). To clarify the subcellular localization, GUX1 and GUX2 proteins were fused to GFP and transiently expressed in *Nicotiana tabacum*. Both were found to colocalize with the Golgi sugar nucleotide transporter, GONST1 (Fig. 1*C*), indicating that the proposed role in plastidic starch synthesis is unlikely.

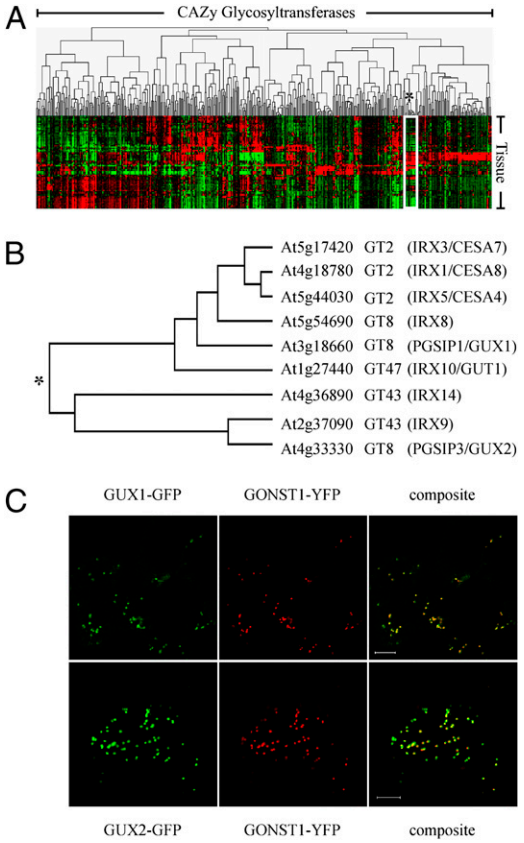

Fig. 1. Identification of candidate secondary cell wall GTs. (*A*) Coexpression clustering of all putative GT genes from the CAZy database, using transcriptomics data from the AtGenExpress expression atlas. Organ expression levels are shown above (green) or below (red) the average for the gene. White boxed section with branch labeled (*) is enlarged in *B*. (*C*) Subcellular localization of GUX1 and GUX2. GFP-tagged GUX1 and GUX2 and Golgi marker GONST1-YFP were transiently expressed in tobacco leaves and examined by laser-scanning confocal microscopy.

To investigate a role for GUX1 and GUX2 in secondary cell wall polysaccharide biosynthesis, two independent T-DNA insertional mutant lines for each gene were identified (*gux1-1*, *gux1-2*, *gux2-1*, and *gux2-2*) and confirmed as transcriptional knockouts (Fig. S4 *A* and *B*). Cell wall alcohol-insoluble residue (AIR) was prepared from the basal stems of these plants, a tissue rich in secondary cell wall. The AIR was characterized with polysaccharide analysis by carbohydrate gel electrophoresis (PACE) using xylan-specific glycosylhydrolases (GHs) (10). The AIR was first deesterified and solubilized with 4 M NaOH, and then digested to completion with excess GH11 β(1,4)-xylanase. Hydrolysis of WT stem AIR released Xyl and $(Xyl)_2$, and [Me]GlcA$(Xyl)_4$ (24) (Fig. 2*A*). Notably, the hydrolysis of *gux1* AIR showed a clear decrease in the intensity of the [Me]GlcA$(Xyl)_4$ band, suggesting the mutant contained reduced [Me]GlcA substitution of the xylan [GlucUronic acid substitution of Xylan (GUX)]. To investigate the modified xylan phenotype further, the frequency of [Me]GlcA substitution of Xyl was estimated by PACE in two alleles of both *gux1* and *gux2* mutants. Xyl, $(Xyl)_2$, and [Me]GlcA$(Xyl)_4$ oligosaccharides released by xylanase digestion were quantitated (Fig. 2*B*). The *gux1* mutants showed a reduction to ~30% of WT frequency of [Me]GlcA substitution. The *gux2* mutants also showed a small but significant reduction to about 80% of WT. We next made double *gux1 gux2* mutants to investigate the combined role of GUX1 and GUX2 in xylan substitution. Even though xylanase digests of AIR released Xyl and $(Xyl)_2$ and some $(Xyl)_3$ indicating xylan was present in the *gux1 gux2* mutants, the [Me]GlcA$(Xyl)_4$ oligosaccharides were almost undetectable (Fig. 2*A*). Previous work has shown that, in some species at least, GlcA side chain addition is closely coupled with xylan backbone synthesis (17, 25). However, quantitation of xylanase-released oligosaccharides indicated that there was no difference in the amount of xylan backbone between WT, single and double *gux* mutants despite the reduction in side chain addition (Fig. 2*C*). Next, the monosaccharide composition of noncellulosic polymers of WT and *gux1 gux2* mutants was analyzed (Fig. 2*D*). Consistent with the finding that the xylan backbone synthesis was unaltered, the neutral monosaccharide composition including Xyl was not significantly changed. In contrast, GlcA was significantly reduced in *gux1 gux2*. The reduction is consistent with loss from GX, and remaining GlcA may arise from pectin, arabinogalactan proteins, and primary cell wall glucuronoarabinoxylan. In all GX synthesis mutants studied to date, the GlcA is partially or entirely replaced by MeGlcA, but together the proportion of Xyl residues substituted in the xylan is unaltered at ~10% (8–10, 12–14). To investigate whether the reduction in [Me]GlcA substitution in the *gux* mutants is due to loss specifically of MeGlcA or GlcA, deuter-

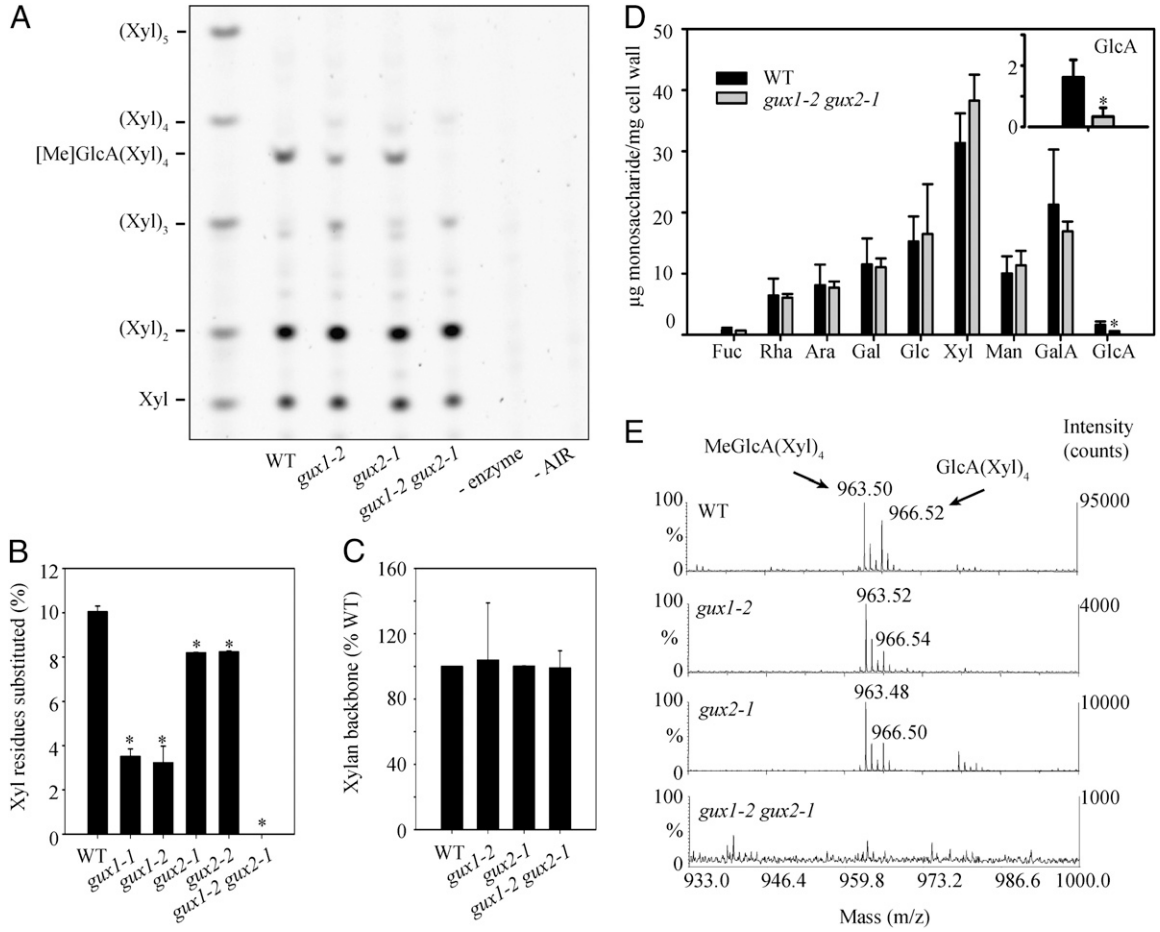

Fig. 2. Xylan structure and quantity in stem of WT and *gux* mutant plants. (*A*) AIR from WT, *gux1*, *gux2,* and *gux1 gux2* stems was digested with xylanase NpXyn11A and analyzed by PACE. (*B*) Quantification of Xyl residues in the xylan backbone substituted with [Me]GlcA by PACE as described in *A*. (*C*) Quantification of the xylan backbone by PACE as described in *A*. (*D*) Monosaccharide analysis of WT and *gux1 gux2* stem. AIR was hydrolyzed to constituent monosaccharide sugars using TFA and analyzed by HPAEC-PAD. (*Inset*) Adjusted scale for GlcA. (*E*) MALDI-TOF MS analysis of xylan structure. Oligosaccharides produced by NpXyn11A digestion of stem AIR were deuteropermethylated. Note the different intensity scales in *E*. All data are from at least three independent biological experiments. (Error bars represent SD.) *Significantly different from WT, $P < 0.05$, Student *t* test.

opermethylated xylanase-released oligosaccharides were analyzed by MALDI-TOF (Fig. 2*E*). Although GlcA appears slightly reduced relative to MeGlcA in *gux1* and in *gux2* single mutants, both types of substitution were affected in each mutant. Consistent with the PACE analysis, both GlcA- and MeGlcA-substituted oligosaccharides were scarcely detectable in *gux1 gux2*. Together, these analyses suggest that GUX1 and GUX2 are responsible for almost all of the substitution of the xylan backbone in Arabidopsis stem GX, and perhaps the remaining GX substitution could arise from the activity of the three other GUX family members.

GUX Proteins Are Required for Xylan GuxT Activity. The absence of [Me]GlcA substitution of xylan in *gux1 gux2* suggests that GUX1 and GUX2 are required for substitution of the β(1-4)-Xyl backbone. A nonradioactive assay was developed to detect GuxT activity in stem microsomes, using UDP-GlcA and the acceptor molecule $(Xyl)_6$ labeled at the reducing end with the uncharged fluorophore 2-aminoacridone (AMAC). In this assay, products containing both a negative charge from GlcA and the fluorescently labeled acceptor molecule are separated and visualized in a polyacrylamide gel, whereas uncharged oligosaccharides such as the acceptor $(Xyl)_6$ are unable to enter the gel. With WT microsomes, a ladder of charged oligosaccharides was produced, putatively $GlcA(Xyl)_n$, from GuxT in combination with xylosyltransferase (XylT) extending the $(Xyl)_6$-AMAC acceptor (Fig. 3*A*). However, the GuxT activity was strongly reduced in the double mutant (Fig. 3*A*). The oligosaccharides appeared in a time-dependent manner and required the presence of WT microsomal protein and $(Xyl)_6$-AMAC acceptor, and it was stimulated by UDP-GlcA (Fig. S5 *A* and *B*). A small amount of product was formed in the absence of exogenous UDP-GlcA, perhaps from nucleotide sugars in the microsome preparation. UDP-Xyl inclusion in the assay was not necessary (Fig. S5*C*), as it arises by conversion of UDP-GlcA to UDP-Xyl in the Golgi (26). The activity of XylT was unaffected in the *gux1 gux2* mutant (Fig S5 *E* and *F*). To confirm that the oligosaccharides were $GlcA(Xyl)_n$-AMAC, we showed that they were hydrolyzed by β(1,4)-xylanase (Fig. 3*B*). The oligosaccharides were also sensitive to β(1,4)-xylosidase, indicating that they have unsubstituted β-Xyl residues at their nonreducing end. The bands were insensitive to a GH67 α-glucuronidase, confirming that they did not have GlcA at the nonreducing end of the oligosaccharide (Fig S5*D*). Importantly, the oligosaccharides disappeared after treatment with α-glucuronidase plus β(1,4)-xylosidase confirming their charge was from GlcA on a xylan oligosaccharide (Fig. 3*B*). Together, these data indicate that *gux1 gux2* stem microsomes have strongly reduced ability to transfer GlcA from UDP-GlcA onto xylooligosaccharide acceptors.

Absence of Sugar Substitution Alters Xylan Properties and Interaction with Other Wall Components. The *gux1 gux2* plants provide a unique opportunity to investigate the biological role of the [Me]GlcA

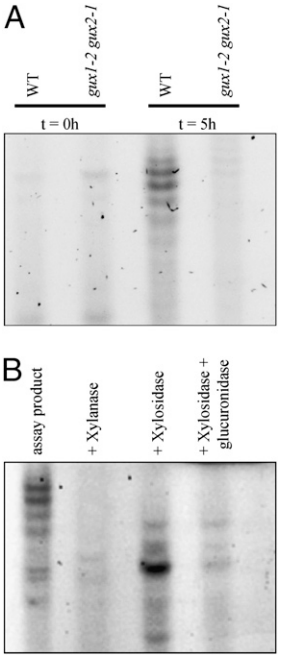

Fig. 3. GuxT activity in WT and *gux1 gux2* stem microsomes. (*A*) Microsomes were incubated with fluorescently labeled acceptor [AMAC-$(Xyl)_6$] and UDP-GlcA. Resulting charged assay product was separated by electrophoresis. (*B*) GuxT assay product characterization. Assay product from WT microsomes was digested with NpXynIIA, β-xylosidase, or β-xylosidase and α-glucuronidase together, and the product analyzed by electrophoresis.

substitution on xylan deposition and interaction with other wall components. Despite the altered xylan structure, there was no observable growth or developmental phenotype in single or double *gux* mutants (Fig. S4*C*). Unlike other xylan synthesis mutants which show collapsed xylem vessels (8–10, 12–14), microscopic analysis of *gux1 gux2* mutant stem sections revealed predominantly normal xylem (Fig. S6 *A* and *B*). Immunofluorescence labeling with two antixylan antibodies confirmed that xylan was present throughout the cell walls in all of the mutants (Fig. S6*B*). Interestingly, labeling was stronger in the double mutants than the WT, suggesting that more epitopes were accessible to the antibodies. The cell walls appeared normal by transmission electron microscopy (TEM) (Fig. S6*B*), and the thickness was not significantly different (WT, 0.236 ± 0.016 μm; *gux1 gux2*, 0.240 ± 0.014 μm; mean ± SEM, n = 38–40). A four-point bending test provided a quantitative assessment of strength of the cell walls in stems, and showed that the double mutant was slightly weaker than WT (Fig. S6*C*). Together these data indicate that despite some reduction in strength, the development and function of the secondary cell walls are largely unaffected by the absence of branching on the xylan.

To investigate any changed interaction between the xylan and other cell wall components, polysaccharides were sequentially chemically extracted from stem AIR. The solubilized polysaccharides were digested with the GH11 xylanase, and analyzed by PACE (Fig. 4*A*). As expected, only trace amounts of xylan were released in the 1,2-cyclohexanediaminetetraacetic acid (CDTA) and Na_2CO_3 fractions, which solubilize mainly pectic polysaccharides. In WT, although the majority of xylan was solubilized by 1 M KOH, a substantial amount was found in the 4 M KOH fraction, and in the residue (Fig. 4*A*). In contrast, nearly all of the xylan in *gux1 gux2* was extracted by 1 M KOH. Monosaccharide analysis of these fractions confirmed that the xylan in *gux1 gux2* was much more easily extracted (Fig. 4*B* and Fig. S7). Interestingly, the solubility at neutral pH of alkali-extracted xylan from the WT and mutants was very different: whereas GX is normally soluble, the unsubstituted *gux* xylan precipitated (Fig. 4*B*). Finally, it is recognized that GX substituents can inhibit the access of hydrolytic enzymes to the xylan backbone, and the glycosidic bond between [Me]GlcA and Xyl is resistant to acid hydrolysis. Thus, after acid hydrolysis or saccharification, oligosaccharides with substitution can remain. Therefore, this substitution can directly prevent complete release of pentose monosaccharide for fermentation. We measured release of Xyl, using a simple enzyme mixture of a GH11 xylanase and β-xylosidase, from WT and *gux1 gux2* AIR after solubilization with alkali. Unlike WT xylan with [Me]GlcA substitution, all of the xylan in *gux1 gux2* could be hydrolyzed to monosaccharide sugar in the presence of two enzymes, more than doubling the yield of free xylose (Fig. 4*C*). The xylan in the *irx9* and *irx14* xylan backbone synthesis mutants have unaltered substitution frequency (10), and therefore show little difference in hydrolysis to the WT xylan.

Discussion

[Me]GlcA Substitution of Xylan Is Not Required for Xylan Synthesis and Deposition in Cell Wall. We have identified two Arabidopsis mutants, *gux1* and *gux2*, that show a large reduction in [Me]GlcA substitution of xylan, and the *gux1 gux2* double mutant shows almost undetectable sugar decoration. Discovery of these mutants has important implications for our understanding of GX synthesis and function and also for the industrial use of xylans.

Reduced branching of xylan with GlcA and MeGlcA in the *gux* mutants is supported by several lines of evidence. PACE revealed the absence of substituted oligosaccharides released by xylanase digests, which was confirmed by MALDI-TOF MS analysis. Moreover, [Me]GlcA were reduced in monosaccharide

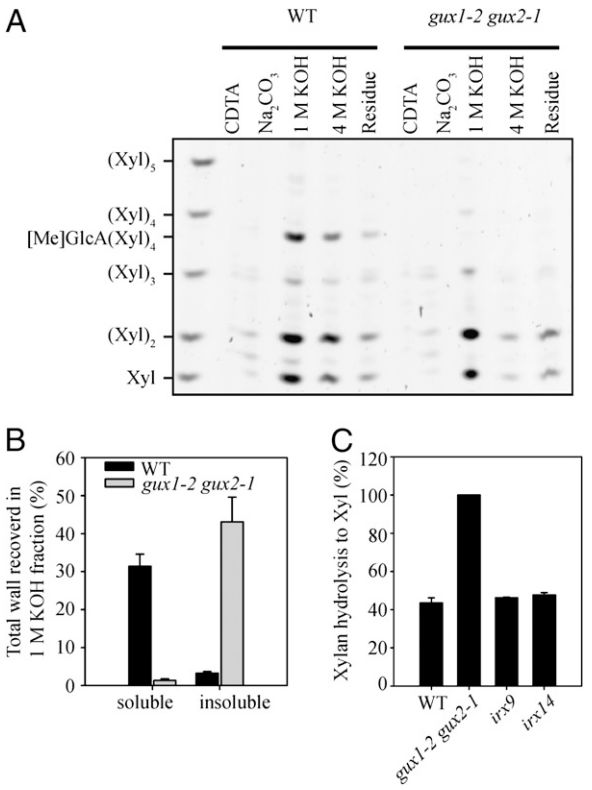

Fig. 4. Properties of WT and *gux1 gux2* xylan. (*A*) WT and *gux1 gux2* AIR was sequentially extracted using CDTA, Na_2CO_3, 1 M KOH, and 4 M KOH. Resulting fractions were analyzed by PACE using NpXyn11A. (*B*) AIR sequentially extracted as in *A*, hydrolyzed to monosaccharide sugars using methanolic HCl, and quantified using GC. Total sugars quantified in 1 M KOH soluble and insoluble fractions (of which the majority are xylan) are shown (complete data in Fig. S7). (*C*) Enzymatic hydrolysis of xylan to Xyl. WT, *gux1 gux2*, *irx9*, and *irx14* AIR were hydrolyzed with NpXyn11A and β-xylosidase and quantified using PACE. Released Xyl monosaccharide is expressed as percent weight of enzyme-hydrolyzed xylan. Error bars represent SD (n = 3).

analysis after acid hydrolysis of cell wall fractions. We could detect significant changes in [Me]GlcA substitution in the single *gux1* and *gux2* mutants, suggesting the GUX1 and GUX2 proteins cannot entirely compensate for each other. The substitution was reduced by approximately two-thirds in *gux1* and by one-third in *gux2*, and was entirely lost in the double mutant. We can rule out the hypothesis that they are each responsible for one type of substitution, as both MeGlcA and GlcA are affected in each single mutant, with little alteration in ratio. The *gux* phenotype is unlike other described xylan synthesis mutants, which have an increased MeGlcA:GlcA ratio but a strictly maintained substitution frequency (8–14). The altered ratio is therefore linked to a decreased rate of xylan backbone synthesis in those mutants, not the quantity of branches. It is unclear how these two different substitutions arise. One hypothesis is that a methyltransferase with limited activity methylates a proportion of GlcA substitutions after GX synthesis (27). However, in the *gux* mutants described here, there are fewer sites for methylation on the GX substrate, but methylation is not increased. This suggests that the methylation mechanism is more complicated and linked to backbone elongation (19).

The specific loss of [Me]GlcA branches of xylan in the mutants, without apparent other effects on xylan synthesis, suggests that the GUX proteins are excellent candidates for the xylan GuxTs. This interpretation is strongly supported by the loss of GuxT activity in the *gux1 gux2* mutant. Moreover, the proteins are localized in the Golgi apparatus where xylan synthesis occurs. A previous GuxT candidate, FRA8/IRX7, has now been shown to be required for synthesis of the xylan reducing end oligosaccharide (9, 28). From our analysis of secondary wall coexpressed CAZy GTs, there are no further apparent candidates in Arabidopsis. The known activities and mechanism of related proteins in the GT8 retaining family indicate that catalysis of an α(1,2)-GlcA linkage with UDP-GlcA as a donor is a possible activity of the protein. Indeed, a related enzyme in GT8 (GAUT1) has been shown to have pectic α(1,4)-GalAT activity (29). Confirmation of this hypothesis could involve establishment of a heterologous assay. Our attempts to achieve this using heterologously expressed GUX proteins in insect or tobacco cells, like those of all of the other putative GTs involved in xylan synthesis, have thus far been unsuccessful. Perhaps this is because the enzymes act indirectly to regulate GuxTs, or they may not function alone, but work as GuxTs in a protein complex with other components of the xylan synthesis machinery (9, 10, 12).

There is some evidence from in vitro assays that GlcA addition and xylan backbone extension may be coupled, as UDP-Xyl can stimulate the GuxT activity in wheat and pea (17, 25), although this has not been observed in all assays and species examined (11, 30). Such coupling of backbone synthesis and substitution is also observed, for example, in xyloglucan synthesis (31). It is therefore surprising that in the *gux1 gux2* mutants, despite the loss of GuxT activity, there was no change in quantity of xylan in the walls. This suggests that xylan chain extension and substitution are not obligatorily coupled during synthesis, and also demonstrates that substitution is not required for xylan solubility, transport, or insertion into the cell wall. It is therefore probable that acetylation of the xylan keeps the polysaccharide soluble during synthesis and transport.

Xylan Lacking Sugar Substitution Confers Altered Lignocellulose Properties and Improved Sugar Release. All xylans isolated thus far from land plants are decorated with additional sugars, most frequently of MeGlcA, GlcA, or Ara, suggesting an important role for branching. The roles of the branches could include structural interactions with other cell wall components to provide strength, and slowing the attack on cell walls by pathogenic microorganisms. Microscopic analysis of stem sections and cell walls of the *gux* mutants revealed that there was no visible change in vascular development or cell wall morphology. Stems were detectably weaker in a bending assay, but this was not nearly as severe as xylan backbone or cellulose synthesis mutants (9, 28, 32). Xylan backbone mutants show collapsed xylem vessels when xylan is reduced by less than one-third, such as in *irx10* and *irx14*, and severe dwarfing is seen if the xylan backbone quantity is reduced further (9, 10, 12, 13). The absence of collapsed xylem or growth reduction in the *gux* mutants shows that xylan without sugar substitution can fulfill much of the structural role of the polysaccharide. The finding raises the prospect that attempts to alter other xylan substitution, such as Ara and Feruloyl-Ara in grasses to reduce crosslinking to lignin, may be successful. Any consequential effect of *gux* mutations on crop resistance to pathogens is difficult to predict. Although greater pathogen susceptibility of plants with simplified cell walls might be predicted, cell wall mutants can show increased resistance (33).

Xylan branches influence incorporation into the lignocellulose. It has been proposed that xylan is covalently attached to lignin through esters to the [Me]GlcA (2, 3). The branches also alter the interaction of the polymer with cellulose, and with other xylan chains (34). Antixylan antibodies revealed that the xylan epitopes are incorporated normally across the entire secondary cell wall of the mutants, and also that the xylan may be more accessible. Altered interaction with other cell wall components may partly explain the change in stem strength. We demonstrated that the xylan in the *gux* mutants was more completely extracted from the cell wall material with 1 M alkali than xylan from the WT cell walls. This extracted unbranched xylan also shows remarkably different solubility properties to GX. Because it is uncharged, it is insoluble at neutral pH. The modification of cell wall properties seen in the *gux* mutants may be beneficial for cellulose and fiber extraction in paper and pulp manufacture, as wood with xylan lacking sugar substitution may be less expensive and easier to process; and the treatment, being less harsh, may yield better-quality cellulose fibrils. In addition, hexenuronic acid would not be generated during pulping, as MeGlcA is absent from the material. Because the double *gux* mutant lacking any sugar decoration on the xylan shows some stem weakness, it may be preferable to obtain plants with intermediate levels of MeGlcA, with proportions optimized for the specific application of the feedstock.

Fermentable sugar release from lignocellulose can be increased by reducing xylan branching. First, the sugar decorations prevent breakdown of xylan to Xyl by simple enzyme mixtures. Although some microbes are able to metabolize Xyl from short oligosaccharides containing [Me]GlcA, yeast is not able to do so (35, 36). The α(1,2)-glycosidic bond between [Me]GlcA and Xyl is the most acid-stable linkage in woody material (37), necessitating α-glucuronidase in the mixture to release all of the Xyl. Enzymes represent a significant cost in lignocellulosic biomass processing into ethanol, and reduction in their usage could have a large economic impact on biofuel production (4). Second, few microbes are able to metabolize MeGlcA, which is 15% of the mass of GX. The absence of MeGlcA in *gux* lignocellulose would increase the proportion of fermentable sugar in feedstocks. Together, the changes to xylan structure could increase the yield of ethanol from *gux* mutant xylan to almost double that of WT.

In conclusion, mutant *gux* plants show remarkably little change in growth, but the properties of the xylan in their cells walls are substantially changed. The GUX genes encode good candidates for xylan GuxTs. These findings make an important contribution to our understanding of xylan synthesis and will allow many studies of the role of GlcA decorations on the xylan backbone. The alterations in *gux* mutants of xylan extraction properties from the lignocellulose, and in the hydrolysis of xylan by enzymes, show that alterations in crop xylan structure could be a feasible goal for the bioprocessing industry. It may become possible to alter the pattern of branching to produce xylan with properties suited to specific end uses.

Materials and Methods

Details of reagents, plant material, microscopy, XylT assay, and testing of stem strength are described in *SI Materials and Methods* and Table S1.

Bioinformatics. GTs of Arabidopsis were classified according to their pattern of expression in the public microarray dataset reported by Schmidt et al. (38), which comprises several isolated tissues and developmental stages. The ex-

pression data were downloaded from ArrayExpress (record E-TABM-17) and the set of GTs as predicted by CAZy (18) (www.cazy.org) were classified with Cluster 2.5 (39). In Cluster 2.5, classification of GTs was performed on mean centered gene profiles using centered correlation as the similarity metric. Further bioinformatics techniques are described in *SI Materials and Methods*.

AIR Preparation and Analysis. AIR was prepared and analyzed by PACE, MS, and high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described elsewhere (10, 40–42) and in *SI Materials and Methods*.

GuxT Assay. Xyl_6 (20 μg; Megazyme) was derivatized with AMAC (Invitrogen) as previously described (42) and purified using a GlykoS column (Prozyme) according to the manufacturer's instructions. The composition of the reaction mixture (60 μL) was 30 μL microsomes (~600 μg protein; prepared as described in *SI Materials and Methods*), 50 mM Hepes-KOH (pH 6.8), AMAC-$(Xyl)_6$ (68.5 mM), DTT (0.5 mM), UDP-GlcA (10 μM), $MnCl_2$ (10 mM), Triton X-100 (0.5% vol/vol), and, if used, UDP-Xyl (100 μM; CarboSource, Complex Carbohydrate Research Center, University of Georgia, Athens, GA). Samples were incubated at 21 °C with shaking. The reaction was stopped by heating (10 min, 95 °C) and lipids removed by phase separation (43). The aqueous fraction was dried under vacuum, and resuspended in 10 μL of 3 M urea. Samples (2 μL) were separated by polyacrylamide gel electrophoresis as described in *SI Materials and Methods*.

Cell Wall Fractionation and Monosaccharide Analysis. Sequential fractionation of the AIR and analysis of monosaccharide composition by GC were carried out as described elsewhere (10) and in *SI Materials and Methods*. Xyl release following digestion with β-xylosidase and NpXynIIA was quantified using PACE as described in *SI Materials and Methods*.

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