GENETIC IDENTIFICATION AND BIOCHEMICAL CHARACTERIZATION OF XYLAN BACKBONE SYNTHASES IN ARABIDOPSIS AND POPLAR

by

CHANHUI LEE

(Under the Direction of Zheng-Hua Ye)

ABSTRACT

Xylan is the second most abundant polysaccharide in plant cell walls. However, its biosynthesis including the initiation and elongation of xylosyl backbone, side chain addition, and sugar modification, is still poorly understood. In an effort to identify xylan biosynthetic enzymes, we first profiled a number of upregulated genes during the process of the secondary cell wall formation. PARVUS was specifically expressed in the fibers and vessels, and its encoding protein was localized in the endoplasmic reticulum. Mutant phenotypes and structure analysis of xylans from the parvus mutant stems clearly showed its involvement in the biosynthesis of reducing end sequence of the xylan. Recent genetic and molecular studies in Arabidopsis have shown that two proteins in glycosyltransferase (GT) 43 family, IRX9 and IRX14, are golgi-localized GTs, genetic defects of which result in a xylan deficient phenotype and a significant reduction in xylan chain length. In addition, the in-vitro activity assay with stem microsomes revealed that irx9 and irx14 mutants possess substantially lower xylosyltransferase activity compared with the wild type, suggesting their possible roles in xylan backbone polymerization. Comprehensive genetic analysis of the four Arabidopsis GT43 members provides evidence that all four Arabidopsis
GT43 members are involved in xylan biosynthesis and suggests that they form two functionally nonredundant groups essential for the normal elongation of xylan backbone. Multiple lines of evidence indicate that GTs involved in xylan biosynthesis are highly conserved between Arabidopsis and Poplar. Complementation studies revealed that PtrGT43 A/B/E are functional homologs of Arabidopsis IRX9, and PtrGT43 C/D are homologs of IRX14, forming two functionally nonredundant groups, which are required for the polymerization of xylan backbone. The studies presented here provide a wealth of genetic information that expands the knowledge of xylan biosynthesis.

INDEX WORDS: Arabidopsis, Poplar, Microsomes, Xylan, Glycosyltransferase, Xylan synthase, Secondary cell walls, Plant cell wall, Cell wall biosynthesis, parvus, irregular xylem 9 (irx9), irregular xylem 14 (irx14)
GENETIC IDENTIFICATION AND BIOCHEMICAL CHARACTERIZATION OF
XYLAN BACKBONE SYNTTHASES IN ARABIDOPSIS AND POPLAR

by

CHANHUI LEE

B.S., Chungnam National University, The Republic of Korea, 2003
M.S., Chungnam National University, The Republic of Korea, 2005

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial
Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2011
GENETIC IDENTIFICATION AND BIOCHEMICAL CHARACTERIZATION OF
XYLAN BACKBONE SYNTHASES IN ARABIDOPSIS AND POPLAR

by

CHANHUI LEE

Major Professor: Zheng-Hua Ye

Committee: Michelle Momany
           Carl M. Deom
           Xiaoyu Zhang
           Wolfgang Lukowitz

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2011
DEDICATION

To my wife and family for their love and support and my son, Hajoon Lee.
ACKNOWLEDGEMENTS

I am exceedingly fortunate to have Dr Zheng-Hua Ye as my major advisor during the past five years. He continues to influence me as a scientist and as a person. Without his willingness to help and support my projects, I would not have achieved this goal. I would like to thank all current and past members in the Ye lab, especially Ruiqin Zhong for giving me endless help and a warm heart. I would like to express many thanks to my committee members, Michelle Momany, Carl M. Deom, Xiaoyu Zhang, and Wolfgang Lukowitz, for their help, great comments and suggestions about my project. I would like to thank all departmental staff for their work, especially Susan Watkins and Kristin Meents. I would like to thank the greenhouse staff, Michael Boyd, Jeff, and Kevin Tarner for taking good care of our plants. I am also very grateful to Beth Richardson for her help and expertise on microscopic analysis and sample sectioning. All the resources of UGA, especially MALDI-TOF from Chemistry department, were great for my research and training. I would like to thank Dr Quincy Teng in EPA for NMR analysis. Finally, I acknowledge my family and friends, who provided unending love and support throughout this journey.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGEMENTS</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>v</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LIST OF TABLES</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ix</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1</td>
</tr>
<tr>
<td>INTRODUCTION AND LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>CELLULOSE BIOSYNTHESIS</td>
<td>2</td>
</tr>
<tr>
<td>XYLOGLUCAN BIOSYNTHESIS</td>
<td>4</td>
</tr>
<tr>
<td>PECTIN BIOSYNTHESIS</td>
<td>5</td>
</tr>
<tr>
<td>MANNAN BIOSYNTHESIS</td>
<td>6</td>
</tr>
<tr>
<td>LIGNIN BIOSYNTHESIS</td>
<td>7</td>
</tr>
<tr>
<td>Xylan BIOSYNTHESIS</td>
<td>9</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>16</td>
</tr>
</tbody>
</table>

| 2.      | 24   |
| THE IRREGULAR XYLEM9 MUTANT IS DEFICIENT IN XYLAN | 24 |
| XYLOSYLTRANSFERASE ACTIVITY | 24 |
| ABSTRACT | 25 |
| INTRODUCTION | 26 |
| RESULTS | 28 |
5. MOLECULAR DISSECTION OF XYLAN BIOSYNTHESIS DURING WOOD FORMATION IN POPLAR

   ABSTRACT ................................................................................................ 161
   INTRODUCTION ....................................................................................... 162
   RESULTS .................................................................................................... 164
   DISCUSSION ............................................................................................. 171
   MATERIALS AND METHODS ................................................................ 174
   ACKNOWLEDGMENTS ........................................................................... 180
   REFERENCES ............................................................................................ 180

6. CONCLUSIONS ..................................................................................................... 217

   REFERENCES ............................................................................................ 223
# LIST OF TABLES

Table 1. 1. Glycosyltransferases involved in the xylan biosynthesis of *Arabidopsis* ................. 23

Table 3. 1. Wall thickness in the stems and roots of wild-type and *parvus* ......................... 91

Table 3. 2. Monosaccharide composition of cell walls of wild-type and *parvus* plants ........ 92

Table 3. 3. Monosaccharide composition of 1 N and 4 N KOH extracts ............................... 93

Table 4. 1. Monosaccharide composition of plants overexpressing GT43 members ............ 139

Table 4. 2. Monosaccharide composition of wild type and GT43 mutants ......................... 140

Table 4. 3. Selected properties of plants overexpressing GT43 members ......................... 141

Table 5. 1. Cell wall composition of plants overexpressing Poplar GT43 members .......... 188

Table 5. 2. Selected properties of plants overexpressing Poplar GT43 members ............ 189

Table 5. 3. Cell wall composition analysis of Poplar, *PoGT43B* and *PoGT8D* RNAi lines .... 190

Table 5. 4. Selected properties of RNAi lines ................................................................. 191

Table 5. 5. Lignin composition analysis of RNAi lines ................................................ 192
LIST OF FIGURES

Figure 2. 1. Biochemical properties of the XylT activity ............................................................. 45
Figure 2. 2. Xylooligomers of different length as acceptors for the XylT activity ......................... 47
Figure 2. 3. Successive transfer of xylosyl residues onto the acceptors by the XylT activity ...... 49
Figure 2. 4. MALDI TOF mass spectrum of the XylT-catalyzed reaction products ............... 51
Figure 2. 5. Confirmation of reaction products by endo/exo-β-(1,4)-xylanase ......................... 53
Figure 2. 6. Biochemical properties of the GlcAT activity ........................................................... 55
Figure 2. 7. The XylT and GlcAT activities in different Arabidopsis organs .............................. 57
Figure 2. 8. The XylT and GlcAT activities in the stems of wild type, irx9, irx8 and fra8 .......... 59
Figure 2. 9. Deficiency in the transfer of xylosyl residues in the irx9 mutant ......................... 61
Figure 3. 1. Expression analysis of the PARVUS gene by real-time quantitative PCR ........... 94
Figure 3. 2. In situ hybridization analysis of the PARVUS mRNA ............................................ 96
Figure 3. 3. Subcellular localization of the PARVUS protein ..................................................... 98
Figure 3. 4. Stem strength and wall thickness of fibers and vessels of the parvus mutant ...... 100
Figure 3. 5. Transmission electron microscopy of secondary walls in the parvus mutant ....... 102
Figure 3. 6. Immunodetection of xylan in fibers and vessels of parvus ............................... 104
Figure 3. 7. MALDI-TOF mass spectra of acidic xylooligosaccharides ................................. 106
Figure 3. 8. Anomeric region of the 1H-NMR spectra of xylooligosaccharides ...................... 108
Figure 3. 9. The XylT and GlcAT activities of the wild type and parvus mutant ...................... 110
Figure 4. 1. Sequence comparison and expression analysis of family GT43 members .......... 142
Figure 4. 2. Expression patterns of I9H, IRX14, and I14H ................................. 144
Figure 4. 3. Subcellular localization of I9H, IRX14, and I14H ............................... 146
Figure 4. 4. Complementation of the irx9 and irx14 mutants ............................... 148
Figure 4. 5. MALDI-TOF mass spectra ................................................................. 150
Figure 4. 6. 1H-NMR spectra ............................................................................... 152
Figure 4. 7. Time course of the xylosyltransferase activity ...................................... 154
Figure 4. 8. Phenotypes of single and double mutations of GT43 members .............. 156
Figure 4. 9. Transmission electron micrographs of the cell walls .............................. 158
Figure 5. 1. Phylogenetic and expression analysis of family GT43 members in Populus .... 193
Figure 5. 2. Subcellular localization of PtrGT43 proteins ......................................... 195
Figure 5. 3. Complementation analysis of PtrGT43 members ..................................... 197
Figure 5. 4. Transmission electron microscopic analysis of secondary walls .......... 199
Figure 5. 5. MALDI-TOF mass spectra of acidic xylooligosaccharides ..................... 201
Figure 5. 6. 1H-NMR spectra of acidic xylooligosaccharides .................................... 203
Figure 5. 7. Time course of the xylosyltransferase activity ........................................ 205
Figure 5. 8. Effects of RNAi downregulation of expression of PoGT43B and PoGT8D .... 207
Figure 5. 9. Transmission electron microscopic analysis of the wood ....................... 209
Figure 5. 10. Immunodetection of xylan in the wood ............................................... 211
Figure 5. 11. 1H-NMR spectra of xylooligosaccharides ............................................ 213
Figure 5. 12. Glucose release by cellulase enzymes .................................................. 215
CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

One of the most distinctive features of plant cells is the deposition of walls outside the plasma membrane during cytokinesis. Since the structural framework of cell walls is mostly composed of a mixture of different polysaccharides, the final architecture of cell walls requires several enzymatic reactions including nucleotide sugar interconversion, polysaccharide biosynthesis, transport, and their assembly. Also, given the fact that the composition and amount of cell walls vary among cell types, the enzymatic reactions should be temporally and spatially controlled for the proper deposition and assembly of cell wall components in order for each cell type to perform its functional role. The cell wall is classified into two types, primary and secondary cell walls, primarily based on its location and polysaccharide composition. Whereas the primary cell wall is mainly composed of cellulose, xyloglucan, and pectin, thick secondary wall is made of cellulose, xylan, mannan, and lignin. With the exception of lignin, all other polymers are synthesized by the actions of glycosyltransferase (GT) that catalyze the attachment of activated donor sugars onto the acceptor substrates. *Arabidopsis* genome contains 445 GTs classified into 40 different families based on amino acid sequence similarity (CAZy; www.cazy.org/). Only a few of them are biochemically characterized in detail. Forward/reverse genetic studies demonstrated that mutation of some of GTs in several species resulted in the wide range of developmental phenotypes, including collapsed xylem, dwarfing, and sterility.
CELLULOSE BIOSYNTHESIS

Cellulose, β-1,4-linked glucose polymer without any substitution, is the most abundant biopolymer produced by vascular plants (Taylor, 2008). Cellulose in the cell wall of higher plants exists as a form of crystallized microfibrils, self-assembled and hydrogen-bonded β-1,4-glucan chains, to serve as a load-bearing network in which other hemicelluloses and pectin are cross-linked for the formation of rigid wall layers. Cellulose synthesizing machinery is located at the plasma membrane, where β-1,4-glucan chains are produced and coalesced to form microfibrils. This machinery has been visualized by freeze fracture electron microscopy and appeared as a hexameric rosette with a diameter of 25-30 nm (Kimura et al., 1999). Cellulose synthase (CesA) proteins originally identified from cotton cDNA on the basis of the sequence similarity with bacterial cellulose synthase belong to GT2 family with an inverting mechanism (Somerville, 2006). Genomic analysis indicate that there are 10 CesA genes in Arabidopsis, 12 in rice, 12 in maize, and 18 in Populus (Burn et al., 2002). All CesA proteins have a zinc-binding domain at the N-terminal and this domain is putatively responsible for protein-protein interaction. Genetic and molecular studies in Arabidopsis have provided the evidence that there are two different groups of CesA proteins required for the cellulose synthesis of primary (CesA1, CesA3, and CesA6) and secondary cell wall (CesA4, CesA7, and CesA8) (Mutwil et al., 2008). Immunoprecipitation experiment revealed that three secondary cell wall-specific CesA proteins appeared to form protein complex (Taylor et al., 2003). Current understanding of the processes of cellulose biosynthesis has been mainly obtained from forward/reverse genetics and transcript analysis. However, several aspects regarding biochemistry of cellulose biosynthesis still remain elusive.
Mutant complementation analysis and domain swapping studies have led to the conclusion that three non-redundant CesA genes are required for correct cellulose synthesis (Taylor, 2008). As seen in secondary-cell wall specific CesA proteins, interaction among three CesA proteins is essential for normal cellulose biosynthesis, although the interaction among primary cell wall CesA proteins has not been experimentally verified (Tanaka et al., 2003). Recent biochemical approach employing dual tagging system (Atanassov et al., 2009) has enabled successful purification of cellulose synthase complex from secondary cell wall and demonstrated that this complex is solely composed of three known secondary cell wall specific CesA proteins (CesA4, 7, and 8). Thus, it is highly plausible that the backbone chain polymerization of cellulose requires only three CesA proteins. However, the mechanism and enzymatic proteins involved in the initiation and termination of cellulose biosynthesis are poorly understood. Early biochemical analysis has failed to detect cellulose synthase activity in microsomes isolated from actively cellulose-synthesizing tissues (Somerville, 2006). Instead, a \( \beta-1,3 \)-linked wound-response polysaccharide, callose, has been detected. Peng et al. reported the biochemical evidence in cotton fiber that the lipid sitosterol-\( \beta \)-glucoside acts as a primer where glucose polymerization is initiated by CesA proteins (Peng et al., 2002). In this model, CesA proteins or unknown UDP-glucosyl transferase transfer glucose residues onto the lipid sitosterol-\( \beta \)-glucoside, forming lipid-linked oligosaccharides. The primer residue linked to oligo-glucose is then cleaved by Korrigan cellulase (Zuo et al., 2000). In vivo and in vitro experimental proofs are still lacking. Sucrose synthase and UDP-glucose pyrophosphorylase-mediated pathways have been shown to be essential for the direct production of UDP-glucose (Mutwil et al., 2008). Taken together, most, if not all, of genes involved in cellulose biosynthesis have been identified, and mutation of each gene results in the severe reduction of cellulose quantity, thereby leading to the
abnormal phenotypes in a number of developmental processes, particularly in vascular tissues. However, there is still huge lack of information regarding the detailed mechanism of its biosynthesis, including initiation, polymerization, and termination of glucan chains.

**XYLOGLUCAN BIOSYNTHESIS**

Xyloglucan in dicot plants is a hemicellulosic polysaccharide abundant only in primary cell wall, which is associated with cellulose microfibrils (Lerouxel et al., 2006). The association and dissociation between cellulose microfibrils and xyloglucan are developmentally regulated and appear to play the critical role during cell expansion. Xyloglucan has a main backbone of β-(1,4)-linked glucose and a side chain of α-1,6-linked xylose (Hoffman et al., 2005). Occasionally, the side chain is further substituted with α-(1,2)-fucose-β-(1,2)-galactose onto xylose residue. The pattern of side chain substitution contributes to the diversity of xyloglucan structure in different species and tissues. All the glycosyltransferases (GTs) responsible for the formation of XG side chain have been identified and biochemically examined (Lerouxel et al., 2006).

Xyloglucan fucosyltransferase (XG FTase) has been initially isolated by protein purification from pea epicotyls, and activity assay of its *Arabidopsis* homolog (*AtFut1*) heterologously expressed in mammalian COS cells also showed XG FTase activity (Faik et al., 2000, 2002; Perrin et al., 1999). The biochemical data have been substantiated by the identification of *mur2* mutant that is deficient of XG fucose (Vanzin et al., 2002). Because this mutant is almost devoid of fucosylated xyloglucan found in the wild type, *AtFUT1/MUR2* has been proposed as a principle XG FTase responsible for all XG fucosylation. MUR3 belonging to GT47 family has been shown to be a XG galactosyltransferase (GTase) (Madson et al., 2003). Structural analysis of XG from *mur3* mutant revealed the absence of two sugars (Galactose, Fucose) found in XG side chain, and activity assay of MUR3 expressed in *Pichia pastoris* demonstrated the enzymatic
activity that transfers galactose onto mur3-derived XG. XG xylosyltrasferase (XTase) in *Arabidopsis* has been identified on the basis of the sequence similarity with fenugreek XTase, belonging to GT34 family. Heterologous expression of AtXT1 in *P. pastoris* confirmed that AtXT1 catalyzes the transfer of xylose form UDP-xylose onto β-1,4-linked glucose oligosaccharide acceptors (Faik et al., 2002). It has been predicted that xyloglucan backbone synthase might be a cellulose synthase like (CSL) protein due to the same structure of β-1,4-linked glucose polymer. This prediction coupled with transcriptional profiling of intensively XG-synthesizing nasturtium seed has led to the identification of XG backbone synthase in *Arabidopsis* (*AtCSLC4*), a member of CSLC gene (Cocuron et al., 2007). Although in-vitro activity of expressed AtCSLC4 protein has not been demonstrated, the observation that the β-1,4-linked glucose is produced in AtCSLC4 expressing *P. Pastoris* provides a strong evidence that AtCSLC4 is responsible for XG backbone synthase.

**PECTIN BIOSYNTHESIS**

Pectin is a structural component in plant cell walls as a galacturnonic acid (GalA)-rich polysaccharide, and its specific deposition in primary cell wall and the middle lamellar has been known to be associated with a number of growth/development-related processes as well as plant defense (O’Neill et al., 2004). Pectin is notorious for the most complex polymer whose biosynthesis requires at least sixty-seven enzymes. In general, pectin is structurally divided into three subclasses, homogalacturonan (HG), rhanogalacturonan I (RG-I), and -II. HG is a homopolymer of α-1,4-linked GalA, RG-I has a repeating backbone of α-1,2-Rha-α-1,4-GalA substituted with several number of oligosaccharides, and RG-II is the most complex pectin with at least eight α-1,4-linked GalA backbone decorated with twelve different sugars in different sugar linkages (Ridley et al., 2001; Mohnen, 2008). Pectin is synthesized, modified (methylation,
acetylation), and assembled in the Golgi as other hemicelluloses, but the detailed mechanism regarding biosynthesis and assembly is poorly understood since only few genes have been identified. Protein purification followed by peptide sequencing has led to the functional identification of an Arabidopsis pectin HG galacturonosyltransferase (AtGAUT1) that catalyzes the transfer GalA from UDP-GalA onto HG acceptors (Sterling et al., 2006). Two homologous genes in Arabidopsis, RGXT1 and RGXT2 belonging to GT77 family, have been identified, and enzyme activity assay with soluble forms of both proteins expressed in insect cells confirmed that both encode α-1,3-xylosyltrasferase that transfers xylose onto fucose, a linkage only found in RG-II side chain (Egelund et al., 2006). Increasing number of genomic/proteomics information together with availabilities of diverse nucleotide sugars and acceptor oligosaccharides will greatly advance our understanding of pectin biosynthesis (Mohnen, 2008).

MANNAN BIOSYNTHESIS

Mannan polysaccharides account of less than 5 % of the dry mass of plant cell walls (Schroder et al., 2001). However, this polymer is the major non-cellulosic component in gymnosperm secondary cell wall (Popper, 2008). Structurally diverse mannan polysaccharides are found in the plant cell walls. The backbone of mannans and galactomannans is solely composed of β-(1,4)-linked mannose whereas mannose and glucose in a nonrepeating pattern have been identified in the backbone of galacto/glucomannans. The mannosyl residues in the backbone are substituted with α-(1,6)-linked galactose in galactomannans and galactoglucomannans. A significant amount of galacto/mannans is present in the seed of some legumes and palms, serving as storage carbohydrates (Buckeridge et al., 2000). Relatively small quantities of glucomannans exist in secondary cell walls of dicot plants and appear to associate non-covalently with cellulose microfibrils (Schroder et al., 2001). Three different
glycosyltransferases are required to complete the biosynthesis of mannan polysaccharides, two backbone synthases (mannan synthase and glucomannan synthase) and a galactosyltransferase for side chain addition. First enzyme identified was a galactosyltransferase from developing fenugreek seed endosperms, in which glucomannans are actively synthesized and accumulated (Edwards et al., 1999). Microsomes isolated from the fenugreek seed showed higher activity of galactosyltransferase onto exogenous mannose oligosaccharides. After protein sequencing, a gene encoding GT34 was heterologously expressed in *Pichia pastoris* and transgenic yeast exhibited high activity of galactosyltransferase. Mannan synthase first isolated from guar seeds is one of the Cellulose Synthase-Like A (CSLA) family (Dhugga et al., 2004). Later, systematic characterization of the biochemical function of a group of CSLA genes in *Arabidopsis* and rice by expressing in *Drosophila* Schneider 2 cells has revealed that CSLA2, 7, and 9 genes possess both mannan and glucomannan synthase activities (Liepman et al., 2005, 2007; Burton et al., 2006; Suzuki et al., 2006; Doblin et al., 2009). Also, several members of CSLA in Poplar were shown to encode both activities, suggesting conservation of enzymatic players of mannan biosynthesis in diverse plant species.

**LIGNIN BIOSYNTHESIS**

Lignin is a complex phenolic polymer primarily found in secondary wall-containing cell types such as vessels and fibers (Zhong and Ye, 2009). The presence of lignin imparts on cell walls the resistibility to both mechanical disruption and the action of cell wall hydrolases (Ralph, 2004). In addition, lignin is impregnated in cellulose and hemicelluloses matrix, providing increased mechanical strength and hydrophobicity. The biosynthesis of lignin is one of the most intensely characterized biosynthetic pathways in plant because of its abundance and economical importance (Boerjan et al., 2003). Lignin is a heteropolymer mainly made of three monolignols,
$p$-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol produced by the phenylpropanoid pathway. After synthesis in the cytoplasm, these monolignols are exported to the extracellular space and subsequently incorporated into the polymer by enzyme-mediated processes that remain poorly understood. Upon formation of the lignin polymer, these monolignols are referred to as final phenylpropanoid units, p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin. The abundance and distribution of lignin vary among plant species and cell types. In general, G and S units are mainly found in angiosperms, whereas lignin of gymnosperms, ferns, and lycophytes is composed of G unit (Campbell and Sederoff, 1996; Zhong and Ye, 2009). Also, lignin polymer contains non-monolignol units, including ferulates, $p$-hydroxyphenyl, and coniferaldehyde (Ralph, 2004). The incorporation of ferulates to the lignin polymer contributes to the formation of crosslinks between lignin and hemicelluloses in secondary cell walls.

The overall reaction to synthesize three monolignols requires the sequential action of ten enzymes, which is well characterized (Boerjan et al., 2003; Rogers and Cambell, 2004)). Deamination of phenylalanine to cinnamic acid is mediated by an ammonia lyase (PAL). Following deamination, a cytochrome P450-dependent monooxygenase (C4H) hydroxylates the aromatic ring of cinnamic acid to form $p$-coumarate, which is then converted to $p$-coumaroyl CoA by an ATP-dependent CoA ligase (4CL). $p$-coumaryl alcohol is then produced from $p$-coumaroyl CoA by two oxidoreductase-mediated successive reductions (CCR and CAD). Coniferyl alcohol is synthesized by further enzymatic reactions including hydroxylation by C3’H, methylation by CCoAOMT, and finally two oxidoreductases (CCR and CAD) of each intermediate. Coniferaldehyde, an intermediate during coniferyl alcohol synthesis is subjected to another hydroxylation by F5H, resulting in the formation of 5-Hydroxyconiferaldehyde. By the sequential actions by a methyltransferase (COMT) and CAD, sinapyl alcohol is produced. All of
genes involved in the monolignol biosynthesis have been identified and biochemically characterized (Humphreys and Chapple, 2002; Rogers and Cambell, 2004; Vanholme et al., 2008). A diverse range of phenotypes including collapsed xylem, dwarfing, and developmental arrest at the early stage has been observed in the lignin-deficient mutants.

**XYLAN BIOSYNTHESIS**

Xylan is a predominant cell wall polymer in all higher plants, as well as some algae (Ebringerová and Heinze, 2000). Its tight association with cellulose microfibrils makes the whole framework of cell walls much stronger. Also, it has been reported that xylan is linked with lignin, pectin, and other hemicelluloses (Barakat et al., 2007). A recent investigation of lignin and carbohydrate complex formation revealed that, during cell wall assembly, non-covalent linkages are formed between xylan and α-carbon of the lignin monomer, and this interaction results in the formation of hydrophobic microdomain. The occurrence and structural diversity of xylan among species, and even in different tissues, indicate its importance in a range of fundamental processes of adaptation and development. Although most of the xylan found in plants commonly possess a backbone of β-(1,4)-linked D-xylosyl (Xyl) residues, several structural varieties primarily derive from different distributions and compositions of the side chain and any functional group substitution (Ebringerová and Heinze, 2000). The existence of side chains might be required to prevent the xylan polymer from self-assembling. Typically, xylan present in a primary cell wall is substituted with arabinose (Ara) and glucuronic acid (GlcA), whereas in a secondary cell wall, xylan has methyl GlcA and/or GlcA as a side chain.

The structure of xylan has been intensively studied in the past several decades because of its biological importance to plant development and commercial usefulness of xylan-derived products for humans and animals (Ebringerová and Heinze, 2000; Ebringerová, 2006).
Deacetylated xylan polymers can be isolated by alkali solution (NaOH and KOH), and a mild hydrolysis or xylanase treatment generates oligoxylosaccharides, that are subject to diverse analytical processes to identify the fine chemical structure. Since an alkali treatment can remove most of the acetyl groups heavily attached to xylosyl residues at O-3 and/or O-2, steam, thermochemical, and microwave oven treatments have been developed, and acetylated xylan has been successfully isolated from some tree species. With the exception of homoxylan, which is only found in some algae and whose backbone is composed of mixed \( \beta-(1,3)/\beta-(1,4) \)-xylan, the backbone of all land plants is \( \beta-(1,4) \)-linked D-Xyl residues of approximately 100-200 degree of polymerization (DP) in length. Xylan is classified into four subgroups in terms of the different distributions and compositions of the side chain sugars and any functional group substitution: arabinoxylan (AX), (methyl)glucurono/arabinoxylan (M/GAX), and (methyl)glucuronoxylan (MGX) (12). Neutral AX is a primary component of grain tissues in crop plants and grasses. More than 30 % of AX has been reported in wheat bran, and about 13 % are present in whole grain flour made from barley and rye. The xylan backbones are substituted with \( \alpha-L-\)arabinofuranose units (Araf) via \( \alpha-(1,3) \) and/or \( \alpha-(1,2) \) linkages in the ratio of 1:1.1-2 (This ratio will vary depending on the plant tissues and extraction methods). The existence of phenolic acids (ferulic and p-coumaric acid) esterifed to the O-5 of some Araf residues appears to provide some potential roles in covalent polysaccharide-polysaccharide or polysaccharide-protein interaction. In addition, another possible role of AX in cereal grain is to inhibit intercellular ice formation during winter time by enhancing the viscosity and the mechanical interference of the cell wall. M/GAX has been found in many tissues of monocot plants (straw, stems, stalks, and outer pericarp of grains), as well as non-lignified tissues in dicot plants. D-glucuronic acid (GlcA) and/or 4-O-methyl-a-D-glucuronic acid (4-O-Me-GlcA) residues are attached at the O-2 of
xylosyl residue (Xyl) (Fincher, 2009). The degree of (GlcA/4-O-Me-GlcA) and Ara substitution depends on the species and tissue types and show considerable variations in the range 3-9 (GlcA):10 (Xyl):1-10 (Araf). In dicots, including woody plants, all tissues undergoing secondary growth have xylan in the form of MGX. Whereas 30 % of GlcA is not methylated in non-woody plants, only methylated GlcA has been found in woody plants.

Although the fine structure of xylan from different species has been sufficiently resolved, the biosynthesis is poorly understood. Radio-isotope labeling experiments demonstrated that xylan biosynthesis takes place in the Golgi complex, and the polymers synthesized are exported outside of the plasma membrane (Fry, 2004). Activity assays have shown that proteins responsible for backbone elongation and the side chain (GlcA and Ara) addition are present in the Golgi fraction (Baydoun et al., 1983; Zeng et al., 2008). Recently, forward and reverse genetics coupled with functional genomics identified several glycosyltransferases (GTs), a group of enzymes that catalyze carbohydrate addition onto acceptor substrates, involved in xylan biosynthesis (Aspeborg et al., 2005; Brown et al., 2005, 2007, 2009; Persson et al., 2005; Zhong et al., 2005; Penan et al., 2007; Lee et al., 2007b, 2009b; Wu et al., 2009; Mortimer et al., 2010). On the basis of information obtained from protein homology search and mutant phenotypes of each GT, their possible roles have been predicted (Table 1.1).

Xylan biosynthesis, including different enzymatic actions for the backbone initiation and elongation, side chain and functional group additions, transportation of the polymer to the cell wall, and assembly with other cell wall components, is poorly understood (Ye et al., 2006; Brown et al., 2007). As mentioned earlier, polysaccharide labeling experiments and immunocytochemistry, as well as in-vitro activity assay with membrane microsomes from actively xylan-synthesizing tissues, indicated that the formation of xylan structure takes place at
the inner face of Golgi membrane by a number of GTs and modifying enzymes. Furthermore, recently identified xylan-specific GTs in *Arabidopsis* demonstrated that all proteins, with the exception of PARVUS, are localized to the Golgi (Zhong et al., 2005; Pena et al., 2007; Lee et al., 2007b; Wu et al., 2009). Thus, as seen in other hemicelluloses (xyloglucan and mannan) and pectin, main events such as backbone polymerization and side chain addition occur at the Golgi complex. Given the fact that PARVUS is predominantly localized endoplasmic reticulum (ER), an unknown initial step of xylan biosynthesis might possibly occur at the ER (Lee et al., 2007b). It is generally believed that as other matrix polymers (xyloglucan and pectin), fully formed xylan polymers in the Golgi are packaged into vesicles, which are then exported to the apoplast along the actin cables (Taylor et al., 2008). Several xylan-deficient mutants that typically show similar phenotypes such as stunted growth and thin secondary cell walls have been genetically identified in *Arabidopsis* and all the mutated genes encode type II-GT in the same or different GT family (Brown et al., 2007). In-depth xylan structural analysis and activity assay with stem microsomes of each mutant proposed the possible role of each protein. To date, no xylan-deficient mutant and xylan-specific GTs have been found in monocot plants although recent bioinformatics using a number of expressed sequence tags (ESTs) in cereal and dicots have found some candidates for xylan synthase, arabinosyl, and feruloyl transferase (Fincher, 2009).

The similarity of backbone structures between cellulose and xylan such as glycosidic linkage (β-1,4) had led to the prediction that one or more of cellulose-synthase like (CSL) enzymes might be responsible for xylan xylosyltransferase (Richmond and Somerville, 2001; Lerouxel et al., 2006). Also, xylan xylosyltransferase would be type I GT (s) with multiple transmembrane helices to perform the processive action during polymerization. However, no xylan xylosyltransferase activity has been detected when recombinant CSL proteins are
heterologously expressed in *Drosophila* Schneider 2 cells (Liepman et al., 2005). Transcriptome studies in an effort to find secondary-cell wall specific genes in *Arabidopsis* and *poplar* uncovered several uncharacterized GTs with single transmembrane domain (Aspeborg et al., 2005; Brown et al., 2005). Thus, different from other hemicelluloses including xyloglucan and glucomannan whose backbone is polymerized by different members of Cellulose synthase like (CSL) gene family, xylan synthase might be non-CSL protein (s) (Lee et al., 2007a). Two proteins belonging to GT43 family, IRX9 and IRX14 have been implicated to be involved in xylan backbone elongation (Pena et al., 2007; Lee et al., 2007a, 2010; Brown et al., 2007; Wu et al., 2010). On the basis of the stereochemical outcome of the enzyme reactions, GT43 family is classified as a family with an inverting mechanism (the anomeric configuration of the product after the enzyme reaction can be inverted) (Lairson et al., 2008). To date, many GTs with inverting mechanism are involved in the β-linked polysaccharides such as cellulose, chitin, and hyaluronan. The implication of the involvement of two proteins (IRX9 and IRX14) in xylan backbone elongation is supported primarily by two experimental evidences: 1) microsomes isolated from each mutant stems exhibited greatly reduced ability to transfer UDP-xylose onto xylooligosaccharide acceptor compared to wild type 2) NMR study revealed that the chain length of *irx9* mutant GX is much shorter than that of WT while detailed GX structure of *irx14* has not been determined (Brown et al., 2007; Pena et al., 2007; Lee et al., 2007a). Our complementation study demonstrated that they are not functional homolog (non-redundant) since 35S promoter-driven IRX14 overexpression in *irx9* background, or vice versa, does not complement the mutant phenotypes (Lee et al., 2010). This result implies that these protein products IRX9 and IRX14 would be essential to make β-(1,4) xylose polymers in all tissues with a lignified secondary cell wall although *IRX14* expression pattern has not been well examined. Brown et al proposed that
similar to cellulose synthase complex, biosynthesis of the xylan backbone may require multiple enzymes or a multienzyme complex in which IRX9 and IRX14 may be part of it (Brown et al., 2007, 2009).

Another interesting observation regarding xylan structure is the existence of tetrasaccharide \( \beta-D-Xyl-(1\rightarrow3)-\alpha-L-Rha-(1\rightarrow2)-\alpha-D-GalA-(1\rightarrow4)-D-Xyl \) at the reducing end of at least 80 % GX molecule in *Arabidopsis*, birch, spruce, and Poplar (Shimizu et al., 1976; Johansson and Samuelson, 1977; Andersson et al., 1983; Pena et al., 2007; Lee et al., 2009a). This reducing end sequence has not been found in monocot AX (Fincher, 2009). Conceivably, the reducing end sequence might serve as either primer or terminator during backbone elongation (Pena et al., 2007; York and O’Neill, 2008). Three GTs, FRA8, IRX8, and PARVUS appear to be involved in the biosynthesis of the reducing end sequences since genetic mutation of each of all three genes lead to a loss of the tetrasaccharide sequence (Zhong et al., 2005; Pena et al., 2007; Lee et al., 2007b). The sugar linkage that the protein is responsible for has not yet been identified. Thus, additional biochemical characterization of each of them is imperative to clarify the exact function in the biosynthesis of the reducing end sequence.

Recently, two Golgi-localized GTs (GUX1 and GUX2) in GT8 family have been suggested to be required for the addition of both GlcA and MeGlcA onto xylan backbone (Mortimer et al., 2010). Substantial reduction of the Me(GlCA) frequency in the isolated xylan polymer was detected in the T-DNA mutants for *GUX1* and *GUX2*. In *gux1gux2* double mutant, no detectable xylan substitution was observed, and xylan glucuronyltransferase activity from stem microsomes was significantly reduced, although the thickness of xylem and fiber cell walls in stems is not affected. It appears that the abundance and chain length of xylan is not altered in *gux1gux2* double mutant, suggesting biosynthetic actions for backbone elongation and side chain
addition are not cooperative. The exact and direct biochemical function of these proteins remains to be determined.

Poplar has been used as a model for understanding tree biology due to the small genome size, rapid growth, close relatedness with other angiosperms, and routine transformation system (Bradshaw et al., 2000; Hawkins et al., 2003). Poplar is an excellent species particularly for studying wood formation. The large size of xylem cells permits detailed descriptive, physiological, molecular characterization of how the secondary cell wall is deposited. Similar to Arabidopsis, secondary cell walls in poplar are composed of cellulose, lignin, and glucuronoxytan (Mellerowicz and Sundberg., 2008). Comparison of transcriptional profiling of genes highly expressed during secondary cell wall formation in poplar and Arabidopsis indicated that cell wall biosynthetic genes involved in cellulose, mannan, and xylan biosynthesis and regulatory genes appear to be highly conserved between them (Aspeborg et al., 2005; Ye et al., 2006). The similarity of amino acid sequences indicates that some of poplar xylem-specific GTs, PoGT43B, GT47C, GT8D, and GT8/E are close homologs of Arabidopsis IRX9, FRA8, IRX8, and PARVUS, respectively, which are essential to glucuronoxytan biosynthesis. To determine if they are also involved in glucuronoxytan biosynthesis, each of poplar GTs was overexpressed in the respective xylan mutants (Zhou et al., 2006, 2007; Lee et al., 2009c). Detailed analysis of transgenic plants revealed that PoGT43B, GT47C, GT8D, and GT8/E are functional orthologs of Arabidopsis IRX9, FRA8, IRX8, and PARVUS, respectively. In situ hybridization revealed that those transcripts are present in the cell types undergoing secondary cell wall thickening. PoGT43B, GT47C, and GT8D are targeted to the Golgi whereas PoGT8/E are localized to ER. Downregulation of PoGT43B, GT47C, and GT8D mediated by RNAi in Poplar caused xylan-
deficient phenotypes (Lee et al., 2009a). Therefore, it is highly plausible that enzyme actions and overall processes of xylan biosynthesis might be conserved between Poplar and Arabidopsis.

REFERENCES


fucosyltransferase that catalyzes the last step of cell wall xyloglucan biosynthesis in pea.


glycosyltransferases are critical for glucuronoxylan biosynthesis during secondary cell wall formation. Plant J 57:718–731.


Table 1. Glycosyltransferases involved in the xylan biosynthesis of *Arabidopsis*

<table>
<thead>
<tr>
<th>Protein name (GT family)</th>
<th>Protein localization</th>
<th>SCW thickness</th>
<th>Reducing end sequence</th>
<th>Xylan chain length</th>
<th>XyIT activity</th>
<th>Predicted function</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRA8 (GT47)</td>
<td>Golgi</td>
<td>Thin</td>
<td>Reduced</td>
<td>80</td>
<td>Unaltered</td>
<td>α-Rha transferase</td>
</tr>
<tr>
<td>IRX8 (GT8)</td>
<td>Golgi</td>
<td>Thin</td>
<td>Reduced</td>
<td>125</td>
<td>Unaltered</td>
<td>α-GalA transferase</td>
</tr>
<tr>
<td>IRX9 (GT43)</td>
<td>Golgi</td>
<td>Thin</td>
<td>Unaltered</td>
<td>28</td>
<td>Reduced</td>
<td>Xylan synthase</td>
</tr>
<tr>
<td>IRX14 (GT43)</td>
<td>Golgi</td>
<td>Thin</td>
<td>Unaltered</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Xylan synthase</td>
</tr>
<tr>
<td>PARVUS (GT8)</td>
<td>ER</td>
<td>Thin</td>
<td>Reduced</td>
<td>N/A</td>
<td>Unaltered</td>
<td>An unknown initial step in the biosynthesis of reducing end sequence</td>
</tr>
</tbody>
</table>

Data derived from Zhong et al., 2005, Pena et al., 2007, Brown et al., 2007, and Lee et al., 2007b. Xylan chain length of the *Arabidopsis* wild type is 93. N/A, not available.
CHAPTER 2

THE IRREGULAR XYLEM9 MUTANT IS DEFICIENT IN XYLAN

XYLOSYLTRANSFERASE ACTIVITY

\(^1\)

ABSTRACT

Xylan is the second most abundant polysaccharide in dicot wood, thus elucidating 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 2 to 6 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 xylan backbone.
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 rationally design strategies 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 pathways 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). GX from birch, spruce and Arabidopsis have been shown to contain a distinct glycosyl sequence \((1\rightarrow4)\)-β-D-Xyl-\((1\rightarrow4)\)-β-D-Xyl-\((1\rightarrow3)\)-α-L-Rhap\((1\rightarrow2)\)-α-D-GalP\(A-(1\rightarrow4)\)-D-Xyl\(A\) 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 and 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 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-kD 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 and 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 Golgi (Zhong et al., 2005; Pena et al., 2007; Zhou et al., 2006 and 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 is 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 Xyl6. No XylT activity was detected without the addition of exogenous acceptors. The transfer of radiolabeled Xyl from UDP-[14C]-Xyl to Xyl6 was dependent on Xyl6 concentration (Fig. 2.1A) with an apparent $K_m$ value of 10.6 mM and $V_{max}$ of 21.8 pmol/min/mg protein. The XylT activity was both time- and protein-concentration-dependent (Fig. 2.1B, C). The optimal pH for the XylT activity was around 6.5 (Fig. 2.1D) and its optimal temperature about 10 to 20 °C (Fig. 2.1E). UDP-GlcA stimulated the XylT activity up to 30% (Fig. 2.1F), 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 length 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. 2.2). The monomer Xyl1-AA was not an acceptor for Xyl transfer (Fig. 2.2B). A low level of Xyl transfer was obtained using Xyl2-AA as an acceptor. The XylT activity readily transferred Xyl residues to acceptors ranging from Xyl3 to Xyl6 (Fig. 2.2D, F, G, H), resulting in the synthesis of xylooligosaccharides with a size up to Xyl12.

To find out whether longer reaction time can lead to the production of longer xylooligosaccharides by the XylTase activity, we did a time course study using Xyl4-AA as acceptors. Up to seven xylosyl residues were added to Xyl4-AA with a 2-hr incubation, leading to the production of a population of xylooligosaccharides ranging from Xyl5 to Xyl11 (Fig. 2.3A).
Prolonged incubation (5 - 8 hr) did not result in the formation of xylooligosaccharides longer than Xyl$_{12}$ but did cause a gradual increase in the proportions of Xyl$_9$ to Xyl$_{11}$ (Fig. 2.3A to C).

We used enzymatic treatment and matrix assisted laser desorption 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-xylooligosaccharides. MALDI-TOF-MS gave masses ($m/z$ 822, 954, 1086, 1218, 1350, 1482 and 1614) for products that differed by 132 Da (Fig. 2.4). 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. 2.4). 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. 2.5). 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 Xyl$_6$ concentration (Fig. 2.6A) with an apparent $K_m$ value of 10.8 mM and $V_{max}$ of 114.6 pmol/min/mg protein. The GlcATase activity was time- and protein concentration-dependent (Fig. 2.6B, C). The optimal pH for GlcAT activity was about 6.5 (Fig. 2.6D), with substantial activity over a temperature range of 10 to 37 °C (Fig. 2.6E). Addition of UDP-Xyl in the reaction mixture did not stimulate GlcAT activity; instead high concentrations (0.5 – 5mM) of UDP-Xyl inhibited its activity (Fig. 2.6F).

**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. 2.7). Because FRA8, IRX8 and IRX9 proteins were previously shown to be targeted to Golgi (Zhong et al., 2005; Pena et al., 2007) and the microsomes we prepared was 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 three mutants compared with the wild type (Fig. 2.8A). 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. 2.8B). 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 wild-type microsomes (Fig. 2.8C), 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, *PoGT47B* gene (Fig. 2.8D). Taken together these results provide strong evidence that IRX9 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 hr to a mixture of xylooligosaccharides ranging from Xyl7-AA to Xyl12-AA, with Xyl7-AA to Xyl9-AA predominating (Fig. 2.9A). After 5-hr of reaction time, the peaks of Xyl10-AA to Xyl12-AA were elevated, whereas those of Xyl6-AA to Xyl8-AA were diminished (Fig. 2.9B). In contrast, in the irx9 mutant, Xyl6-AA was only elongated to a mixture of Xyl7-AA to Xyl9-AA, and even after 5-hr of reaction time Xyl7-AA remained the predominant peak (Fig. 2.9C, 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 biosynthesis of the xylan backbone have remained elusive. 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 \textit{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 \textit{irx9} mutant suggest that the primary effect of the \textit{irx9} mutation may be on the elongation of GX chains rather than on the initiation of GX biosynthesis. However, the \textit{irx9} mutation also decreases 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 \textit{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 \textit{irx9} mutation causes a reduction in XylT activity. The decreased XylT activity in the \textit{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. 2.9). 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 \textit{irx9} mutant (Pena et al., 2007). Thus, our study provides compelling evidence that the decreased GX chain length caused by the \textit{irx9} mutation is due to a deficiency in XylT activity.

It is important to note that the \textit{irx9} mutation does not completely eliminate the XylT activity. Because the T-DNA insertion (SALK\_058238) in the \textit{irx9} mutant disrupts an \textit{IRX9} exon that encodes part of the putative catalytic domain, \textit{irx9} is presumably a null mutant. Therefore, the low level of XylT activity remained in the \textit{irx9} mutant is likely contributed by its functional homolog(s), which may also result in a limited elongation of xylan backbone.

The \textit{fra8} and \textit{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 \((\rightarrow 4\)-\(\beta\)-d-Xylp-\((1\rightarrow 4)\)-\(\beta\)-d-Xylp-\((1\rightarrow 3)\)-\(\alpha\)-L-Rhap-\((1\rightarrow 2)\)-\(\alpha\)-d-GalpA-\((1\rightarrow 4)\)-d-Xylp\), 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 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 suggest 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 of 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 on the requirement of exogenous acceptors is due to the difference of microsome preparations or the nature of XylITs from different species. In any case, it is clear that with Arabidopsis microsomes, exogenous xylooligomers are required for the XylIT 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 preformed 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 Xyl₆ acceptor used is elongated to Xyl₁₂ by the XylIT 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 residues for every 10 xylosyl residues (Ebringerová and Heinze, 2000). Our finding that the GlcAT activity can transfer GlcA onto preformed xylooligomers is in contrast to the previous report showing that pea epicotyl microsomes can only add GlcA onto nascent xylan but not onto preformed 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 XylIT activity. Previous attempts to demonstrate XylIT activity using recombinant IRX9 protein have been unsuccessful (Pena et al., 2007). Thus, it is essential to design strategies to further biochemically
characterize IRX9. 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 irx9-complemented with PoGT43B (Zhou et al., 2007) plants were grown in a greenhouse. Inflorescence stems, roots, leaves used for microsome isolation were collected from 6-week-old plants, and seedlings used for microsome isolation were two weeks old.

Enzymes and chemicals

Endo-β-xylanase M6 and xylooligomers (Xyl₁ to Xyl₆) 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-[¹⁴C]-Xyl (specific activity, 264 mCi/mmM) and UDP-[¹⁴C]-GlcA (specific activity, 300 mCi/mmM) 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 (Xyl₁ to Xyl₆) were labeled at their reducing termini with anthranilic acid (AA) according to Ishii et al. (2002). Briefly, oligosaccharides were reacted for 2 hr at 65 °C with 0.2 M AA in 20 mM sodium acetate, pH 5.5, containing 1M NaBH₃CN. 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 NaBH₃CN by chromatography on a column (1.5 x 100 cm) of Toyopearl HW-40F and eluted with 10 mM ammonium acetate (pH 7.0). The A₂₅₄nm 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 by Kuroyama and Tsumuraya (2001). Briefly, plant materials were homogenized with a mortar and pestle in the grinding solution (50 mM Hepes-NaOH, pH 7.3, 0.4 M sucrose, 10 μg/ml aprotinin and 10 μg/ml leupeptin). After centrifugation at 3,000 g for 15 min to remove debris, the supernatant was further centrifuged at 100,000 g for 1 hr. 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 microsomal fraction was measured using the BioRad protein assay kit with 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 MnCl₂, 1 mM DTT, 0.5% Triton X-100, 0.2 μg/μl Xyl₆, and UDP-[¹⁴C]-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 MnCl₂, 1 mM DTT, 0.5% Triton X-100, 0.1 mM cold UDP-Xyl, 0.2 μg/μl Xyl₆, and UDP-[¹⁴C]-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-[¹⁴C]-Xyl by paper chromatography according to Ishikawa et al. (2000). The terminated reaction mixture
was spotted onto a strip of Whatmann 3MM paper and developed with 95% ethanol:1M 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 0.1N 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**

XylT activity with Xyl\(_n\)-AA acceptors was carried out in a reaction mixture (a total volume of 30 \(\mu\)l) 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 \(\mu\)g of microsomes. After incubation at 21 \(^\circ\)C for various times, the reaction was terminated with 0.1 M acetic acid, and filtered with an Amicon Ultrafree-MC filter (0.22 \(\mu\)m). The products formed were analyzed by reversed-phase HPLC analysis. HPLC was performed with an Agilent 1100 series LC system and a Shimadzu RF-10AXL fluorescence detector (Ex\(_{320\text{nm}}\), Em\(_{420\text{nm}}\)). The incorporated Xyl\(_n\)-AA products were separated using a Luna C18 (5 \(\mu\)m) column (250 mm long, 4.6 mm i.d.). The column was eluted at 0.5 ml/min with aq 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 \(\beta\)-xylanase and \(\beta\)-xylosidase**

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

**Matrix-Assisted Laser-Induced/Ionization-Time-Of-Flight Mass Spectrometry (MALDI-TOF MS)**

The AA-labeled xylooligosaccharides from the XylT-catalyzed reactions were analyzed by MALDI-TOF mass spectrometry 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 approx 8 x 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 Xyl₅-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.

**ACKNOWLEDGMENTS**

We thank Nicholas S. Hill for use of his lyophilizer, and the reviewers for their constructive comments and suggestions. This work was supported by grants from the U.S. Department of Energy-Bioscience Division (Grant DE-FG02-03ER15415 to Z.-H. Y. and Grants DE-FG05-93ER20097 and DE-FG02-96ER20220 to M.A.O. and A.G.D.).
REFERENCES


Figure 2.1. Biochemical properties of the XylT activity in microsomes from *Arabidopsis* stems. Microsomes were incubated with UDP-[^14]C]-Xyl and Xyl₆ for 20 min unless otherwise indicated, and the XylT activity was measured by counting the radioactivity (CPM) 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 Xyl₆ acceptor. (B) Time course of the transfer of radiolabeled Xyl onto the Xyl₆ acceptor by the XylT activity. Note that the XylT activity is linear in the first 40 min and reached 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.
Figure 2.2. Xylooligomers of different length as acceptors for the XylT activity. Microsomes were incubated with UDP-Xyl and the fluorescent acceptors Xyl1-AA (B), Xyl2-AA (C), Xyl3-AA (D), Xyl4-AA (F), Xyl5-AA (G), or Xyl6-AA (H) for 5 hr, and the reaction products were analyzed by reverse-phase HPLC for separation and detection of the fluorescent xylooligosaccharides. A chromatogram of standard Xyl1,6-AA is shown in (A) and (E) for reference. The XylT activity can transfer xylosyl residues onto xylooligomers as short as xylobiose.
Figure 2.3. Successive transfer of xylosyl residues onto the acceptors by the XylT activity.

Microsomes were incubated with UDP-Xyl and the fluorescent Xyl4-AA acceptor for 2 hr (A), 5 hr (B) and 8 hr (C), and the reaction products were analyzed by reverse-phase HPLC. The XylT activity is able to successively elongate Xyl4 up to Xyl12.
Figure 2.4. MALDI TOF mass spectrum of the XylT-catalyzed reaction products. Microsomes were incubated with UDP-Xyl and the acceptor Xyl4-AA, and the reaction products were purified and analyzed by MALDI TOF MS. Inset shows the chemical structure of Xyl₄-AA. A series of ions with a mass increment of 132 D corresponding to one xylosyl residue were observed in the spectrum. These ions correspond to AA-labeled oligosaccharides [Xyl₄-AA + Na]⁺ composed of 5 to 11 Xyl residues.
Figure 2.5. 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 Xyl₁-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 Xyl₁ to Xyl₃, indicating that the reaction products have β-(1,4)-linkage.
Figure 2.6. 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 (CPM) 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 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.
Figure 2.7. The XylT and GlcAT activities in different *Arabidopsis* organs. Microsomes isolated from stems, seedlings, roots and leaves were incubated with UDP-[\(^{14}\)C]-Xyl and Xyl6 for 20 min, and the XylT activity was measured by counting the radioactivity (CPM) 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.
Figure 2.8. 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 Xyl6 and UDP-[¹⁴C]-GlcA or UDP-[¹⁴C]-Xyl for 20 min unless otherwise indicated, and the GlcAT and XylT activities were measured by counting the radioactivity (CPM) 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-hr incubation. (D) Complementation of *irx9* with the *Arabidopsis* IRX9 or its poplar homolog PoGT43B restored the XylT activity.
Figure 2.9. Deficiency in the transfer of xylosyl residues onto xylooligomer acceptors in the irx9 mutant. Microsomes from the wild type (A, B) and irx9 (C, D) were incubated with UDP-Xyl and the fluorescent Xyl₆-AA acceptor for 2 hr (A, C) and 5 hr (B, D), and the reaction products were analyzed by reverse-phase HPLC. Note that the XylT activity in the wild type transferred up to 6 xylosyl residues onto the acceptors, whereas only up to three xylosyl residues were transferred by the XylT activity in irx9.
CHAPTER 3

THE PARVUS GENE IS EXPRESSED IN CELLS UNDERGOING SECONDARY WALL THICKENING AND ESSENTIAL FOR GLUCURONOXYLAN BIOSYNTHESIS

\(^{1}\)

ABSTRACT

Xylan together with cellulose and lignin are the three major components of secondary walls in wood and elucidation of the biosynthetic pathway of xylan is of importance for potential modification of secondary wall composition to produce wood with improved properties. So far, three Arabidopsis glycosyltransferases, FRAGILE FIBER8, IRREGULAR XYLEM8 and IRREGULAR XYLEM9, have been implicated in glucuronoxylan (GX) biosynthesis. In this study, we demonstrate that PARVUS, which is a member of family GT8, is required for the biosynthesis of the tetrasaccharide primer sequence, β-D-Xyl-(1→3)-α-L-Rha-(1→2)-α-D-GalA-(1→4)-D-Xyl, located at the reducing end of GX. The PARVUS gene is expressed during secondary wall biosynthesis in fibers and vessels, and its encoded protein is predominantly localized in the endoplasmic reticulum. Mutation of the PARVUS gene leads to a drastic reduction in secondary wall thickening and GX content. Structural analysis of GX using 1H NMR spectroscopy revealed that the parvus mutation causes a loss of the tetrasaccharide primer sequence at the reducing end of GX and an absence of glucuronic acid side chains in GX. Activity assay showed that the xylan xylosyltransferase and glucuronyltransferase activities were not affected in the parvus mutant. Together, these findings implicate a possible role of PARVUS in the initiation of biosynthesis of the GX tetrasaccharide primer sequence and provide novel insights into the mechanisms of GX biosynthesis.
INTRODUCTION

Cellulose, xylan and lignin are the three principal components of dicot wood. Because of the importance of wood in human life, it is imperative to elucidate the mechanisms involved in the biosynthesis and regulation of wood components. Knowledge gained from such studies will potentially allow us to modify cell wall composition on the basis of our needs, such as engineering walls more suitable for biofuel production. Although the biosynthetic genes for cellulose and lignin have been well characterized, our understanding of genes involved in xylan biosynthesis is limited.

Xylan is composed of a linear backbone of (1,4)-linked β-D-xylosyl (Xyl) residues with an average degree of polymerization from 90 to 120 (Johansson and Samuelson, 1977; Pena et al., 2007). About 10% of Xyl residues on the backbone are substituted with α-D-glucuronic acid (GlcA), 4-O-methyl-α-D-glucuronic acid (MeGlcAT), and/or α-D-arabinose at O-2 and/or O-3, and the Xyl residues may be acetylated on C-2 or C-3 (Ebringerová and Heinze, 2000). Based on the nature of substitutions, xylan is typically named (methyl)glucuronoxylan (GX), which is the main hemicellulose in dicot wood, arabinoxylan and glucuronoarabinoxylan, which are the most abundant hemicelluloses in grass cell walls (Ebringerová and Heinze, 2000). The biosynthesis of the xylan backbone and its side chains requires at least five types of enzymes, including xylosyltransferases (XylT), glucuronyltransferases (GlcAT), arabinosyltransferases, methyltransferases, and acetyltransferases. The activities of XylT, GlcAT, arabinosyltransferase, and methyltransferases involved in xylan biosynthesis have been biochemically studied in a number of plant species (Dalessandro and Northcote, 1981; Baydoun et al., 1983 and 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). Recently, the *IRREGULAR XYLEM9 (IRX9)*
gene, which encodes a putative glycosyltransferase (GT) belonging to family GT43 (Coutinho et al., 2003), has been found to be essential for normal xylan backbone biosynthesis. The \textit{IRX9} gene is expressed in cells undergoing the biosynthesis of secondary walls including GX, and its encoded protein is localized in Golgi, where GX is synthesized (Pena et al., 2007). Mutation of the \textit{IRX9} gene causes a reduction in GX level (Brown et al., 2005; Bauer et al., 2006) as well as a substantial decrease in GX chain length (Pena et al., 2007). The defective GX chain elongation caused by the \textit{irx9} mutation is correlated with a deficiency in xylan XylT activity (Lee et al., 2007). IRX9 is the first GT known to be required for normal xylan XylT activity. Both genetic and biochemical analyses suggest that IRX9 is a putative xylan synthase required for GX backbone elongation (Pena et al., 2007; Lee et al., 2007). The poplar GT43A/B, which is a homolog of IRX9 and expressed during wood formation (Aspeborg et al., 2005), is also likely responsible for GX backbone elongation in the secondary walls of wood because PoGT43B can functionally complement the GX and XylTase deficiency caused by the \textit{irx9} mutation (Zhou et al., 2007; Lee et al., 2007).

In addition to the β-(1,4)-linked D-Xyl residues in the backbone, the reducing end of GX from birch, spruce and \textit{Arabidopsis} has been found to contain a distinct tetrasaccharide primer sequence β-D-Xyl-(1→3)-α-L-Rha-(1→2)-α-D-GalA-(1→4)-D-Xyl (Shimizu et al., 1976; Johansson and Samuelson, 1977; Anderson et al. 1983; Pena et al., 2007). Conceivably, at least four additional GTs are required for the biosynthesis of the tetrasaccharide primer sequence. So far, two GT genes, \textit{FRAGILE FIBER8 (FRA8)} and \textit{IRX8}, which encode proteins belonging to GT families 47 and 8 (Coutinho et al., 2003), respectively, have been implicated in the biosynthesis of the tetrasaccharide primer sequence at the GX reducing end. Both \textit{FRA8} and \textit{IRX8} are specifically expressed in cells undergoing secondary wall biosynthesis, and their encoded
proteins are localized in Golgi (Zhong et al., 2005b; Pena et al., 2007). Mutations of the FR48 and IRX8 genes result in a reduction in GX level (Brown et al., 2005; Zhong et al., 2005b; Pena et al., 2007; Persson et al., 2007) and a near absence of the tetrasaccharide primer sequence, indicating that FR48 and IRX8 are involved in the biosynthesis of the tetrasaccharide primer sequence at the GX reducing end (Pena et al., 2007). Because family GT47 enzymes to which FR48 belongs have inverting mechanisms and catalyze the formation of β-glycosidic linkages using α-linked nucleotide sugars or vice-versa, FR48 was proposed to catalyze the β-linkage of Xyl to O3 of rhamnose using UDP-α-D-Xyl as a substrate or the α-linkage of rhamnose to O2 of galacturonic acid (GalA) using UDP-β-L-rhamnose (Pena et al., 2007). Since family GT8 enzymes of which IRX8 is a member have retaining mechanisms and typically catalyze the formation of α-glycosidic linkages using α-linked nucleotide sugars, IRX8 was proposed to catalyze the α-linkage of GalA to O4 of the reducing Xyl residue of the tetrasaccharide primer sequence (Pena et al., 2007). The poplar GT47C, which is a homolog of FR48 and expressed during wood formation, is also likely involved in the biosynthesis of the tetrasaccharide primer sequence at the reducing end of GX in secondary walls of wood (Zhou et al., 2006).

As described above, three GT genes have been implicated in various steps of GX biosynthesis. However, at least nine different enzymes are required for the biosynthesis of GX, arabinoxylan and glucuronoarabinoxylan. To further our understanding of the xylan biosynthetic pathway, it is necessary to identify and characterize additional xylan biosynthetic genes. In this study, we report that the PARVUS gene, which encodes a protein belonging to family GT8, is another GT required for GX biosynthesis. We show that the PARVUS gene is expressed during secondary wall biosynthesis and its encoded protein is localized in the endoplasmic reticulum (ER). We demonstrate that mutation of the PARVUS gene leads to a reduction in GX content and a loss of
the tetrasaccharide primer sequence at the GX reducing end. Considering the fact that PARVUS is associated with ER, it is tempting to propose that PARVUS is involved in the first transfer of the reducing Xyl residue of the tetrasaccharide primer sequence at the GX reducing end to an unknown acceptor at ER.

RESULTS

Expression of the PARVUS gene is associated with secondary wall thickening

The interfascicular fibers of Arabidopsis inflorescence stems deposit a large amount of cellulose, xylan and lignin in secondary walls, which have been used as a model for studying genes involved in secondary wall biosynthesis (Zhong et al., 2001 and 2005b; Pena et al., 2007). To find genes responsible for xylan biosynthesis, we analyzed GTs that were up-regulated during secondary wall thickening using interfascicular fibers isolated by laser microdissection (Zhong et al., 2006). In this report, we studied the function of a putative GT gene, At1g19300, which was induced during secondary wall thickening of fibers. The At1g19300 gene is a member of GT family 8 (Coutinho et al., 2003) and was previously named PARVUS/GLZ1/GATL1 (Lao et al., 2003; Shao et al., 2004; Sterling et al., 2006) and proposed to be involved in pectin biosynthesis (Lao et al., 2003; Lerouxel et al., 2006). We have found that the PARVUS gene is highly expressed in fibers and xylem cells undergoing secondary wall thickening but not in parenchymatous pith cells (Fig. 3.1A). At the organ level, the PARVUS gene is predominantly expressed in stems in which abundant secondary wall-containing fibers and xylem cells are produced (Fig. 3.1B). In addition, the expression of the PARVUS gene was shown to be co-induced with three known xylan biosynthetic genes, FRA8, IRX8 and IRX9, by overexpression of MYB46 (Fig. 3.1C), which results in ectopic deposition of secondary walls (Zhong et al., 2007a). Moreover, its expression was down-regulated together with FRA8, IRX8 and IRX9 in
SND1/NST1 RNAi lines (Fig. 3.1D), which exhibit a loss of secondary wall thickening in fiber cells (Zhong et al., 2007b). Therefore, we reasoned that PARVUS is likely involved in the biosynthesis of a secondary wall component, such as GX, rather than pectin as previously proposed.

We next performed in situ mRNA localization to investigate the developmental expression pattern of the PARVUS gene in Arabidopsis stems and roots. In rapidly elongating internodes in which protoxylem is the only secondary wall-containing tissue present, the PARVUS mRNA signal was seen in the developing vessels of the protoxylem (Fig. 3.2A). At this stage, interfascicular fiber cells are undergoing rapid elongation and no secondary wall thickening is evident (Ye et al., 2002). Concomitantly, no PARVUS mRNA signal was seen in interfascicular fiber cells (Fig. 3.2A). In nonelongating internodes in which secondary wall thickening is evident in interfascicular fibers, strong PARVUS mRNA signal was detected in interfascicular fiber cells in addition to developing vessels and xylary fibers in metaxylem (Fig. 3.2B). The PARVUS mRNA signal was also observed in developing vessels and xylary fibers in secondary xylem of roots (Fig. 3.2C). The control stem section hybridized with the sense PARVUS RNA probe did not show any positive signals (Fig. 3.2D). These results demonstrate that the PARVUS gene is specifically expressed in cells undergoing secondary wall thickening, including interfascicular fibers, vessels, and xylary fibers. The secondary wall-associated expression pattern of the PARVUS gene revealed in this study differs from that reported in a previous study, which employed the promoter-GUS reporter gene analysis and showed its expression in almost all cell types in stems (Shao et al., 2004). It is apparent that the promoter used in the expression study lacks some regulatory elements required for the endogenous gene expression.

The PARVUS protein is associated with the endoplasmic reticulum
The PARVUS protein is predicted to have a hydrophobic signal peptide sequence at the N-terminus (Fig. 3.3A; Lao et al., 2003) by both protein hydropathy analysis and the prediction of protein sorting signals and localization sites program (PSORT; http://psort.ims.u-tokyo.ac.jp/).

To examine the subcellular localization of the PARVUS protein, we co-expressed yellow fluorescent protein (YFP)-tagged PARVUS with a cyan fluorescent protein (CFP)-tagged ER marker and a Golgi marker in carrot (Daucus carota) protoplasts and studied their colocalization patterns. It was found that the fluorescent signals of PARVUS-YFP displayed a network-like pattern, which clearly overlapped with the localization pattern of the ER marker (Fig. 3.3D to G). In contrast, the network-like signals of PARVUS-YFP displayed apparent differences from the punctate localization pattern of FRA8-CFP (Fig. 3.3H to K) that is known to be localized in Golgi (Zhong et al., 2005a) although some overlap of signals was observed. The control protoplasts expressing YFP alone had fluorescent signals distributed throughout the cytoplasm (Fig. 3.3B, C). These results indicate that the PARVUS protein is predominantly associated with ER.

**Mutation of the PARVUS gene causes a severe reduction in secondary wall thickening of fibers and vessels**

The finding that the PARVUS gene is specifically expressed in cells undergoing secondary wall thickening suggests that it is likely involved in secondary wall biosynthesis. To find out whether the PARVUS gene is essential for secondary wall thickening, we obtained a homozygous T-DNA insertion knockout line of PARVUS (SALK_045368; obtained from ABRC, Columbus, OH, USA)) for examination of stem strength and wall thickness of fibers and vessels. The homozygous T-DNA knockout plants were smaller in rosette size and their stems exhibited a dwarf phenotype, which is identical to the previously reported observations for parvus mutations.
(Lao et al., 2003; Shao et al., 2004). It was found that mutation of the PARVUS gene led to a severe reduction in the mechanical strength of stems compared with the wild type (Fig. 3.4A). Examination of fibers and vessels in stems and roots revealed that the wall thickness of fibers and vessels in the parvus mutant was drastically decreased compared with the wild type (Fig. 3.4B to G). It was apparent that the vessels in the parvus mutant exhibited a collapsed phenotype, which is most likely caused by the reduced wall strength as observed in other known secondary wall defective mutants (Brown et al., 2005; Zhong et al., 2005b; Persson et al., 2005).

Transmission electron microscopy showed that the wall thickness of interfascicular fibers, vessels and xylary fibers was reduced by 50 to 66% compared with the wild type (Fig. 3.5; Table 3.1). These results suggest that PARVUS is required for normal secondary wall thickening in both fibers and vessels.

**Mutation of the PARVUS gene results in a reduction in GX content in stems and a loss of LM10 antibody labeling of xylan in secondary walls of fibers and vessels**

The observation that PARVUS is associated with ER indicates that it is involved in the biosynthesis of a non-cellulosic polysaccharide. Because the major non-cellulosic polysaccharide in secondary walls of Arabidopsis fibers and vessels is GX, we postulate that PARVUS might play a role in GX biosynthesis. To investigate which secondary wall components are affected by the parvus mutation, we first analyzed the composition of total cell walls isolated from stems and found that the amount of xylose and glucose, which are the respective main components of GX and cellulose, was reduced by 46.7% and 23.5%, respectively, compared with the wild type (Table 3.2). No significant alterations were seen in the amount of other cell wall neutral sugars, including mannose, galactose, arabinose, rhamnose and fucose. Further composition analysis of 1 N and 4 N KOH extracts of cell walls from stems of wild type and parvus showed that the most
drastically reduced sugar content was xylose (Table 3.3). Because the xylose in 1 N and 4 N KOH extracts of Arabidopsis stems primarily comes from GX (Zhong et al., 2005b), these findings strongly suggest that PARVUS is involved in GX biosynthesis. The reduction in cellulose content in the parvus mutant is likely due to an indirect effect, which has also been observed in three other known xylan deficient mutants (Brown et al., 2005; Zhong et al., 2005b). The parvus mutation has previously been shown to reduce the xylose content in leaf cell walls, which was attributed to an indirect effect due to its presumed role in pectin biosynthesis (Lao et al., 2003). Because PARVUS is a GT believed to be involved in polysaccharide synthesis but unlikely in lignin synthesis, we did not analyze the possible effect of its mutation on lignin alterations.

To substantiate the role of PARVUS in GX biosynthesis, we next examined the effect of the parvus mutation on GX deposition by immunolocalization using monoclonal antibody LM10 that binds to 4-O-methylglucuronoxylan (McCartney et al., 2005). Immunolabeling of wild-type stem and root sections showed intensive fluorescent signals in the walls of interfascicular fibers and xylem cells including vessels and xylary fibers (Fig. 3.6A, D), all of which are known to contain abundant GX in secondary walls. In contrast, almost no fluorescent signals were observed in the secondary wall-containing tissues in parvus stems and roots (Fig. 3.6B, E). Expression of the wildtype PARVUS gene in the parvus mutant restored the xylan signals to the wild-type level in all secondary wall-containing tissues in stems and roots (Fig. 3.6C, F). These results demonstrate that PARVUS is required for the normal biosynthesis and deposition of GX in secondary walls of fibers and vessels.

The parvus mutation leads to an absence of nonmethylated GlcA side chains in GX
To determine whether mutation of the PARVUS gene affects the GX structure, the KOH solubilized GX from stems of wild type and the parvus mutant was digested with endoxylanase and the acidic xylooligosaccharides produced by the digestion were analyzed by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). MALDI-TOF MS of the wild-type acidic xylooligosaccharides revealed two major ion peaks [M + Na]+ at mass to-charge ratios (m/z) of 743 and 759 (Fig. 3.7A), which are attributed to xylooligosaccharides with four Xyl residues substituted with one non-methylated α-D-GlcA or one 4-O-Me-α-D-GlcA, respectively (Fig. 3.7C; Zhong et al., 2005b). The xylooligosaccharides bearing 4-O-Me-α-D-GlcA side chains are more abundant than those bearing α-D-GlcA side chains, which is a typical pattern observed in the GX of wild-type Arabidopsis (Zhong et al., 2005b). In contrast, the MALDI-TOF spectrum of parvus acidic xylooligosaccharides contains only the ion peak at m/z of 759 (Fig. 3.7B). The parvus mutant lacked the ion with m/z of 745 that is attributed to xylotetrasaccharides substituted with one nonmethylated α-D-GlcA. The MALDI-TOF analysis demonstrates that the parvus mutation results in a loss of α-D-GlcA side chains in GX.

The parvus mutation causes a loss of the tetrasaccharide primer sequence located at the reducing end of GX

The Arabidopsis GX contains a tetrasaccharide primer sequence located at the reducing end, which differs from the GX backbone (Pena et al., 2007). Previous study showed that FRA8 and IRX8 are required for the biosynthesis of this tetrasaccharide primer sequence (Pena et al., 2007). To further investigate the roles of PARVUS in GX biosynthesis, we examined whether the parvus mutation affects the biosynthesis of the tetrasaccharide primer sequence using NMR spectrometry. The 1H-NMR spectrum of wild-type acidic xylooligosaccharides contained anomeric resonances of H1 and H5 of α-D-GlcA and H1 of branched β-D-Xyl residues bearing
an α-D-GlcA residue as well as H1 and H5 of 4-O-Me-α-D-GlcA residues and H1 of branched β-D-Xyl residues bearing a 4-O-Me-α-D-GlcA residue (Fig. 3.8A). The spectrum also includes resonances of H1 of α-Xyl and β-Xyl residues at the reducing ends of oligoxylosaccharides and H1 of unbranched β-Xyl residues (Fig. 3.8A). Furthermore, the resonances assigned to the tetrasaccharide primer sequence β-D-Xyl-(1→3)-α-L-Rha-(1→2)-α-D-GalA-(1→4)-D-Xyl that is located at the reducing end of GX was apparent in the spectrum of wild-type acidic xylooligosaccharides (Fig. 3.8A). These resonances include 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. The observed 1H-NMR spectrum of wild-type acidic xylooligosaccharides is the same as that previously reported for wild-type Arabidopsis acidic xylooligosaccharides (Zhong et al., 2005; Pena et al., 2007). Examination of the 1H-NMR spectrum of parvus acidic xylooligosaccharides showed an apparent lack of resonances of H1 of α-D-GalA, H1 of α-L-Rha, H1 of 3-linked β-DXyl, H4 of α-D-GalA, and H2 of α-L-Rha, which are assigned to the tetrasaccharide primer sequence β-D-Xyl-(1→3)-α-L-Rha-(1→2)-α-D-GalA-(1→4)-D-Xyl that is located at the reducing end of GX (Fig. 3.8B). In addition, the resonances assigned to H1 and H5 of α-D-GlcA and H1 of branched β-D-Xyl residues bearing an α-D-GlcA residue were absent in parvus (Fig. 3.8B), which is consistent with the MALDI-TOF data (Fig. 3.7). These results demonstrate that the parvus mutation leads to a loss of the tetrasaccharide primer sequence at the GX reducing end, which suggests that PARVUS is involved in the biosynthesis of the tetrasaccharide primer sequence.

*The xylan xylosyltransferase and glucuronyltransferase activities are not affected by parvus mutation*
To investigate whether the reduced GX content and altered GX structure were caused by a reduction in xylan xylosyltransferase (XylT) or glucuronyltransferase (GlcAT) activities, we performed comparative analysis of their activities using microsomes isolated from wild-type and parvus stems. It was found that there was no decrease in the XylT and GlcAT activities in the parvus mutant; instead they were elevated compared with the wild type (Fig. 3.9). The elevation of XylT and GlcAT activities was also observed in three other xylan deficient mutants, fra8, irx8 and irx9 (Lee et al., 2007), probably due to a compensatory effect caused by these mutations. These results suggest that PARVUS is not required for the normal activities of xylan XylT and GlcAT.

DISCUSSION

Xylan is the second most abundant polysaccharide in dicot wood and thus understanding its biosynthetic pathway is of importance for rationally modifying secondary wall composition to produce wood with improved properties. At least nine different enzymes are needed in the multiple steps of xylan biosynthesis, including the initiation and elongation of xylan backbone, and addition and modification of xylan side chains. So far, one GT, IRX9, has been demonstrated to be required for normal xylan XylT activity and normal elongation of the xylan chain, and two other GTs, FRA8 and IRX8, have been shown to be required for the biosynthesis of the tetrasaccharide primer sequence at the reducing end of GX. Our identification of PARVUS as another GT essential for the biosynthesis of the tetrasaccharide primer sequence at the reducing end of GX marks another important step toward the elucidation of the complex process of xylan biosynthesis.

*PARVUS is expressed during secondary wall biosynthesis and its encoded protein is associated with the endoplasmic reticulum*
Gene expression analyses revealed that the PARVUS gene is expressed predominantly in cells undergoing secondary wall thickening (Figs. 3.1 and 3.2), a pattern similar to that of three other known xylan biosynthetic genes (Zhong et al., 2005b; Pena et al., 2007; Persson et al., 2007). The secondary wall-associated expression pattern indicates that PARVUS is unlikely involved in the biosynthesis of a primary wall component, such as pectin, because there is little pectin present in secondary walls (Willats et al., 2001). The previous notion that PARVUS might be involved in pectin biosynthesis was primarily based on its sequence homology to other members of family GT8, QUASIMODO1 (Bouton et al., 2001) and GAUT1 (Sterling et al., 2006), and no genetic or biochemical data are available to substantiate such a proposed function. As discussed below, the secondary wall-associated expression pattern of PARVUS is consistent with its essential role in the biosynthesis of GX in secondary walls. The poplar GT8E/F, which is a close homolog of PARVUS, has also been shown to be expressed during wood formation (Aspeborg et al., 2005). PARVUS was found to be predominantly localized in ER, a localization pattern different from that of other known xylan biosynthetic enzymes, FRA8, IRX8 and IRX9, which are located in Golgi (Zhong et al., 2005b; Pena et al., 2007). This difference is consistent with the sequence analysis showing that whereas FRA8, IRX8 and IRX9 are type II membrane proteins, PARVUS is predicted to contain a hydrophobic signal peptide sequence but no transmembrane helices. The ER localization of PARVUS suggests that it catalyzes a step that is different from FRA8, IRX8 and IRX9 in GX biosynthesis.

**PARVUS is likely involved in the initiation of the biosynthesis of the GX tetrasaccharide primer sequence at the endoplasmic reticulum**

Structural analysis of GX from the parvus mutant clearly demonstrates that PARVUS is involved in the biosynthesis of the tetrasaccharide primer sequence at the reducing end of GX. Thus,
PARVUS joins FRA8 and IRX8 in the list of GTs required for the synthesis of the tetrasaccharide sequence. Based on their catalytic mechanisms, FRA8 has previously been proposed to catalyze the transfer of Xyl to rhamnose or rhamnose to GalA, whereas IRX8 was proposed to transfer GalA to the reducing Xyl residue at the GX reducing end (Pena et al., 2007). Because PARVUS belongs to family GT8 in which known members have retaining catalytic mechanisms and it is phylogenetically related to IRX8, it is possible that PARVUS and IRX8 function redundantly in catalyzing the addition of α-D-GalA to O4 of the reducing Xyl residue at the GX reducing end using UDP-α-D-GalA as a sugar donor. However, the fact that PARVUS is predominantly localized in ER and IRX8 in Golgi suggests that PARVUS catalyzes an enzymatic step earlier than IRX8 in the GX biosynthetic pathway. One appealing hypothesis is that PARVUS is involved in the initiation of the tetrasaccharide primer biosynthesis by catalyzing the transfer of the reducing Xyl residue onto an unknown acceptor in ER. This scenario bears analogy with the biosynthesis of animal glycosaminoglycans. It has been shown that the biosynthesis of animal glycosaminoglycans requires a tetrasaccharide primer sequence, and the synthesis of the primer is initiated in ER by transferring of the reducing Xyl residue to protein and the subsequent steps of the tetrasaccharide primer synthesis occur in Golgi (Prydz and Dalen, 2000). Previous study has shown that GX is covalently attached to protein (Crosthwaite et al., 1994). Thus, it is possible that the GX tetrasaccharide primer synthesis is initiated in ER where PARVUS might catalyze the transfer of the reducing Xyl residue to protein, which is then transported to Golgi where subsequent additions of sugar residues are catalyzed by IRX8, FRA8 and other uncharacterized GTs. It was previously proposed that the tetrasaccharide sequence at the reducing end of GX might function as a primer or as a chain terminator for GX biosynthesis (Pena et al., 2007). Our finding that PARVUS is predominantly localized in ER and
required for the synthesis of the tetrasaccharide sequence at the reducing end of GX favors the
tetrasaccharide sequence as a primer for GX biosynthesis.

**PARVUS is essential for the biosynthesis of normal amount of GX and GlcA side chains**

In addition to a loss of the tetrasaccharide sequence at the reducing end of GX, the *parvus*
mutation also leads to a reduction in GX content and a loss of GlcA side chains. These
phenotypes, which are also observed in three other xylan mutants, *fra8, irx8* and *irx9* (Brown et
al., 2005; Zhong et al., 2005b; Bauer et al., 2006; Pena et al., 2007; Persson et al., 2007), are
likely an indirect effect caused by the defects in the biosynthesis of the tetrasaccharide primer
sequence. As proposed above, the tetrasaccharide sequence most likely functions as a primer for
xylan backbone elongation, and thus a disruption in the biosynthesis of the primer sequence by
the *parvus* mutation leads to an inhibition of GX biosynthesis. The loss of GlcA but not
methylated GlcA side chains in GX could be explained by the saturation mechanism (Zhong et
al., 2005; Pena et al., 2007). Because GlcA is methylated after it is transferred onto the GX
backbone (Baydoun et al., 1989), it is possible that in wild type, the methyltransferase activity
could not keep up with the rate of GlcA addition and thus a proportion of GlcA side chains are
not methylated. In contrast, the GX biosynthesis rate is greatly reduced in the *parvus* mutant, and
thus the available methyltransferase activity could sufficiently methylate all GlcA side chains.
This is consistent with the activity data showing that the *parvus* mutation does not affect the
GlcAT activity (Fig. 3.9).

In summary, our study reveals an essential role of PARVUS in GX biosynthesis in secondary
walls. We have demonstrated that PARVUS is required for the biosynthesis of the
tetrasaccharide primer sequence, β-D-Xyl-(1→3)-α-L-Rha-(1→2)-α-D-GalA-(1→4)-D-Xyl,
located at the reducing end of GX. Based on our findings, we propose that PARVUS is involved
in the initiation of the biosynthesis of the tetrasaccharide sequence, which functions as a primer for subsequent GX biosynthesis. Our work provides foundation knowledge for further dissecting the mechanisms involved in the initiation of GX biosynthesis.

MATERIALS AND METHODS

Gene expression analysis

For analysis of gene expression in different cell types, interfascicular fiber cells, xylem cells and pith cells from inflorescence stems of 6-week-old *Arabidopsis* plants were isolated using the PALM microlaser system (PALM Microlaser Technologies, Bernried, Germany) and used for RNA isolation and amplification as described (Zhong et al., 2006). For analysis of gene expression in different organs, total RNA was isolated from leaves, roots, stems, and flowers of 6-week-old *Arabidopsis* plants using a Qiagen RNA isolation kit (Valencia, CA, USA). For quantitative PCR analysis, total RNA was treated with DNase I and used for first strand cDNA synthesis. The first strand cDNA was then used as template for real-time quantitative PCR analysis using gene specific primers (5’-gtacacgtcagcagtcgag-3’ and 5’-agaatccagcaagagctgagttg-3’) with the QuantiTect SYBR Green PCR Kit (Clontech, Mountain View, CA, USA). The relative mRNA levels were determined by normalizing the PCR threshold cycle number of each gene with that of the *EF1α* reference gene. The expression level of each gene in the wild-type control or in the sample with the lowest expression level was set to 1 and the data were the average of three replicates.

In situ localization of mRNAs

Wild-type *Arabidopsis* inflorescence stems were used for *in situ* mRNA localization as described (McAbee et al., 2005). Tissues were fixed in 2.5% formaldehyde and 0.5% glutaraldehyde and then embedded in paraffin. Ten-micrometer-thick sections were cut, mounted onto slides, and
hybridized with digoxygenin-labeled \textit{PARVUS} antisense RNA probe synthesized using the DIG RNA labeling mix (Roche, Mannheim, Germany). The \textit{PARVUS} cDNA sequence used for RNA probe synthesis was PCR-amplified using gene-specific primers (5’-ccgacgttcctctagag-3’ and 5’-ggtttaatccgcttacc-3’) and this sequence shows no significant homology with other GTs. The hybridization and washing conditions used only allowed specific hybridization of the RNA probe to the \textit{PARVUS} mRNA. The hybridization signals were detected with alkaline phosphatase-conjugated antibodies against digoxigenin and subsequent color development with alkaline phosphatase substrates.

\textbf{Subcellular localization of fluorescent protein-tagged proteins}

The colocalization of fluorescent protein-tagged PARVUS with the Golgi and ER markers was carried out in carrot protoplasts as described (Zhong et al., 2005a). The PARVUS cDNA was fused in frame with the yellow fluorescent protein (YFP) cDNA and ligated between the cauliflower mosaic virus 35S promoter and the nopaline synthase terminator in a high copy vector. The fusion protein thus generated will have the YFP tag at the C-terminus. The PARVUS-YFP construct was co-transfected into carrot protoplasts together with cyan fluorescent protein (CFP)-tagged FRA8, a protein known to be localized in Golgi (Zhong et al., 2005a) or with a CFP-tagged ER marker that was generated by fusing an \textit{Arabidopsis} chitinase signal peptide to the N-terminus and an HDEL sequence to the C-terminus of CFP (Batoko et al., 2000). After 20-hr incubation, the transfected protoplasts were examined for yellow and cyan fluorescent signals using a Leica TCs SP2 spectral confocal microscope.

\textbf{Immunolocalization of xylan}

Basal stem internodes and roots of 10-week-old plants were fixed with 2\% glutaraldehyde
in phosphate buffered saline (PBS), dehydrated through a gradient of ethanol, and then embedded in LR White Resin (Electron Microscopy Sciences, Fort Washington, PA, USA). One-micrometer-thick sections were cut with a microtome and incubated with the LM10 monoclonal antibody, which recognizes glucuronoxylan (Plantprobes, Leeds, UK; McCartney et al., 2005) and then with fluorescein isothiocyanate-conjugated secondary antibodies. The fluorescence-labeled sections were observed using a Leica TCs SP2 spectral confocal microscope. Images from single optical sections were collected and processed with Adobe Photoshop.

**Breaking force measurement**

The main inflorescence of 10-week-old wild type *Arabidopsis* and *parvus* plants were divided into three equal segments, which were measured for their breaking force using a digital force/length tester (Model DHT4-50; Larson System, Minneapolis, MN, USA). The breaking force (g) was calculated as the force needed to break apart a stem segment (Zhong et al., 1997).

**Histology**

Basal stem internodes and roots of 10-week-old plants were fixed with 2% glutaraldehyde in PBS at 4°C overnight. After fixation, tissues were postfixed in 1% (v/v) OsO4, dehydrated through a gradient of ethanol, embedded in Spurr’s resin (Electron Microscopy Sciences), and then subjected to sectioning with a microtome (Burk et al., 2006). One-micrometer-thick sections were stained with toluidine blue for light microscopy. For transmission electron microscopy, 85-nm thick sections were stained with uranyl acetate and lead citrate, and visualized using a Zeiss EM 902A transmission electron microscope.

**Cell wall isolation and extraction**

Inflorescence stems of 10-week-old plants were collected for cell wall isolation. Stems were ground into fine powder in liquid nitrogen with a mortar and pestle, homogenized sequentially in
70% ethanol and 100% acetone with a polytron. The resulting cell wall residues were dried in a vacuum oven at 60°C. The wall preparations were subsequently subjected to sequential extractions with ammonium acetate (50 mM, 24 h), 1 N KOH containing 1% (w/v) NaBH4 (24 hr), and 4 N KOH containing 1% (w/v) NaBH4 (24 hr) according to Zhong et al. (2005b). The 1N and 4 N KOH soluble extracts were passed through a glass-fiber filter, neutralized to pH 6.0 with glacial acetic acid, dialyzed in a dialysis tubing (3,500 Mr cutoff; Fisher Scientific, Pittsburgh, PA, USA) against deionized water, and lyophilized.

**Cell wall composition analysis**

Cell wall sugars (as alditol acetates) were determined following the procedure described by Hoebler et al. (1989). Briefly, cell walls were incubated with 70% sulfuric acid at 37°C for 60 minutes followed by addition of inositol as the internal standard and dilution with water to 2N sulfuric acid. After heating for 120 minutes at 100°C, the solution was cooled and treated with 25% ammonium solution. After reduction with sodium borohydride in dimethyl sulfoxide, the solution was heated for 90 minutes at 40°C, followed by sequential treatment with glacial acetic acid, acetic anhydride, 1-methylimidazole, dichloromethane, and water. The organic layer containing the alditol acetates of the hydrolyzed cell wall sugars was washed three times with water, and sugars were analyzed on an Agilent 6890N gas-liquid chromatography (Wilmington, DE, USA) equipped with a 30 m x 0.25 mm (i.d.) silica capillary column DB 225 (Alltech Assoc., Deerfield, IL, USA).

**Generation of xylooligosaccharides with β-endo-xylanase**

The 1 N and 4 N KOH solubilized wall preparations were digested with β-xylanase M6 (Megazyme, Wicklow, Ireland) for generation of xylooligosaccharides as described (Zhong et al., 2005b). The released xylooligosaccharides were desalted and separated by size-exclusion.
chromatography on a Sephadex G-25 column (100 x 2.5 mm). Fractions containing the oligosaccharides were determined by the phenol-sulfuric assay (DuBois et al., 1956), pooled and lyophilized.

**Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)**

The acidic xylooligosaccharides released from β-xylanase digestion were analyzed using a MALDI-TOF mass 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 x 10-7 torr. The aqueous sample was mixed (1:1, v/v) with the MALDI matrix (0.2 M 2,5-dihydroxybenzoic acid and 0.06 M 1-hydroxyisoquinoline in 50% acetonitrile) and dried on the stainless steel target plate. Spectra are the average of 100 laser shots.

**1H-NMR spectroscopy**

NMR spectra of the acidic xylooligosaccharides from β-xylanase digestion were recorded at 298 K with an NMR spectrometer. Chemical shifts were measured relative to internal acetone at δ 2.225. The 1H-NMR assignments were done by comparison with the NMR spectra data for *Arabidopsis* acidic xylooligosaccharides (Zhong et al., 2005b; Pena et al., 2007) and further confirmed by two-dimensional gCOSY, HSQC, HMBC, and TOCSY spectra.

**Assay of GlcAT and XylT activities**

Microsomes were isolated and the GlcAT and XylT activities were determined following the procedures by Kuroyama and Tsumuraya (2001). 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 MnCl2, 1 mM DTT, 0.5% Triton X-100, 0.2 μg/μl Xyl6 (Megazyme), and UDP-[14C]-GlcA (0.1 μCi; American Radiolabeled Chemical, St. Louis, MO,
USA). 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 MnCl2, 1 mM DTT, 0.5% Triton X-100, 0.1 mM cold UDP-Xyl (CarboSource Service, Athens, GA, USA; supported in part by NSF-RCN grant #0090281), 0.2 μg/μl Xyl6, and UDP-[14C]-Xyl (0.1 μCi; American Radiolabeled Chemical). After incubation at 21 oC for 20 min, the reactions were stopped by addition of the termination solution (0.3 M acetic acid containing 20 mM EGTA). The radiolabeled xyloooligosaccharides were separated from UDP-[14C]-Xyl or UDP-[14C]-GlcA by paper chromatography according to Ishikawa et al. (2000), and the incorporation of radiolabeled Xyl or GlcA onto xyloooligosaccharides was determined with a PerkinElmer scintillation counter (Waltham, MA, USA).

ACKNOWLEDGMENTS

We thank N.S. Hill for his help on the lyophilization of samples, M.A. O’Neill for his suggestions on GX analysis, and the editor and the reviewers for their comments and suggestions. C.H. Lee was partly supported by Korea Science and Engineering Foundation (Grant # KRF-2005-215-F00002). This work was supported by a grant from the U.S. Department of Energy-Bioscience Division (Grant # DE-FG02-03ER15415).
REFERENCES


which encodes a putative glucuronyltransferase, is essential for normal secondary wall synthesis. *Plant Cell* 17: 3390-3408.


Table 3.1. Wall thickness of fibers and vessels in the stems and roots of wild-type and *parvus* mutant plants

<table>
<thead>
<tr>
<th>Sample</th>
<th>Interfascicular Fibers</th>
<th>Stem</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vessels</td>
<td>Xylary fibers</td>
</tr>
<tr>
<td>Wild Type</td>
<td>2.10±0.36</td>
<td>1.01±0.09</td>
<td>0.71±0.18</td>
</tr>
<tr>
<td><em>parvus</em></td>
<td>0.71±0.21</td>
<td>0.45±0.15</td>
<td>0.36±0.07</td>
</tr>
</tbody>
</table>

Wall thickness was measured from transmission electron micrographs of fibers and vessels. Data are means (μm) ± SE from 20 cells.
Table 3.2. Monosaccharide composition of cell walls from the stems of wild-type and *parvus* plants

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Mannose</th>
<th>Galactose</th>
<th>Arabinose</th>
<th>Rhamnose</th>
<th>Fucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>306±26</td>
<td>122±4</td>
<td>20.7±0.6</td>
<td>14.9±2.4</td>
<td>13.7±1.7</td>
<td>8.0±1.0</td>
<td>2.5±0.6</td>
</tr>
<tr>
<td><em>parvus</em></td>
<td>234±22</td>
<td>65±5</td>
<td>25.2±0.5</td>
<td>18.3±0.7</td>
<td>17.4±1.7</td>
<td>5.0±0.6</td>
<td>1.6±0.3</td>
</tr>
</tbody>
</table>

Cell wall residues used for composition analysis were prepared from stems of 10-week-old plants. Data are means (mg/g dry cell wall) ± SE of three independent assays.
Table 3.3. Monosaccharide composition of 1 N and 4 N KOH extracts from the cell walls of wild-type and *parvus* plants

<table>
<thead>
<tr>
<th>Cell wall sample</th>
<th>Xylose</th>
<th>Mannose</th>
<th>Galactose</th>
<th>Arabinose</th>
<th>Rhamnose</th>
<th>Fucose</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1N KOH extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>44.5±10.5</td>
<td>0.7±0.1</td>
<td>3.5±0.4</td>
<td>3.2±0.7</td>
<td>2.7±0.7</td>
<td>0.6±0.1</td>
<td>11.4±0.6</td>
</tr>
<tr>
<td><em>parvus</em></td>
<td>9.9±0.5</td>
<td>1.4±0.1</td>
<td>4.9±0.4</td>
<td>4.5±0.4</td>
<td>2.8±0.2</td>
<td>0.5±0.2</td>
<td>6.9±0.1</td>
</tr>
<tr>
<td>4N KOH extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>30.2±4.0</td>
<td>8.6±0.5</td>
<td>6.6±0.7</td>
<td>2.8±0.3</td>
<td>2.3±0.2</td>
<td>2.1±0.2</td>
<td>16.2±1.0</td>
</tr>
<tr>
<td><em>parvus</em></td>
<td>13.2±1.6</td>
<td>7.8±1.1</td>
<td>4.8±0.6</td>
<td>2.2±0.2</td>
<td>1.4±0.1</td>
<td>1.4±0.1</td>
<td>12.2±1.5</td>
</tr>
</tbody>
</table>

Cell walls were extracted sequentially with 1 N and 4 N KOH and the alkali-soluble extracts were used for monosaccharide composition analysis. Data are means (mg/g dry cell wall) ± SE of two independent assays.
Figure 3.1. Expression analysis of the *PARVUS* gene by real-time quantitative PCR. Error bars represent SE of three replicates. (A) The *PARVUS* gene is highly expressed in interfascicular fibers and xylem cells but absent in pith cells. The cells used for expression analysis were lasermicrodissected from *Arabidopsis* stem sections. (B) The *PARVUS* gene exhibits predominant expression in stems undergoing secondary wall thickening. (C) The expression of *PARVUS* together with three other secondary wall biosynthetic genes, *FRA8, IRX8* and *IRX9*, is induced by MYB46 overexpression, which results in ectopic deposition of secondary walls. (D) The expression of *PARVUS, FRA8, IRX8* and *IRX9* is down-regulated in SND1/NST1 RNAi lines, which exhibit a loss of secondary wall thickening in fiber cells.
Figure 3.2. In situ hybridization analysis of the PARVUS mRNA in Arabidopsis stems and roots. Cross sections of stems and roots of wild-type plants were hybridized with digoxigenin-labeled PARVUS antisense (A-C) or sense (D) RNA probe, and subsequently detected with alkaline phosphateconjugated antibodies. The hybridization signals are shown as purple color. (A) Cross section of the elongating part of a stem showing the predominant expression of PARVUS in developing vessels (arrows). (B) Cross section of the non-elongating part of a stem showing the expression of PARVUS in interfascicular fibers, xylary fibers (arrow) and developing vessels (arrowheads), all of which undergoing extensive secondary wall thickening. (C) Cross section of a root showing the predominant expression of PARVUS in the developing secondary xylem. (D) A control section of the non-elongating part of a stem hybridized with the sense PARVUS probe showing the absence of hybridization signals. if, interfascicular fiber; mx, metaxylem; px, protoxylem; sx secondary xylem. Bars = 204 μm.
Figure 3.3. Subcellular localization of the PARVUS protein. Yellow fluorescent protein (YFP)-tagged PARVUS together with cyan fluorescent protein (CFP)-tagged subcellular organelle markers were co-expressed in carrot protoplasts, and their fluorescent signals were visualized with a laser confocal microscope. (A) Hydropathy plot of the PARVUS protein showing the presence of a signal peptide sequence at the N terminus. (B) and (C) Differential interference contrast (DIC) image (B) and the corresponding fluorescent signals (C) of a carrot protoplast expressing YFP alone. (D-G) DIC image (D) and the corresponding PARVUS-YFP signals (E), ER-CFP signals (F) and the merged image (G) of a carrot cell expressing PARVUS-YFP and the ER marker ERCFP. Note the close superimposition of PARVUS-YFP and ER-CFP signals. (H-K) DIC image (H) and the corresponding PARVUS-YFP signals (I), FRA8-CFP signals (J) and the merged image (K) of a carrot cell expressing PARVUS-YFP and the Golgi-localized FRA8-CFP. Note the differences between the network localization pattern of PARVUS-YFP and the punctate pattern of FRA8-CFP. Bars = 35 μm.
Figure 3.4. Mutation of the *PARVUS* gene results in a dramatic reduction in stem strength and wall thickness of fibers and vessels. Inflorescence stems of 10-week-old plants were used for breaking strength measurement, and the bottom internodes and roots were sectioned for examination of fibers and vessels. (A) Breaking force measurement showing a drastic reduction in stem strength in the *parvus* mutant compared with the wild type. Error bars represent SE of measurements of 20 plants. (B) and (C) Cross sections of stems showing vascular bundles of the wild type (B) and *parvus* mutant (C). Note that the vessels in the *parvus* mutant are severely deformed (arrows). (D) and (E) Cross sections of interfascicular regions of stems showing the drastically reduced fiber wall thickness in *parvus* (E) compared with the wild type (D). (F) and (G) Cross sections of roots showing the thin-walled vessels and fibers in secondary xylem in the *parvus* mutant (F) compared with the wild type (G). Note the severely deformed vessels in the *parvus* mutant (arrows). Co, cortex; if, interfascicular fiber; ph, phloem; ve, vessel; xf, xylary fiber. Bars = 141 μm.
Figure 3.5. Transmission electron microscopy of secondary walls of fibers and vessels in the *parvus* mutant. (A) and (B) Interfascicular fiber cells of stems showing their thin walls in *parvus* (B) compared with the wild type (A). (C) and (D) Vessels and xylary fibers of stems showing their thin walls in *parvus* (D) compared with the wild type (C). Note the deformation of vessels. (E) and (F) Vessels and xylary fibers in root secondary xylem showing their thin walls in *parvus* (F) compared with the wild type (E). Note the deformation of vessels. ve, vessel; xf, xylary fiber. Bars = 7.9 μm.
Figure 3.6. Immunodetection of xylan in fibers and vessels of parvus stems and roots. Stem and root sections were probed with the monoclonal antibody LM10 that was generated against plant cell wall (1,4)-β-D-xylan. Xylan signals were detected with fluorescein isothiocyanate-conjugated secondary antibodies and visualized with a laser confocal microscope. (A) to (C) Xylan immunofluorescent signals in stem sections of the wild type (A), parvus mutant (B), and parvus complemented with the wild-type PARVUS gene (C). Note the absence of xylan signals in the walls of interfascicular fibers and xylem in the parvus mutant compared with the wild type. (D) to (F) Xylan immunofluorescent signals in root sections of the wild type (D), parvus mutant (E), and parvus complemented with the wild-type PARVUS gene (F). Note the absence of xylan signals in the walls of secondary xylem in the parvus mutant. if, interfascicular fiber, sx, secondary xylem; xy, xylem. Bars = 159 μm.
Figure 3.7. MALDI-TOF mass spectra of acidic xylooligosaccharides generated by $\beta$-endoxyylanase digestion of GX from the stems of the wild type (A) and parvus mutant (B). The ions at $m/z$ 743 and 759 correspond to xylooligosaccharides composed of four Xyl residues bearing a nonmethylated GlcA residue (X4G) and a methylated GlcA residue (X4M), respectively (C). Note the absence of the ion at $m/z$ 743 corresponding to X4G in the parvus mutant.
A

Wild type

\[ X_M \]

739

\[ X_G \]

745

650

m/z

1100

B

parvus

\[ X_M \]

759

650

m/z

1100

C

\[ X_G: \]

\[ \beta-D-Xyl(1\rightarrow4)\beta-D-Xyl(1\rightarrow4)\beta-D-Xyl(1\rightarrow4)D-Xyl \]

\[ \alpha-D-GlcA \]

\[ X_M: \]

\[ \beta-D-Xyl(1\rightarrow4)\beta-D-Xyl(1\rightarrow4)\beta-D-Xyl(1\rightarrow4)D-Xyl \]

\[ 4-O-Me-\alpha-D-GlcA \]
Figure 3.8. Anomeric region of the $^1$H-NMR spectra of xylooligosaccharides generated by β-endoxylanase digestion of GX from wild type (A) and parvus (B) stems. Resonances are labeled with the position of the assigned proton and the identity of the residue containing that proton. G denotes the resonance of H1 of branched β-Xyl residues bearing an α-GlcA side chain. M denotes the resonance of H1 of branched β-Xyl residues bearing a 4-O-Me-α-GlcA side chain. The resonances of H5 of 4-O-Me-α-GlcA in parvus are shifted relative to those of the wild type. It is apparent that compared with the wild type, the parvus mutant lacks the resonances of H1 of α-DGalA, H1 of α-L-Rha, H1 of 3-linked β-D-Xyl, H4 of α-D-GalA and H2 of α-L-Rha from the tetrasaccharide primer sequence located at the reducing end of GX as well as the resonances of H1 and H5 of α-GlcA residues, and H1 of branched β-Xyl residues bearing an α-GlcA residues.
Figure 3.9. The XylT and GlcAT activities in the stems of the wild type and *parvus* mutant.

Microsomes isolated from the stems of the wild type and *parvus* mutant were incubated with Xyl6 and UDP-[14C]-Xyl or UDP-[14C]-GlcA, and the XylT and GlcAT activities were detected by counting the radioactivity (CPM) of the reaction products. Error bars represent SE of two separate assays. (A) XylT activity in the *parvus* mutant compared with the wild type. (B) GlcAT activity in the *parvus* mutant compared with the wild type.
CHAPTER 4

THE ARABIDOPSIS FAMILY GT 43 GLYCOSYLTRANSFERASES FORM TWO FUNCTIONALLY NON-REDUNDANT GROUPS ESSENTIAL FOR THE ELONGATION OF GLUCURONOXYLAN BACKBONE

ABSTRACT

There exist four members of family GT43 glycosyltransferases in the Arabidopsis genome and mutations of two of them, IRX9 or IRX14, have previously been shown to cause a defect in glucuronoxylan (GX) biosynthesis. However, it is currently unknown whether IRX9 and IRX14 perform the same biochemical function and whether the other two GT43 members are also involved in GX biosynthesis. In this report, we performed comprehensive genetic analysis of the functional roles of the four Arabidopsis GT43 members in GX biosynthesis. The I9H (IRX9 homolog) and I14H (IRX14 homolog) genes were shown to be specifically expressed in cells undergoing secondary wall thickening and their encoded proteins were targeted to the Golgi, where GX is synthesized. Overexpression of I9H but not IRX14 or I14H rescued the GX defects conferred by the irx9 mutation, whereas overexpression of I14H but not IRX9 or I9H complemented the GX defects caused by the irx14 mutation. Double mutant analyses revealed that I9H functioned redundantly with IRX9 and that I14H was redundant with IRX14 in their functions. In addition, double mutations of IRX9 and IRX14 were shown to cause a loss of secondary wall thickening in fibers and a much more severe reduction in GX amount than their single mutants. Together, these results provide genetic evidence demonstrating that all four Arabidopsis GT43 members are involved in GX biosynthesis and suggest that they form two functionally non-redundant groups essential for the normal elongation of GX backbone.
INTRODUCTION

Secondary walls constitute the bulk of cellulosic biomass produced by vascular plants. Cellulosic biomass in the form of fibers and wood is an important raw material for a myriad of industrial uses, such as timber, pulping, paper-making, and textiles. Due to the dwindling of non-renewable fossil fuels and the detrimental effects of burning fossil fuels on the global environment, there has been an urgent call to develop alternative renewable energy sources and the lignocellulosic biomass from plants is considered to be an attractive renewable source for biofuel production (Somerville, 2006). However, lignocellulosic biomass is recalcitrant to the enzymatic conversion of cellulose into sugars because cellulose is embedded in a complex mixture of polysaccharides and lignin polymers that block the accessibility of degrading enzymes. It has been shown that reduction of lignin and xylan by chemical or enzymatic treatment or by the transgenic approach reduces the recalcitrance of the lignocellulosic biomass to saccharification (Himmel et al., 2007; Chen and Dixon et al., 2007; Lee et al., 2009a). Therefore, a complete understanding of how individual components of lignocellulosic biomass are biosynthesized will potentially allow us to design novel strategies for genetic modification of cell wall composition, hence reduction in biomass recalcitrance to biofuel production.

Xylan is the main hemicellulose that cross-links with cellulose in the secondary walls of dicot plants (Carpita and McCann, 2000). It is made of a linear backbone of β-(1,4)-linked xylosyl residues, about 10% of which are attached with side chains of single residues of glucuronic acid (GlcA) and/or 4-O-methylglucuronic acid (MeGlcA) via α-(1,2)-linkages. The backbone xylosyl residues may also be substituted with arabinosyl and acetyl groups. Based on the nature of the side chains, xylan is generally grouped as (methyl)glucuronoxylan (GX), which is the main hemicellulose in dicots, and arabinoxylan and glucuronoroarabinoxylan, which are the
most abundant hemicelluloses in grass cell walls (Ebringerová and Heinze, 2000). In addition to
the xylosyl backbone, the reducing end of xylan from birch (*Betula verrucosa*), spruce (*Picea
abies*), Arabidopsis, and poplar (*Populus alba X tremula*) contains a unique tetrasaccharide
sequence β-D-Xylp-(1→3)-α-L-Rhap-(1→2)-α-D-GalpA-(1→4)-D-Xylp (Shimizu et al., 1976;
Johansson and Samuelson, 1977; Andersson et al., 1983; Pena et al., 2007; Lee et al., 2009).

The biosynthesis of xylan requires multiple glycosyltransferases and other modifying
enzymes. Early biochemical studies revealed the activities of xylosyltransferases,
glucuronosyltransferases, arabinosyltransferases, methyltransferases, and acetyltransferases that
are likely involved in the biosynthesis of xylan (Baydoun et al., 1983 and 1989; Kuroyama and
Tsumuraya, 2001; Gregory et al., 2002; Porchia et al., 2002; Urahara et al., 2004; Zeng et al.,
2008). However, none of the genes corresponding to these xylan biosynthetic enzymes have been
identified. Recent molecular and genetic studies in Arabidopsis and poplar have led to the
identification of a number of glycosyltransferases that are essential for GX biosynthesis. Among
them, several members of the families GT47 and GT8 from Arabidopsis (FRA8, F8H, IRX8, and
PARVUS) and poplar (GT47C, GT8D, and GT8E/8F) are implicated in the biosynthesis of the
GX reducing end sequence (Aspeborg et al., 2005; Zhong et al., 2005; Brown et al., 2005 and
2007; Zhou et al., 2006 and 2007; Pena et al., 2007; Persson et al., 2007; Lee et al., 2007b, 2009b
and 2009c). These glycosyltransferase genes are specifically expressed in vessels and fibers and
their encoded proteins are targeted to Golgi where GX is synthesized except for PARVUS and
GT8E/8F that are predominantly located in the endoplasmic reticulum (Lee et al., 2007b and
2009c). Mutations of the Arabidopsis *FRA8*, *IRX8*, and *PARVUS* genes all led to a near loss of
the reducing end tetrasaccharide sequence and a reduction in GX amount (Brown et al., 2007;
Pena et al., 2007; Lee et al., 2007), indicating their essential roles in the biosynthesis of the GX reducing end sequence although their exact enzymatic activities are still unknown.

The genetic studies have also identified roles of two members of family GT43 glycosyltransferases, IRX9 and IRX14, from Arabidopsis and GT43B from poplar in the biosynthesis of the GX xylosyl backbone (Brown et al., 2007; Pena et al., 2007; Zhou et al., 2007). The expression of IRX9 has been shown to be associated with cells undergoing secondary wall biosynthesis and its encoded protein is targeted to the Golgi. Mutation of the IRX9 gene causes a drastic reduction in xylan xylosyltransferase activity (Brown et al., 2007; Lee et al., 2007) and concomitantly a substantial decrease in the GX chain length and GX amount (Pena et al., 2007). Mutation of IRX14 was shown to result in a reduction in the GX level and the xylosyltransferase activity (Brown et al., 2007). In addition, two functionally redundant glycosyltransferases IRX10 and IRX10-like, which belong to family GT47, were also demonstrated to be required for the normal GX level and xylan xylosyltransferase activity, suggesting their involvement in the biosynthesis of the GX xylosyl backbone (Brown et al., 2009; Wu et al., 2009).

In this report, we performed comprehensive molecular and genetic studies of the roles of all members of the Arabidopsis family GT43 glycosyltransferases in GX biosynthesis. We show that like IRX9, the other three GT43 members, I9H (IRX9 homolog), IRX14 and I14H (IRX14 homolog), are expressed in secondary wall-containing cells and their encoded proteins are targeted to the Golgi. We have found that the GX defects in the irx9 mutant can be rescued by overexpression of I9H but not IRX14 and I14H. Similarly, overexpression of I14H but not IRX9 and I9H is able to complement the GX defects caused by the irx14 mutation. Furthermore, genetic analysis of an array of double mutants revealed redundant and non-redundant roles of
GT43 members in GX biosynthesis. Our findings demonstrate that the Arabidopsis family GT43 glycosyltransferases form two functionally non-redundant groups essential for the normal elongation of GX backbone.

RESULTS

Family GT43 glycosyltransferase genes are expressed in cells undergoing secondary wall thickening

The previous findings that two members of the Arabidopsis family GT43 glycosyltransferases are involved in GX biosynthesis raised a question as to whether other GT43 members perform a similar function. There exist four GT43 members, IRX9 (At2g37090), I9H (IRX9 homolog; At1g27600), IRX14 (At4g36890), and I14H (IRX14 homolog; At5g67230), in the Arabidopsis genome. Among them, IRX14 and I14H share the highest sequence similarity (81.1%), and I9H shares a relatively higher sequence similarity with IRX9 than with IRX14 and I14H (Fig. 4.1, A and B). Phylogenetic analysis further indicates that IRX14 and I14H are more closely related to each other than to IRX9 and I9H, and vice versa (Fig. 4.1C). IRX9 was previously shown to be specifically expressed in vessels and fibers (Pena et al., 2007), but the expression patterns of other GT43 members are not known. Quantitative PCR analysis showed that I9H was ubiquitously expressed in all organs examined, whereas IRX14 and I14H were preferentially expressed in the inflorescence stems (Fig. 4.1D). Further expression analysis in the isolated cells from stems revealed that I9H, IRX14, and I14H were preferentially expressed in xylem and/or interfascicular fibers, the cell types undergoing secondary wall thickening (Fig. 4.1E). We next tested whether the expression of I9H, IRX14, and I14H was regulated by SND1, a master transcriptional switch activating the biosynthetic pathways of xylan, cellulose, and lignin (Zhong et al., 2006 and 2007). It was found that their expression was reduced in the
SND1/NST1 RNAi lines and that SND1 overexpression induced the expression of *I9H* and *IRX14* (Fig. 4.1F).

We further examined the expression patterns of *I9H*, *IRX14*, and *I14H* in transgenic Arabidopsis plants using the β-glucuronidase (GUS) reporter gene. Consistent with the quantitative PCR analysis, these genes were found to be specifically expressed in interfascicular fibers and xylem cells in the inflorescence stems (Fig. 4.2, A, B, D, E, G and H). In the roots, *IRX14* and *I14H* were expressed in both developing vessels and xylary fibers in the secondary xylem (Fig. 4.2, F and I), whereas *I9H* expression was only evident in developing vessels but not in xylary fibers (Fig. 4.2C). These expression studies demonstrate a close association of *I9H* and *I14H* with cells undergoing secondary wall thickening, suggesting their possible involvement in secondary wall biosynthesis.

**Family GT43 glycosyltransferases are targeted to the golgi**

IRX9 was previously demonstrated to reside in the Golgi (Pena et al., 2007), but the subcellular locations of other GT43 members have not yet been examined. Sequence analysis of *I9H*, *IRX14*, and *I14H* using the TMHMM2.0 program for prediction of transmembrane helices in proteins (http://www.cbs.dtu.dk/service/TMHMM-2.0/) predicts that they are membrane proteins with a single (*I9H* and *IRX14*) or double (*I14H*) transmembrane helices (Fig. 4.3, A to C). To study their actual subcellular locations, yellow fluorescent protein (YFP)-tagged GT43 members were co-expressed with cyan fluorescent protein (CFP)-tagged FRA8 in carrot (*Daucus carota*) cells. Examination of the fluorescent signals revealed that *I9H*, *IRX14*, and *I14H* exhibited a punctate distribution, a pattern matched with FRA8 (Fig. 4.3, F to Q) that is known to be localized in the Golgi (Zhong et al., 2005). The control cells expressing YFP alone showed
signals throughout the cytoplasm (Fig. 4.3, D and E). These results demonstrate that I9H, IRX14, and I14H are Golgi-localized proteins.

**Overexpression of I9H but not IRX14 and I14H complements the irx9 mutant defects**

Previous genetic studies showed that mutation of either IRX9 or IRX14 caused a partial reduction in the xylosyltransferase activity and GX content (Brown et al., 2007; Lee et al., 2007; Pena et al., 2007), which may be due to functional compensation by other GT43 members. To test this possibility, we investigated whether the defects caused by either irx9 or irx14 could be rescued by overexpression of GT43 members driven by the cauliflower mosaic virus (CaMV) 35S promoter. One of the prominent phenotypes of irx9 was the weakened stem strength due to the reduced secondary wall thickness (Fig. 4.4, A and B). Overexpression of I9H and IRX9 in the irx9 mutant effectively restored the stem breaking strength and the secondary wall thickness of fibers to the wild-type levels (Fig. 4.4, A, C, D, and G), indicating that I9H is a functional homolog of IRX9. It was noted that not all I9H-complemented irx9 plants exhibited the same breaking strength as that of the wild type, which is likely due to the variation of the degree of complementation among different individuals. In contrast, overexpression of IRX14 and I14H did not complement the stem strength and secondary wall thickness defects conferred by irx9 (Fig. 4.4, A, E and F).

The reduced secondary wall thickness in irx9 was previously found to be caused by GX defects, including a decrease in GX level, a loss of non-methylated GlcA, and a reduced GX chain length (Pena et al., 2007). Analysis of the GX content revealed that I9H overexpression in the irx9 mutant restored the cell wall xylose level to that of the wild type (Table 4.1), indicating that excess I9H could complement the loss of IRX9 to restore the GX level. It should be noted that similar to fra8, irx9 and irx14 cause not only a reduction in xylose but also a reduction in
glucose, which might be attributed to an indirect impediment of cellulose synthesis caused by a
reduction in GX (Zhong et al., 2005). The observed increase in the levels of some other cell wall
sugars might be simply due to the relative decrease in the levels of cellulose and GX or an
increased synthesis of other wall polysaccharides in response to the reduced level of cellulose, a
phenotype commonly observed in cellulose-deficient mutants (Fagard et al., 2000; His et al.,
2001). Overexpression of GT43 members in irx9 also resulted in a change in the levels of other
sugars in addition to xylose, which might reflect the complexity of cell wall metabolism. The
structure of GX in the complemented plants was examined by analyzing the acidic
xyloooligosaccharides using matrix-assisted laser-desorption ionization time-of-flight mass
spectrometry (MALDI-TOF MS). Acidic xyloooligosaccharides from the wild type contain two
prominent ion peaks [M+Na]+ at mass-to-charge ratios (m/z) of 745 and 759, which correspond
to xylotetrasaccharides substituted with one GlcA and one MeGlcA, respectively (Fig. 4.5A).
The loss of GlcA side chains caused by the irx9 mutation (Fig. 4.5B) was rescued by
overexpression of I9H and IRX9 (Fig. 4.5, C and D) but not by IRX14 and I14H (Fig. 4.5, E and
F).

The structure of GX from the complemented plants was further analyzed using nuclear
magnetic resonance (NMR) spectroscopy. The resonances that are assigned to the GX reducing
end tetrasaccharide sequence, β- D-Xyl-(1→3)-α-L-Rha-(1→2)-α-D-GalA-(1→4)-D-Xyl, include
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
(Pena et al., 2007). It was previously found that the resonances for the GX reducing end
tetrasaccharide sequence in irx9 were highly elevated compared with those of the wild type,
which is caused by a significant reduction in the GX chain length and thereby a relative increase
in the ratio of the GX reducing end sequence over the backbone xylosyl residues (Pena et al.,
2007). It was evident that overexpression of I9H but not IRX14 and I14H in *irx9* restored the resonance intensity for the GX reducing end tetrasaccharide sequence to the wild-type level (Fig. 4.6; Table 4.3), indicating that excess I9H is sufficient to complement the GX chain length defect conferred by *irx9*. Consistent with the MALDI-TOF MS results showing that I9H overexpression restores the α-D-GlcA side chains in GX (Fig. 4.5), the resonances assigned to H1 of α-D-GlcA residues, which were absent in *irx9*, were restored to the wild-type level in the I9H-complemented *irx9* plants (Fig. 4.6).

The restoration of GX level and structure in I9H-complemented *irx9* plants was accompanied by a partial rescue of the GX xylosyltransferase activity (Fig. 4.7A). Overexpression of IRX14 and I14H did not restore the xylosyltransferase activity in the *irx9* mutant. It was interesting to note that overexpression of IRX9 in *irx9* led to an increased xylosyltransferase activity compared with the wild type. Together, these results suggest that I9H can perform the same biochemical function as IRX9 in the biosynthesis of GX backbone and that they are functionally distinct from IRX14 and I14H.

**Overexpression of I14H but not IRX9 and I9H complements the *irx14* mutant defects**

Our finding that overexpression of IRX14 and I14H does not complement the *irx9* mutant defects prompted us to test whether IRX14 and I14H form another pair of functional homologs involved in GX backbone biosynthesis. It was found that overexpression of I14H and IRX14 but not IRX9 and I9H in the *irx14* mutant rescued the defects in the stem strength, secondary wall thickness, the GX level, the GlcA side chains, and the GX xylosyltransferase activity (Fig. 4.4, A, H to M; Fig. 4.5, G to L; Fig. 4.7; Table 4.1). The *irx14* mutation was previously shown to cause a reduction in the GX level (Brown et al., 2007), but it is unknown whether the GX chain length is affected in the mutant. NMR analysis showed that similar to those in *irx9*, the resonances for
the GX tetrasaccharide reducing end sequence in *irx14* were highly increased compared to those in the wild type (Fig. 4.6), indicating that the GX chain length in *irx14* is significantly reduced (Table 4.3). Overexpression of I14H in *irx14* apparently restored the resonance signals for the GX tetrasaccharide reducing end sequence to the wild-type level, indicating its ability to complement the GX chain length defect conferred by *irx14* (Fig. 4.6; Table 4.3). These results demonstrate that I14H and IRX14 form another group of functional homologs involved in GX biosynthesis.

**Double mutant analysis reveals redundant roles of GT43 members in GX biosynthesis**

The findings that *I9H* is expressed in the same cell types as *IRX9* and excess I9H is able to complement the *irx9* mutant defects indicate that they function redundantly in GX biosynthesis. Likewise, I14H might function redundantly with IRX14 in GX biosynthesis. To test these hypotheses, we generated an array of double mutants with all possible combinations of the GT43 members and examined their effects on plant growth and GX biosynthesis. Although the *irx9* or *irx14* mutant exhibited a reduction in stem strength, secondary wall thickness, and the GX level (Brown et al., 2007; Pena et al., 2007), T-DNA insertion mutation of *I9H* or *I14H* alone did not have any discernable effects (Figs. 4.8 and 9; Table 4.2). However, homozygous double mutations of *IRX9* and *I9H* led to a strong retardation of plant growth (Fig. 3.8D). Even after prolonged growth (4 months), no inflorescence stems grew out from the rosettes, which prevented us from further analysis of the double homozygous mutants. The double mutant that is homozygous for *irx9* and heterozygous for *i9h (+/-)* did grow out short inflorescence stems after prolonged growth. Examination of the *irx9 i9h (+/-)* inflorescence stems revealed that the interfascicular fibers had thinner walls (Figs. 4.8J and 4.9F) than did *irx9* (Figs. 4.8F and 4.9B). In addition, cell wall composition analysis showed that the xylose content in *irx9 i9h (+/-)* was
more severely reduced than that in \textit{irx9} (Table 4.2). These results demonstrate that IRX9 and I9H play redundant roles in GX biosynthesis and secondary wall thickening.

Similar to \textit{irx9 i9h}, double homozygous mutations of \textit{IRX14} and \textit{I14H} cause a much stronger effect on plant growth compared with \textit{irx14} (Fig. 4.8D). The \textit{irx14 i14h} grew out short inflorescence stems, allowing us to examine the secondary wall thickening and the GX level. The \textit{irx14 i14h} stems had little secondary wall thickening in the interfascicular fibers (Figs. 4.8O and 4.9K) and a much lower xylose content (Table 4.2) than those of \textit{irx14} (Figs. 4.8H and 4.9D). The double mutant that is homozygous for \textit{irx14} and heterozygous for \textit{i14h} (+/-) also had a more severe retardation in plant growth compared with \textit{irx14} (Fig. 4.8D).

We next examined the plant growth and secondary wall thickening in the double mutants, including \textit{irx9 i14h}, \textit{i9h irx14}, and \textit{i9h i14h}. The plant growth (Fig. 4.8D) and secondary wall thickening phenotypes of the \textit{irx9 i14h} (Figs. 4.8L and 4.9H) and the \textit{i9h irx14} (Figs. 4.8M and 4.9I) double mutants were similar to those of \textit{irx9} (Figs. 4.8F and 4.9B) and \textit{irx14} (Figs. 4.8H and 4.9D), respectively. Cell wall composition analysis revealed that the xylose content in \textit{irx9 i14h} had a slight reduction compared with \textit{irx9}, whereas the xylose content in \textit{i9h irx14} was similar to that in \textit{irx14} (Table 4.2). Although no significant alterations in plant growth and secondary wall thickening were seen in the \textit{i9h i14h} double mutant (Figs. 4.8D, 8N and 4.9J) compared with the wild type (Figs. 4.8E and 4.9A), the xylose content in \textit{i9h i14h} was slightly reduced (Table 4.2).

We further investigated the effects of double mutations of \textit{IRX9} and \textit{IRX14} on plant growth and GX biosynthesis. Both \textit{irx9 irx14} and \textit{irx9 irx14} (+/-) exhibited strong retardation in plant growth (Fig. 4.8D). The \textit{irx9 irx14} plants grew out small inflorescence stems after 6-month growth, and examination of these stems revealed the absence of discernable secondary walls in
the interfascicular fibers (Figs. 4.8K and 4.9G), a phenotype much more severe than that in the
irx9 (Fig. 4.8F) or irx14 (Fig. 4.8H) single mutant. Further cell wall composition analysis of irx9
irx14 stems revealed a drastic reduction in the xylose content (Table 4.2), indicating a more severe effect on GX biosynthesis than the irx9 or irx14 mutation alone.

The results from these comprehensive genetic analyses demonstrate that all four GT43 members are involved in GX biosynthesis although IRX9 and IRX14 play major roles. Together with the mutant complementation studies, our findings suggest that the family GT43 glycosyltransferases form two functionally non-redundant groups, IRX9 together with I9H and IRX14 together with I14H, both of which are essential for GX biosynthesis.

**DISCUSSION**

**IRX9 and IRX14 function non-redundantly in the elongation of GX backbone**

Our findings that IRX9 can not functionally complement the loss of IRX14 and vice versa suggest that IRX9 and IRX14 are two functionally non-redundant GT43 members essential for GX biosynthesis. Considering the fact that both IRX9 and IRX14 are required for the elongation of GX backbone (Pena et al., 2007; Fig. 4.6), it is reasonable to propose that IRX9 and IRX14 function cooperatively in the elongation of GX backbone. Such a cooperative function might have important implications in understanding the biochemical mechanism of GX backbone biosynthesis. The GX backbone is composed of β-(1,4)-linked xylosyl residues, and its biosynthesis needs to overcome a steric problem because each xylosyl residue is flipped nearly 180° with respective to its neighboring residues. Similar to a model proposed for cellulose biosynthesis in which two glycosyltransferase activities operate cooperatively from opposite sites to add cellobiosyl units to the β-(1,4)-glucan chain (Carpita and Vergara, 1998), the biosynthesis of GX backbone might also require two enzyme activities that operate in such a mechanism.
Although their exact biochemical functions await further investigation, IRX9 and IRX14 are strong candidates for such two glycosyltransferase activities involved in the elongation of the β-(1,4)-xylan chain. This hypothesis is consistent with the facts that mutation of either \textit{irx9} or \textit{irx14} results in a defect in the GX chain length and a reduction in the xylosyltransferase activity and that IRX9 and IRX14 are non-redundant in their functions.

**Redundancy of family GT43 genes in GX biosynthesis**

We have demonstrated that I9H and I14H are redundant in function with IRX9 and IRX14, respectively based on the following lines of evidence. First, all four GT43 members are expressed in the same cell types that undergo secondary wall thickening (Fig. 4.2) and their proteins are targeted into the Golgi (Fig. 4.3), where GX is synthesized. Second, overexpression of I9H and I14H can effectively rescue the GX defects, including the GX chain length, caused by the mutations of \textit{IRX9} and \textit{IRX14}, respectively (Figs. 4.4 to 6; Tables 4.1 and 4.3). Third, double mutation of \textit{IRX9} and \textit{I9H} results in a more severe defect in the GX level and a strong retardation of plant growth and likewise, double mutation of \textit{IRX14} and \textit{I14H} leads to similar phenotypes (Fig. 4.8; Table 4.2). Together, these results suggest that IRX9 and I9H are a pair of functional homologs and IRX14 and I14H are another pair of functional homologs. This finding explains the early observations that the \textit{irx9} or \textit{irx14} mutant only exhibits partial reductions in GX content and secondary wall thickness (Brown et al., 2005 and 2007; Pena et al., 2007). However, the \textit{i9h} or \textit{i14h} mutation alone does not cause any GX defects, indicating that IRX9 and IRX14 are functionally dominant in GX biosynthesis in vivo. The gene redundancy appears to be common in GX biosynthesis in Arabidopsis. For example, FRA8 and F8H are a pair of redundant genes required for GX reducing end sequence biosynthesis (Lee et al., 2009b), and IRX10 and IRX10-L are another pair involved in GX backbone biosynthesis (Brown et al., 2009; Wu et al., 2009).
In both of these cases, single mutation of FRA8 or IRX10 results in a defect in GX biosynthesis but single mutation of its homolog does not. Double mutations of these genes, either FRA8 and F8H or IRX10 and IRX10-L, cause a strong retardation in plant growth, a phenotype that is also observed in the double mutants, irx9 i9h and irx14 i14h. Since GX is a critical component required for cross-linking cellulose microfibrils in secondary walls, gene redundancy in GX biosynthesis likely presents an evolutionary advantage to safeguard the proper making of secondary walls, which are the key structure of vessels essential for water transport in the plant body.

**Genes involved in GX backbone biosynthesis**

It was previously proposed that some of the cellulose synthase-like (CSL) genes might encode xylan synthase (Richmond and Somerville, 2001), which is congruent with the findings that several hemicellulose backbones, such as xyloglucan β-1,4-glucan (Cocuron et al., 2007), β-1,4-mannan (Dhugga et al., 2004; Liepman et al., 2005), and β-(1,3;1,4)-mix-linked glucan (Burton et al., 2006), are synthesized by members of the CSL gene families. However, no recombinant CSL proteins were shown to exhibit any xylan synthase activity (Liepman et al., 2005). Transcription profiling analysis of wood formation in poplar revealed no correlations between secondary wall synthesis and the expression of CSL genes with the exception of the CSLA (a mannan synthase) gene (Aspeborg et al., 2005; Suzuki et al., 2006). Although mutation of CSLD5 has been reported to cause a decrease in xylosyltransferase activity and xylan immunolabeling, the CSLD5 gene is not expressed in cells undergoing secondary wall thickening and thus the observed phenotypes were considered to be indirect (Bernal et al., 2007). Therefore, genes responsible for the biosynthesis of GX backbone remain elusive. Because GT43 members are specifically expressed in cells undergoing secondary wall thickening and GX
biosynthesis (Pena et al., 2007; Fig. 4.2) and IRX9 and IRX14 are required for the normal GX xylosyltransferase activity and the normal elongation of GX chains (Brown et al., 2007; Lee et al., 2007; Pena et al., 2007; Fig. 4.6), GT43 members are apparently strong candidates for GX xylosyltransferases or part of the complexes required for the xylosyltransferase activities. However, an attempt of assaying the xylosyltransferase activity of recombinant IRX9 proteins was not successful (Pena et al., 2007). Our finding that IRX9 and IRX14 are non-redundant in function suggests that expression of both IRX9 and IRX14 would be required for the GX xylosyltransferase activity. Furthermore, the recent finding that IRX10 and IRX10-L are also required for the normal GX xylosyltransferase activity (Brown et al., 2009; Wu et al., 2009) indicates that the biosynthesis of GX backbone is much more complicated than expected. As suggested previously, a complex composed of multiple glycosyltransferases and other proteins might be involved in GX backbone synthesis (Pena et al., 2007). Further biochemical characterization of GT43 members together with other players will be necessary to unravel the biochemical process of GX biosynthesis.

MATERIALS AND METHODS

Plant growth conditions

Plants were grown in a greenhouse under 14-h-light/10-h-dark cycles. Common garden potting soil was used for growing plants with biweekly application of plant fertilizers.

Gene expression analysis

Total RNA from Arabidopsis thaliana (ecotype Columbia) plants was isolated with a Qiagen RNA isolation kit (Qiagen). The organs used were from 7-week-old plants grown in a greenhouse. RNA from different cell types (interfascicular fibers, xylem, and pith cells) was isolated and amplified as described previously (Zhong et al., 2006). First strand cDNA was
synthesized from total RNA treated with DNase I and then used as a template for real-time quantitative PCR analysis with the QuantiTect SYBR Green PCR kit (Clontech). The PCR primers for \textit{I9H} are 5’-tccaggagacatcatttatagagc-3’ and 5’-ctatttcatatgagagcttg-3’, those for \textit{IRX14} are 5’- aagatcaaatgccattatcccaag-3’ and 5’-tcagtttctttcttgatgcttagacga-3’, and those for \textit{I14H} are 5’- cctggctggatcataaagtcacc-3’ and 5’-tcagctagttgctgacactttgac-3’. The relative expression level was calculated by normalizing the PCR threshold cycle number of each gene with that of the \textit{EF1\textalpha} reference gene. The data were the average of three biological replicates.

RT-PCR was applied to examine the transcript level in the GT43 mutants as described previously (Zhong et al., 2005). The expression of the \textit{EF1\textalpha} gene was used as an internal control for determining the RT-PCR amplification efficiency among different samples.

\textbf{GUS reporter gene analysis}

To ensure the inclusion of all sequences required for the expression of the endogenous genes, the \textit{I9H}, \textit{IRX14}, or \textit{I14H} gene containing a 3-kb 5’ upstream sequence, the entire coding region, and a 2-kb 3’ downstream sequence was used for the GUS reporter gene analysis. The GUS reporter gene was inserted in frame right before the stop codon of these genes and then cloned into the binary vector pBI101 (Clontech) to create the GUS reporter constructs. The constructs were transformed into wild-type \textit{Arabidopsis} plants by the agrobacterium-mediated transformation (Bechtold and Bouchez 1994) to generate the GUS reporter transgenic plants. Inflorescence stems and roots from 7-week-old first generation of the transgenic plants were examined for the GUS activity as described previously (Zhong et al. 2005). At least 30 independent transgenic plants were tested and a consistent GUS staining pattern was observed for each GUS reporter construct.

\textbf{Subcellular localization}
The subcellular localization of I9H, IRX14, and I14H was performed by co-transfecting yellow fluorescent protein (YFP)-tagged fusion proteins and the cyan fluorescent protein (CFP)-tagged Golgi marker (FRA8) into carrot (D. Carota) protoplasts (Liu et al. 1994). The full-length cDNAs of I9H, IRX14, and I14H were fused in frame with the YFP cDNA and ligated between the CaMV 35S promoter and the nopaline synthase terminator in pBI221 (Clontech). Fluorescence signals in transfected protoplasts were visualized using a Leica TCs SP2 spectral confocal microscope (Leica Microsystems). At least 20 fluorescence-positive protoplasts were examined and the same subcellular localization pattern was observed for each construct. Images were saved and processed with Adobe Photoshop Version 7.0 (Adobe Systems).

Complementation of the irx9 and irx14 mutants

The full-length cDNAs of GT43 members driven by the CaMV 35S promoter were cloned into the pGPTV binary vector. The constructs were introduced into the Arabidopsis irx9 or irx14 mutant by the agrobacterium-mediated transformation (Bechtold and Bouchez, 1994). Transgenic plants were selected on hygromycin, and the first generation of transgenic plants was used for breaking strength and anatomical analyses. Basal parts of the main inflorescence of 10-week-old plants were measured for the breaking force using a digital force/length tester (Zhong et al., 1997). The breaking force was calculated as the force needed to break apart a stem segment. For each construct, at least 96 transgenic plants were generated and examined.

Histology

Tissues were fixed in 2% formaldehyde and embedded in Low Viscosity (Spurr's) resin (Electron Microscopy Sciences) as described (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 902A transmission electron microscope (Carl Zeiss). For each construct, stems from at least 8 transgenic plants (first generation) with representative phenotypes were sectioned and representative data were shown.

**Cell wall sugar composition analysis**

Inflorescence stems from at least 20 independent lines of Arabidopsis plants were used for cell wall isolation according to Zhong et al. (2005). 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 an Agilent 6890N gas-liquid chromatography (Wilmington) equipped with a 30 m x 0.25 mm (i.d.) silica capillary column DB 225 (Alltech Assoc.).

**Matrix-Assisted Laser-Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)**

Xylooligosaccharides were prepared by digestion of 4 N KOH solubilized wall preparations with β-xylanase M6 (Megazyme) as described (Zhong et al., 2005). The acidic xylooligosaccharides were analyzed using a MALDI-TOF mass 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 x 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 the stainless steel target plate. Spectra are the average of 100 laser shots.

**¹H-NMR spectroscopy**

NMR spectra of the acidic xylooligosaccharides from β-xylanase digestion were acquired at 20 °C on a Varian Inova 600 MHz spectrometer (599.7 MHz, ¹H) using a 5 mm cryogenic
triple resonance probe (Varian). All NMR samples were prepared using 100% D₂O in 3 mm standard NMR tube. ¹H 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-seconds. The residual water resonance was suppressed by a 1-second presaturation 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 ¹H NMR assignments were done by comparison with the NMR spectra data for *Arabidopsis* acidic xylooligosaccharides (Zhong et al., 2005; Peña et al., 2007).

**Assay of xylosytransferase activity**

Microsomes were isolated from the inflorescence stems of at least four individual 8-week-old plants for each genotype following the procedure of Kuroyama and Tsumuraya (2001) and stored at -80°C until used. For assay of the xylosyltransferase activity, microsomes (100 μg per 30 μl reaction) were incubated with the reaction mixture containing 50 mM HEPES-KOH, pH 6.8, 5 mM MnCl₂, 1 mM dithiothritol, 0.5% Triton X-100, 0.1 mM cold UDP-Xyl (CarboSource Service), 0.2 μg/μl Xyl₆ (Megazyme) and UDP-[¹⁴C]Xyl (0.1 μCi; American Radiolabeled Chemical). The reaction products were separated from UDP-[¹⁴C]Xyl by paper chromatography according to Ishikawa et al. (2000) and counted for the amount of radioactivity with a PerkinElmer scintillation counter.

**Accession numbers**

The *Arabidopsis* Genome Initiative locus identifiers for the Arabidopsis genes investigated in this study are IRX9 (At2g37090), I9H (At1g27600), IRX14 (At4g36890), and I14H (At5g67230).
ACKNOWLEDGMENTS

We thank the ABRC (Columbus, OH) for the T-DNA knockout lines of IRX9, I9H, IRX14, and I14H, and the editor and the reviewers for their constructive comments and suggestions. This work was supported by grants from the U.S. Department of Energy-Bioscience Division (DE-FG02-03ER15415 to Z.H.Y.), and the U.S. EPA Office of Research and Development and the U.S. EPA Office of Science Council Policy (Q.T.). This work has been subjected to review by the National Exposure Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

REFERENCES


Table 4.1. Monosaccharide composition of cell walls from inflorescence stems of wild type, *irx9*, *irx14*, and the *irx9* and *irx14* plants overexpressing GT43 members

<table>
<thead>
<tr>
<th>Sample</th>
<th>Xylose</th>
<th>Glucose</th>
<th>Mannose</th>
<th>Galactose</th>
<th>Arabinose</th>
<th>Rhamnose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>108.7 ± 4.6</td>
<td>412.1 ± 4.7</td>
<td>18.9 ± 1.3</td>
<td>17.5 ± 1.3</td>
<td>12.6 ± 2.2</td>
<td>11.1 ± 1.2</td>
</tr>
<tr>
<td><em>irx9</em></td>
<td>60.8 ± 0.3</td>
<td>337.9 ± 0.4</td>
<td>39.0 ± 0.1</td>
<td>38.4 ± 1.3</td>
<td>28.6 ± 0.2</td>
<td>16.4 ± 0.6</td>
</tr>
<tr>
<td><em>irx9</em> + IRX9</td>
<td>91.6 ± 3.1</td>
<td>370.4 ± 3.0</td>
<td>19.1 ± 3.2</td>
<td>16.7 ± 0.8</td>
<td>16.7 ± 0.9</td>
<td>14.9 ± 0.9</td>
</tr>
<tr>
<td><em>irx9</em> + I9H</td>
<td>107.4 ± 2.8</td>
<td>446 ± 3.8</td>
<td>24.1 ± 0.9</td>
<td>18.2 ± 0.8</td>
<td>18.2 ± 0.6</td>
<td>12.7 ± 0.6</td>
</tr>
<tr>
<td><em>irx9</em> + IRX14</td>
<td>60.2 ± 5.9</td>
<td>366.9 ± 35.0</td>
<td>44.2 ± 5.0</td>
<td>25.8 ± 3.4</td>
<td>28.8 ± 3.2</td>
<td>19.3 ± 2.9</td>
</tr>
<tr>
<td><em>irx9</em> + I14H</td>
<td>52.7 ± 11.6</td>
<td>393.7 ± 42.2</td>
<td>43.1 ± 5.6</td>
<td>24.9 ± 3.6</td>
<td>23.9 ± 2.2</td>
<td>16.4 ± 1.2</td>
</tr>
<tr>
<td><em>irx14</em></td>
<td>49.4 ± 5.0</td>
<td>334 ± 8.4</td>
<td>23.6 ± 0.7</td>
<td>32.9 ± 2.9</td>
<td>12.0 ± 1.5</td>
<td>12.2 ± 0.9</td>
</tr>
<tr>
<td><em>irx14</em> + IRX9</td>
<td>45.9 ± 3.9</td>
<td>338.5 ± 11.7</td>
<td>23.6 ± 2.2</td>
<td>28.6 ± 0.8</td>
<td>13.7 ± 1.3</td>
<td>17.1 ± 0.8</td>
</tr>
<tr>
<td><em>irx14</em> + I9H</td>
<td>56.0 ± 2.0</td>
<td>372.1 ± 9.6</td>
<td>31.5 ± 2.6</td>
<td>29.1 ± 0.2</td>
<td>14.6 ± 0.1</td>
<td>18.2 ± 0.9</td>
</tr>
<tr>
<td><em>irx14</em> + IRX14</td>
<td>98.5 ± 4.3</td>
<td>385.4 ± 3.8</td>
<td>27.7 ± 1.3</td>
<td>24.9 ± 1.4</td>
<td>13.9 ± 0.2</td>
<td>12.7 ± 0.8</td>
</tr>
<tr>
<td><em>irx14</em> + I14H</td>
<td>96.9 ± 1.2</td>
<td>397.5 ± 6.0</td>
<td>18.0 ± 1.3</td>
<td>22.7 ± 3.3</td>
<td>11.5 ± 0.1</td>
<td>15.2 ± 0.2</td>
</tr>
</tbody>
</table>

The wall residues for cell wall composition analysis were prepared from stems. The data are means (mg/g dry cell wall) ± SE of duplicate assays.
Table 4.2. Monosaccharide composition of cell walls from inflorescence stems of wild type and GT43 mutants

<table>
<thead>
<tr>
<th>Sample</th>
<th>Xylose</th>
<th>Glucose</th>
<th>Mannose</th>
<th>Galactose</th>
<th>Arabinose</th>
<th>Rhamnose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>110.4 ± 1.4</td>
<td>402.0 ± 0.2</td>
<td>18.0 ± 0.4</td>
<td>14.6 ± 0.2</td>
<td>11.0 ± 0.2</td>
<td>11.7 ± 0.4</td>
</tr>
<tr>
<td>irx9</td>
<td>57.2 ± 0.3</td>
<td>336.9 ± 0.4</td>
<td>35.1 ± 0.1</td>
<td>39.9 ± 1.3</td>
<td>23.8 ± 0.2</td>
<td>13.9 ± 0.6</td>
</tr>
<tr>
<td>i9H</td>
<td>106.6 ± 3.8</td>
<td>418.1 ± 4.5</td>
<td>17.5 ± 0.7</td>
<td>13.9 ± 0.6</td>
<td>9.0 ± 0.1</td>
<td>10.4 ± 0.1</td>
</tr>
<tr>
<td>irx14</td>
<td>59.5 ± 2.1</td>
<td>346.5 ± 7.5</td>
<td>24.6 ± 2.5</td>
<td>16.2 ± 1.3</td>
<td>11.2 ± 0.1</td>
<td>10.9 ± 0.5</td>
</tr>
<tr>
<td>i14h</td>
<td>102.3 ± 4.8</td>
<td>391.8 ± 7.6</td>
<td>17.9 ± 2.3</td>
<td>15.1 ± 1.1</td>
<td>10.3 ± 0.5</td>
<td>10.6 ± 0.5</td>
</tr>
<tr>
<td>irx9  i9h(+/-)</td>
<td>40.2 ± 1.1</td>
<td>329.7 ± 8.4</td>
<td>40.4 ± 2.1</td>
<td>43.4 ± 1.4</td>
<td>35.8 ± 1.1</td>
<td>19.4 ± 2.0</td>
</tr>
<tr>
<td>irx9  irx14</td>
<td>26.5 ± 0.5</td>
<td>292.4 ± 5.3</td>
<td>20.2 ± 4.8</td>
<td>49.3 ± 3.4</td>
<td>26.0 ± 1.9</td>
<td>12.1 ± 0.2</td>
</tr>
<tr>
<td>irx9  i14h</td>
<td>49.2 ± 1.7</td>
<td>337.9 ± 13.4</td>
<td>30.6 ± 1.5</td>
<td>23.1 ± 1.8</td>
<td>15.2 ± 1.3</td>
<td>13.7 ± 0.9</td>
</tr>
<tr>
<td>i9h  irx14</td>
<td>59.3 ± 3.9</td>
<td>358.0 ± 15.7</td>
<td>25.7 ± 2.9</td>
<td>15.6 ± 1.7</td>
<td>11.2 ± 0.5</td>
<td>11.9 ± 0.3</td>
</tr>
<tr>
<td>i9h  i14h</td>
<td>94.2 ± 21.9</td>
<td>366.7 ± 24.3</td>
<td>12.6 ± 0.4</td>
<td>12.3 ± 1.0</td>
<td>8.9 ± 0.4</td>
<td>9.5 ± 0.5</td>
</tr>
<tr>
<td>irx14  i14h</td>
<td>29.1 ± 1.8</td>
<td>251.7 ± 11.1</td>
<td>24.3 ± 1.8</td>
<td>26.9 ± 2.2</td>
<td>19.3 ± 1.1</td>
<td>13.9 ± 0.6</td>
</tr>
</tbody>
</table>

The wall residues for cell wall composition analysis were prepared from stems. The data are means (mg/g dry cell wall) ± SE of duplicate assays.
Table 4.3. Relative abundance of the reducing end tetrasaccharide sequence and side chains, and the degree of polymerization of GX from the wild type and GT43 mutants overexpressing various GT43 members

<table>
<thead>
<tr>
<th>Sample</th>
<th>Frequency of occurrence of GlcA and MeGlcA side chains&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Relative abundance of the reducing end tetrasaccharide sequence&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Average DP&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>21.6%</td>
<td>100%</td>
<td>93.0</td>
</tr>
<tr>
<td>irx9</td>
<td>21.3%</td>
<td>31.9%</td>
<td>29.7</td>
</tr>
<tr>
<td>irx9 + IRX9</td>
<td>20.3%</td>
<td>88.1%</td>
<td>81.9</td>
</tr>
<tr>
<td>irx9 + I9H</td>
<td>19.0%</td>
<td>79.2%</td>
<td>73.7</td>
</tr>
<tr>
<td>irx9 + IRX14</td>
<td>20.3%</td>
<td>23.1%</td>
<td>21.5</td>
</tr>
<tr>
<td>irx9 + I14H</td>
<td>20.8%</td>
<td>34.0%</td>
<td>31.6</td>
</tr>
<tr>
<td>irx14</td>
<td>22.0%</td>
<td>37.6%</td>
<td>35.0</td>
</tr>
<tr>
<td>irx14 + IRX9</td>
<td>23.4%</td>
<td>40.9%</td>
<td>38.0</td>
</tr>
<tr>
<td>irx14 + I9H</td>
<td>23.3%</td>
<td>40.9%</td>
<td>38.0</td>
</tr>
<tr>
<td>irx14 + IRX14</td>
<td>21.5%</td>
<td>70.3%</td>
<td>65.4</td>
</tr>
<tr>
<td>irx14 + I14H</td>
<td>20.4%</td>
<td>82.3%</td>
<td>76.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>The frequency of occurrence of the GlcA and MeGlcA side chains was calculated from the ratios of the NMR resonance of the branched β-Xyl to that of total β-Xyl.

<sup>b</sup>The relative abundance of the reducing end tetrasaccharide sequence was determined from the ratios of the NMR resonance of the reducing end sequence to that of the branched β-Xyl. The abundance of the reducing end tetrasaccharide sequence in the wild type was taken as 100%.

<sup>c</sup>The average degree of polymerization (DP) was calculated based on the ratios of relative abundance of the reducing end sequence of a given sample to that of the wild type. The average DP of the wild type was taken as 93 (Pena et al., 2007).
Figure 4.1. Sequence comparison and expression analysis of family GT43 members. (A) Amino acid sequence alignment of the four Arabidopsis GT43 proteins. The numbers shown at the left of each sequence are the positions of amino acid residues in the corresponding proteins. Identical and similar residues are shaded with black and gray, respectively. (B) Percentage of identity and similarity of GT43 proteins. (C) Phylogenetic relationship of Arabidopsis GT43 members. The GT43 sequences were aligned using the ClustlW program (Thompson et al., 1994) and the phylogenetic tree was displayed using the TREEVIEW program (Page, 1996). The 0.1 scale denotes 10% change, and the bootstrap values are presented in percentages at the nodes. (D) Quantitative PCR analysis of the expression of *I9H*, *IRX14*, and *I14H* in different Arabidopsis organs. The highest expression level in an organ was set to 100. (E) Quantitative PCR analysis of the expression of *I9H*, *IRX14*, and *I14H* in pith cells, xylem cells, and interfascicular fibers that were laser-microdissected from wild-type Arabidopsis stems. The expression level in pith cells was taken as 1. (F) Quantitative PCR analysis of the expression of *I9H*, *IRX14*, and *I14H* in the SND1 overexpressors (SND1-OE) (left panel) and SND1/NST1 RNAi lines (right panel). The expression of each gene in the wild type was used as a control. Error bars in (D) to (F) denote SE of three biological replicates.
Figure 4.2. Expression patterns of *I9H*, *IRX14*, and *II4H* in elongating and non-elongating internodes of inflorescence stems, and roots. Cross sections of stems and roots from transgenic Arabidopsis plants expressing the *I9H*, *IRX14*, and *II4H* genes fused with the GUS reporter genes were stained for the GUS activity (shown as blue). if, interfascicular fiber; ve, vessels; xf, xylary fiber; xy, xylem. Bar in (A) = 130 μm in (A) to (I).
Figure 4.3. Subcellular localization of I9H, IRX14, and I14H. Fluorescent protein-tagged fusion proteins were expressed in carrot protoplasts, and the signals were visualized with a laser confocal microscope. (A) to (C) I9H, IRX14, and I14H are membrane proteins as predicted by the TMHMM2.0 program. Inside, the cytoplasmic side of the membrane; outside, the noncytoplasmic side of the membrane. (D) and (E) A carrot protoplast (D; differential interference contrast image) expressing YFP alone showing the fluorescent signals throughout the cytoplasm (E). (F) to (I) A carrot protoplast (F) co-expressing I9H-YFP (G) and the Golgi-localized FRA8-CFP (H). (J) to (M) A carrot protoplast (J) co-expressing IRX14-YFP (K) and FRA8-CFP (L). (N) to (Q) A carrot protoplast (N) co-expressing I14H-YFP (O) and FRA8-CFP (P). Note the colocalization of I9H-YFP (I), IRX14-YFP (M), and I14H-YFP (Q) with the Golgi marker FRA8-CFP.
Figure 4.4. Complementation of the *irx9* and *irx14* mutants by overexpression of GT43 members. The full-length cDNAs of the four Arabidopsis GT43 members driven by the CaMV 35S promoter were introduced into *irx9* or *irx14* and the bottom parts of inflorescence stems of 10-week-old transgenic overexpressors were examined for the stem breaking strength (A) and the secondary wall thickness in interfascicular fibers (B to M). Bar in (B) = 5.4 μm in (B) to (M). (A) Measurement of the breaking strength of stems of the wild type, *irx9*, *irx14*, and the mutants overexpressing GT43 members. Each bar represents the breaking force of the inflorescence stem of individual plants. It was evident that the stem breaking strength of *irx9* was restored by overexpression of IRX9 or I9H and that of *irx14* was restored by overexpression of IRX14 or I14H. (B) to (M) Transmission electron microscopy showing the restoration of the secondary wall thickness of the interfascicular fibers in the stems of the *irx9* mutant by overexpression of IRX9 and I9H, and that of the *irx14* mutant by overexpression of IRX14 and I14H.
Figure 4.5. MALDI-TOF mass spectra of acidic xylooligosaccharides generated by β-endoxylanase digestion of GX from the stems of the wild type, \textit{irx9}, \textit{irx14}, and the mutants overexpressing GT43 members. The ions at $m/z$ 745 and 759 correspond to xylotetrasaccharides bearing a GlcA residue (X$_4$G) or a methylated GlcA residue (X$_4$M). The ion at $m/z$ 781 corresponds to the doubly sodiated X$_4$M. Note that the missing ion at $m/z$ 745 corresponding to X$_4$G in \textit{irx9} was restored by overexpression of IRX9 or I9H and that in \textit{irx14} was restored by overexpression of IRX14 or I14H.
Figure 4.6, \(^1\)H-NMR spectra of acidic xylooligosaccharides generated by \(\beta\)-endoxylanase digestion of GX from the stems of the wild type, \textit{irx9}, \textit{irx14}, and the mutants overexpressing GT43 members. Resonances are labeled with the position of the assigned proton and the identity of the residue containing that proton. Note the restoration of the resonance of H1 of \(\alpha\)-GlcA in \textit{irx9} complemented with IRX9 and I9H and in \textit{irx14} complemented with IRX14 and I14H (arrows). The resonances of H1 of \(\alpha\)-D-GalA, H1 of \(\alpha\)-L-Rha, H1 of 3-linked \(\beta\)-D-Xyl, H4 of \(\alpha\)D-GalA, and H2 of \(\alpha\)-L-Rha are from the GX reducing end tetrasaccharide sequence. Note the elevated relative resonance intensity of the GX reducing end tetrasaccharide sequence in \textit{irx9} and \textit{irx14} and the reduction in its resonance intensity in \textit{irx9} complemented with IRX9 and I9H and in \textit{irx14} complemented with IRX14 and I14H. The doublet resonance peak at 4.39 ppm in the wild type is assigned to H5 \(\alpha\)-GlcA and that at 4.35 ppm is assigned to H5 Me-\(\alpha\)-GlcA. The resonance shift of H5 proton in other samples is likely attributed to its high sensitivity to a slight variation of pH and concentration of the samples because of its close proximity to the carboxyl group. A similar cause may lead to a slight resonance shift of H1 and H4 \(\alpha\)-GalA. HDO, hydrogen deuterium oxide.
Figure 4.7. Time course of the xylosyltransferase activity in the wild type, *irx9, irx14*, and the mutants overexpressing GT43 members. (A) Overexpression of I9H or IRX9 but not IRX14 or I14H in *irx9* rescued the deficiency in the xylosyltransferase activity caused by the *irx9* mutation. Note the increased xylosyltransferase activity in *irx9* overexpressing IRX9. (B) Overexpression of I14H or IRX14 but not IRX9 or I9H in *irx14* rescued the deficiency in the xylosyltransferase activity caused by the *irx14* mutation.
Figure 4.8. Effects of single and double mutations of GT43 members on plant growth and fiber wall thickening. (A) Diagrams showing the sites of T-DNA insertions in the $I9H$ and $I14H$ genes. (B) PCR identification of homozygous $i9h$ and $i14h$ mutants. T-DNA indicates the amplified DNA fragment with a T-DNA left border primer and an $I9H$ or $I14H$ primer flanking the T-DNA insertion site. Endogenous indicates the amplified $I9H$ or $I14H$ DNA fragment with primers spanning the T-DNA insertion site. (C) Breaking strength measurement showing that the stem strength of $i9h$ and $i14h$ is similar to that of the wild type. Each bar represents the breaking force of the inflorescence stem of individual plants. (D) Morphology of 10-week-old single and double GT43 mutants. Insets show the enlarged images of the rosette plants of $irx9\, i9h$ and $irx9\, irx14$. (E) to (O) Cross sections of stems showing the interfascicular fibers in single and double GT43 mutants. Note the extremely thin fiber walls in $irx9\, irx14$ and $irx14\, i14h$. The bottom parts of inflorescence stems of 10-week-old plants except for $irx9\, i9h\, (+/-)$ and $irx9\, irx14$, which were from 4-month and 6-month old plants, respectively, were sectioned and stained with toluidine blue for anatomy. if, interfascicular fiber. Bar in (E) = 65 μm for (E) to (O).
Figure 4.9. Transmission electron micrographs of the cell walls of interfascicular fibers in the stems of the wild type and GT43 mutants. The bottom parts of the inflorescence stems of 10-week-old plants except for \textit{irx9 i9h (+/-)} and \textit{irx9 irx14}, which were from 4-month and 6-month old plants, respectively, were examined for fiber wall thickness. Bar in (A) = 7.2 μm for (A) to (K).
CHAPTER 5

MOLECULAR DISSECTION OF XYLAN BIOSYNTHESIS DURING WOOD FORMATION IN POPLAR

\(^1\)

ABSTRACT

Xylan, being the second most abundant polysaccharide in dicot wood, is considered to be one of the factors contributing to wood biomass recalcitrance for biofuel production. To better utilize wood as biofuel feedstock, it is crucial to functionally characterize all the genes involved in xylan biosynthesis during wood formation. In this report, we investigated roles of poplar families GT43 and GT8 glycosyltransferases in xylan biosynthesis during wood formation. There exist seven GT43 genes in the genome of poplar (Populus trichocarpa), five of which, namely PtrGT43A, PtrGT43B, PtrGT43C, PtrGT43D, and PtrGT43E, were shown to be highly expressed in the developing wood and their encoded proteins were localized in the Golgi. Comprehensive genetic complementation coupled with chemical analyses demonstrated that overexpression of PtrGT43A/B/E but not PtrGT43C/D was able to rescue the xylan defects conferred by the Arabidopsis irx9 mutant, whereas overexpression of PtrGT43C/D but not PtrGT43A/B/E led to a complementation of the xylan defects in the Arabidopsis irx14 mutant. The essential roles of poplar GT43 members in xylan biosynthesis was further substantiated by RNAi downregulation of GT43B in the hybrid poplar (Populus alba x tremula) leading to reductions in wall thickness and xylan content in wood, and an elevation in the abundance of the xylan reducing end sequence. Wood digestibility analysis revealed that cellulase digestion released more glucose from the wood of poplar GT43B RNAi lines than the control wood, indicating a decrease in wood biomass recalcitrance. Furthermore, RNAi downregulation of another poplar wood-associated glycosyltransferase, PoGT8D, was shown to cause decreases in wall thickness and xylan content as well as in the abundance of the xylan reducing end sequence. Together, these findings demonstrate that the poplar GT43 members form two functionally nonredundant groups, i.e., PtrGT43A/B/E as functional orthologs of Arabidopsis IRX9 and
PtrGT43C/D as functional orthologs of Arabidopsis IRX14, all of which are involved in the biosynthesis of xylan backbones, and that the poplar GT8D is essential for the biosynthesis of the xylan reducing end sequence.

INTRODUCTION

Wood as the most abundant biomass produced by land plants is indispensable to our daily lives. Wood not only provides abundant raw materials for energy, construction, paper-making, and many other industrial uses, but also has recently been considered to be an attractive source for biofuel production (Carroll and Somerville, 2009). There has been a long history of active research in wood biology in the hope of better utilizing this abundant, renewable biomass for our ever-expanding needs (Plomion et al., 2001). Although wood is well characterized anatomically and chemically, our understanding of the genes participating in wood formation is still in infancy and only a small number of wood-associated genes from tree species have been characterized functionally (Mellerowicz and Sundberg, 2008). Further investigation of the functions of genes involved in wood formation in tree species is necessary in order to provide foundation knowledge for genetic improvement of wood quantity and quality.

Previous transcriptome profiling of genes expressed during wood formation have revealed a number of regulatory and biosynthetic genes that are likely involved in the biosynthesis of wood components, including cellulose, hemicellulose (xylan and glucomannan), and lignin (Aspeborg et al., 2005; Prassinos et al., 2005; Andersson-Gunneras et al., 2006; Bedon et al., 2007; Pavy et al., 2008; Wilkins et al., 2009; Grant et al., 2010; Zhong et al., 2010a and 2010b). Noteworthy is the transcriptome analysis of the secondary wall thickening zone in poplar wood that identified 25 wood-associated glycosyltransferases (GTs) (Aspeborg et al., 2005). Among these GTs, four (CesA1, CesA2, CesA3 and CesA9) are cellulose synthases (Joshi et al.,
and one (GT2A) is a mannan synthase (Suzuki et al., 2006; Liepman et al., 2007). A number of other poplar wood-associated GTs, including GT47C, GT43B, and GT8E/F, have recently been demonstrated to be involved in the biosynthesis of xylan, the second most abundant polysaccharide in wood.

In poplar wood, xylan is made of a linear chain of β-1,4-linked xylosyl residues with a degree of polymerization around 120 (Jacobs and Dahlman, 2001). Some of the xylosyl residues in the xylan backbone are substituted by α-1,2-linked 4-O-methylglucuronic acid residues and acetylated at C-2 or C-3 (Timell, 1967). As in other woody species, the reducing end of xylan in poplar contains a unique tetrasaccharide sequence consisting of β-D-Xylp-(1→3)-α-L-Rhap-(1→2)-α-D-GalpA-(1→4)-b-Xylp (Shimizu et al., 1976; Johansson and Samuelson, 1977; Andersson et al., 1983; Pena et al., 2007; Lee et al., 2009). Complementation studies have shown that poplar GT47C and GT8E/F are functional orthologs of the Arabidopsis thaliana FRA8 (Zhong et al., 2005) and PARVUS (Lee et al., 2007b), respectively, which are involved in the biosynthesis of the xylan reducing end sequence (Zhou et al., 2006; Lee et al., 2009c). The essential role of poplar GT47C in xylan biosynthesis has been demonstrated directly by RNA interference (RNAi) analysis showing that downregulation of PoGT47C expression causes reductions in both xylan content and the abundance of the xylan reducing end sequence (Lee et al., 2009a). Complementation study has also revealed that the poplar GT43B is a functional ortholog of the Arabidopsis IRX9 participating in the elongation of xylan backbone (Zhou et al., 2007). The functional roles of the remaining poplar wood-associated GTs identified by Asprborg et al. (2005) have not been analyzed experimentally. Elucidation of the functions of all wood-associated GTs will help unravel the complex process of wood formation.
In the *Arabidopsis* genome, there exist four GT43 genes, all of which have been shown to be involved in xylan biosynthesis (Brown et al., 2007; Pena et al., 2007; Keppler and Showalter, 2010; Lee et al., 2010; Wu et al., 2010). Genetic studies have revealed that the *Arabidopsis* GT43 members form two functionally non-redundant groups, IRX9/I9H and IRX14/I14H, that are required for the normal elongation of xylan backbone (Lee et al., 2010). The poplar (*Populus trichocarpa*) genome contains seven GT43 genes (Tuskan et al., 2006), namely *PtrGT43A-G*, of which only *GT43B* was previously shown to be implicated in xylan biosynthesis (Zhou et al., 2007). In this report, we show that three additional poplar GT43 members, *PtrGT43C/D/E*, are specifically expressed in developing secondary xylem and their encoded proteins are targeted into Golgi where xylan is synthesized. We demonstrate by complementation analysis that *PtrGT43A/B/E* are functional orthologs of the *Arabidopsis* IRX9, whereas *PtrGT43C/D* are functional orthologs of the *Arabidopsis* IRX14. Furthermore, we show that RNA interference (RNAi) downregulation of *GT43B* expression in poplar leads to a defect in xylan biosynthesis and a reduction in wood recalcitrance to cellulase digestion. We also reveal that RNAi downregulation of the expression of *GT8D* in poplar causes a reduction in xylan content and a decrease in the abundance of the xylan reducing end sequence. Our studies provide direct evidence showing that the poplar GT43 members form two functionally distinct groups involved in the elongation of xylan backbone and that the poplar GT8D participates in the biosynthesis of the xylan reducing end sequence during wood formation.

**RESULTS**

**Poplar GT43 genes are expressed in developing secondary xylem**

There exist seven GT43 genes in the genome of *Populus trichocarpa* (Tuskan et al., 2006). Besides the wood-associated *PtrGT43A/B*, which are close homologs of *IRX9* (Aspeborg
et al., 2005), the other five GT43 genes, namely \( \text{PtrGT43C/D/E/F/G} \), show less sequence similarity to \( \text{IRX9} \). Phylogenetic analysis revealed that \( \text{PtrGT43C/D} \) are closely related to \( \text{IRX14 and I14H} \) (also named \( \text{IRX14L} \)), whereas \( \text{PtrGT43E/F/G} \) are more closely grouped together with \( \text{I9H} \) (also named \( \text{IRX9L} \)) (Fig. 5.1A). Previous transcriptome analysis showed that \( \text{PtrGT43A-E} \) but not \( \text{PtrGT43F/G} \) are highly expressed during wood formation (http://www.bar.utoronto.ca/efppop/cgi-bin/efpWeb.cgi; Wilkins et al., 2009), indicating that they might be involved in the biosynthesis of wood components. Therefore, we decided to perform functional analysis to elucidate the functions of these wood-associated GT43 genes.

We first employed in situ mRNA hybridization approach to examine the cell-type level expression pattern of GT43 genes in the developing wood of \( \text{Populus trichocarpa} \). It was found that strong hybridization signals for \( \text{PtrGT43A/C/E} \) were detected not only in developing vessels and fibers in secondary xylem but also in ray parenchyma cells (Fig. 5.1B to D). Hybridization signals were also evident in phloem fibers. Since \( \text{PtrGT43A/B} \) are duplicated genes with high sequence identity (87%), the probe for \( \text{PtrGT43A} \) was able to recognize both \( \text{PtrGT43A} \) and \( \text{PtrGT43B} \) transcripts. Similarly, \( \text{PtrGT43C/D} \) are duplicated genes with high sequence identity (86%), the probe for \( \text{PtrGT43C} \) recognized both \( \text{PtrGT43C} \) and \( \text{PtrGT43D} \) transcripts. The control sections hybridized with the sense probes did not show any positive signals in developing xylem (Fig. 5.1F). These results demonstrate that these poplar GT43 genes are expressed in vessels, fibers and ray parenchyma cells in developing secondary xylem, an expression pattern similar to genes known to be involved in xylan biosynthesis in poplar (Zhou et al., 2006 and 2007).

To further substantiate their involvement in the biosynthesis of wood components, we determined the subcellular locations of these poplar GT43 proteins. Poplar GT43B was
previously shown to be targeted to the Golgi (Zhou et al., 2007). Sequence analysis using the
TMHMM2.0 program for the prediction of transmembrane helices in proteins
(http://www.cbs.dtu.dk/service/TMHMM-2.0/) predicted that these poplar GT43 proteins are
typical type II membrane proteins containing one or two transmembrane helices (Fig. 5.2A to D).
Subcellular localization studies using fluorescence protein tagging in carrot protoplasts
demonstrated that the GT43 proteins were co-localized with FRA8 (Fig.5.2G to V), a family
GT47 glycosyltransferase known to be located in the Golgi (Zhong et al., 2005). The control
protoplasts expressing YFP alone showed a uniform distribution of fluorescent signals
throughout the cytoplasm (Fig. 5.2E and F). These results indicate that these poplar GT43
proteins are located in the Golgi where non-cellulosic polysaccharides, such as xylan, are
synthesized.

**Expression of PtrGT43A/B/E but Not PtrGT43C/D rescues the irx9 mutant phenotypes**

The results from gene expression and protein subcellular localization analyses suggest
that similar to poplar GT43B, other poplar GT43 members are also likely involved in the
biosynthesis of xylan during wood formation. To test this hypothesis, we examined the ability for
poplar GT43 genes to complement the xylan defects conferred by the *Arabidopsis irx9* mutant.
The *irx9* inflorescence stems exhibited a strong reduction in their mechanical strength due to a
defect in secondary wall thickening in xylem and interfascicular fibers (Brown et al., 2005; Pena
et al., 2007). Complementation analysis revealed that overexpression of PtrGT43A/B/E in *irx9*
effectively restored the stem mechanical strength to the wild-type level, whereas overexpression
of PtrGT43C/D did not (Fig. 5.3A). Examination of xylem and interfascicular fibers in stems
further showed that overexpression of PtrGT43A/B/E but not PtrGT43C/D complemented the
collapsed vessel and reduced secondary wall thickening phenotypes caused by the *irx9* mutation (Fig. 5.3B to I and 5.4A to H).

To find out whether the restored secondary wall phenotype was due to complementation of the xylan defects in *irx9*, we analyzed the xylan content and structure in the PtrGT43-complemented *irx9* plants. The *irx9* mutant is deficient in xylose content caused by the reduced chain length of xylan backbone (Pena et al., 2007). Cell wall composition analysis demonstrated that overexpression of PtrGT43A/B/E but not PtrGT43C/D significantly restored the xylose content close to the wild-type level (Table 5.1). Further analysis of xylan structure showed that the loss of the glucuronic acid (GlcA) side chains conferred by *irx9* was restored by overexpression of PtrGT43A/B/E but not PtrGT43C/D (Fig. 5.5A to H).

We next examined the xylan chain length in *irx9* with the overexpression of GT43 members. The *irx9* mutation leads to a reduction in xylan chain length from an average of 93 in the wild type to 30, thereby resulting in a relative increase in the abundance of the reducing end sequence as revealed by nuclear magnetic resonance spectroscopy (NMR) (Pena et al., 2007; Figure 5.6). Analysis of the xylan structure using NMR showed a significant reduction in the relative abundance of the reducing end sequence due to a restoration of the xylan chain length in *irx9* with the overexpression of PtrGT43A/B/E but not PtrGT43C/D (Fig. 5.6; Table 5.2). Consistent with the restored chain length, overexpression of PtrGT43A/B/E partially complemented the defective xylosyltransferase activity conferred by the *irx9* mutation (Fig. 5.7A). Taken together, these chemical and structural analyses indicate that PtrGT43A/B/E are functional orthologs of IRX9 and they are involved in the biosynthesis of xylan backbone.

Expression of *PtrGT43C/D* but not *PtrGT43A/B/E* rescues the *irx14* mutant phenotypes
PtrGT43C/D are more closely related to the *Arabidopsis* IRX14 instead of IRX9 based on the phylogenetic analysis (Fig. 5.1A), which may explain why PtrGT43C/D failed to rescue the irx9 mutant phenotypes. To find out whether PtrGT43C/D are functional orthologs of IRX14, we overexpressed them in the irx14 mutant. Comprehensive histological, chemical and biochemical analyses of the transgenic plants demonstrated that PtrGT43C/D but not PtrGT43A/B/E complemented the irx14 mutant phenotypes, including the restoration of vessel morphology (Fig. 5.3J to 5.3Q), secondary wall thickness (Fig. 5.4I to 5.4P), stem mechanical strength (Fig. 5.3A), xylan content (Table 5.1), GlcA side chains (Fig. 5.5I to 5.5P), xylan chain length (Fig. 5.6; Table 5.2), and xylosyltransferase activity (Fig. 5.7B). It was interesting to note that although overexpression of PtrGT43E did not rescue the xylan deficient phenotypes conferred by the irx14 mutation, it partially restored the xylosyltransferase activity (Fig. 5.7B). Together with the results from the irx9 complementation test, these data indicate that the poplar GT43 members are involved in xylan biosynthesis and they form two functionally distinct groups, i.e., PtrGT43A/B/E as IRX9 functional orthologs, and PtrGT43C/D as IRX14 functional orthologs.

**Downregulation of the expression of Poplar **GT43B** and **GT8D** results in defects in xylan biosynthesis in poplar**

We further investigated the effects of downregulation of the expression of PoGT43B and PoGT8D on xylan biosynthesis in the hybrid poplar *Populus alba x tremula*. PoGT43B was previously shown to be a functional ortholog of *Arabidopsis* IRX9 by complementation study and PoGT8D is an ortholog of *Arabidopsis* IRX8 that is known to be involved in the biosynthesis of the xylan reducing end sequence, but their functional roles in wood formation in poplar have not been studied (Zhou et al., 2007). We generated and examined over 40 transgenic poplar RNAi lines for downregulation of expression of PoGT43B or PoGT8D. Quantitative PCR
analysis revealed that the transgenic poplar plants expressing PoGT43B RNAi transcripts exhibited a significant reduction in the expression level of PoGT43A/B and that those expressing PoGT8D RNAi transcripts had a severe reduction in the expression level of PoGT8D and its duplicated gene, PoGT8D-2 (Fig. 5.8A). Examination of the wood of transgenic poplar plants showed that RNAi downregulation of the expression of PoGT43B and PoGT8D caused defects in wood cell structure and morphology, i.e., a deformation of vessel shapes (Fig. 5.8B to 5.8F) and a reduction in the wall thickness of fibers (Fig. 5.9A to 5.9E). These results indicate that PoGT43B and PoGT8D are required for the normal development of secondary walls and the mechanical strength of vessels.

To find out whether the observed secondary wall defects in PoGT43B and PoGT8D RNAi lines were correlated with alterations in xylan content and structure, we isolated wood cell walls from the RNAi lines. Cell wall composition analysis demonstrated that the xylose content was significantly reduced in both PoGT43B- and PoGT8D-RNA lines compared with the control (Table 5.3), indicating that RNAi downregulation of the expression of PoGT43B and PoGT8D causes a defect in xylan biosynthesis. This conclusion was further substantiated by the immunolocalization of xylan with the xylan monoclonal antibody LM10 showing that the xylan signals were reduced in the walls of wood cells but not phloem fiber cells in the PoGT8D RNAi lines (Fig. 5.10C). No drastic reduction in xylan signals was observed in the PoGT43B RNAi lines (Fig. 5.10B), which is consistent with the observation in the Arabidopsis irx9 mutant (Pena et al., 2007). It was noticed that the amount of some other monosaccharides, noticeably arabinose, was also altered in both PoGT43B and PoGT8D RNAi lines (Table 5.3), which could be a result of indirect effects on primary wall biosynthesis in ray parenchyma cells of wood.
In addition to the reduction in xylan amount, RNAi downregulation of the expression of *PoGT43B* and *PoGT8D* also caused a change in xylan chain length or the abundance of its reducing end sequence (Fig. 5.11). NMR spectroscopic analysis demonstrated that the resonance signals for the xylan reducing end sequence in the *PoGT43B* RNAi lines were elevated compared to those in the control (Fig. 5.11; Table 5.4). This phenomenon is similar to that observed in the *Arabidopsis irx9* mutant (Pena et al., 2007), which is caused by the shortening of chain length of the xylan backbone, thus leading to a relative elevation in the signals for the reducing end sequence. In contrast, the resonance signals for the xylan reducing end sequence in the *PoGT8D* RNAi lines were decreased compared to those in the control (Fig. 5.11; Table 5.4), an observation similar to that in the *Arabidopsis irx8* mutant due to a defect in the biosynthesis of the xylan reducing end sequence.

To find out whether defects in xylan biosynthesis affected the synthesis of other wall components, we analyzed lignin composition in the wood of the wild type and transgenes. It was found that the amount of extractable guaiacyl lignin in the *PoGT43B* RNAi lines was significantly reduced, whereas that in the *PoGT8D* RNAi lines was slightly affected compared with the control (Table 5.5). A similar but milder reduction in the amount of syringyl lignin was also detected. These results indicate that alterations in xylan biosynthesis negatively impact the deposition of lignin polymers in transgenic wood.

**Increased cellulose digestibility by cellulase in the *PoGT43B* RNAi lines**

Lignin and xylan were considered to be critical factors contributing to the recalcitrance of biomass for the conversion of cellulose to fermental sugars (Himmel et al., 2007). To find out whether RNAi downregulation of the expression of *PoGT43B* and *PoGT8D* had any effects on the recalcitrance of wood, we determined the digestibility of wood cell walls by cellulase. To do
so, wood cell walls were incubated with cellulase and the released glucose was quantitatively determined. The results showed that more glucose was released from wood cell walls of *PtrGT43B* RNAi lines but no change from those of *PtrGT8D* RNAi lines compared to those of the control (Fig. 5.12), demonstrating that the alterations in xylan content and structure in *PtrGT43B* RNAi lines lead to an increased digestibility of wood cell walls by cellulase.

**DISCUSSION**

Glycosyltransferase families are classified based on their amino acid sequence similarity rather than on their uses of common acceptors and/or donors (Campbell et al., 1997). Therefore, members in the same GT family may perform different biochemical activities. One such example is family GT47 comprising of 39 members (Zhong and Ye, 2003), several of which have been demonstrated to be involved in the biosynthesis of different cell wall polysaccharides, such as xylan (Zhong et al., 2005; Brown et al., 2005, 2007 and 2009; Pena et al., 2007; Wu et al., 2009), xyloglucan (Madson et al., 2003), and pectin (Harholt et al., 2006). Furthermore, FRA8 and IRX10, two phylogenetically closely-related members of GT47 (Zhong and Ye, 2003), have distinct roles in xylan biosynthesis; FRA8 being involved in the biosynthesis of the xylan reducing end sequence (Pena et al., 2007) and IRX10 being required for the normal biosynthesis of xylan backbone (Brown et al., 2009). Thus, genetic and biochemical studies are necessary to assign the biochemical functions of different glycosyltransferases in a particular GT family. In this study, we performed functional characterization of five poplar wood-associated family GT43 members and revealed that they form two functionally distinct groups involved in the biosynthesis of xylan backbone. Our study enriches the understanding of family GT43 glycosyltransferases and their roles in xylan biosynthesis during wood formation.
Our findings that the five poplar wood-associated GT43 members are able to complement the xylan defects conferred by the irx9 or irx14 mutation provide genetic evidence that these five poplar GT43 members are involved in the xylan backbone biosynthesis during wood formation. Furthermore, the complementation study has demonstrated that these poplar GT43 members form two functionally non-redundant groups, PtrGT43A/B/E as functional orthologs of IRX9 and PtrGT43C/D as functional orthologs of IRX14. The remaining two members, PtrGT43F/G, which have little expression in developing wood, are phylogenetically grouped together with Arabidopsis I9H (Fig. 5.1A) and likely have the same biochemical functions as IRX9/I9H. It has previously been proposed that IRX9 and IRX14 are two xylosyltransferases that function cooperatively in the elongation of β-(1,4)-xylan chain and such a cooperative mechanism, which is similar to that proposed for cellulose biosynthesis (Carpita and Vergara, 1998), may be essential to overcome the steric problem for the transfer of each xylosyl residue because one xylosyl residue is flipped nearly 180° with respect to its neighboring residue (Lee et al., 2010). The finding that PtrGT43A/B/E and PtrGT43C/D are functional orthologs of IRX9 and IRX14, respectively, indicates that the biochemical mechanism for xylan biosynthesis is well conserved between herbaceous Arabidopsis and woody trees, both of which employing two functionally non-redundant groups of GT43 members for the elongation of the xylan backbone.

It is interesting to note that although overexpression of PtrGT43C/D and PtrGT43E in irx9 and irx14, respectively, did not complement the defects in xylan structure, their overexpression caused a slight elevation in xylosyltransferase activity (Fig. 5.7). Assuming that GT43 members are xylosyltransferases, one possible explanation for this phenomenon is that their overexpression leads to an increase in the addition of a single xylosyl residue onto the exogenous acceptor, thereby an elevated xylosyltransferase activity in the in vitro assay.
However, this elevated xylosyltransferase activity could not overcome the steric problem for the successive transfer of xylosyl residues with different orientation next to each other, thus unable to result in normal elongation of the xylan backbone. So far, it is still unknown whether GT43 members themselves possess xylosyltransferase activity or are accessory enzymes required for xylosyltransferase activity. Although an early attempt to detect xylosyltransferase activity using recombinant IRX9 protein in yeast cells was unsuccessful (Pena et al., 2007), available evidence from genetic and biochemical analyses in both Arabidopsis and poplar favor the hypothesis that GT43 members are xylosyltransferases responsible for the elongation of xylan backbone. The findings that two functionally non-redundant groups of GT43 members are involved in the elongation of xylan backbone in both Arabidopsis and poplar indicate that at least these two groups of GT43 members are required for future attempts of uncovering the identity of long-sought xylan synthase.

In addition to the finding that poplar GT43 members are involved in the elongation of xylan backbone, our study demonstrates that another poplar wood-associated GT, PoGT8D, is required for the biosynthesis of the xylan reducing end sequence. RNAi downregulation of PoGT8D expression was shown to cause a significant reduction in the abundance of the xylan reducing end sequence, an effect opposite to that caused by PoGT43B RNAi downregulation in which the abundance of the xylan reducing end sequence is elevated (Fig. 5.11; Table 5.4). This finding indicates that PoGT8D is a functional ortholog of Arabidopsis IRX8 involved in the biosynthesis of the xylan reducing sequence (Pena et al., 2007). Together with the previous studies showing that two other members of poplar wood-associated GTs, GT47C (Lee et al., 2009a) and GT8E/8F (Lee et al., 2009c), are functional orthologs of Arabidopsis FRA8 and PARVUS, respectively, that are required for the biosynthesis of xylan reducing end sequence,
these results indicate that the biochemical mechanisms underlying the biosynthesis of both xylan backbone and reducing end sequence are evolutionarily conserved between Arabidopsis and tree species.

Wood is a renewable, abundant forest product important for a myriad of commercial applications and a potential feedstock for biofuel production (Carroll and Somerville, 2009). Our findings that two functionally non-redundant groups of poplar GT43 members are involved in the biosynthesis of xylan backbone and that a poplar family GT8 member is implicated in the biosynthesis of the xylan reducing end sequence not only expands our understanding of the biochemical process of wood formation and but also provides additional tools for the genetic manipulation of wood components tailored to our needs. For example, we have demonstrated that alterations of xylan content and chain length in the PoGT43B RNAi lines lead to an apparent increase in the digestibility of wood by cellulase, which may have important implications in designing strategies for genetic modification of wood components for biofuel production. Further investigation of the biochemical activities of these xylan biosynthesis-related glycosyltransferases together with functional characterization of other wood-associated glycosyltransferases (Aspeborg et al., 2005) will undoubtedly help dissect the complex process of wood formation.

MATERIALS AND METHODS

Plant growth conditions

Arabidopsis thaliana, Populus trichocarpa (a gift from Dr. G.A. Tuskan) and Populus alba x tremula (a gift from Dr. S. Strauss) plants were grown in a greenhouse under 14-h-light/10-h-dark cycles. Common garden potting soil was used for growing plants with biweekly application of plant fertilizers.
In situ hybridization

Stems of *Populus trichocarpa* were fixed in 2.5% formaldehyde and 0.5% glutaraldehyde, embedded in paraffin, and sectioned for *in situ* mRNA localization according to Zhou et al. (2007). Digoxigenin-labeled antisense and sense RNA probes of *PtrGT43* cDNAs were generated using the DIG RNA labeling mix (Roche). Stem sections were hybridized with the antisense and sense probes and the hybridization signals were detected by incubating with alkaline phosphatase-conjugated antibodies against digoxigenin and subsequent color development with alkaline phosphatase substrates.

Subcellular localization

Yellow fluorescent protein (YFP)-tagged *PtrGT43* fusion proteins and cyan fluorescent protein (CFP)-tagged Golgi marker (FRA8; Zhong et al., 2005) were co-transfected into carrot (*D. carota*) protoplasts to determine the subcellular localization of *PtrGT43* proteins according to Liu et al. (1994). The full-length cDNAs of *PtrGT43* were fused in frame with the *YFP* cDNA and ligated between the CaMV 35S promoter and the nopaline synthase terminator in pBI221 (Clontech). Fluorescence signals in transfected protoplasts were visualized using a Leica TCs SP2 spectral confocal microscope (Leica Microsystems). At least 5 fluorescence-positive protoplasts were examined and the same subcellular localization pattern was observed for each construct. Images were saved and processed with Adobe Photoshop Version 7.0 (Adobe Systems).

Complementation analysis

The full-length *PtrGT43* cDNAs driven by the cauliflower mosaic virus (CaMV) 35S promoter were cloned into the pGPTV binary vector (Becker et al., 1992). The *PtrGT43* overexpression constructs were introduced into the *Arabidopsis irx9* or *irx14* mutant by the
agrobacterium-mediated transformation (Bechtold and Bouchez, 1994). Transgenic plants were selected on hygromycin, and the first generation of transgenic plants was used for breaking strength and anatomical analyses. Basal parts of the main inflorescence of 10-week-old plants were measured for the breaking force using a digital force/length tester (Zhong et al., 1997). The breaking force was calculated as the force needed to break apart a stem segment. For each construct, at least 64 transgenic plants were generated and examined.

**Histology**

Stems were fixed in 2% formaldehyde and embedded in Low Viscosity (Spurr's) resin (Electron Microscopy Sciences) as described (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 902A transmission electron microscope (Carl Zeiss). For each construct, stems from at least 8 transgenic plants were examined and representative data were shown.

**Cellwall sugar composition analysis**

Cell walls were isolated from stems of *Arabidopsis* and poplar plants according to Zhong et al. (2005). Neutral sugar composition of cell walls (as alditol acetates) was analyzed following the procedure described by Hoebler et al. (1989). The alditol acetates of the hydrolyzed cell wall sugars were determined on a PerkinElmer Clarus 500 gas-liquid chromatography equipped with a 30 m x 0.25 mm (i.d.) silica capillary column DB 225 (Alltech Assoc.). The minor fucose peak was not discerned due to its overlap with a noise peak.

**Matrix-Assisted Laser-Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)**
Xylan was solubilized from cell walls by incubation with 1 N KOH, and the solubilized xylan was digested with \( \beta \)-xylanase M6 (Megazyme) to release xylooligosaccharides as described (Zhong et al., 2005). The acidic xylooligosaccharides were analyzed using a MALDI-TOF mass 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 x 10^{-7} \text{ 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 the stainless steel target plate. Spectra are the average of 100 laser shots.

**\(^1\)H-NMR Spectroscopy**

NMR spectra of the acidic xylooligosaccharides from \( \beta \)-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 using 100% D\(_2\)O in 3 mm standard NMR tube. \(^1\)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-seconds. The residual water resonance was suppressed by a 1-second presaturation 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 comparison with the NMR spectra data for *Arabidopsis* acidic xylooligosaccharides (Zhong et al., 2005; Peña et al., 2007).

**Assay of xylosyltransferase activity**

Microsomes were isolated from the inflorescence stems of 7-week-old *Arabidopsis* plants and used for the xylosyltransferase activity assay (Kuroyama and Tsumuraya, 2001). For each assay, microsomes (100 \( \mu \)g) were incubated with the reaction mixture containing 50 mM
HEPES-KOH, pH 6.8, 5 mM MnCl$_2$, 1 mM dithiothreitol, 0.5% Triton X-100, 0.1 mM cold UDP-Xyl (CarboSource Service), 0.2 µg/µl Xyl$_6$ (Megazyme) and UDP-[14C]Xyl (0.1 µCi; American Radiolabeled Chemical). The reaction products were separated from UDP-[14C]Xyl by paper chromatography according to Ishikawa et al. (2000) and counted for the amount of radioactivity with a PerkinElmer scintillation counter.

**Generation of transgenic poplar plants**

The *PoGT43B* and *PoGT8D* RNA interference (RNAi) constructs were generated by cloning their cDNAs into pBI121 in opposite orientations on both sides of the β-glucuronidase (GUS) spacer, which is located between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (NOS) terminator. The constructs were introduced into *Agrobacterium tumefaciens* GV3101, which was further used to transform the hybrid poplar (*Populus tremula X Populus alba*) as described by Leple et al. (1992). The transgenic poplar seedlings were selected on Murashige and Skoog medium containing 100 µg/L kanamycin and 500 µg/L carbenicillin. After rooting, transgenic seedlings were transferred to soil and grown in the greenhouse. The wild-type control plants were transgenic poplar plants transformed with an empty vector.

**Gene expression analysis**

Total RNA from poplar stems was isolated with a Qiagen RNA isolation kit (Qiagen). First strand cDNA was synthesized from total RNA treated with DNase I and then used as a template for real-time quantitative PCR analysis with the QuantiTect SYBR Green PCR kit (Clontech). The PCR primers for *PoGT43A* are 5’-gttcgatcctttgggatcctgaga-3’ and 5’-tcatagttttctctgtagcatc-3’, those for *PoGT43B* are 5’-ctcaatcctctgggatcctgagag-3’ and 5’-catgtaaagaattgtaatctgctc-3’, those for *PoGT8D* are 5’-tcgagcaaagcctttggctagatatagc-3’ and 5’-agatggcctaatagctcgccttc-3’, and those for *PoGT8D-2* are 5’-tcgagcaaagcctttggctagatatagc-3’ and 5’-agatggcctaatagctcgccttc-3’.
and 5’-tcatgtcctaatatgacagcccgtaat-3’. The relative expression level was calculated by normalizing the PCR threshold cycle number of each gene with that of a poplar actin reference gene (Lee et al., 2009a). The data were the average of three replicates from three separate RNA extractions.

**Immunodetection of xylan**

Sections (1 µm thick) of poplar stems were subjected to immunodetection of xylan by incubating with the LM10 monoclonal xylan antibody (Plantprobes, Leeds, UK; McCartney et al., 2005) followed with fluorescein isothiocyanate-conjugated secondary antibodies (Sigma) according to Pena et al. (2007). The sections were observed for fluorescence signals using a Leica TCs SP2 spectral confocal microscope. Images from single optical sections were collected and processed with Adobe Photoshop.

**Wood digestibility analysis**

Poplar wood cell walls were digested with cellulase enzymes according to Chen and Dixon (2007). Briefly, the milled, ethanol-insoluble wood cell walls (equivalent to 100 mg of glucose) were incubated with cellulase (52 units; Celluclast 1.5L from Sigma) and Cellobiase (16 units; Sigma) in 0.1 M sodium acetate buffer (pH 4.8) at 37°C for 72 h. Under the same conditions, the enzyme mixture was able to digest 90% of Whatmann No. 1 filter paper cellulose into glucose. The cellobiose released by cellulose was converted to glucose by the addition of cellobiase in the digestion solution. After centrifugation, the supernatant was used for quantitation of neutral sugars released from wood cellulose using gas chromatography as described above. The digestion of wood with cellulase and cellobiase resulted in little release of xylose. The enzyme mixture without the addition of wood cell walls was used as a blank. All samples were assayed independently twice.
Accession numbers

The GenBank accession numbers and gene models for poplar GT genes are as follows:
PtrGT43A (JF518934; eugene3.00280138), PtrGT43B (JF518935; fgenesh1_kg.C_LG_XVI000045),
PtrGT43C (JF518936; gw1.VII2855.1), PtrGT43D (JF518937; estExt.Genewise1_v1.C_LG_V4069),
PtrGT43E (JF518938), PoGT43B (EF501825), and PoGT8D (EF501824). The Arabidopsis Genome Initiative locus identifiers for the Arabidopsis genes investigated in this study are IRX9 (At2g37090), I9H (At1g27600), IRX14 (At4g36890), and I14H (At5g67230).

ACKNOWLEDGMENTS

We thank two anonymous reviewers for their constructive comments and suggestions. This work was funded by the Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences of the U.S. Department of Energy through Grant DE-FG02-03ER15415. This paper has been reviewed in accordance with the U.S. 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. No conflict of interest declared.

REFERENCES


identifies biochemical and developmental regulators in secondary wall biosynthesis. Plant J. 45, 144-165.


galactosyltransferase that is evolutionarily related to animal exostosins. Plant Cell. 15, 1662-1670.


which encodes a putative glucuronyltransferase, is essential for normal secondary wall synthesis. Plant Cell. 17, 3390-3408.


Table 5.1. Cell wall composition analysis of stems of wild type, *irx9*, *irx14*, and the *irx9* and *irx14* plants overexpressing Poplar GT43 members

<table>
<thead>
<tr>
<th>Sample</th>
<th>Xylose</th>
<th>Glucose</th>
<th>Mannose</th>
<th>Galactose</th>
<th>Arabinose</th>
<th>Rhamnose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>111.9 ± 14.0</td>
<td>415.7 ± 0.5</td>
<td>17.7 ± 2.0</td>
<td>13.9 ± 0.4</td>
<td>11.7 ± 1.0</td>
<td>10.9 ± 0.6</td>
</tr>
<tr>
<td><em>irx9</em></td>
<td>57.9 ± 3.3</td>
<td>333.3 ± 4.9</td>
<td>37.8 ± 1.4</td>
<td>31.6 ± 8.1</td>
<td>23.7 ± 5.1</td>
<td>15.1 ± 1.8</td>
</tr>
<tr>
<td><em>irx9</em> +PtrGT43A</td>
<td>88.9 ± 0.6</td>
<td>396.6 ± 20.2</td>
<td>18.4 ± 2.8</td>
<td>19.3 ± 1.9</td>
<td>15.9 ± 1.0</td>
<td>11.2 ± 0.8</td>
</tr>
<tr>
<td><em>irx9</em> +PtrGT43B</td>
<td>94.1 ± 4.5</td>
<td>386.3 ± 1.5</td>
<td>19.7 ± 0.9</td>
<td>18.7 ± 0.1</td>
<td>18.2 ± 0.6</td>
<td>13.0 ± 0.1</td>
</tr>
<tr>
<td><em>irx9</em> +PtrGT43C</td>
<td>53.8 ± 3.1</td>
<td>328.9 ± 8.9</td>
<td>28.1 ± 3.9</td>
<td>28.3 ± 2.0</td>
<td>26.8 ± 2.9</td>
<td>11.9 ± 0.8</td>
</tr>
<tr>
<td><em>irx9</em> +PtrGT43D</td>
<td>47.9 ± 3.4</td>
<td>316.1 ± 9.5</td>
<td>29.1 ± 0.1</td>
<td>27.5 ± 0.3</td>
<td>24.4 ± 0.5</td>
<td>12.0 ± 0.3</td>
</tr>
<tr>
<td><em>irx9</em> +PtrGT43E</td>
<td>81.1 ± 5.2</td>
<td>351.9 ± 13.0</td>
<td>19.2 ± 1.8</td>
<td>18.5 ± 1.6</td>
<td>16.1 ± 0.8</td>
<td>10.6 ± 0.7</td>
</tr>
<tr>
<td><em>irx9</em> + IRX9</td>
<td>120.1 ± 0.2</td>
<td>381.8 ± 1.7</td>
<td>15.4 ± 0.5</td>
<td>15.8 ± 0.2</td>
<td>14.8 ± 0.5</td>
<td>11.0 ± 0.9</td>
</tr>
<tr>
<td><em>irx14</em></td>
<td>55.4 ± 2.1</td>
<td>345.9 ± 2.6</td>
<td>24.8 ± 2.0</td>
<td>27.5 ± 7.0</td>
<td>14.8 ± 1.1</td>
<td>14.7 ± 0.6</td>
</tr>
<tr>
<td><em>irx14</em> +PtrGT43A</td>
<td>76.6 ± 2.4</td>
<td>415.4 ± 14.8</td>
<td>34.3 ± 0.7</td>
<td>23.8 ± 0.2</td>
<td>17.9 ± 0.1</td>
<td>14.9 ± 0.4</td>
</tr>
<tr>
<td><em>irx14</em> +PtrGT43B</td>
<td>70.5 ± 4.6</td>
<td>415.3 ± 11.4</td>
<td>33.2 ± 0.6</td>
<td>22.8 ± 0.5</td>
<td>18.4 ± 0.7</td>
<td>15.8 ± 0.2</td>
</tr>
<tr>
<td><em>irx14</em> +PtrGT43C</td>
<td>99.9 ± 0.2</td>
<td>375.1 ± 19.7</td>
<td>17.2 ± 1.0</td>
<td>14.6 ± 0.5</td>
<td>13.0 ± 0.5</td>
<td>11.1 ± 0.1</td>
</tr>
<tr>
<td><em>irx14</em> +PtrGT43D</td>
<td>96.7 ± 3.0</td>
<td>374.5 ± 9.8</td>
<td>17.4 ± 1.9</td>
<td>15.3 ± 1.7</td>
<td>13.4 ± 0.7</td>
<td>10.6 ± 0.4</td>
</tr>
<tr>
<td><em>irx14</em> +PtrGT43E</td>
<td>64.1 ± 0.1</td>
<td>399.0 ± 15.0</td>
<td>31.6 ± 2.3</td>
<td>21.5 ± 1.2</td>
<td>16.7 ± 0.5</td>
<td>14.8 ± 0.3</td>
</tr>
<tr>
<td><em>irx14</em> + IRX14</td>
<td>99.4 ± 5.2</td>
<td>384.1 ± 22.4</td>
<td>16.8 ± 1.5</td>
<td>16.4 ± 0.8</td>
<td>12.9 ± 0.7</td>
<td>10.1 ± 0.7</td>
</tr>
</tbody>
</table>

The wall residues for cell wall composition analysis were prepared from mature inflorescence stems. The data are means (mg/g dry cell wall) ± SE of duplicate assays.
Table 5.2. Relative abundance of the xylan reducing end sequence and the DP of xylan in wild type, *irx9*, *irx14*, and the *irx9* and *irx14* plants overexpressing Poplar GT43 members

<table>
<thead>
<tr>
<th>Sample</th>
<th>Frequency of occurrence of GlcA and MeGlcA side chains&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Relative abundance of the reducing end sequence&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Average DP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>20.6%</td>
<td>100%</td>
<td>93</td>
</tr>
<tr>
<td><em>irx9</em></td>
<td>20.3%</td>
<td>335%</td>
<td>28</td>
</tr>
<tr>
<td><em>irx9</em> + PtrGT43A</td>
<td>17.4%</td>
<td>114%</td>
<td>82</td>
</tr>
<tr>
<td><em>irx9</em> + PtrGT43B</td>
<td>19.1%</td>
<td>130%</td>
<td>72</td>
</tr>
<tr>
<td><em>irx9</em> + PtrGT43C</td>
<td>16.6%</td>
<td>299%</td>
<td>31</td>
</tr>
<tr>
<td><em>irx9</em> + PtrGT43D</td>
<td>19.9%</td>
<td>287%</td>
<td>32</td>
</tr>
<tr>
<td><em>irx9</em> + PtrGT43E</td>
<td>18.3%</td>
<td>116%</td>
<td>80</td>
</tr>
<tr>
<td><em>irx9</em> + IRX9</td>
<td>20.3%</td>
<td>110%</td>
<td>85</td>
</tr>
<tr>
<td><em>irx14</em></td>
<td>22.1%</td>
<td>265%</td>
<td>35</td>
</tr>
<tr>
<td><em>irx14</em> + PtrGT43A</td>
<td>20.5%</td>
<td>229%</td>
<td>41</td>
</tr>
<tr>
<td><em>irx14</em> + PtrGT43B</td>
<td>20.6%</td>
<td>209%</td>
<td>44</td>
</tr>
<tr>
<td><em>irx14</em> + PtrGT43C</td>
<td>17.6%</td>
<td>96%</td>
<td>97</td>
</tr>
<tr>
<td><em>irx14</em> + PtrGT43D</td>
<td>19.3%</td>
<td>121%</td>
<td>77</td>
</tr>
<tr>
<td><em>irx14</em> + PtrGT43E</td>
<td>20.7%</td>
<td>222%</td>
<td>42</td>
</tr>
<tr>
<td><em>irx14</em> + IRX14</td>
<td>21.5%</td>
<td>137%</td>
<td>69</td>
</tr>
</tbody>
</table>

<sup>a</sup>The frequency of occurrence of glucuronic acid (GlcA) and methylated glucuronic acid (MeGlcA) side chains was calculated from the ratios of the NMR resonance of the branched β-Xyl to that of total β-Xyl. <sup>b</sup>The relative abundance of the reducing end sequence was determined from the ratios of the NMR resonance of the reducing end sequence to that of the branched β-Xyl. The abundance of the reducing end sequence in the wild type was taken as 100%. <sup>c</sup>The average degree of polymerization (DP) was calculated according to Pena et al. (2007).
Table 5.3. Cell wall composition analysis of the wood of control Poplar, *PoGT43B* and *PoGT8D* RNAi lines

<table>
<thead>
<tr>
<th>Sample</th>
<th>Xylose</th>
<th>Glucose</th>
<th>Mannose</th>
<th>Galactose</th>
<th>Arabinose</th>
<th>Rhamnose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>127.0 ± 2.6</td>
<td>368.8 ± 6.7</td>
<td>17.5 ± 2.0</td>
<td>13.3 ± 0.2</td>
<td>17.1 ± 0.2</td>
<td>9.9 ± 0.4</td>
</tr>
<tr>
<td>PoGT43B-RNAi-3</td>
<td>88.9 ± 5.2</td>
<td>357.0 ± 5.4</td>
<td>18.0 ± 0.6</td>
<td>18.1 ± 0.6</td>
<td>20.2 ± 0.7</td>
<td>9.3 ± 0.1</td>
</tr>
<tr>
<td>PoGT43B-RNAi-5</td>
<td>77.0 ± 1.3</td>
<td>357.4 ± 0.4</td>
<td>21.3 ± 0.3</td>
<td>15.1 ± 0.2</td>
<td>25.1 ± 0.1</td>
<td>10.8 ± 0.1</td>
</tr>
<tr>
<td>PoGT43B-RNAi-11</td>
<td>73.3 ± 4.7</td>
<td>362.5 ± 6.5</td>
<td>21.6 ± 1.7</td>
<td>14.9 ± 0.4</td>
<td>22.2 ± 1.0</td>
<td>10.9 ± 0.3</td>
</tr>
<tr>
<td>PoGT43B-RNAi-32</td>
<td>92.3 ± 1.0</td>
<td>338.1 ± 4.7</td>
<td>16.4 ± 0.9</td>
<td>17.1 ± 0.4</td>
<td>20.8 ± 0.4</td>
<td>11.1 ± 0.5</td>
</tr>
<tr>
<td>PoGT8D-RNAi-1</td>
<td>69.4 ± 2.1</td>
<td>370.2 ± 5.3</td>
<td>19.1 ± 0.7</td>
<td>22.4 ± 0.5</td>
<td>11.3 ± 0.5</td>
<td>8.1 ± 0.3</td>
</tr>
<tr>
<td>PoGT8D-RNAi-5</td>
<td>92.7 ± 1.4</td>
<td>369.2 ± 8.0</td>
<td>14.0 ± 0.5</td>
<td>15.3 ± 0.5</td>
<td>8.2 ± 0.4</td>
<td>8.4 ± 0.2</td>
</tr>
<tr>
<td>PoGT8D-RNAi-15</td>
<td>99.8 ± 0.8</td>
<td>387.6 ± 2.7</td>
<td>17.7 ± 0.9</td>
<td>15.7 ± 0.9</td>
<td>11.5 ± 1.1</td>
<td>7.6 ± 0.7</td>
</tr>
<tr>
<td>PoGT8D-RNAi-26</td>
<td>79.5 ± 1.1</td>
<td>336.4 ± 26.4</td>
<td>14.5 ± 0.9</td>
<td>11.9 ± 1.2</td>
<td>10.4 ± 0.3</td>
<td>7.4 ± 0.2</td>
</tr>
</tbody>
</table>

The wall residues for cell wall composition analysis were prepared from stems. The data are means (mg/g dry cell wall) ± SE of duplicate assays.
Table 5.4. Relative abundance of the xylan reducing end sequence and the frequency of occurrence of side chains on the xylan backbone

<table>
<thead>
<tr>
<th>Sample</th>
<th>Frequency of occurrence of MeGlcA side chains\textsuperscript{a}</th>
<th>Relative abundance of the xylan reducing end sequence\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.5%</td>
<td>100%</td>
</tr>
<tr>
<td>PoGT8D-RNAi-1</td>
<td>23.9%</td>
<td>62%</td>
</tr>
<tr>
<td>PoGT8D-RNAi-26</td>
<td>24.3%</td>
<td>45%</td>
</tr>
<tr>
<td>PoGT43B-RNAi-3</td>
<td>22.4%</td>
<td>110%</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The frequency of occurrence of the MeGlcA side chains was calculated from the ratios of the NMR resonance of the branched $\beta$-Xyl to that of total $\beta$-Xyl.

\textsuperscript{b}The relative abundance of the reducing end tetrasaccharide sequence was determined by the ratios of the NMR resonance of the reducing end sequence to that of the branched $\beta$-Xyl, and the abundance of the reducing end sequence in the control was taken as 100%.
Table 5.5. Lignin composition analysis of the wood of control Poplar, *PoGT43B* and *PoGT8D* RNAi lines

<table>
<thead>
<tr>
<th>Sample</th>
<th>Guaiacyl lignin</th>
<th>Syringyl lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.7 ± 0.1</td>
<td>5.5 ± 0.4</td>
</tr>
<tr>
<td>PoGT43B-RNAi-3</td>
<td>3.4 ± 0.4</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>PoGT43B-RNAi-5</td>
<td>1.7 ± 0.2</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>PoGT43B-RNAi-11</td>
<td>1.7 ± 0.1</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>PoGT43B-RNAi-32</td>
<td>6.1 ± 1.1</td>
<td>4.4 ± 0.5</td>
</tr>
<tr>
<td>PoGT8D-RNAi-1</td>
<td>4.5 ± 0.5</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>PoGT8D-RNAi-5</td>
<td>3.8 ± 0.6</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>PoGT8D-RNAi-15</td>
<td>5.8 ± 0.4</td>
<td>4.9 ± 0.7</td>
</tr>
<tr>
<td>PoGT8D-RNAi-26</td>
<td>6.8 ± 0.9</td>
<td>4.6 ± 0.7</td>
</tr>
</tbody>
</table>

Guaiacyl lignin is the sum of vanillin, acetovanillin and vanillic acid. Syringyl lignin is the sum of syringaldehyde, acetosyringaldehyde and syringic acid. The data are means (mg/g dry cell wall) ± SE of two independent assays.
Figure 5.1. Phylogenetic and expression analysis of family GT43 members in *Populus trichocarpa*. (A) Phylogenetic relationship of PtrGT43 members together with the *Arabidopsis* GT43 members (IRX9, I9H, IRX14 and I14H). The amino acid sequences were aligned using the ClustlW program (Thompson et al., 1994) and the phylogenetic tree was shown using the TREEVIEW program (Page, 1996). The 0.1 scale indicates 10% change. (B) to (E) In situ mRNA localization of *PtrGT43* genes in the developing wood of poplar stems. Cross sections of stems were hybridized with digoxigenin-labeled antisense (B-D) or sense (E) RNA probes, and the hybridization signals were detected with alkaline phosphatase-conjugated antibodies and are shown as purple color. Note the strong hybridization signals for *PtrGT43A* (B), *PtrGT43C* (C), and *PtrGT43E* (D) in vessels, xylary fibers, and ray parenchyma in developing wood. The hybridization signals for *PtrGT43A* (B) and *PtrGT43C* (C) were also evident in phloem fibers. The section hybridized with the sense RNA probe of *PtrGT43A* exhibited no apparent hybridization signals (E). pf, phloem fiber; rp, ray parenchyma; ve, vessel; xf, xylary fiber. Bars = 81 µm.
Figure 5.2. Subcellular localization of PtrGT43 proteins. Fluorescent protein-tagged fusion proteins were expressed in carrot protoplasts, and the fluorescent signals were detected with a laser confocal microscope. (A) to (D) PtrGT43 are type II membrane proteins as predicted by the TMHMM2.0 program. Inside, the cytoplasmic side of the membrane; outside, the noncytoplasmic side of the membrane. (E) and (F) A carrot protoplast (E; differential interference contrast image) expressing YFP alone showing the fluorescent signals throughout the cytoplasm (F). (G) to (J) A carrot protoplast (G) co-expressing PtrGT43A-YFP (H) and the Golgi-localized FRA8-CFP (I). (K) to (N) A carrot protoplast (K) co-expressing PtrGT43C-YFP (L) and FRA8-CFP (M). (O) to (R) A carrot protoplast (O) co-expressing PtrGT43D-YFP (P) and FRA8-CFP (Q). (S) to (V) A carrot protoplast (S) co-expressing PtrGT43E-YFP (T) and FRA8-CFP (U). Note the colocalization of PtrGT43A-YFP (J), PtrGT43C-YFP (N), PtrGT43D-YFP (R), and PtrGT43E (V) with the Golgi marker FRA8-CFP. Bars = 24 µm.
Figure 5.3. Complementation analysis of PtrGT43 members in the *Arabidopsis irx9* and *irx14* mutants. The full-length *PtrGT43* cDNAs driven by the CaMV 35S promoter were introduced into *irx9* or *irx14* and the bottom parts of inflorescence stems of 10-week-old transgenic plants were examined for the stem breaking strength (A) and vessel morphology (B to Q). Bar in (B) = 58 µm in (B) to (Q). (A) Measurement of the breaking strength of stems of the wild type, *irx9*, *irx14*, and the mutants overexpressing PtrGT43 members. Each bar represents the breaking force of the inflorescence stem of individual plants. Note that the stem breaking strength of *irx9* was restored by overexpression of PtrGT43A/B/E and that of *irx14* was restored by overexpression of PtrGT43C/D. (B) to (I) The collapsed vessel phenotype (arrow) exhibited in the *irx9* mutant was rescued by overexpression of PtrGT43A/B/E but not PtrGT43C/D. (J) to (Q) The collapsed vessel phenotype (arrow) exhibited in the *irx14* mutant was rescued by overexpression of PtrGT43C/D but not PtrGT43A/B/E.
Figure 5.4. Transmission electron microscopic analysis of secondary walls in the *irx9* and *irx14* mutants overexpressing PtrGT43. The bottom parts of inflorescence stems of 10-week-old transgenic plants were examined for the secondary wall thickness of interfascicular fibers. Bar in (A) = 8.7 µm in (A) to (P). (A) to (H) Restoration of the secondary wall thickness of interfascicular fibers in the stems of the *irx9* mutant by overexpression of PtrGT43A/B/E but not PtrGT43C/D. (I) to (P) Restoration of the secondary wall thickness of interfascicular fibers in the stems of the *irx14* mutant by overexpression of PtrGT43C/D but not PtrGT43A/B/E.
Figure 5.5. MALDI-TOF mass spectra of acidic xyooligosaccharides generated by β-endoxylanase digestion of xylan from stems of the wild type, *irx9*, *irx14*, and the mutants Overexpressing PtrGT43. The ions at *m/z* 745 and 759 correspond to xylotetrasaccharides bearing a GlcA residue (X₄G) or a methylated GlcA residue (X₄M). The ion at *m/z* 891 corresponds to xylopentasaccharide bearing a methylated GlcA residue (X₅M). (A) to (H) Restoration of the missing ion at *m/z* 745 corresponding to X₄G (arrows) in *irx9* by overexpression of PtrGT43A/B/E but not PtrGT43C/D. (I) to (P) Restoration of the missing ion at *m/z* 745 corresponding to X₄G (arrows) in *irx14* by overexpression of PtrGT43C/D but not PtrGT43A/B/E.
Figure 5.6. $^1$H-NMR spectra of acidic xylooligosaccharides generated by $\beta$-endoxylanase digestion of xylan from stems of the wild type, $irx9$, $irx14$, and the mutants overexpressing PtrGT43. Resonances are labeled with the position of the assigned proton and the identity of the residue containing that proton. Note the restoration of the resonance of H1 of $\alpha$-GlcA in $irx9$ complemented with PtrGT43A/B/E and in $irx14$ complemented with PtrGT43C/D (arrows). The resonances for the xylan reducing end tetrasaccharide sequence are shown as H1 of $\alpha$-D-GalA, H1 of $\alpha$-L-Rha, H1 of 3-linked $\beta$-D-Xyl, H4 of $\alpha$-D-GalA, and H2 of $\alpha$-L-Rha. Note that $irx\ 9$ and $irx14$ exhibit an elevated resonance intensity of the xylan reducing end tetrasaccharide sequence compared with the wild type and that this elevation is reduced in $irx9$ overexpressing PtrGT43A/B/E and in $irx14$ overexpressing PtrGT43C/D. The doublet resonance peak at 4.39 ppm in the wild type is assigned to H5 $\alpha$-GlcA and that at 4.36 ppm is assigned to H5 Me-$\alpha$-GlcA. The resonance shift of H5 proton in some of the other samples is likely attributed to its high sensitivity to a slight variation of pH and concentration of the samples because of its close proximity to the carboxyl group. HDO, hydrogen deuterium oxide.
Figure 5.7. Time course of the xylosyltransferase activity in the wild type, irx9, irx14, and the mutants overexpressing PtrGT43. Microsomes were isolated from inflorescence stems of 7-week-old Arabidopsis plants and subjected to the assay of xylosyltransferase activity using xylohexaose as an acceptor and UDP-[14C]Xyl as a donor. (A) Partial restoration of the xylosyltransferase activity in irx9 overexpressing PtrGT43A/B/E. A slight increase in the xylosyltransferase activity was also seen in irx9 overexpressing PtrGT43C/D. (B) Partial restoration of the xylosyltransferase activity in irx14 overexpressing PtrGT43C/D. Note that overexpression of PtrGT43E in irx14 also resulted in an elevation in the xylosyltransferase activity.
Figure 5.8. Effects of RNAi downregulation of expression of *PoGT43B* and *PoGT8D* on Poplar wood development. **(A)** Real-time quantitative PCR analysis showing a reduction in the expression of *PoGT43A/B* in *PoGT43B* RNA lines and that of *PoGT8D/8D-2* in *PoGT8D* RNA lines. The expression level of *PoGT43A/B* or *PoGT8D/8D-2* in the control is taken as 100. Error bars represent the SE of three independent replicates. **(B) to (F)** Cross sections of the secondary xylem of stems showing deformed vessel morphology (arrows) and thin walls of xylary fibers in the RNAi lines of *PoGT43B* (C and D) and *PoGT8D* (E and F) compared with the control (B). Stems from 9-month-old plants were sectioned and stained with toluidine blue for examination of wood cells. rp, ray parenchyma; ve, vessel; xf, xylary fiber. Bar in (B) = 73 µm for (B) to (F).
Figure 5.9. Transmission electron microscopic analysis of the wood of the control, PoGT43B- and PoGT8D-RNAi lines. Note the significant reduction in the wall thickness of fibers in the RNAi lines of PoGT43B (B and C) and PoGT8D (D and E) compared with the control (A). Bar in (A) = 6.9 µm for (A) to (E).
Figure 5.10. Immunodetection of xylan in the wood of the control, *PoGT43B*- and *PoGT8D*-RNAi lines. Stems from 9-month-old wild type (A), *PoGT43B* (B) and *PoGT8D* (C) RNAi lines were sectioned and probed with the monoclonal xylan antibody LM10. Xylan signals were detected with fluorescein isothiocyanate-conjugated secondary antibodies and visualized with a laser confocal microscope. Note the drastic reduction in the xylan signals in the secondary xylem of *PoGT8D* RNAi line (C) compared with the control (A). pf, phloem fiber; sx, secondary xylem. Bar in (A) = 260 µm for (A) to (C).
Figure 5.11. $^1$H-NMR spectra of xylooligosaccharides generated by β-endoxylanase digestion of xylan from the wood of the control, PoGT43B- and PoGT8D-RNAi lines. Resonances are labeled with the position of the assigned proton and the identity of the residue containing that proton. It is evident that compared with the control, 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 from the xylan reducing end sequence were reduced in PoGT8D RNAi lines but elevated in PoGT43B-3 RNAi lines. The $^1$H-NMR spectrum of xylooligosaccharides from Arabidopsis xylan is included for comparison. Note that the resonances of α-GlcA residues are prominent in Arabidopsis xylooligosaccharides (asterisks) but nearly absent in poplar xylooligosaccharides.
Figure 5.12. Glucose release by cellulase enzymes from wood cell walls of the control, PoGT43B and PoGT8D RNAi lines. Wood cell walls of the control, PoGT43B- and PoGT8D-RNAi lines were incubated with cellulase and cellobiase. The enzymatic hydrolysis efficiency is presented by the percentage of glucose released by the enzymes over the total cellulose amount in the cell walls. Data are means ± SE of two separate assays and the quantitative difference of the data between the control and PoGT43-RNAi lines was found to be statistically significant using the Student’s t test program (http://www.graphpad.com/quickcalcs/ttest1.cfm) (p < 0.001).
Graph showing enzymatic hydrolysis efficiency (%).
CHAPTER 6
CONCLUSIONS

Xylan is an abundant hemicellulose and plays pivotal functions in the proper secondary cell wall formation. Xylan has recently attracted much attention since it might provide an important source of sugars for biofuel production from higher plants. Thus, understanding xylan biosynthesis is crucial not only to illuminate the basic features of cell wall biology, but also to lead us to design a better strategy for plant biotechnology.

The biosynthesis of xylan requires a number of glycosyltransferases and modifying enzymes. Given that more than 500 glycosyltransferases exist in the Arabidopsis genome, it is unrealistic to use traditional biochemical approaches to identify xylan biosynthetic enzymes. Recently, transcriptional profiling of genes specifically expressed in cells undergoing secondary cell wall deposition in Arabidopsis and Poplar has contributed to the identification of the candidate genes involved in xylan biosynthesis (Aspeborg et al., 2005; Brown et al., 2005). Detailed characterization of those T-DNA mutants has led to the identification of multiple glycosyltransferase that are essential for xylan biosynthesis. Among them, three proteins, FRA8, IRX8, and PARVUS appear to be responsible for the reducing end sequence (Pena et al., 2007; Lee et al., 2007a, 2010; Brown et al., 2007). Two functionally redundant glycosyltransferases, GUX1 and GUX2 in family GT8, seem to be required for the substitution of glucuronic acid (Mortimer et al., 2010).
Xylan backbone is composed of β-(1,4)-linked xylosyl residues in which each xylosyl residue is inverted nearly 180° with respect to each neighboring xylose. Thus, biosynthesis of xylan backbone needs to overcome this steric problem by either iterative addition of disaccharide units, β-(1,4)-linked xylose dimer, or involvement of at least two enzymes with opposing catalytic sites (Carpita, 2011). It was previously proposed that a member of the cellulose synthase-like (Csl) gene family may encode xylan backbone synthase (XylTase) because the only difference between glucose and xylose is the absence of the primary alcohol group at C6 in xylose (Richmond and Somerville, 2001; Lerouxel et al., 2006). However, heterologous expression of Csl proteins did not show the activity of XylTase (Liepman et al., 2005).

As an initial step to unambiguously identifying xylan backbone synthase, we established in vitro activity assay to detect XylTase activity from *Arabidopsis* stem microsomes. Early biochemical studies revealed that microsomal membrane fractions from different plants possess XylTase, which catalyzes the transfer of xylose from UDP-xylose to either an endogenous acceptor or exogenous xyloooligosaccharide (Lee et al., 2007a). We determined that microsomes from *Arabidopsis* inflorescence stem, where xylan is actively synthesized, also contained XylTase, which is dependent on the presence of exogenous oligosaccharides. We confirmed that the reaction products are identical to the β-(1,4)-linked elongating oligomers by MALDI-TOF and endo-β-(1,4)-xylanase treatment.

We next determined if microsomes from any of xylan mutants (*fra8, irx8, irx9*, and *parvus*) are deficient in XylTase activity (Lee et al., 2007a). XylTase activities measured from *fra8, irx8*, and *parvus* mutant microsomes are comparable to that of wild type. In contrast, *irx9* exhibited a substantial reduction in XylTase activity, suggesting that IRX9 is essential for xylan chain elongation. These findings are consistent with the previous NMR data that the shortened
chain length of xylan backbone was found in \textit{irx9}, whereas \textit{fra8}, \textit{irx8}, and \textit{parvus} mutations resulted in the loss of reducing end sequence, but no effect on the xylan chain length.

In order to characterize the exact biochemical function of IRX9, we heterologously expressed IRX9 in yeast cells and Tobacco Bright Yellow-2 (BY-2) cells (Unpublished data). No XylTase activity was detected using recombinant IRX9 protein, leading to the possibility that more enzymatic/regulatory components are required for xylan backbone elongation.

Brown et al. identified an additional xylan biosynthetic gene \textit{IRX14} encoding a member of family GT43 (Brown et al., 2007). Mutation of IRX14 resulted in similar defects in xylan chain length and XylTase activity as \textit{irx9}. Thus, IRX14 is likely to have a role in xylan backbone elongation.

There exist four GT43 members in the \textit{Arabidopsis} genome IRX9, I9H, IRX14, and I14H. Two (IRX9 and IRX14) of them appear to be essential for xylan backbone elongation. Thus, we questioned whether IRX9 and IRX14 perform the same biochemical function and whether the other two GT43 members are also involved in xylan biosynthesis. In order to address these questions, we performed comprehensive molecular and genetic studies of the role of all members of family GT43 (Lee et al., 2010). We found that I9H and I14H are redundant in function with IRX9 and IRX14, respectively, based on complementation tests and double mutant analysis. No xylan deficient phenotypes were observed in the single mutation of \textit{I9H} and \textit{I14H}, indicating that IRX9 and IRX14 are functionally dominant in xylan biosynthesis in vivo. We also found that IRX9 and IRX14 are two functionally nonredundant glycosyltransferases in xylan backbone elongation.

Multiple lines of evidence indicate that GTs involved in xylan biosynthesis are highly conserved between \textit{Arabidopsis} and Poplar. First, all \textit{Arabidopsis} GTs participating in xylan
biosynthesis have close homologs in Poplar. Previous transcriptional analysis in Poplar has shown that these homologs are specifically expressed in wood-forming tissues (Aspeborg et al., 2005; Ye et al., 2006). Second, overexpression of the Poplar homologs (PoGT47C, PoGT8D, PoGT43B, and PoGT8E/F) in the corresponding *Arabidopsis* mutants leads to the complete restoration of mutant phenotypes and chemotypes to wild type level (Zhou et al., 2006, 2007; Lee et al., 2009b). Third, down-regulation of PoGT47C, homologous to *Arabidopsis* FRA8, resulted in a reduction of the thickness of secondary walls, a decreased amount of xylose, and a reduction in the abundance of the reducing end sequence (Lee et al., 2009a). Fourth, considering the structural similarity of xylan polymer found in *Arabidopsis* and Poplar, it is conceivable that the overall enzymatic actions to complete xylan biosynthesis are performed by homologous GTs.

There exist seven members of GT43 family in the Poplar genome. Five of them are highly expressed during wood formation and their encoded proteins are targeted to the Golgi. Genetic complementation experiments demonstrated that PtrGT43 A/B/E are functional homologs of *Arabidopsis* IRX9, and PtrGT43 C/D are homologs of IRX14 (Lee et al., 2011). Downregulation of PtrGT43B expression through RNAi resulted in the xylan deficient phenotypes that included thin secondary cell walls and a reduced level of xylose content (Lee et al., 2011). Also, structural analysis of the xylan isolated from RNAi lines showed an elevation in the abundance of the xylan reducing end sequence, a chemotype similar to the *Arabidopsis irx9* mutant. Thus, our findings provide the genetic evidence that the poplar GT43 members form two functionally nonredundant groups, all of which are involved in the biosynthesis of xylan backbones.

Genetic studies and in vitro activity assay using mutant microsomes led to the hypothesis that *Arabidopsis* IRX9 or IRX14 function as xylan backbone synthase. However, polymerization
activity was not detected using the recombinant IRX9 or IRX14. Hence, we investigated whether the coexpression of IRX9 and IRX14 is required for xylan backbone polymerization. IRX9 and IRX14 were concomitantly expressed in tobacco BY-2 cells, and the resulting transformed lines were screened for in vitro xylan synthase activity. Notably, we detected an increased activity with the incorporation of four to five xylose residues into the acceptors (Lee, C and Ye, Z.H. manuscript in preparation). We confirmed that the reaction products are elongated β-(1,4)-linked xylooligomers by MALDI-TOF and endo- β-(1,4)-xylanase treatment. These findings will provide the first convincing biochemical evidence that successive addition of a xylose unit in β-(1,4) linkage into elongating xylooligosaccharide is cooperatively catalyzed by two golgi localized glycosyltransferases, IRX9 and IRX14, in *Arabidopsis*.

Glycosyltransferases (GTs) are a large family of enzymes that are essential to constructing sugar blocks in higher organisms (Lerouxel et al., 2006). Due to the presence of complex cell walls in plants, more GTs have been found (approximately 450 GTs in *Arabidopsis* and 800 GTs in Poplar). In order to make different complex polysaccharides found in the plant cell wall, it is apparent that the concerted and coordinated series of action of a number of GT are required. Given the fact that the classification of GTs into families is mainly derived from amino acid sequence similarities, not from catalytic activities, only limited information (fold types and stereochemical outcomes) can be obtained for the precise enzymatic roles. Genetic and molecular studies are indispensible to gain a better understanding of the possible roles of GTs and thereby design a better approach for its biochemical characterization.

In dicot plants, secondary cell walls are predominantly composed of cellulose, xylan, mannan, and lignin. Molecular analysis of GT-encoding genes and detailed characterization of those T-DNA mutants have led to the identification of several xylan biosynthetic genes in
*Arabidopsis*. We showed the genetic and biochemical evidence that the cooperative activities of IRX9 and IRX14 are required for xylan backbone polymerization. However, the precise biochemical function of other GTs is presently still unknown. Thus, the next important challenge to increase our knowledge on the xylan biosynthesis is to provide biochemical evidence of FRA8, IRX8, PARVUS, GUX1/2, and IRX10/10L. In addition, understanding how the xylan polymers synthesized in the Golgi are transported and assembled during secondary cell wall formation is also an important issue that must be addressed.
REFERENCES

Aspeborg, H., Schrader, J., Coutinho, P.M., Stam, M., Kallas, A., Djerbi, S., Nilsson, P.,
Denman, S., Amini, B., Sterky, F., Master, E., Sandberg, G., Mellerowicz, E., Sundberg,

novel genes in Arabidopsis involved in secondary cell wall formation using expression

(2007). Comparison of five xylan synthesis mutants reveals new insight into the


xylem9 mutant is deficient in xylan xylosyltransferase activity. Plant Cell Physiol 48:
1624–1634.

The PARVUS Gene is Expressed in Cells Undergoing Secondary Wall Thickening and is

expression in poplar results in a reduced glucuronoxylan content and an increased wood


