

THE ROLE OF GALACTURONOSYLTRANSFERASE12/IRREGULAR XYLEM8 IN
ARABIDOPSIS SECONDARY CELL WALL FORMATION AND ANTHER DEHISCENCE

by

ZHANGYING HAO

(Under the Direction of Debra Mohnen)

ABSTRACT

GAUT12 (GalactUronosylTransferase12)/IRX8 (IRregular Xylem8) is a putative glycosyltransferase involved in Arabidopsis secondary cell wall biosynthesis. Prior work has shown that Arabidopsis mutant *irregular xylem8* (*irx8*) has collapsed xylem due to the lack of xylan and a small fraction of homogalacturonan (HG). The initial goal of this thesis research was to identify the biochemical function of GAUT12 by establishing an *in vitro* enzymatic reaction. Two hypotheses were tested: 1) GAUT12 is a galacturonosyltransferase (GalAT) that adds the galacturonic acid (GalA) into the xylan reducing-end oligosaccharide sequence, and 2) GAUT12 is an HG:GalAT that synthesizes a subfraction of HG. An anti-GAUT12 polyclonal antibody that specifically recognizes GAUT12 was generated and used to immunoabsorb GAUT12. The immunoabsorbed-GAUT12 fraction contained little activity for incorporation of D-[¹⁴C]GalA from UDP-D-[¹⁴C]GalA onto HG oligosaccharides or heat-inactivated *irx8* microsomes, although it remains possible that GAUT12 utilizes other pectic structures as acceptors. During the course of this study, further characterization of the *irx8* mutant revealed a reduction in lignin biosynthesis and deposition in mutant stems, which was also reflected in *irx8* suspension culture cells grown on xylogenic medium. A reduction of lignin and xylan was also observed in the

anther endothecium causing indehiscent anthers in *irx8*. The pollen grains of *irx8*, despite their smaller size compared to wild type, were viable upon manual release. Therefore, the *irx8* sterility was solely due to indehiscent anthers. Detailed analyses of lignin using NMR spectroscopy confirmed the loss of G lignin and altered lignin extractability in *irx8* stem cell walls, revealing potential changes in xylan and G lignin in the *irx8* mutant. In addition, known genes involved in xylan biosynthesis were investigated for their expression levels by qPCR in *irx8* suspension culture cells grown on xylogenic medium. Genes tightly correlated with tracheary element formation and those that may associate with GAUT12 function in xylan biosynthesis were identified.

INDEX WORDS: *Arabidopsis thaliana*, secondary cell wall, GAUT12, *irregular xylem*, xylan, pectin, lignin, biosynthesis, xylogenic, suspension cell culture, anther dehiscence

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DEDICATION

I dedicate this work to my parents and Haibao, for their continuous love, support, and motivation over the years; and to my dear friends, for the memorable time at the Complex Carbohydrate Research Center.

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INTRODUCTION

The overall goal of the research in this dissertation was to extend our understanding of secondary cell wall biosynthesis and wall polymer interactions through the study of GAUT12, a putative glycosyltransferase involved in Arabidopsis secondary cell wall formation.

Chapter 1 starts with an introduction of the plant cell wall, *irregular xylem* mutants, the GAUT gene family, and transcriptional regulation of secondary wall formation via a network of transcription factors. This chapter then reviews the structures and biosynthesis of xylan, lignin, and pectin, including biochemical and genetic analyses of enzyme activities required, and confirmed and putative genes encoding the enzymes that produce these polymers. Chapter 1 also summarizes current evidence supporting the interaction of pectin with lignin and xylan, which indicates that pectin may have a role in secondary cell wall formation. An index of acronyms and abbreviations used in Chapter 1 can be found in Appendix A, and proposed criteria to distinguish between confirmed and putative enzyme activity are summarized in Appendix B.

Chapter 2 describes the characterization of the *irx8* mutant and reveals that lignin defects together with the known xylan defects cause collapsed xylem and indehiscent anthers in this mutant. The indehiscent anthers lead to the male sterility of *irx8*. We analyzed the chlorite fraction of *irx8* cell walls using NMR spectroscopy and confirmed the loss of G lignin signals in this mutant. The NMR analyses of other cell wall fractions also revealed that *irx8* mutant walls show a different lignin extractability compared with wild-type walls. The role for GAUT12 in lignin biosynthesis and/or deposition was also studied in the *irx8 wrky12* double mutant

(Appendix E). An anti-GAUT12 polyclonal antibody was also generated, characterized, and used to study the enzymatic activity of immunoabsorbed-GAUT12 fractions. Enzyme assays not presented in Chapter 2 are summarized in Appendix D. Using the anti-GAUT12 antibody, initial analyses of the proteins that co-immunoabsorbed with GAUT12 are summarized in Appendix C.

Chapter 3 describes the generation of *Arabidopsis irx8* suspension cell cultures and the characterization of their growth on xylogenic and control media compared to growth of wild-type and *GAUT12*-overexpression cell lines on the corresponding media. The reduction of lignin in *irx8* cells grown on xylogenic medium was consistent with the reduction of lignin in *irx8* stems. Compared to the wild type, the altered expression of xylan biosynthesis-related genes in *irx8* and in *GAUT12*-overexpression cell lines revealed possible gene cassettes involved in xylan biosynthesis. Some details of the generation and optimization of the suspension cell culture are presented in Appendix F.

Chapter 4 summarizes the major conclusions from the previous chapters and proposes future research directions.

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Plant cell walls and glycosyltransferases

Plant cell walls are the most abundant biomass on this planet, and they are naturally recalcitrant, i.e. resistant to microbial or mechanical degradation. Over the past 80 years, people have tried to utilize plant biomass for biofuel production, however recalcitrant walls have necessitated biomass pretreatment, a procedure in which large amounts of acid or base, and hydrolytic enzymes are required to loosen the biomass to facilitate the release of soluble oligosaccharides for downstream fermentation. (Himmel et al., 2007).

The plant cell wall is a highly complex yet dynamic structure composed of polysaccharides and diverse proteins. During plant cell division, the middle lamella and primary walls are first laid down at the cell plate. Primary cell walls retain their ability to expand and re-shape as plant cells grow. The rather rigid secondary cell walls are laid down by some differentiated cells to add mechanical strength (**Figure1.1**). Both primary and secondary cell walls contain cellulose, hemicelluloses, and pectin, however, the percentage and composition of these diverse wall polymers vary in these two types of walls (Popper and Fry, 2008). Cellulose provides the major tensile strength for primary cell walls. Hemicelluloses are cellulose-like structures which, with pectin, form a matrix to stabilize cellulose microfibrils. Pectin is a major component of the middle lamella that can form hydrated gels which are believed to facilitate

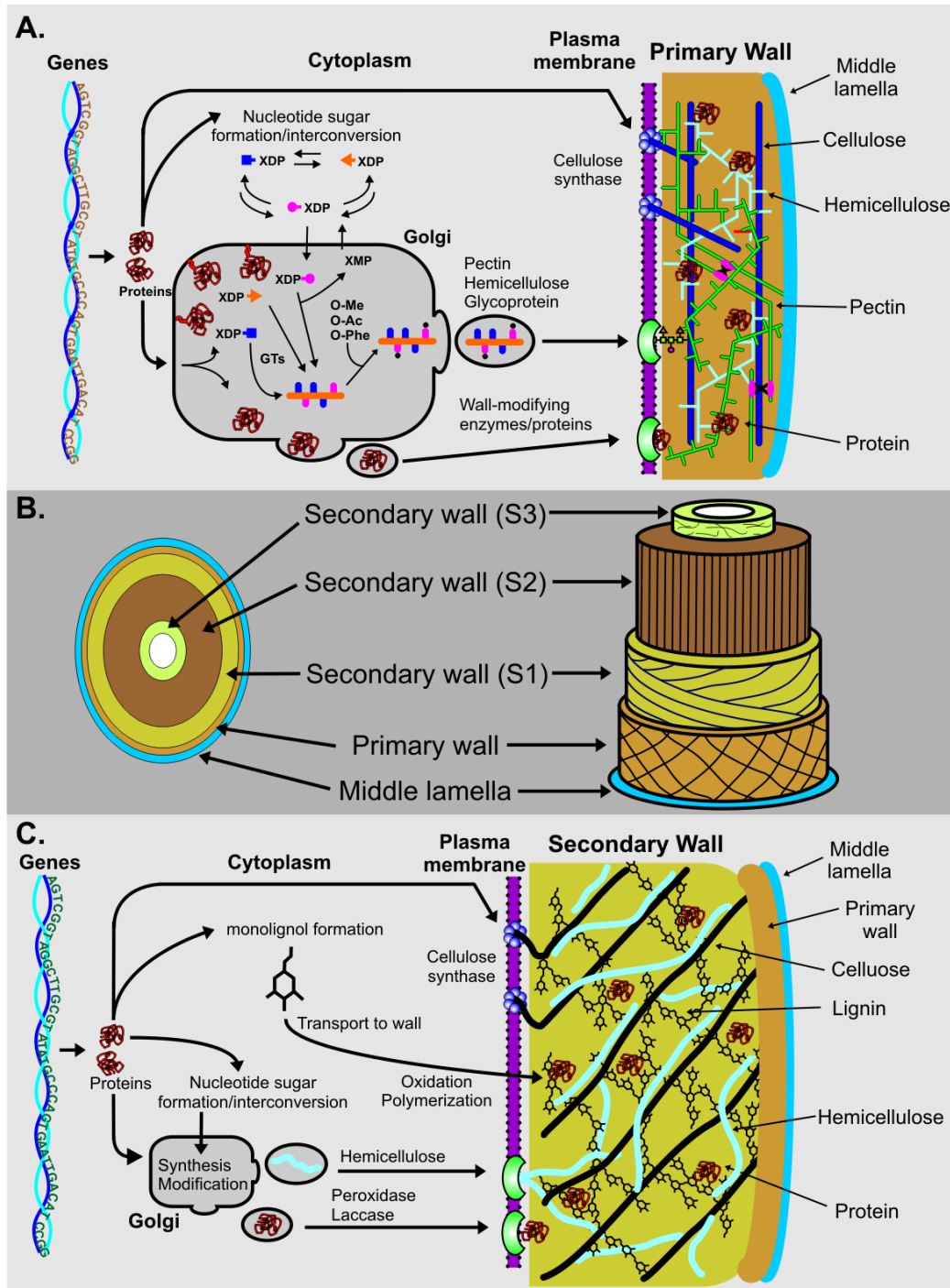


Figure 1.1. Schematic diagrams of plant cell wall structure and biosynthesis.

Adapted from Mohnen et al. (2008); originally generated by Malcolm O'Neill, CCRC.

(A) Plant cells are surrounded by primary cell walls with middle lamella between neighboring cells. While cellulose is produced by particle-rosette-like cellulose synthase complexes (CesAs)

at the plasma membrane, matrix polysaccharides including pectins and hemicelluloses are synthesized in the Golgi apparatus, transported by vesicles to the wall and further assembled *in muro*.

(B) Differentiated plant cells such as vessels and fibers have secondary thickening once the cells cease primary growth. Three layers (S1, S2, and S3) of secondary walls with different cellulose orientation are laid down sequentially inward from primary walls to the plasma membrane.

(C) Secondary wall cellulose is produced by secondary CesAs at the plasma membrane, while secondary hemicelluloses are synthesized in the Golgi apparatus and then transported to the wall. Monolignols are generated in the cytoplasm, deposited and polymerized in the wall to form lignin that waterproofs water-conducting vessels and structurally strengthens fiber cells to support the plant.

slippage of cellulose microfibrils during cell growth and hold them in place once growth ceases (Cosgrove, 2005). Pectin also determines the porosity of the wall and provide selective passage of molecules during intercellular transport and pathogen defense (Mohnen, 2008). Cellulose, xylan, and lignin are three major components of secondary cell walls in dicot woody plants. Secondary cell walls constitute the majority of biomass, in which cellulose is most beneficial for producing biofuel. Xylan, on the other hand, is still not the preferable sugar source despite extensive efforts to engineer microbes for more efficient and simultaneous fermentation of both hexoses and pentoses (Ragauskas et al., 2006). Lignin is a vital component required to maintain cell wall structural integrity and to act as a glue to condense cellulose and hemicelluloses (Jones et al., 2001). The properties of the phenolic compounds that comprise lignin contribute to difficulties in papermaking, wood quality improvement, and in reducing biomass recalcitrance (Ragauskas et al., 2006). Lignin can be altered by manipulation of lignin biosynthetic genes and may be removed to some extent. However, the residual lignin prevents the walls from collapsing and helps maintain a spatial structure, which allows access of chemicals and enzymes during biomass pretreatment.

The plant cell walls are synthesized by the concerted effort of enzymes encoded by over 2000 genes in which a majority code for glycosyltransferases, methyl- and acetyl transferases, and glycosidases (Albersheim et al., 2011). To date, only a small number of glycosyltransferases have been biochemically characterized and their enzymatic functions confirmed. For example, glycosyltransferases involved in pectin biosynthesis with proven functions are GAUT1 (Sterling et al., 2006), RGXT1 and RGXT2 (Egelund et al., 2006), and XGD1 (Jensen et al., 2008). The difficulties associated with studying the biochemical activity of plant glycosyltransferases include their low abundance, the fact that many are membrane-bound proteins and thus difficult

to isolate and express, and the lack of acceptors for specific enzyme assays. In addition, at least some glycosyltransferases appear to function in protein complexes (Mutwil et al., 2008; Zeng et al., 2010; Atmodjo et al., 2011; Harholt et al., 2012b). Thus, studying an individual protein may not yield full *in vivo* enzyme activity, which may partially explain the challenge of recovering enzyme activity from heterologously expressed individual plant glycosyltransferases. Proper folding and modification of the target glycosyltransferases in a heterologous system may also be problematic. Heterologous expression systems that have proven successful for recovering enzymatically active plant glycosyltransferases include *Pichia pastoris* (Edwards et al., 1999; Faik et al., 2002; Madson et al., 2003; Cocuron et al., 2007; Egelund et al., 2008; Maris et al., 2009; Liu et al., 2011), insect cells (Liepman et al., 2005; Egelund et al., 2006), COS cells (Perrin et al., 1999), human embryonic kidney (HEK) 293 cells (Vanzin et al., 2002; Sterling et al., 2006), and *Nicotiana benthamiana* (Jensen et al., 2008). Finally, the lack of availability or knowledge of the *in vivo* acceptor substrates often limits biochemical assay development for glycosyltransferases.

It is important to distinguish confirmed versus putative enzyme activities to avoid drawing misleading conclusions. Confirmation of an enzyme activity requires demonstration of the *in vitro* enzyme activity upon heterologous expression of the candidate gene or a production of the specific polysaccharide upon expression of the candidate gene in a heterologous host. In this thesis, only those genes with this level of proof will be considered to encode proven wall biosynthetic enzymes (see **Appendix B**). All other genes will be considered putative. For putative wall biosynthetic genes, the data supporting the proposed enzyme activity can be classified into strong, medium, and weak, depending upon the experimental evidence provided.

Appendix B lists our criteria for strong, medium, and weak evidence of a gene as encoding putative wall biosynthetic enzymes.

It is a general strategy to establish wall biosynthetic enzyme assays first using crude microsomal membrane preparations and then to purify the active component to identify the specific enzyme (Perrin et al., 1999; Sterling et al., 2006; Zeng et al., 2008). Subsequently, amino acid or cDNA sequences from such proven GTs can be aligned to identify homologous enzymes in other plant species (Faik et al., 2002; Cocuron et al., 2007). Screening putative glycosyltransferase mutants for reduction in enzyme activity has become another methodology to identify putative GTs (Brown et al., 2007; Lee et al., 2007a; Lee et al., 2007b). A more challenging strategy is to test for enzyme activity of putative GTs based on their mutant cell wall defects (Vanzin et al., 2002; Madson et al., 2003). In such cases, the more precise the information is about the linkage/wall polymer alteration in the mutant walls, the more likely that the enzymatic activity may be discovered. The acceptors for the enzyme assays may also be prepared from corresponding mutants (Egelund et al., 2006). However, negative results are often obtained using this approach and it can be difficult to distinguish whether a negative result means that the hypothesized enzyme activity is incorrect, or rather, that the enzyme assay conditions were inappropriate. The above mentioned strategies are often combined in wall glycosyltransferase characterization studies. However, proving that a putative plant cell wall glycosyltransferase gene encodes the proposed enzyme is not trivial.

Irregular xylem mutants and other secondary cell wall mutants

Fifteen *irregular xylem* mutants have been characterized and a summary of their phenotypes and proven or putative gene functions is given in **Table 1.1**. The term “*irregular*

Table 1.1. Summary of mutants with *irx* phenotypes and their inferred gene functions based on published data.

Mutant name	Gene ID	Protein family	Stem cross-section phenotypes			Stem cell wall alteration compared to corresponding wild type	Lignin alteration compared to wild type	Xylan reducing end glycosyl sequence 1	Inferred Gene Function
			2° xylem vessel	2° inter-fascicular fiber	1° metaxylem				
<i>irx1</i>	<i>IRX1/AtCESA8</i>	GT2	Collapsed and deformed	Slightly thinner cell walls	~ Normal	> 80% loss of cellulose	Not apparent	ND	SCW cellulose synthase subunit
<i>irx2/kor</i>	<i>KOR/IRX2/RSW2</i>	GH9	Collapsed and deformed	More walls deposited in cell corners	~ Normal	~ 70% loss of crystalline cellulose	Similar to <i>irx1</i>	ND	Cellulose deposition in PCW and SCW; $\beta(1,4)$ -EGase
<i>irx3</i>	<i>IRX3/AtCESA7</i>	GT2	Collapsed and deformed	Uneven and thinner cell walls	~ Normal	~ 70-80% loss of celluloses	Similar to <i>irx1</i> ; little effect on COMT RNA level	ND	SCW cellulose synthase subunit
<i>irx4</i>	<i>IRX4/CCR1</i>	CCR	Collapsed and deformed	Uneven, diffused cell wall	ND	Unchanged % of cellulose/hemicellulose	> 50% lignin loss of both G and S lignin	ND	Cinnamoyl-CoA reductase
<i>irx5</i>	<i>IRX5/AtCESA4</i>	GT2	Collapsed and deformed	Uneven and thinner cell walls	ND	~ 70% loss of cellulose	Similar to <i>irx3</i>	ND	SCW cellulose synthase subunit
<i>irx6</i>	<i>IRX6/COBL4</i>	GT47	Slightly deformed	ND	ND	~ 80% loss of cellulose (Ws-0)	ND	ND	Cellulose synthesis
<i>irx7/fra8</i>	<i>IRX7/FRA8</i>	GT47	Severely deformed	Thinner cell walls	ND	25% loss of cellulose; ~ 60% loss of xylose; ~ 70% loss of xylan †	ND	Absent	Synthesis of xylan reducing end sequence
<i>irx8</i>	<i>IRX8/GAUT12</i>	GT8	Severely deformed	Thinner cell walls	Collapsed	~ 20% loss of cellulose ~ 60% loss of xylan †	ND	Absent	Synthesis of xylan reducing end sequence, or a sub-fraction of pectin
<i>irx9</i>	<i>IRX9</i>	GT43	Severely deformed	Thinner cell walls	ND	~ 20% loss of cellulose; ~ 60% loss of xylan † ; 30% of WT xylan DP	ND	present	Xylan backbone synthesis; $\beta(1,4)$ -xylan:XylIT
<i>*irx10 irx10-L</i>	<i>IRX10/GUT2, IRX10-L/GUT1</i>	GT47	Collapsed and deformed	Thinner cell walls	Collapsed	~ 30% loss of cellulose; ~ 60% loss of xylose	ND	present	Xylan backbone synthesis; $\beta(1,4)$ -xylan:XylIT
<i>irx11</i>	<i>IXR11/KNAT7</i>	HD1	Slightly deformed	ND	ND	Slight reduction in cellulose•	ND	ND	Transcription factor; target of MYB46, and SND1
<i>irx12</i>	<i>IRX12/LAC4</i>	LAC	Slightly deformed	ND	ND	Little reduction in cellulose•	ND	ND	Putative laccase; phenylpropanoid metabolism

<i>irx13</i>	<i>IRX13/FLA11</i>	AGP	Slightly deformed	ND	ND	~ 20% loss of cellulose	ND	ND	Fasciclin-like AGP
<i>irx14</i>	<i>IRX14</i>	GT43	Collapsed and deformed	ND	ND	Slight loss of cellulose; ~ 30% loss of xylose; ~ 40% loss of xylan † ; 36% of WT xylan DP	ND	present	Xylan backbone synthesis; β (1,4)-xylan:XylIT
<i>parvus3</i>	<i>GLZ1/GATL1/PARVUS</i>	GT8	Collapsed and deformed	Thinner cell walls	RNA was detected	~ 20% loss of cellulose; > 50% loss of xylose; ~ 55% loss of xylan †	ND	absent	Synthesis of xylan reducing end sequence
<i>*irx15</i> <i>irx15-L</i>	<i>IRX15</i> , <i>IRX15-L</i>	not a GT, DUF579	Slightly deformed	irregular wall margin	ND	~35% loss of xylose, ~50% loss of xylan † ; 50% of WT xylan DP	no effect	present	Unclear function in xylan biosynthesis

1°, primary cell wall; 2°, secondary cell wall; PCW, primary cell wall; SCW, secondary cell wall; COMT, caffeic acid O-methyltransferase; ND, not determined in these studies; *, double mutant; •, not significant; AGP, arabinogalactan protein; †, PACE (polysaccharide analysis by carbohydrate gel electrophoresis) of xylan; **, β -D-Xylp-(1-3)- α -L-Rhap-(1-2)- α -D-GalpA-(1-4)-D-Xylp found at the reducing end of xylan (Pena et al., 2007).

xylem (irx)” was first used to describe three Arabidopsis mutants, namely *irx1*, *irx2*, *irx3*, with collapsed and deformed xylem vessels obtained from a screen of ethyl methanesulfonate (EMS)-mutagenized Arabidopsis M2 seeds (Turner and Somerville, 1997). *IRX1* and *IRX3* were identified as secondary cell wall cellulose synthase subunits (Taylor et al., 2000). *IRX2* was identified as allelic to *KORRIGAN (KOR)* and is believed to have roles in cellulose deposition in both primary and secondary cell walls (Szyjanowicz et al., 2004). All three mutants do not have apparent alterations in lignin based on phloroglucinol-HCl staining, and there was no reduction in the total phenolic content of stems based on total lignin measurements using the acetyl bromide method (Turner and Somerville, 1997). The *irx4* mutant has a severe lignin reduction but unchanged cell wall sugar composition, resulting in a phenotype of diffused interfascicular fiber cell walls (Jones et al., 2001). *IRX4* was mapped as allelic to *CCR* that encodes a cinnamoyl-CoA reductase (Jones et al., 2001). Different lines of *irx5* were isolated from EMS-mutagenized seeds and Ds-insertion lines. These mutants display collapsed xylem phenotypes as well as thinner cell walls in interfascicular fibers. *IRX1*, *IRX3*, and *IRX5* co-immunoprecipitate from whole stem extracts, and transcripts of *IRX1* and *IRX3* were undetectable in *irx5* extracts. These data revealed that all three proteins form a secondary cell wall cellulose synthase complex and are indispensable for proper complex assembly (Taylor et al., 2003). These early forward genetic mutant studies have led to the notion that the *irx* phenotype is indicative for any mutation related to secondary cell wall formation (Taylor et al., 2003).

The annotation of the Arabidopsis genome in 2000 made reverse genetic studies possible and supported larger scale targeted screening of T-DNA insertion lines for *irx* phenotypes. Persson et al. (2005) used a regression method to investigate the correlation of Arabidopsis gene expression with *CESA* genes using publicly available microarray data. The tight co-expression of

IRX8 and *IRX13* led to the isolation of *irx8* and *irx13* mutants. Simultaneously, Brown et al. (2005) generated Arabidopsis stem microarray data and cross-referenced it with public microarray data resulting in the identification of novel *irregular xylem* mutants, designated *irx6* to *irx12*. The *irx6* mutant is the only one of these seven mutants that exhibits greatly reduced cellulose due to the disruption of *COBRA-LIKE4* (*COBL4*) in the Wassilewskija (Ws) background (Brown et al., 2005). The *irx10* single mutant has a close-to-wild type phenotype (Brown et al., 2005), whereas the *irx10 irx10-L* double mutant displays a severe *irx* phenotype. The two homologous genes are speculated to synthesize the β -(1,4)-xylan backbone, since microsomes from the double mutant, but not single mutants, contained reduced xylan:xylosyltransferase (XylT) activity (Brown et al., 2009; Wu et al., 2009a). The *irx11* mutation disrupts *KNAT7*, which encodes a homeodomain (HD1) transcription factor that serves as a direct target of SND1. Studies by Zhong et al. (2008) demonstrated that *KNAT7*-dominant repression caused reduced cell wall thickness in both xylary and interfascicular fibers, while over-expression of *KNAT7* did not result in any changes in secondary cell walls. In general, *irx6*, *irx10*, and *irx11* exhibit less severe *irx* phenotypes compared to *irx3* (Brown et al., 2005). The *irx12* mutant stood out from the rest in Fourier transformation infrared (FTIR) spectroscopy, possibly due to its one-of-a-kind defect in phenylpropanoid metabolism (Brown et al., 2005). The *irx13* has a 20% loss of cellulose and loss of non-cellulosic polymers, which is similar to the *irx8* (Persson et al., 2005).

Despite the slight reduction of cellulose in the *irx7*, *irx8*, and *irx9*, they accumulate cellulose during stem development, and exhibit more severe *irx* phenotypes than some of the cellulose-deficient mutants such as the *irx2-2* (Brown et al., 2005). The *fra8/irx7* mutant displays a dramatic loss of xylan and especially lacks GlcA residues in its cell walls. *FRA8/IRX7* was thus

thought to encode a glucuronyltransferase (GlcAT) that adds GlcA onto the glucuronoxylan (GX) backbone (Zhong et al., 2005). However, GlcAT activity was not reduced in *fra8/irx7* microsomes compared to the wild-type counterpart. Microsomes isolated from *irx9* had reduced xylan:XylT activity but did not affect GlcAT activity (Lee et al., 2007a), suggesting its role in synthesizing the xylan backbone. Neither XylT nor GlcAT activity was affected in *irx8* stem microsomes, indicating that *GAUT12/IRX8* is not involved in the synthesis of the xylan backbone or xylan substitution (Brown et al., 2007; Lee et al., 2007a). Besides these three mutants, Brown et al. (2007) also studied two other mutants that exhibited severe *irx* phenotypes: *irx14* and *parvus-3*. No loss of xylan:XylT activity could be detected in *parvus-3* microsomes while dramatic loss of xylan:XylT activity was detected in *irx9* and *irx14* (Brown et al., 2007; Lee et al., 2007a). These results indicate that these *irregular xylem* mutants mentioned above have different functions in xylan biosynthesis.

The recently identified *irx15 irx15-L* double mutant represents a new class of xylan mutants in which xylan synthesis is not directly affected. Both genes are not typical glycosyltransferases defined by CAZy and there was no significant reduction of xylan:XylT activity in stem microsomes (Brown et al., 2011b; Jensen et al., 2011). Surprisingly, the *irx15 irx15-L* double mutant has increased LM10 epitopes (low-substituted xylan) in its stems. However, the double mutant has reduced xylan chain length and replacement of GlcA with meGlcA substitution on the xylan backbone. These results suggest that xylan biosynthesis is altered in the *irx15 irx15-L* double mutant.

It should be noted that all of the *irx* mutants have been documented for collapsed secondary xylem (e.g. mature xylem vessels) and reduced wall thickness in xylary fibers and interfascicular fibers. However, only the *irx8* and *irx10 irx10-L* were reported to have

deformation in primary xylem (i.e. protoxylem and metaxylem). Collapsed metaxylem in *irx8* was proposed to reflect its earlier role in Arabidopsis vasculature development (Turner and Somerville, 1997; Persson et al., 2007). It was also recognized, however, by Persson et al. (2007), that in addition to reduced xylan, *irx8* mutant cell walls are also reduced in a sub-fraction of pectin, leading these authors to propose hypothetical functions for *GAUT12/IRX8* in the biosynthesis of xylan or a pectin sub-fraction. Noteworthy for the work reported in this thesis, *irx7*, *irx8*, *irx9*, *irx14*, and *parvus-3* have not been studied in regards to lignin alterations.

Inferred gene functions in xylan biosynthesis revealed by these mutants (**Table 1.1**) are discussed in detail later in this chapter (see XYLAN BIOSYNTHESIS).

ARABIDOPSIS GALACTURONOSYLTRANSFERASE12 AND THE GAUT GENE FAMILY

GAUT12 and characterization of irx8 mutants

Both *IRX8* and *GAUT12* refer to *A. thaliana* gene *At5g54690*. *IRX8* is the gene name based on the mutant phenotype (Brown et al., 2005; Persson et al., 2005), while *GAUT12* is the other gene name based on its membership in the *GAUT1*-related gene family (Sterling et al., 2006; Persson et al., 2007). Herein we will refer to the gene as *GAUT12* and its protein as GAUT12.

GAUT12 encodes a protein of 535 amino acids with a calculated molecular mass of 60.88 kDa and a pI of 9.17. GAUT12 and GAUT1 share 40% amino acid sequence identity and 61% sequence similarity (Sterling et al., 2006). *GAUT12* is strongly expressed in xylem and interfascicular fiber cells in Arabidopsis stems, which have been studied using semi-quantitative RT-PCR (Persson et al., 2007), GUS reporter systems (Pena et al., 2007; Persson et al., 2007), *in*

situ hybridization (Pena et al., 2007), and quantitative RT-PCR analysis (Brown et al., 2005). GAUT12 is predicted to be a type II transmembrane protein with its presumed C-terminal catalytic domain facing the Golgi lumen. Its sub-cellular localization in the Golgi apparatus was indicated by co-localization of GAUT12-YFP and MUR4-CFP (Burget et al., 2003) in carrot protoplasts (Pena et al., 2007). *GAUT12* T-DNA insertion mutant lines (**Figure 1.2A**) are severely dwarfed (**Figure 1.2B**), sterile, and have collapsed xylem due to thinner secondary cell walls in stems and roots (**Figure 1.2C**) (Brown et al., 2005; Pena et al., 2007; Persson et al., 2007). The published data on various alleles of *irx8* mutants is summarized in **Table 1.2**. The *irx8* mutants are maintained by selecting homozygote mutants from heterozygous plants.

GAUT12 is a member of the GAUT1-related gene family that is divided into seven sub-clades (**Figure 1.2D**). The GAUT family belongs to glycosyltransferase family 8 (GT8) as classified by the CAZy (<http://www.cazy.org/>) database (Cantarel et al., 2009) and a more in-depth phylogenetic tree of the GT8 family has been developed by Yin et al. (Yin et al., 2010). Arabidopsis GAUT12 has two orthologs in *Populus trichocarpa*, POPTR 0001s44250 and POPTR 0011s13600 (**Figure 1.2D**). Transgenic poplar plants in which both gene products have been impaired by RNA-interference display brittle stems and reduced wall in vessels and fibers, although the collapsed xylem and dwarfed phenotype of the Arabidopsis *irx8* mutants are not recapitulated in poplar (Li et al., 2011). GAUT12 does not have any orthologs in the rice genome (**Figure 1.2D**), indicating that the function associated with GAUT12 is either missing in grasses or carried out by other GAUT genes. Interestingly, the xylan reducing-end sequence (XRES) has thus far only been found in eudicots and gymnosperms but not in basal land plants including *P. patens*, *S. kraussiana*, *E. hyemale*, or in grass species including rice (Kulkarni et al., 2012).

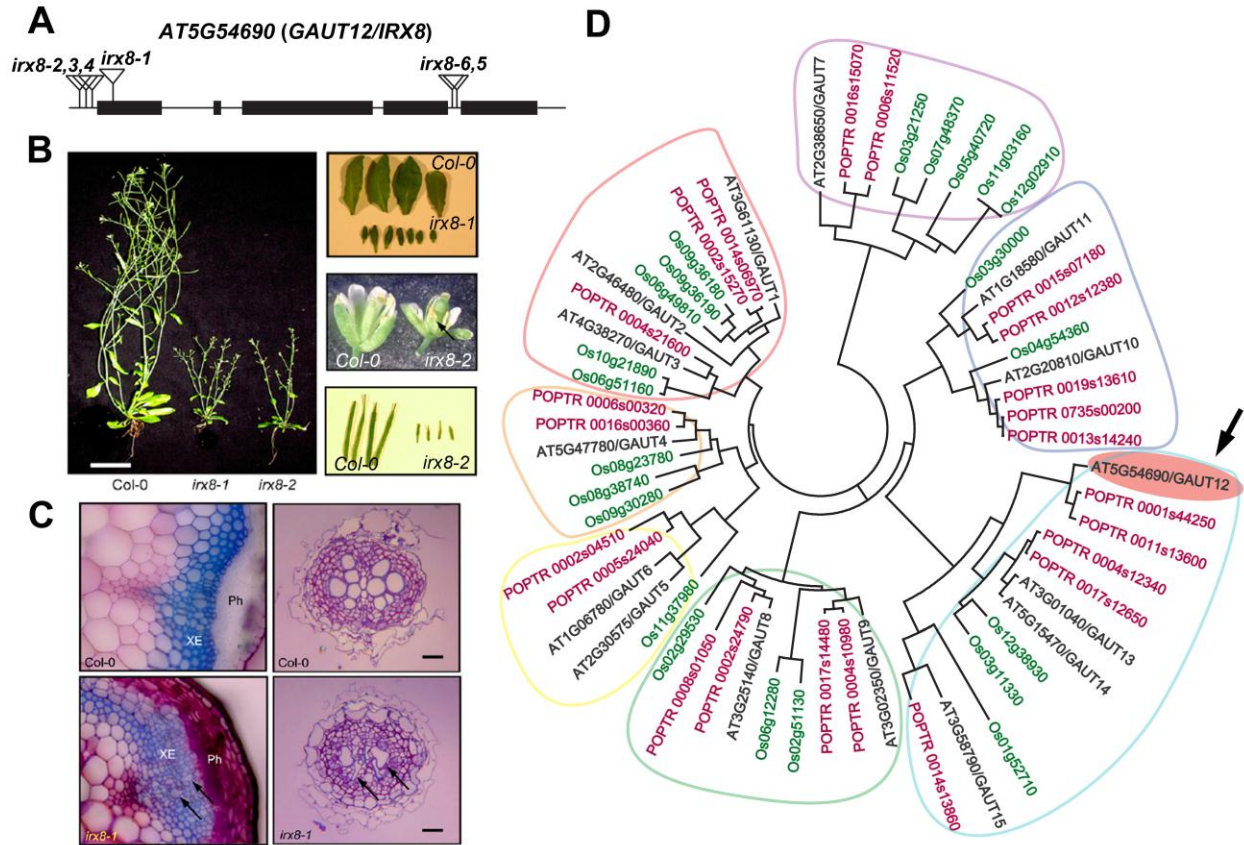


Figure 1.2. Characterization of *irx8* mutants and phylogenetic tree of the GAUT protein family.

(A) Location of various *irx8* T-DNA insertion mutant alleles.

(B) Seven-week-old WT and *irx8* mutants, bar = 5 cm, rosette leaves, flowers, and siliques (adapted from Persson et al. 2007);

(C) Toluidine blue stained hand-cut cross-sections of WT and *irx8* stems and roots (adapted from Persson et al. 2007);

(D) Phylogenetic tree of the GAUT protein family in Arabidopsis, rice, and poplar (adapted from Atmodjo, Hao, and Mohnen, Annual Review Plant Biology Vol.64, 2013, in press). GAUT12 is highlighted and indicated by the black arrow.

Table 1.2. Summary of *irx8* mutant alleles.

Name	Accession No.	Position of insertion	Transcript level	Primer used	Phenotypes	Cell wall defects	Reference
<i>irx8-1</i>	SALK_014026	exon	null	start to end	Stunted growth, smaller leaves, siliques, flowers, same trichome size; no seeds obtained from HM mutants; shorter anther filament, reduced number of pollen; collapsed xylem in stems and roots; lost xylem cell organization in roots; collapsed xylem in both initiating and mature xylem cells; similar across all alleles	Neutral sugar composition of stems and roots (7-wk-old): increased Rha, Ara, Man, Gal, Glc; decreased Xyl; significant reduction of cellulose in 7-wk-old stem;	Persson et al., 2007; Pena et al., 2007 (<i>irx8-2</i>); Persson et al., 2005
<i>irx8-2</i>	SAIL_603_G02	5' UTR	null*	start to end			Persson et al., 2007; Persson et al., 2005
<i>irx8-3</i>	SAIL_1262_H02	5' UTR	significant reduction	start to end		N/A	Persson et al., 2007
<i>irx8-4</i>	SAIL_1214_C12	5' UTR	significant reduction	start to end		N/A	Persson et al., 2007
<i>irx8-5</i>	SALK_044387	intron	significant reduction	start to end		Fractionated stem wall of <i>irx8-1</i> and <i>irx8-5</i> : decrease in Xyl, and EPG/PME-released GalA	Persson et al., 2007
<i>irx8-6</i>	SALK_008642	intron	reduced transcript	Designed to prime RNA close to 5' and 3' ends of gene	Dwarf plant with decreased cell wall thickness in stem and root vessels and fiber cells by 60% to WT; reduced stem strengths; HM mutants were obtained from heterozygous plants	35% Xyl of WT; significant decrease in cellulose but showed increase in cellulose during development; lack of the glycosyl sequence 1 (XRES) at xylan reducing end	Brown et al., 2005; Pena et al., 2007 (named as <i>irx8-1</i>)

*Persson et al. (2007) reported the *irx8-2* is a null mutant; however, in this thesis residual full length *GAUT12* transcript in this mutant was detected (see Chapter 2). HM, homozygous.

Therefore, the currently available phylogenetic data suggests a possible correlation between the presence of GAUT12 orthologs and the presence of the xylan reducing-end sequence.

Quantification of cellulose in *Arabidopsis irx8* revealed a ~25% reduction, which is mild compared to cellulose defective mutants such as *irx1* and *irx3* (Brown et al., 2005; Persson et al., 2005). Analyses of neutral sugar composition of total *irx8* walls isolated from stems and roots showed a >50% reduction in Xyl, while the amounts of Ara, Rha, Man, Gal, and Glc were increased compared with wild type (Persson et al., 2007). Trimethylsilyl (TMS)-glycosyl residue composition analyses of fractionated walls of 7-week-old stems of *irx8* revealed a major (~50-60%) mole percent (mol%) loss of Xyl in the 4 N KOH-soluble fraction, and a reduction of GalA in the EPG- and EPG/PME- released fractions. Pectin methylesterase (PME) removes the methyl esters from the C-6 of GalA residues in HG backbone, and the “smooth” HG can then be cleaved by endopolygalacturonase (EPG). EPG/PME releases enzyme-accessible pectins of the wall, while 4 N KOH extracts most of the hemicelluloses, leaving cellulose-enriched pellets (Caffall, 2008). Linkage analyses revealed reduced amounts of 4-linked Xylp, confirming a dramatic loss of $\beta(1\rightarrow4)$ -xylan in *irx8*. The relative mol% of Rha increased in the EPG- and EPG/PME- fractions in accordance with the increase of Rha identified in the total cell walls. However, 2-linked Rhap was found to be reduced in the EPG-fraction, which suggests an altered RG-I structure in the walls of *irx8* mutants. However, not all pectins were affected, as the EGTA/Na₂CO₃-released fraction contained similar amounts of GalA compared with WT, indicating that only a sub-fraction of HG was affected. EGTA/Na₂CO₃ releases a fraction of pectin that is not accessible to EPG/PME or that may be calcium cross-linked (Caffall, 2008). The reduced 4-linked and 3,4-linked GalAp, despite the increased terminal GalAp, indicated that the reduced pectin in the EPG-fraction was likely HG (Persson et al., 2007). No significant

difference was identified in the 4 N KOH-insoluble fraction of *irx8*, which includes mostly crystalline cellulose and its bound proteins.

The reduction of xylan was also demonstrated by the significant loss of LM10 and LM11 labeling in 7-week-old *irx8* stem and roots (Pena et al., 2007; Persson et al., 2007). LM10 binds to 4-*O*-methylglucuronoxylan but not to arabinoxylans or glucuronoarabinoxylan (McCartney et al., 2005). There was almost no LM10 labeling in *irx8* vessels and fiber cells. Conversely LM11, which binds to a broader range of branched xylans, showed some residual labeling (Persson et al., 2007).

Further ¹H-NMR analyses of the acidic xylo-oligosaccharide fraction of the endoxylanase-treated 1 N KOH-soluble wall material from *irx8* showed a near absence of the glycosyl sequence (XRES) signal that was present at the reducing-end of wild type and *irx9* xylan (Pena et al., 2007). The glycosyl sequence has been resolved to be D-Xylp-β(1→4)-D-Xylp-β(1→3)-L-Rhap-α(1→2)-D-GalpA-α(1→4)-D-Xylitol. The xylitol is produced from xylose during xylan extraction using the KOH buffer containing NaBH₄, which prevents degradation by base-catalyzed peeling reactions. The molecular weight (MW) of *irx8* xylan was determined by size-exclusion chromatography to be heterodisperse, with some xylan appearing to be larger than that found in wild type, while the *irx9* xylan chains appeared to be much smaller in MW than wild type. These results suggest that the mutation in *GAUT12* affects the production/presence of the xylan reducing-end glycosyl sequence, and that *GAUT12* may transfer the α-D-GalA into this sequence (Pena et al., 2007).

The ultimate solution to understanding the role of *GAUT12* in secondary wall biosynthesis is to identify its enzymatic activity. Based on reported studies of *irx8*, two hypotheses have been proposed for *GAUT12* enzymatic activity. *Hypothesis 1*: *GAUT12*

functions in the synthesis of the *Arabidopsis* glucuronoxylan reducing-end tetrasaccharide by catalyzing the addition of α -D-GalA onto the xylose. The moiety onto which the reducing-end xylose is attached is not known. *Hypothesis 2*: The reduced amounts of GalA in the *irx8* mutant walls are due to the loss of an acceptor/primer required for xylan synthesis and GAUT12 synthesizes a sub-fraction of HG (or possibly RG-I) onto which xylan is covalently attached.

The GAUT gene family and functions of other GAUTs besides GAUT12

The *GAUT1*-related gene family (**Figure 1.2D**) was identified by blasting *GAUT1* sequence against the *Arabidopsis* genome, which is composed of *GAUT1* and fourteen *GAUT1* homologs encoding GAUTs 2-15 with 36-68% amino acid sequence identity and 56-84% sequence similarity to *GAUT1* (Sterling et al., 2006). Additionally, another ten related *Arabidopsis* genes encoding proteins with 23-29% amino acid sequence identity and 42-53% sequence similarity to *GAUT1* were also identified and named the *Arabidopsis* GAUT-like (GATL) family. Both GAUTs and GATLs belong to CAZy (<http://www.cazy.org/>) GT family 8 (Cantarel et al., 2009), albeit with distinct features. While the GAUTs are predicted to be type II transmembrane proteins (61-78 kDa) with variable N-terminal sequences, the GATLs are smaller proteins (39-44 kDa) with no transmembrane domains but with N-terminal signal peptides that direct them through the secretory pathway (Sterling et al., 2006; Kong et al., 2011). Some GATLs were demonstrated to be Golgi-localized (Kong et al., 2011) or ER-localized (Lee et al., 2007b). Molecular analyses of the GATL gene family including characterization of several mutants have been reported (Lao et al., 2003; Shao et al., 2004; Lee et al., 2007b; Kong et al., 2009; Kong et al., 2011). The GATL family will not be reviewed here, except those members with functions related to *GAUT12*, such as *GATL1*.

Functional identification of the GAUT1 was achieved by combined proteomic and biochemical analyses. Liquid chromatography-tandem mass spectrometry sequencing of a trypsin-digested, homogalacturonan (HG):galacturonosyltransferase (GalAT)-enriched, partially purified solubilized Arabidopsis membrane enzyme preparation revealed two related glycosyltransferases, At3g61130 and At2g28650, named GAUT1 and GAUT7, respectively (Sterling et al., 2006). GAUT1, but not GAUT7, was demonstrated to have HG:GalAT activity when transiently transfected into human embryonic kidney (HEK) cells.

GAUT1 contains 673 amino acids and is predicted to be a 77.4 kDa protein having a basic pI of 9.95. However, GAUT1 isolated from Arabidopsis suspension culture by immunoprecipitation from detergent-solubilized microsomes is a 58.6 kDa protein. N-terminal sequencing of this protein revealed that the GAUT1 N-terminal sequence including the transmembrane (TM) domain is removed, indicating a cleavage between Met₁₆₇ and Arg₁₆₈ (Atmodjo et al., 2011). Interestingly, without its TM domain, GAUT1 is retained in the Golgi by covalent and non-covalent interactions with GAUT7 which serves to anchor GAUT1 and forms a protein complex with GAUT1 (Atmodjo et al., 2011). Evidence from bi-molecular fluorescence complementation (BiFC) experiments, in which the split halves of the YFP protein were separately fused to GAUT1 and GAUT7 and then transiently co-expressed in tobacco leaf cells, yielded fluorescence and indicated that GAUT1 and GAUT7 are co-localized in a complex in the Golgi apparatus. However, in the absence of GAUT7, GAUT1 could not be retained in the Golgi on its own. In addition, *GAUT1*- and *GAUT7*-promoter::GUS expression confirmed co-expression of *GAUT1* and *GAUT7* in various tissues in Arabidopsis (Atmodjo et al., 2011). The immunoprecipitated GAUT1:GAUT7 complex has high selectivity towards oligogalacturonide (OGA) accepters compared to RG-I oligomers of similar sizes and to RG-II monomers from

wine, confirming that the GAUT1:GAUT7 complex is an HG:GalAT (Atmodjo et al., 2011). While the available *gaut7* knock-down mutants have no obvious growth phenotypes (Caffall et al., 2009a), recovery of homozygous *gaut1* mutants has been unsuccessful, as they appear to be lethal at germination. This indicates a vital role for *GAUT1* in plant development (Atmodjo, 2010). *GAUT7* forms its own clade with two poplar and five rice orthologs in the phylogenetic tree (**Figure 1.2D**), possibly indicating a specific function for *GAUT7* among *GAUTs* and a more important function in monocots (Atmodjo, 2010).

Other *GAUTs* that are likely to function in HG biosynthesis based on high sequence similarity to *GAUT1* and/or mutant cell wall composition analyses are *GAUT4*, *GAUT6*, and *GAUT8*. Having 83% amino acid (a.a.) similarity to *GAUT1*, *GAUT4* may also function in pectin biosynthesis, however homozygous *gaut4* mutants have not been recovered since the gene may also be crucial for the survival of plants (Caffall et al., 2009a). Generation of knock-down or knock-out mutants of both *GAUT1* and *GAUT4*, possibly via an inducible RNAi system (Guo et al., 2003) that down-regulates *GAUT1* or *GAUT4* at different developmental stages, may help to evaluate the functions of these genes *in vivo*.

GAUT6 has 64% a.a. similarity to *GAUT1*, and characterization of the *gaut6* mutants revealed reduced GalA in its cell walls and an overall reduction in water-extractable seed mucilage production in *gaut6* mutants (Caffall, 2008). The pectin-enriched fractions of *gaut6* had an increased ratio of HG to RG-I as indicated by the GalA:Rha ratio in these fractions. This may be due to the loss of the more readily released RG-I of *gaut6* walls in aqueous solution during sequential cell wall extraction. It is hypothesized that *GAUT6* may be involved in the synthesis of a specific HG that is somehow cross-linked to RG-I, the structure of which remains to be determined (Caffall, 2008). Transient expression of *GAUT6* and *GAUT6-HIS* in *Nicotiana*

benthamiana via *Agrobacterium*-mediated transformation resulted in microsomes with elevated levels of HG:GalAT activity compared to those prepared from non-transformed or empty vector-transformed plants (Caffall, 2008). Two truncated forms of GAUT6 (Δ 1-27, removal of TM; and Δ 1-149, removal of both TM and stem region) expressed in *E.coli* also had some HG:GalAT activity (Caffall, 2008). These preliminary results from heterologous expression studies indicate a role for GAUT6 as an HG:GalAT.

GAUT8/QUA1 has 77% a.a. similarity to GAUT1 and appears to encode a putative glycosyltransferase that affects cell adhesion as well as HG and xylan biosynthesis (Bouton et al., 2002; Leboeuf et al., 2005; Orfila et al., 2005). While some homozygous *gaut8* knockout (KO) mutants are lethal, the *qua1-1* (knockdown, KD) and *qua1-2* (KO) mutants have been used in all reported studies, with the latter having a severely dwarfed phenotype (Bouton et al., 2002). The microsomes from *qua1-1* stems had a ~30% reduction in HG:GalAT activity and 40% reduction in β (1 $\rightarrow$ 4)-xylan:XylT activity (Orfila et al., 2005). In addition, *GAUT8/QUA1* was shown to be co-expressed with *QUA2* and with one of its 29 homologs. *QUA2* encodes a putative methyltransferase and *qua2-1* has ~50% reduction in HG (Mouille et al., 2007). These results led Mouille et al. (2007) to propose either that GAUT8/QUA1 and QUA2 are part of a protein complex or that QUA2 provides a methylated GalA acceptor for the GAUT8/QUA1 GalAT function. Demonstration of GAUT8/QUA1 enzymatic activity is required to confirm its role in pectin synthesis.

GAUT11 was found to affect seed mucilage production. The *gaut11* mutants have reduced GalA in cell walls from multiple tissues including siliques, inflorescence and leaves. Ruthenium red-stained seeds showed a 50% reduction in the thickness of the extruded mucilage in ~30% of *gaut11-2* seeds, while the remaining ~70% of *gaut11-2* seeds contained little

extruded mucilage (Caffall et al., 2009a). Despite the reduced mucilage detected by ruthenium red staining, the amount of total carbohydrates in the water-soluble mucilage is similar in *gaut11-2* compared with wild type. This water-soluble mucilage of *gaut11-2* has a 19% reduction in the uronic acid content, which was consistent with the reduction in GalA in this fraction determined by glycosyl residue composition analysis. These results suggest a function for GAUT11 in pectin biosynthesis and seed mucilage production.

Mutations of *GAUT9* and *GAUT10* have no obvious overall growth or cell adhesion defects. However, the walls of *gaut9* and *gaut10* have reduced GalA. Therefore, *GAUT9* and *GAUT10* may also have a role in pectin biosynthesis (Caffall et al., 2009a).

Belonging to the same clade as *GAUT12*, *GAUT13*, -14, and -15 share high sequence similarity with *GAUT12* but are located in different branches of the phylogenetic tree (**Figure 1.2D**). Cell walls of *gaut13* and *gaut14* have increased GalA and Gal and reduced Xyl and Rha compared with wild type walls, suggesting a potential role for both genes in affecting xylan synthesis which may partially overlap with the presumed function of *GAUT12*. However, it has been reported that double mutants between *irx8* (*gaut12*) and *gaut13*, *gaut14*, or *gaut15* did not provide additive phenotypes (Wu et al., 2010). Generation of other combinations of double or even triple mutants among these genes may provide additional information of the corresponding gene function.

Last but not least, few significant changes were found in *gaut2*, *gaut3*, and *gaut5* mutants, suggesting less important or redundant roles for these genes in plant development. Except for *GAUT2*, which appears to be a non-functional homolog of *GAUT1* (Sterling et al., 2006; Caffall et al., 2009a), *GAUT3* and *GAUT5* may serve as functionally redundant backups

to GAUT1 and GAUT6, respectively. Complementation experiments can be designed to test this hypothesis.

All GAUTs and their orthologs share a family motif of H-x (2)-[ILV]-x-[ST]-D-N-[IV]-[IL]-A-[ASTV]-S-V-V-[AIV]-x-S-x-[AIV]-x (2)-[AS]-x (2)-[PS]-x (3)-V-[FL]-H-[ILV]-[ILV]-T-[DN]-x (2)-[NST]-x (2)-[AGP]-[IM]-x (3)-F in *Arabidopsis*, rice, tobacco, and chickpea, as well as the characteristic DxD motif for glycosyltransferases shared between GAUTs and GATLs (Sterling et al., 2006). In depth phylogenetic analyses of GAUTs and GATLs to other GT8 family members showed that GAUTs, GATLs, and a lower plant-specific GAUT and GATL-related (GATR) group fall into a distinct GT8 family class (class I), distinguishable from the galactinol synthase (GolS) and plant glycogenin-like starch initiation proteins (PGSIP) clades (class II) and from the bacterial GT8 proteins (class III) (Yin et al., 2010). The phylogeny suggests that the three classes are unlikely to share similar functions. Indeed, recently four proteins from the PGSIP clades (renamed GUX1-4) have been shown to add the GlcA and meGlcA residues onto xylan (Mortimer et al., 2010; Lee et al., 2012b; Rennie et al., 2012). The presence of the GATR group in mosses/green algae and the increased number of GAUTs and GATLs from lower plants to higher plants indicates that these homologs may have conserved functions in pectin biosynthesis, consistent with the presence of pectin found in Charophytes and Chlorophytes (Popper and Fry, 2003; Popper, 2008; Sorensen et al., 2011), Bryophytes (Roberts et al., 2012), and Lycopodiophytes (Harholt et al., 2012a).

TRANSCRIPTIONAL REGULATION OF SECONDARY CELL WALL FORMATION

Master switches

Secondary cell wall formation is precisely controlled as it takes place in vascular-tissue and specialized cells in stems, roots, anthers, and siliques. The transcription factor (TF) network of secondary cell wall thickening is highly complex as shown in **Figure 1.3**. Several master transcriptional switches were identified as governing the process of secondary thickening, often in a tissue-specific manner. For example, SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1 (SND1) is expressed in interfascicular fibers (IFs) and xylary fibers (Zhong et al., 2006), while VASCULAR-RELATED NAC-DOMAIN6 (VND6) and VND7 are localized in the developing metaxylem and protoxylem, respectively (Kubo et al., 2005). The NAC SECONDARY WALL THICKENING PROMOTING FACTOR2 (NST2) shows high expression in anthers, whereas NST1 appears to be expressed in a similar pattern as NST3/SND1, i.e. in fiber cells (Mitsuda et al., 2005; Mitsuda et al., 2007). In addition, both NST1 and NST3 are also expressed in specific cells (endocarp *b* layer) controlling secondary wall formation during silique dehiscence, leading to the regulation of pod shattering in a partially redundant manner (Mitsuda and Ohme-Takagi, 2008).

Master switches are located at the top of the TF regulation hierarchy (**Figure 1.3**) and their ectopic expression may lead to massive deposition of secondary wall thickening throughout the plant. Overexpression of SND1 in *Arabidopsis* caused ectopic secondary wall formation in leaf and stem epidermal cells and stems cortical cells (Zhong et al., 2006). Overexpression of NST1 and NST2 induced secondary wall thickening in anthers, stamens, ovules, stems, and less frequently, in roots (Mitsuda et al., 2005). In the case of VND6 and VND7, for which overexpression by a constitutive promoter such as the CaMV 35S promoter led to lethal

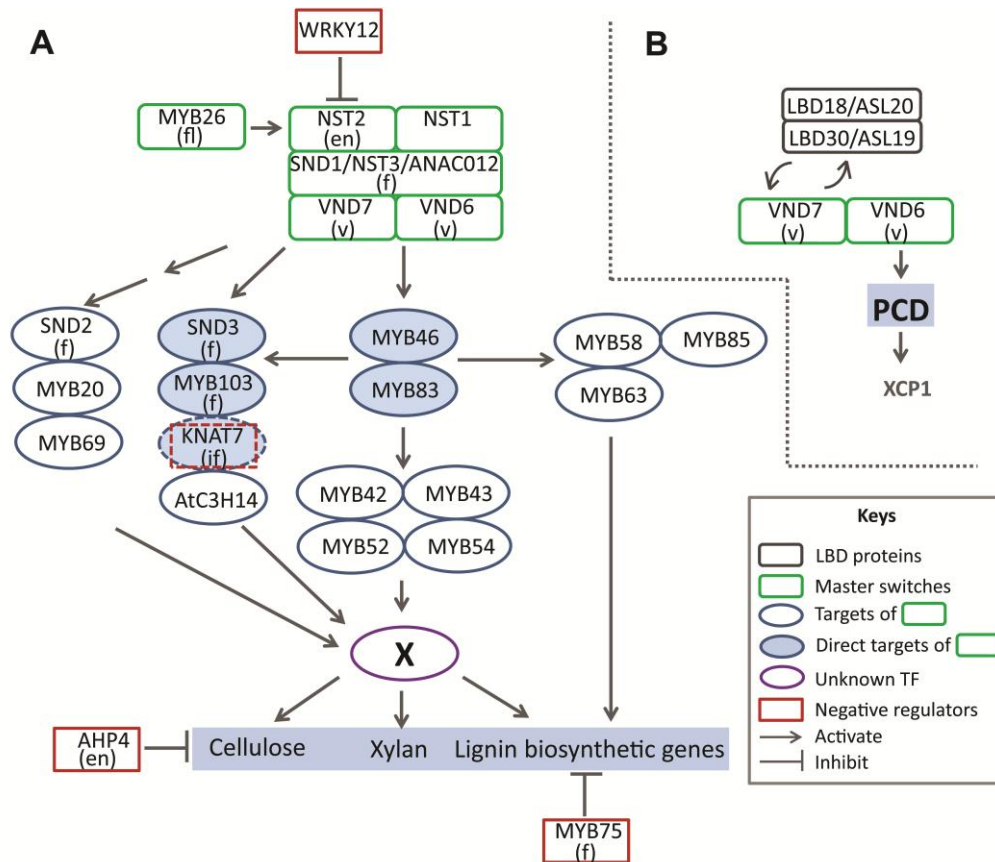


Figure 1.3. Schematic diagram of transcription network controlling Arabidopsis secondary cell wall formation.

(A) Transcription factor regulation as indicated. X represents unknown downstream transcription factors that directly regulate biosynthetic genes for cellulose, xylan, and lignin. MYB75, WRKY12, and AHP4 are negative regulators. MYB26 regulates both NST1/2; WRKY12 inhibits NST2 by direct binding to its promoter. KNAT7 appears to have a controversial role in vessels and fibers.

(B) VND7 and LOB domain protein (LBD18 and LBD30) form a positive feedback loop; PCD, programmed cell death; en, endothecium; f, fibers; fl, flower; if, interfascicular fiber; v, vessels. Stacked symbols do not indicate protein-complex formation. Adapted from Demura and Ye, 2010 and Zhong and Ye, 2009.

transgenic plants (Yamaguchi et al., 2010), a glucocorticoid-mediated posttranslational induction system (Lloyd et al., 1994; Sablowski and Meyerowitz, 1998) was used to bypass the problem. Briefly, the transcription factor was fused to a glucocorticoid receptor (GR) and inactivated in the cytosol by binding to heat shock proteins via the GR (Aoyama and Chua, 1997). When the glucocorticoid binds the GR, the transcription factor-GR fusion protein is released, translocated to the nucleus and functions as a transcription factor. Being a strong synthetic glucocorticoid, dexamethasone- (DEX) induced constitutive expression of VND6 and VND7 in Arabidopsis and poplar led to ectopic TE formation in multiple cell types causing plant death in Arabidopsis (Yamaguchi et al., 2010). Moreover, DEX-induced overexpression of VND7, but not VND6, in tobacco BY-2 cells resulted in transdifferentiation of 90% of the cells into tracheary elements (Yamaguchi et al., 2010).

All master switches regulate secondary wall formation via a network of downstream transcription factors that further direct downstream transcription factors which eventually up-regulate the expression of biosynthetic genes (**Figure 1.3A**). Reported direct transcription factor targets of SND1 are MYB46 (Zhong et al., 2007), MYB83 (McCarthy et al., 2009), SND3, MYB103, and KNAT7, the five of which were also found to be direct targets of VND6, VND7, NST1, and NST2 (Zhong et al., 2008). SND1 also regulates the expression of SND2, MYB20, MYB69, MYB42, MYB43, MYB52, MYB54, and MYB85, however the regulation is not direct (Zhong et al., 2006; Zhong et al., 2008). Overexpression of SND1 did not have any effect on VND6 and VND 7 transcripts, suggesting that the latter two are not downstream genes of SND1 (Zhong et al., 2006). Both DEX-induced VND6 and VND7 expression were shown to up-regulate the expression of cell wall biosynthetic genes *CesA4/IRX5*, *CesA7/IRX3*, *CesA8/IRX1*,

IRX8/GAUT12, *IRX10*, *CCoAOMT7*, *IRX12/LAC4*, and *XCP1* in Arabidopsis (Yamaguchi et al., 2010).

Besides sharing common transcription factor targets involved in secondary thickening, VND6 specifically regulates genes involved in programmed cell death (PCD) such as *XCP1*, which encodes a xylem-specific papain-like cysteine peptidase, that SND1 does not regulate (**Figure 1.3B**). Whereas SND1 controls expression of particular lignin biosynthesis genes such as *PAL1* and *C4L3* that VND6 does not regulate (Ohashi-Ito et al., 2010). This suggests that VND6 and VND7 are involved in vessel development, and SND1, NST1 and NST2 are rather involved in regulating fiber secondary cell wall formation. In addition, similar to SND1, NST1 and NST2 appear to control only the secondary wall thickening but not the entire process of tracheary element formation since TE cells formed ectopically by overexpression of NST1 do not go through PCD (Mitsuda et al., 2005). In addition, the *snd1 nst1* double KO mutant lost the ability to form secondary walls in xylary and interfascicular fibers but had intact vessels (Zhong and Ye, 2007). These results suggest that master transcription factors share common targets in producing secondary walls but exert distinct functions during tissue differentiation, in accordance with their individual expression patterns as discussed above.

MYB26/MALE STERILE35 (MS35) is essential for secondary thickening in anther endothecium (Yang et al., 2007). MYB26 is expressed in endothecium, style and nectaries, however *ms35* mutants are defective in anther dehiscence but not in female fertility (Steiner-Lange et al., 2003; Yang et al., 2007). Overexpression of MYB26 resulted in ectopic secondary wall thickening in epidermal tissues in both Arabidopsis and tobacco (*Nicotiana tabacum*). MYB26 controls secondary thickening via the regulation of NST1 and NST2 but not via MYB61 or MYB32 (Yang et al., 2007), the latter two of which are involved in the phenylpropanoid

pathway and ectopic lignification (Newman et al., 2004; Preston et al., 2004). MYB26 was also shown to regulate the expression of *IRX1*, *IRX3*, *GAUT12/IRX8*, and *IRX12* but not *IRX5*, or lignin genes *PAL1*, *C4H*, and *IRX4* (Yang et al., 2007). These results suggest that MYB26 is involved in the cellulose and xylan deposition in the anther endothecium cell layer, and that polysaccharide and lignin deposition in secondary wall thickening are two separately controlled processes.

It has been reported that ASYMMETRIC LEAVES2-LIKE19 (ASL19)/LATERAL ORGAN BOUNDARIES DOMAIN LBD30 (LBD30) and ASL20/LBD18 have VND6- and VND7-dependent expression in immature TEs (Soyano et al., 2008). Overexpression of LBD18 and LBD30 resulted in TE transdifferentiation through, probably indirect, activation of both VND6 and VND7. LBD18 overexpression also caused ectopic expression of VND7 suggesting that these two ASL/LBD proteins are in a positive feedback loop with VND7 during TE differentiation (Soyano et al., 2008) (**Figure 1.3B**). The LOB (LATERAL ORGAN BOUNDARIES) protein has been shown to be a transcriptional activator in yeast (Husbands et al., 2007). However, the majority of the LBD proteins have not been proven to be transcription factors. By promoting the expression of VND6/7, ASL19/LBD30 and ASL20/LBD18 may stabilize and coordinate the expression of downstream target genes during TE differentiation (Soyano et al., 2008).

Mid-level transcription factors

Being common downstream targets of SND1, VND6/7, and NST1/2, both MYB46 and MYB83 are second tier transcription factors that act redundantly in the regulation of secondary cell wall formation in *Arabidopsis* (Zhong et al., 2007; McCarthy et al., 2009). Gene redundancy

brings about challenges for transcription factor studies since mutation of a single gene is unlikely to produce a visible phenotype. To avoid this issue, a dominant repression (DR) system was employed. In brief, the transcription factor of interest is fused to the EAR domain, a 12-amino acid sequence (LDLDLELRGFA, designated SRDX) that acts as a sufficient repressor of the target gene as well as its homologs to achieve loss-of-function phenotypes of the target transcription factors (Hiratsu et al., 2003).

Dominant repression of MYB46 and MYB83, two common targets of master switches, caused a reduction of secondary wall thickening in both vessels and fibers, and similar phenotypes were observed with the MYB46 MYB83 double RNAi line. The double T-DNA KO mutant *myb46 myb83* has a more severe phenotype in that it only develops a pair of leaves which prevents further analysis of the mature plant (Zhong et al., 2007; McCarthy et al., 2009). Overexpression of the two TFs caused increased secondary cell wall thickening and ectopic lignin deposition in cortex, epidermal and pith cells. Despite the tissue specificity of their upstream master switches, MYB46 and MYB83 function in both vessels and fibers in a redundant manner (McCarthy et al., 2009). They regulate the expression of transcription factors including MYB58, MYB63 and MYB85 that are involved in lignin biosynthesis, and SND3, MYB103, KNAT7, AtC3H14, MYB52, and MYB54 involved in secondary wall formation (Zhong et al., 2007; Zhong et al., 2008; Ko et al., 2009; McCarthy et al., 2009). However, they do not control the expression of *INTERFASCICULAR FIBERLESS1 (IFL1)* (Zhong and Ye, 1999) or *ARABIDOPSIS THALIANA HOMEODOMAIN GENE 8 (ATHB-8)* (Baima et al., 2001) that are involved in interfascicular fiber and xylem differentiation, respectively (Zhong et al., 2007). It has been reported that TFs at the bottom of the hierarchy are more indispensable to the

viability of the cell, and that midlevel TFs control a greater number of targets than TFs of higher levels (Yu and Gerstein, 2006).

Dominant repression of MYB85, which is regulated by both MYB46 and MYB83, also caused distorted vessels and reduced thickness of fiber cells, and overexpression of MYB85 resulted in ectopic lignin deposition in stem cortical and epidermal cells. Moreover, MYB85 controls the expression of *4CL1* but not *IRX9* or *CesA8*, suggesting its role in regulating lignin biosynthesis (Zhong et al., 2008). Similarly, being regulated by MYB46 and MYB83 (McCarthy et al., 2009), dominant repression of MYB58 and MYB63 also reduced cell wall thickness in both vessels and fibers, with a more severe phenotype in the MYB63-dominant repression plants. MYB58 and MYB63 control expression of lignin biosynthetic genes but not those involved in xylan or cellulose biosynthesis (Zhou et al., 2009). These results suggest that MYB85, MYB58, and MYB63 are downstream of MYB46 and MYB83 and regulate lignin biosynthesis in both vessel and fiber cells.

Dominant repression of SND2, SND3, and MYB103 led to normal vessels but xylary and interfascicular fibers with reduced cell wall thickness. Overexpression of these three TFs caused increased thickness of fiber cell walls (Zhong et al., 2008). These TFs regulate the expression of *CesA8* but not *IRX9* or *4CL1* suggesting that they affect secondary thickening by regulation of cellulose biosynthesis. Dominant repression of MYB52, MYB54, and MYB 69 yielded similar phenotypes, but their overexpression did not induce ectopic secondary wall deposition indicating that they may be further downstream of the TF network. Transactivation analyses showed that overexpression of MYB52 and MYB54 appeared to slightly induce the expression of cellulose (*CESA8*), xylan (*IRX9*), and lignin (*4CL1*) biosynthetic genes, while overexpressing MYB69 did not (Zhong et al., 2008).

The functional characterization of the KNOX family transcription factor *KNAT7* (*KNOTTED1-like* homeodomain protein7) was controversial, as different conclusions were drawn using different molecular methods. The *knat7* mutant was first identified as an *irx* mutant (*irx11*) for its collapsed xylem phenotype despite its normal stature (Brown et al., 2005). Multiple transcriptional analyses have suggested a positive role of *KNAT7* in secondary thickening. (1) During TE induction in *Arabidopsis* suspension cell cultures, *KNAT* transcript was induced together with *VND6*, *VND7*, *MYB52*, *MYB43*, and *MYB85* (Kubo et al., 2005). (2) Overexpression of *MYB46* induced *KNAT7* transcript together with *MYB85* (Zhong et al., 2007). (3) *KNAT7* transcript was also up-regulated by overexpression of *SND1* (Zhong et al., 2006), and *KNAT7* was shown, via direct target analyses, to be a direct downstream target of *SND1*, vessel-specific *VND6/7*, and *NST1/2* (Zhong et al., 2008). However, the amount of *KNAT7* transcript did not increase as much as other transcription factors such as *MYB85*, *SND2*, *MYB103*, and *SND3* (Zhong et al., 2007).

Further characterization of *knat7* loss-of-function mutants, however, revealed increased interfascicular fiber (IF) cell walls in addition to thinner and collapsed xylem vessel walls (Li et al., 2012). Moreover, overexpression of *KNAT7* caused reduced IF cell walls leading to the conclusion that *KNAT7* acts as a negative regulator for secondary cell wall formation (Li et al., 2012). This notion is supported by evidence that *KNAT7* physically interacts with *MYB75* (also known as *PRODUCTION OF ANTHOCYANIN PIGMENT1*, *PAP1*), a regulator of anthocyanin biosynthesis in *Arabidopsis* (Borevitz et al., 2000). KO of *MYB75* in *Arabidopsis* also has reduced IF cell walls, suggesting that both *MYB75* and *KNAT7* are involved in secondary wall formation in IF cells, possibly by re-allocation of the phenolic compounds.

Negative regulators

Several negative regulators have been identified related to secondary wall formation. MYB75 was previously identified as a positive regulator of anthocyanin biosynthesis in *Arabidopsis* as its overexpression resulted in anthocyanin accumulation (Borevitz et al., 2000; Gonzalez et al., 2008). However, the *myb75* loss-of-function mutant had increased secondary wall thickness in interfascicular fiber cells, suggesting that it may also act as a repressor of lignin production in the phenylpropanoid pathway (Bhargava et al., 2010). Although genes involved in secondary wall biosynthesis had increased expression in *myb75*, MYB75 only displayed weak trans-activation activity in a protoplast reporter system, and hence the repression effect on secondary wall formation is likely to be indirect (Bhargava et al., 2010).

The *mtstp-1* (secondary wall thickening in pith) mutant was found through a forward genetic mutant screen of an *Medicago truncatula* *Tnt1* retrotransposon insertion population for ectopic lignification in pith cells (Wang et al., 2010). The *Arabidopsis* homolog of *MtSTP* is *AtWRKY12*, the T-DNA insertion mutants of which also have ectopic lignin deposition in pith parenchyma cells. WRKY12 represses the expression of NST2, C3H14 and C3H14L. However, WRKY12 only directly binds to the NST2 promoter, suggesting that the regulation of the other two TFs is likely indirect. The expression of *NST2*, *C3H14* and *C3H14L* was found to be tightly correlated with secondary thickening and highly up-regulated in the pith cells of the *wrky12-1* mutant. It has been concluded that WRKY12 represses NAC and C3H zinc finger transcription factors which further control secondary wall formation (Wang et al., 2010).

Arabidopsis histidine phosphotransfer proteins (AHPs) are mediators involved in multistep phosphorelay signaling pathways for cytokinin signaling. AHP1, AHP2, AHP3, and AHP5 have positive roles in vascular development in *Arabidopsis* roots, while AHP4 may act as

a negative regulator in the cytokinin signaling pathway (Hutchison et al., 2006). AHP4 is expressed in young developing flowers and negatively affects secondary wall thickening in anther endothecium (Jung et al., 2008). Overexpression of AHP4 reduced the expression of *IRX1*, *IRX6*, and *IRX8*. The exact mechanism of AHP4 effects on secondary wall thickening remains unresolved since the negative regulation appeared to be indirect via the cytokinin signaling pathway. However, this finding suggests a role of cytokinin signaling in secondary wall formation (Jung et al., 2008).

Transcription factors that regulate GAUT12 during secondary cell wall formation

A number of secondary cell wall specific transcription factors (TFs), as shown in **Figure 1.3**, have been reported to regulate *GAUT12* transcript levels in Arabidopsis plants. Ko et.al (2009) investigated TF MYB46 using an inducible promoter system and identified downstream TFs AtC3H14, MYB52, and MYB63, all of which up-regulate *GAUT12* along with other secondary cell wall biosynthetic genes such as *CESA4*, *IRX7*, and *IRX9* (Ko et al., 2009). However, *GAUT12* transcript did not increase to the same levels as *IRX9*, or to the levels of genes involved in lignin biosynthesis. Overexpression of SND1-regulated MYB83 caused an increase in *GAUT12* transcript level (McCarthy et al., 2009), whilst overexpression of lignin-related SND1-regulated TFs MYB58 and MYB63 did not affect the transcript levels of *GAUT12*, *IRX9*, or secondary *CESA* genes (Zhou et al., 2009).

Kubo et al. (2005) established an Arabidopsis xylogenic suspension cell culture system and used microarray analysis to study up-and down-regulation of genes in response to tracheary element formation induced by brassinolide (BL). Among up-regulated genes in Set 3 were secondary cell wall biosynthetic genes including *GAUT12*, *CESA4*, *CESA7*, *CESA8*,

GATL1/PARVUS, *IRX10*, and lignin biosynthetic genes *CCoAOMT* and *4CL1*, PCD-related gene *XND1*, as well as TFs including KNAT7, four NAC-domain proteins VND3, VND4, VND5, and VND7, and three MYB-domain proteins MYB52, MYB85, and MYB43. It is noted that no other *GAUT* genes besides *GAUT12* were identified in this study, indicating a specific role for *GAUT12* in Arabidopsis secondary cell wall formation.

Recently, a detailed characterization of VND6 and VND7 suggests that they are master transcription factors that regulate tracheary element formation and up-regulate *GAUT12* expression when overexpressed (Yamaguchi et al., 2010). Another master transcription factor, MYB26/MS35, specific for secondary wall thickening in anther endothecium required for anther dehiscence, also up-regulates *GAUT12* expression along with *IRX1*, *IRX3*, and *IRX12*, indicating a role for *GAUT12* during secondary thickening in the endothecium layer (Yang et al., 2007).

XYLAN STRUCTURE AND BIOSYNTHESIS

The main reason that many cell wall researchers have studied *GAUT12* is because it affects xylan biosynthesis, a major hemicellulose that constitutes lignocellulosic biomass. *GAUT12 loss-of-function* mutants have collapsed xylem with walls that contain reduced amounts of xylan and a specific minor fraction of pectin (Pena et al., 2007; Persson et al., 2007). Xylan structures, genes involved in xylan biosynthesis, and current views on the mechanisms of xylan biosynthesis are reviewed below. Knowledge of xylan biosynthesis has mostly been gained through biochemical and molecular characterization of xylan mutants.

Xylan structure and function

Xylans are a group of polysaccharides that constitute a major part of graminaceous primary and secondary walls and of dicot secondary walls. Xylans of higher plants share a common linear backbone of $\beta(1\rightarrow4)$ -D-xylan (**Figure 1.4**). However, xylan is not a simple repeating structure due to diverse substitutions on the backbone xyloses including D-GlcA, 4-O-MeGlcA, L-Ara and/or D-Xyl, L-Ara, D-/L-Gal, and D-Glc (Ebringerova and Heinze, 2000). Xylan is therefore often termed heteroxylan.

Grass xylans are frequently substituted with arabinosyl residues at O-2 or O-3, and/or double branched at both O-2 and O-3, as typically found in cereal grains (Ebringerova and Heinze, 2000). Such xylans are known as arabinoxylans (AX) and as glucuronoarabinoxylan (GAX) (**Figure 1.4A**). The arabinosyl residue at the xylose O-2 is often 5-O-feruloylated (Ishii, 1997a; Wende and Fry, 1997a), which may serve as a connecting point from grass xylan to other wall polymers such as lignin (Grabber et al., 2000). Although the arabinoxylans in the cereal endocarp contain very little GlcA, vegetative tissues of grasses contain more GlcA and MeGlcA residues, making GAX a more suitable general term for graminaceous monocot xylans (Scheller and Ulvskov, 2010). The GAX polymer contains a repeating structure of four unsubstituted xylose residues separated by two arabinosylated xyloses, although the position of GlcA substitutions is unknown (Faik, 2010). Multiple side chain structures have been found in xylan such as L-Araf- $\alpha(1\rightarrow2)$ -L-Araf linked to the O-3 of xylose in sorghum GAX (Verbruggen et al., 1998), D-Galp- $\alpha(1\rightarrow2)$ -D-MeGlcA linked to O-2 of xylose in eucalyptus xylan (Shatalov et al., 1999), and D-Xylp- $\beta(1\rightarrow2)$ -L-Araf linked to O-3 of xylose in xylan isolated from corn cobs and barley husks (Ebringerova et al., 1992; Ebringerova et al., 1998; Hoijs et al., 2006; Pastell et al., 2009). Highly substituted GAX is more soluble and presumably ideal for initial transport to the

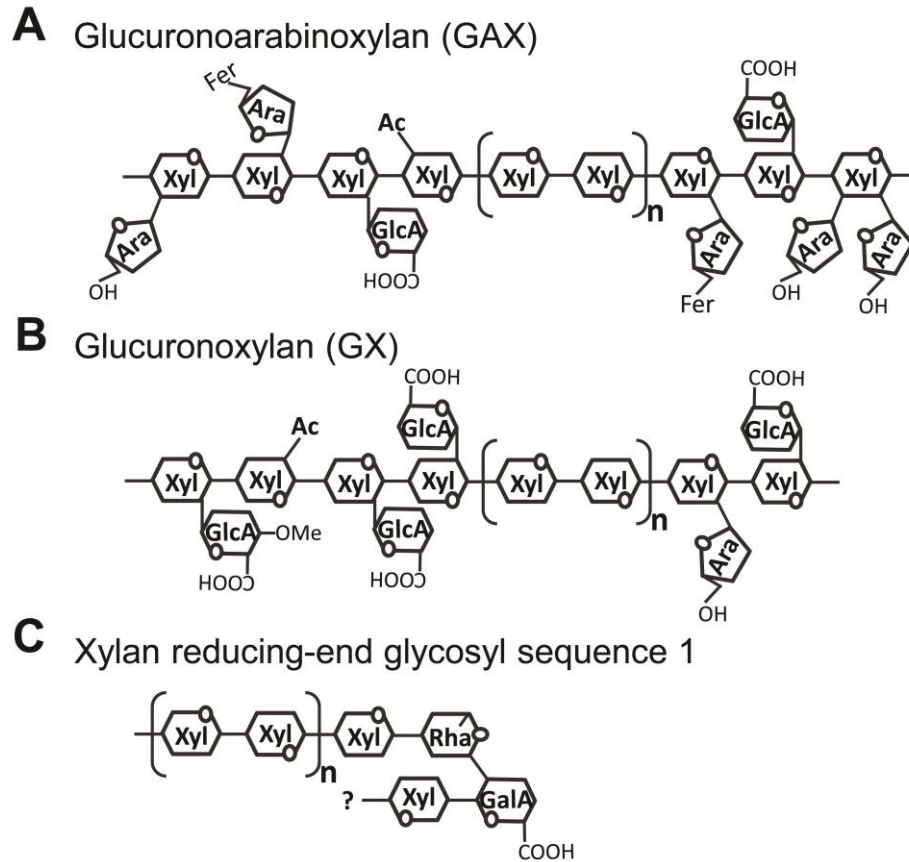


Figure 1.4. Cartoon illustration of the basic xylan structures.

(A) Glucuronoarabinoxylan (GAX) predominantly found in grasses. Arabinosyl residues can be found either at *O*-2, *O*-3 or at *O*-2 and *O*-3 of xylose. Fer, esterification with ferulic acid; Ac, acetylation.

(B) Glucuronoxylan (GX) typically found in dicots. The xylosyl residues are substituted with D-GlcA and 4-*O*-methyl-GlcA and occasionally arabinosyl residues at the *O*-2 or *O*-3 position.

(C) The reducing-end glycosyl sequence 1 (XRES) found in Arabidopsis, birch and spruce xylan. It remains unclear whether the XRES D-Xylp- β (1 $\rightarrow$ 4)-D-Xylp- β (1 $\rightarrow$ 3)-L-Rhap- α (1 $\rightarrow$ 2)-D-GalpA- α (1 $\rightarrow$ 4)-D-Xylp structure is attached to any moiety *in muro*. Adapted from Ebringerova and Heinze, 1999 and Scheller and Ulvskov, 2010.

expanding primary wall. It has been suggested that GAX is trimmed by arabinofuranosidases in the wall resulting in fewer arabinosyl residues than originally synthesized in the cell, consistent with the observation that less substituted AX interacts with celluloses in mature walls (Gibeaut and Carpita, 1991; Carpita and Gibeaut, 1993; Obel et al., 2002; Scheller and Ulvskov, 2010).

Dicot xylans usually contain single α -D-GlcpA or 4-O-methyl-GlcpA (MeGlcA) moieties at the O-2 of xylose and less frequently arabinosyl residue substitutions. Glucuronoxylan (GX) (**Figure 1.4B**) refers mainly to the xylans of hard wood species, a major component of secondary walls that contains 4-O-MeGlcA at O-2 of xylose, with a ratio of MeGlcA:Xyl ranging from 1:4 to 1:16 in different wood species (Timell, 1964; Ebringerova and Heinze, 2000). GX is also found as a small part of dicot primary walls, accounting for 4% of *Arabidopsis* leaf walls (Zabackis et al., 1995) and 5% of sycamore suspension cell walls (Darvill et al., 1980). Arabinosyl substitution in GX is usually at the O-2 of xylosyl residues (Darvill et al., 1980; Zabackis et al., 1995), however, arabinosyl side chains were also found as double branched arabinosyl residues attached to the O-2 and O-3 of one xylosyl residue in flax seed mucilage (Naran et al., 2008), or a trisaccharide sequence of L-Araf- α (1 $\rightarrow$ 3)-D-Xylp- β (1 $\rightarrow$ 3)-L-Araf found at O-3 of xylose in psyllium (*Plantago Ovata* Forsk) seed husk (Fischer et al., 2004) and *Plantago asiatica* L. seeds (Yin et al., 2012). Other uncommon side chains such as L-Fucp- α (1 $\rightarrow$ 2)-D-Xylp at O-3 of xylose was identified in *Hyptis suaveolens* seed coat (Aspinall et al., 1991).

A glycosyl sequence (**Figure 1.4C**) was found at the reducing end of xylans (XRES) in some dicots and conifers such as birch (*Betula verrucosa*), spruce (*Picea abies*), and *Arabidopsis* (Andersson et al., 1983; Doelling and Pikaard, 1993; Dhugga et al., 2004; Pena et al., 2007). The structure D-Xylp- β (1 $\rightarrow$ 4)-D-Xylp- β (1 $\rightarrow$ 3)-L-Rhap- α (1 $\rightarrow$ 2)-D-GalpA- α (1 $\rightarrow$ 4)-D-Xylp, termed

xylan reducing-end glycosyl sequence 1 (XRES), has not been reported from grass species (Scheller and Ulvskov, 2010), and it remains unclear whether the structure is attached to any moiety *in muro* since the reducing reagent used to isolate xylan left this structure with a free reducing-end xylitol (Pena et al., 2007). This structure has not been found in basal land plants including *P. patens*, *Selaginella Kraussiana*, *E. hyemale*, rice, and other grasses (Kulkarni et al., 2012).

Xylans of higher plants are partially acetylated (Wende and Fry, 1997b; Ebringerova and Heinze, 2000). About half of the xylose backbone was shown to be O-acetylated in aspen, eucalyptus, and hybrid *Paulownia elongate*/*Paulownia fortunei* xylan, at O-2, O-3, or O-2 and O-3 (Teleman et al., 2000; Evtuguin et al., 2003; Goncalves et al., 2008). The acetyl groups are labile to alkaline extraction and partially resistant to steaming or hot water treatment (Ebringerova and Heinze, 2000). The acetates released from xylan acetyl groups inhibit the microbial fermentation of glucose, and more severely, of xylose (Helle et al., 2003).

As a major type of hemicellulose, xylan provides mechanical strength for plants and has many applications that affect our daily life. For example, arabinoxylans in rye and wheat affect the processing, quality, and nutritional values of breads, and the brewing properties of grains (Vinkx and Delcour, 1996). Corn fiber-bran arabinoxylans, by-products from starch production, have drawn interest for potential uses as gum arabic replacers to serve as food additives, thickeners, and stabilizers as well as film formers and emulsifiers (Wolf et al., 1953; Doner and Hicks, 1999; Yadav et al., 2007; Yadav et al., 2009). Being the second most abundant polysaccharide after cellulose, xylan serves as a major constituent of lignocellulosic biomass for biofuel production (Carroll and Somerville, 2009), animal feed (Bedford and Partridge, 2010), and is a major component that must be removed during pulping in the papermaking industry

(Sjöström and Alén, 1999). Xylan derivatives can be turned into bioactive reagents such as Larchwood xylan sulphate (Doctor et al., 1991) and pentosan polysulphate (Anees, 1996). Improving the water solubility of arabinoxylan by carboxymethylation benefits pharmaceutical applications of bioactive xylans (Saghir et al., 2008).

Biosynthesis of xylan

A number of enzymes have been identified as potentially involved in the biosynthesis of the xylan backbone and its side chains. **Table 1.3** summarizes the major structural linkages present in xylan and candidate genes thought to encode xylan biosynthetic enzymes.

Xylan backbone $\beta(1\rightarrow4)$ -xylosyltransferase (XylT) activity has been identified in microsomes isolated from wheat (*Triticum aestivum* L.), Arabidopsis, and French bean (*Phaseolus vulgaris*) (Porchia and Scheller, 2000; Kuroyama and Tsumuraya, 2001; Gregory et al., 2002; Brown et al., 2007; Lee et al., 2007a), however, specific genes that may encode these enzymes were identified only recently using genetic and biochemical techniques.

Much information about GX biosynthesis was gained from mutant studies. Previously described Arabidopsis mutant studies (**Table 1.1**) demonstrated a significant loss of xylan in *irx7*, *irx8*, *irx9*, *parvus-3*, and *irx14* (Brown et al., 2007), as well as in the *irx10 irx10-L* double mutant (Brown et al., 2009; Wu et al., 2009a), implicating roles for these genes in xylan synthesis. Compared with wild type, stem microsomes from *irx9*, *irx10 irx10-L*, and *irx14* had reduced xylan:XylT activity, suggesting that IRX9 (GT43), IRX10 (IRX10-L, GT47), and IRX14 (GT43) function to synthesize the $\beta(1\rightarrow4)$ -xylan backbone (Brown et al., 2005; Brown et al., 2007; Lee et al., 2007a). Genetic studies revealed that *IRX9* and *IRX14* are not functionally

Table1.3 Proven and putative xylan biosynthesis enzymes and genes

Type of enzyme activity	GT names ¹	Gene ID	CAZy ²	Notes	References
Xylan backbone					
β(1→4)-D-XylT	IRX9	At2g37090	GT43	Co-expression but not individual expression in tobacco cells yields xylan:XylT activity (microsomes)	(Brown et al., 2007; Lee et al., 2007a; Pena et al., 2007; Lee et al., 2010; Wu et al., 2010)
	IRX14	At4g36890		Homolog of IRX9	
	IRX9-L/I9H	At1g27600		Homolog of IRX14	
	IRX14-L/I14H	At5g67230			
	IRX10/GUT2	At1g27440	GT47	Catalytic activity not demonstrated	(Brown et al., 2009; Wu et al., 2009b)
	IRX10-L/GUT1	AT5g61840		Homolog of IRX10	
	IRX15	At3g50220	DUF579	Not a GT	(Brown et al., 2011a; Jensen et al., 2011)
	IRX15-L	At5g67210		homolog of IRX15	
Xylan reducing-end sequence					
β(1→3)-D-XylT	?			Possibly involved in making xylan reducing end sequence, corresponding linkages are predicted based on the GT family function. No catalytic activities have been demonstrated.	(Shao et al., 2004; Zhong et al., 2005; Brown et al., 2007; Lee et al., 2007b; Pena et al., 2007; Persson et al., 2007; Kong et al., 2009; Lee et al., 2009)
α(1→2)-L-RhaT	IRX7/FRA8	At2g28110	GT47		
	F8H	At5g22940			
α(1→4)-D-GalAT	GAUT12/IRX8	At5g54690	GT8		
D-XylT?	GATL1/PARVUS	At1g19300	GT8		
Xylan side chains					
α(1→2)-D-GlcAT α(1→2)-D-meGlcAT	GUX1	At3g18660	GT8	Transient expression of GUX1, 2, 4 in tobacco leaf cells yielded xylan:GlcAT activity (microsomes and purified); overexpression of GUX1-3 in tobacco BY2 cells yielded GlcAT activity (microsomes); both used Xyl ₆ as acceptors. GUX5 has not been shown to have activity.	(Mortimer et al., 2010; Lee et al., 2012b; Rennie et al., 2012)
	GUX2	At4g33330			
	GUX3	At1g77130			
	GUX4	At1g54940			
GlcA MT ³	GXMT1	At1g33800	DUF579	Golgi-localized, requires Co ²⁺ for optimal activity <i>in vitro</i> ; also altered lignin structure	(Urbanowicz et al., 2012)
α(1→2) L-ArafT	?		GT61 ?		
α(1→3)-L-ArafT	TaXAT1	FR873610.1	GT61	Gain-of-function was achieved in transgenic Arabidopsis	(Anders et al., 2012)
	TaXAT2	FR846232.1			
β(1→2)-D-XylT	XAX1	Os02g22380	GT61	Loss-of-function in the rice <i>xax1</i> mutant; has reduction in ferulic acid esters	(Chiniquy et al., 2012)

Table 1.3 Cont'd.

Type of enzyme activity	GT names ¹	Gene ID	CAZy ²	Notes	References
Xylan side chains					
UDP-L-Arap↔UDP-L-Araf mutase	UAM1	Os03g40270	GT75	E.coli-expressed UAM1 and UAM3 had activity, UAM2 did not	(Konishi et al., 2007)
	UAM2	Os04g56520			
	UAM3	Os07g41360			
5-O-feruloyltransferase		Os05g08640	PF02458	RNAi of these four genes showed reduced FA in leaves of rice	(Piston et al., 2010)
		Os06g39470			
		Os01g09010			
		Os06g39390			
Xylan acetylation					
O-acetyltransferase	RWA1	At5g46340		May not directly be involved in O-acetylation of xylan, catalytic activity not demonstrated. RWA2 also affects O-acetylation of pectins and xyloglucan. Proposed to function as a translocator for acetylated intermediates.	(Lee et al., 2011; Manabe et al., 2011)
	RWA2	At3g06550			
	RWA3	At2g34410			
	RWA4	At1g29890			

1. Enzymes with confirmed activities are bolded. The rest are enzymes with putative functions as indicated.
2. Glycosyltransferase (GT) family and protein family classified by CAZy database.
3. MT: methyltransferase

redundant in xylan backbone biosynthesis since *IRX9* could not rescue the *irx14* phenotype or vice versa (Lee et al., 2010; Wu et al., 2010). However, it is still unclear whether they each make a sub-fraction of xylan polymers or form a XylT complex *in vivo* (Brown et al., 2007). To this end, it has recently been demonstrated that microsomes isolated from tobacco suspension cell cultures that co-express *IRX9* and *IRX14* contain significantly higher xylan:XylT activity than those expressing the single genes (Lee et al., 2012a). These results suggest that *IRX9* and *IRX14* may encode non-redundant XylTs that function in complexes during xylan backbone synthesis (York and O'Neill, 2008), which may explain why heterologous expression of individual genes has not yielded xylan:XylT activity (Pena et al., 2007; Scheller and Ulvskov, 2010). Being functional homologs of *IRX9* and *IRX14*, *IRX9-L/I9H* and *IRX14-L/I14H* are, respectively, partially redundant to their homologs and appear to have a minor role (Keppler and Showalter, 2010; Lee et al., 2010; Wu et al., 2010). The recently identified *IRX15* and *IRX15-L* genes encode DUF579-containing proteins that are not typical GTs classified by CAZy. The *irx15 irx15-L* double mutant has defective xylem cell walls with reduced xylan backbone length and the replacement of GlcA with MeGlcA xylan side chains (Brown et al., 2011a; Jensen et al., 2011). However, the *irx15 irx15-L* double mutant stem microsomes exhibited only a small reduction in xylan:XylT activity compared with wild type, suggesting that the reduced xylan chain length was not a result of reduction in the rate of xylan backbone elongation (Brown et al., 2011b; Jensen et al., 2011). Therefore, *IRX15* and *IRX15-L* define a new class of genes involved in xylan biosynthesis, whose function remains elusive.

No reduction in either XylT or GlcAT activity was found in *irx7*, *irx8*, or *parvus-3* mutants (Brown et al., 2007; Lee et al., 2007a), despite the loss of xylan reducing end glycosyl sequence 1 (**Figure 1.4C**) in all three mutants. Thus, the functions of these genes remain unclear.

The XRES has been speculated to act as either a primer or terminator for glucuronoxyran biosynthesis (York and O'Neill, 2008). It has been speculated that *IRX7* (GT47, inverting enzymes), *IRX8/GAUT12* (GT8, retaining enzymes), and *PARVUS/GATL1* (GT8) makes the linkage of L-Rha- α (1 $\rightarrow$ 2)-D-GalA, D-GalA- α (1 $\rightarrow$ 4)-D-Xyl, and the reducing-end Xyl to an unknown lipid acceptor in ER (Scheller and Ulvskov, 2010), respectively, in the XRES (Brown et al., 2007; Lee et al., 2007b; Pena et al., 2007). One possibility is that such genes form a xylan biosynthesis complex.

Based on the studies of these GX mutants, two possible GX biosynthesis models were proposed (York and O'Neill, 2008). The first model proposes that glycosyl sequence 1 (XRES) acts as a primer and xylan chain elongation takes place via the transfer of D-xylose from UDP-xylose onto the non-reducing end of the primer to form a growing xylan chain (**Figure 1.5A**). In this model, glycosyl sequence 1 is also proposed to be synthesized by non-reducing end addition of glycosyl residues to the initial xylose residue which is presumably anchored to an unknown moiety. The anchor moiety appears to be removed when the glycosyl sequence 1 is isolated from the wall, leaving a free xylitol at the reducing end (Pena et al., 2007). This model for xylan elongation is supported by *in vitro* xylo-oligosaccharide elongation using labeled xylo-oligomers as sugar acceptors and UDP-xylose as the sugar donor. Elongation of xylo-oligosaccharides can be achieved, apparently from the non-reducing end (Brown et al., 2007; Lee et al., 2007a). Precedence for this primer-dependent non-reducing end elongation mechanism is seen in the synthesis of glycogen in which the glycogenin core protein first transfers a glycosyl residue from UDP-Glc to its own Tyr194 and sequentially catalyzes the addition of seven more glycosyl residues. This oligosaccharide serves as a primer for further non-reducing end extension of the glycogen chain by the glycogen synthase (Smythe and Cohen, 1991).

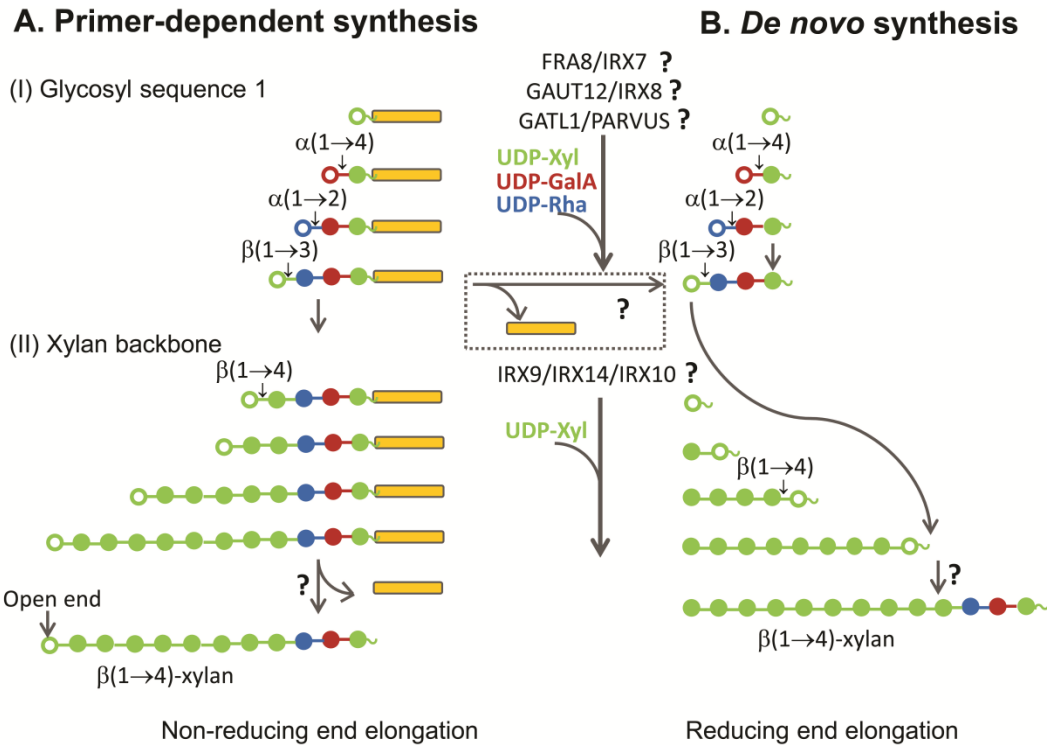


Figure 1.5. Hypothetical models for glucuronoxylan (GX) biosynthesis.

The proposed steps may be catalyzed by the indicated GT gene product. However, enzyme activities of these putative genes remain to be demonstrated experimentally.

(A) The primer-dependent synthesis model envisions that glycosyl sequence 1 acts as a primer and that the xylan chain is elongated from the non-reducing end, leaving the non-reducing end open for further elongation. The yellow rectangle represents an unknown moiety that serves as an anchor for glycosyl sequence 1.

(B) The *de novo* synthesis model envisions that xylan chain elongation takes place at the reducing end and is later transferred onto glycosyl sequence 1, which is possibly produced either *de novo* or in a primer-dependent fashion (shown in dashed box), thereby terminating further backbone elongation. The reducing-end is on the right side of each sugar chain, and open circles represent the most recently added sugar residues. Unknown players are indicated as “?”. Adapted with modification from York and O’Neill (2008).

The second model proposes *de novo* synthesis of xylan backbone by reducing-end elongation with glycosyl sequence 1 as a terminator for chain length control (**Figure 1.5B**). In this model, glycosyl sequence 1 is envisioned to be synthesized *de novo*, with no primer and via elongation from the non-reducing end. In this scenario, xylan chain elongation is achieved by inserting single D-xylose from UDP-xylose or blocks of xylo-oligomers onto the reducing end of the nascent xylan chain. Xylan chain elongation is terminated via the transfer of the final glycosyl sequence 1 onto the reducing end of the growing xylan chain once the desired backbone chain length is achieved. Reducing-end elongation requires an activated sugar chain. The hydroxyl group of the NDP-sugar is the nucleophile that attacks the anomeric carbon of the reducing-end sugar residue, resulting in the transfer of the sugar chain to the NDP-sugar, i.e. the addition of the new sugar residue occurs on the reducing end of the existing sugar chain. This process is very much like the elongation of polypeptides. Enzymes that adopt this mechanism usually contain two substrate-binding sites that alternatively process transfer of the existing sugar chain to the NDP-sugar (two-site insertion mechanism). In terms of xylan biosynthesis, this mechanism is supported by the heterodisperse xylan backbone lengths found in mutants lacking the glycosyl sequence 1 (Pena et al., 2007). Such mutant phenotypes are consistent with a model in which the xylan chain is transferred last to the reducing end of the glycosyl sequence 1 to terminate xylan chain elongation (Pena et al., 2007). Two substrate-binding sites can possibly be achieved by GTs forming xylan synthase complexes (York and O'Neill, 2008). The reducing-end elongation of monosaccharides or oligosaccharides was previously demonstrated in syntheses of lipopolysaccharide (LPS) O-antigen (Raetz and Whitfield, 2002) and *E.coli* group 1 and 4 capsular polysaccharide (CPS) K-antigen (Whitfield, 2006). The components involved in LPS biosynthesis may also have a chain length determination function (Raetz and Whitfield, 2002).

The two proposed xylan biosynthesis mechanisms are not mutually exclusive and can occur in diverse ways in different species, tissues, or cell types (York and O'Neill, 2008). For example, the glycosyl sequence 1 may be synthesized in a primer-dependent manner but removed from the anchor moiety prior to the incorporation onto the reducing end of a *de novo* synthesized xylan backbone chain (**Figure 1.5**, dashed box).

Glucuronosyltransferase (GlcAT) activity has been demonstrated in microsomes harvested from pea (*Pisum sativum*) epicotyls (Waldron and Brett, 1983), Arabidopsis stems (Lee et al., 2007a), and wheat (*Triticum aestivum*) etiolated seedlings (Zeng et al., 2008). It has been reported that GlcAT is dependent on the presence of exogenous UDP-xylose (Waldron and Brett, 1983), however, prior UDP-xylose-incubated pea microsomes did not act as a better acceptor for GlcAT activity than non-incubated microsomes, suggesting that the GlcA substitution may happen shortly after xylan chain elongation rather than onto preformed xylan (Baydoun et al., 1989). The enhancement of GlcAT activity in the presence of UDP-xylose was also shown in wheat microsomes (Zeng et al., 2008). In addition, arabinosyltransferase (AraT) activity was also greatly enhanced by the presence of UDP-xylose (Zeng et al., 2010). The cooperative action of co-immunoprecipitated wheat XylT, AraT, and GlcAT suggests that the three GTs may function in a xylan synthase complex (Zeng et al., 2010).

Recent reverse genetic studies shed light on the genes involved in making the glucuronosyl and arabinosyl residues of xylan. GlucUronic acid substitution of Xylan (GUX)-1 and GUX2 which belong to GT8, were found to be co-expressed with xylan biosynthetic genes and required for the addition of GlcA onto xylan in Arabidopsis (Mortimer et al., 2010). Interestingly, the two genes were previously predicted to be the starch initiation proteins PGSIP1 and PGSIP3 based on sequence homology to yeast and mammalian glycogenin proteins

(Chatterjee et al., 2005). The *gux1 gux2* double mutant contained nearly no detectable GlcA substitution on xylan due to the loss of GlcAT activity. The double mutant had no collapsed xylem and looked normal overall, albeit with weaker stems. However, heterologous expression of GUX1 and GUX2 in tobacco or insect cells failed to demonstrate *in vitro* activity, possibly due to the lack of proper components or complex members (Mortimer et al., 2010). Recently, transiently overexpressed YFP-HA-tagged GUX1 and GUX2 proteins in *Nicotiana benthamiana* leaf cells demonstrated *in vitro* GlcAT activity by catalyzing the addition of GlcA from UDP-GlcA onto xylohexaose acceptors (Rennie et al., 2012). Increased GlcAT activity was also observed in microsomes harvested from the *GUX1*- and *GUX2*-overexpression tobacco BY2 cells compared to the microsomes harvested from the empty vector transformed cells (Lee et al., 2012b). Two wheat Xylan Arabinosyltransferases (TaXAT) candidate genes that are highly expressed in wheat endosperm were shown to be responsible for xylan arabinosylation (Anders et al., 2012). In addition, GUX3 (Lee et al., 2012b) and GUX4 (Rennie et al., 2012) have been demonstrated to be functionally active homologs of GUX1 and GUX2. The Arabidopsis glucuronoxylan methyltransferase 1 (GXMT1) that catalyzes the 4-O-methylation of GlcA substitutions has recently been characterized (Urbanowicz et al., 2012). The *gxmt1* mutants have a 75% reduction in the 4-O-methylation of GlcA residues. GXMT1 was shown to be a Golgi-localized protein and requires Co^{2+} for optimal *in vitro* enzymatic activity (Urbanowicz et al., 2012).

Wheat *TaXAT1*-RNAi transgenic plants, in which *TaXAT1* transcript was suppressed by 6- to 30-fold but *TaXAT2* down-regulation was not consistent, had a large reduction in $\alpha(1\rightarrow3)$ -linked Araf (Anders et al., 2012). Moreover, heterologous expression of *TaXAT2* in both WT and *gux1 gux2* mutant plants (Mortimer et al., 2010) led to increased amounts of arabinosylated

xylan in transgenic *Arabidopsis* cell walls compared with wild type, providing evidence for a gain-of-function of $\alpha(1\rightarrow3)$ -AraT activity due to the GT61 genes (Anders et al., 2012). The arabinofuranose residues on xylan chains are donated by UDP-Araf, which is produced from UDP-Arap by UDP-Arap mutases (UAMs) in a reversible reaction that favors the production of the UDP-Araf (Konishi et al., 2007). UAMs were first identified and characterized in rice but are widespread in green plants (Konishi et al., 2007). RNAi down-regulation of UAMs in rice resulted in transgenic rice plants with reduced amounts of Araf in the walls, as well as a reduction in the degree of xylan backbone substitution, and a 25~80% reduction of ferulic acid and *p*-coumaric acid contents. Dwarfism and sterility were observed in the transgenic rice plants with a >25% reduction in Araf contents (Konishi et al., 2011). Recently, the xylan in the rice *xylosyl arabinosyl substitution of xylan 1* (*xax1*) mutant cell walls was characterized by enzymatic fingerprinting, revealing a reduction in the structure of $(\beta\text{-}1,4\text{-Xyl})_4$ with a $\beta\text{-Xylp-}(1\rightarrow2)\text{-}\alpha\text{-Araf-}(1\rightarrow3)$ substitution (Chiniquy et al., 2012). The XAX1 gene encodes a putative $\beta\text{-}1,2\text{-xylosyltransferase}$ and when transiently expressed in tobacco leaves catalyzes the addition of Xyl from UDP-Xyl onto endogenous acceptors in tobacco (Chiniquy et al., 2012).

Another important feature of grass GAX is the 5-*O*-feruloylation of arabinosyl residues that is catalyzed by feruloyl transferases that are yet to be discovered. The transfer is likely to take place in the Golgi (Fry et al., 2000; Obel et al., 2003), using feruloyl-CoA (or possibly feruloyl-glucose) as a donor substrate. It was previously thought that intermediates such as RGPs (reversibly glycosylated polypeptides) may exist and that the feruloyl-arabinose is transferred as a unit to the GAX acceptor (Scheller and Ulvskov, 2010). RGPs are plant-specific proteins that form reversible complexes with UDP-sugars (Konishi et al., 2007), and recently all five *Arabidopsis* RGP proteins have been studied. RGP1, RGP2, and RGP3 expressed in *E.coli* have

the UDP-Ara mutase activity (Rautengarten et al., 2011). Interestingly, a reduction of feruloyl and coumaroyl esters were also identified in the rice *xax1* mutant mentioned above, in which a putative β -1,2-xylosyltransferase responsible for the β -Xylp-(1 $\rightarrow$ 2)- α -Araf-(1 $\rightarrow$ 3) substitution was defective (Chiniquy et al., 2012). This result led to speculations that feruloylation of the arabinosyl residue may occur after the Xyl residue is transferred to the Ara residue rather than to a UDP-Araf generating a UDP-feruloyl-arabinose intermediate. It remains possible that the Xyl residue stabilizes the ferulic acid esters by protecting them from enzymatic removal (Chiniquy et al., 2012). Candidate genes for AX feruloylation have been predicted to be in the PF02458 family (Mitchell et al., 2007), and down-regulation of four PF02458 family members in rice resulted in reduced feruloyl esters in rice cell walls (Piston et al., 2010).

Xylan acetylation has been thought to be catalyzed by O-acetyltransferases in the Golgi apparatus (Ishii, 1997b; Pauly and Scheller, 2000), the process of which is not well understood. It is reported that the *Cryptococcus neoformans* *CAS1* encodes a membrane-bound protein that catalyzes the O-acetylation of glucuronoxylopolysaccharide, its main capsular polysaccharide (Janbon et al., 2001). One out of the four Arabidopsis *CAS1* homologs, namely *REDUCED WALL ACETYLATION (RWA)2*, was identified to be involved in cell wall acetylation (Manabe et al., 2011). Single *rwa2* loss-of-function mutants are affected in acetylation of several pectic polymers and xyloglucan. The ER localization of RWA2 via ten predicted transmembrane domains led the authors to speculate a role for RWA2 in transporting an acetylated intermediate at an earlier step in the acetylation process (Manabe et al., 2011). Interestingly, various *rwa2* T-DNA insertion alleles did not show any obvious phenotypes, while the quadruple *rwa1/2/3/4* mutant showed a 40% reduction in xylan acetylation (Lee et al., 2011). The quadruple *rwa1/2/3/4* mutant has slightly deformed vessels, reduced stem strength and secondary wall

thickness in fiber cells, and alterations in the methylation of GlcA residues (Lee et al., 2011). Recently, a forward genetic study identified *Arabidopsis altered xyloglucan4 (axy4)* mutants with a specific reduction in xyloglucan *O*-acetylation (Gille et al., 2011). The corresponding gene *AXY4* encodes a type II transmembrane protein with a Trichome Birefringence-Like (TBL) domain and a domain of unknown function (DUF231) that is speculated to be a xyloglucan-specific *O*-acetyltransferase. The *axy4 rwa2* double mutant displayed further reduced xyloglucan *O*-acetylation in a non-additive and non-synergistic way, suggesting that RWA2 partially acts in the same xyloglucan *O*-acetylation pathway (Gille et al., 2011). By comparison to the bacterial, fungi, and mammal *O*-acetylation mechanism, it has been suggested that RWA proteins may be the acetyl-donor translocator which provides substrates for the *AXY/TBL* proteins that presumably encode polysaccharide specific *O*-acetyltransferases (Gille and Pauly, 2012). Specific enzyme activities remain to be demonstrated for these proteins.

LIGNIN BIOSYNTHESIS AND LIGNIN-CARBOHYDRATE COMPLEXES

Lignin is a complex polymer formed by oxidative polymerization of the hydroxycinnamyl alcohol derivatives, *p*-coumaryl, coniferyl, and sinapyl alcohol (**Figure 1.6A**), to give rise to *p*-hydroxylphenyl (H), guaiacyl (G), and syringyl (S) lignin, respectively (**Figure 1.6B**). These lignin monomers are coupled by random oxidization in the presence of oxidases and peroxidases in the wall, forming intermolecular ether or carbon-carbon linkages such as β -*O*-4', β '- β ', β -5', β -1', 5-5', and 4-*O*-5' (**Figure 1.7**), which increase the complexity of lignin polymers. The β -*O*-4', β '- β ', β -5' coupling are computed to be the most thermodynamically favorable while the β -1', 5-5', and 4-*O*-5' are less favorable (Sangha et al., 2012). Diverse lignin

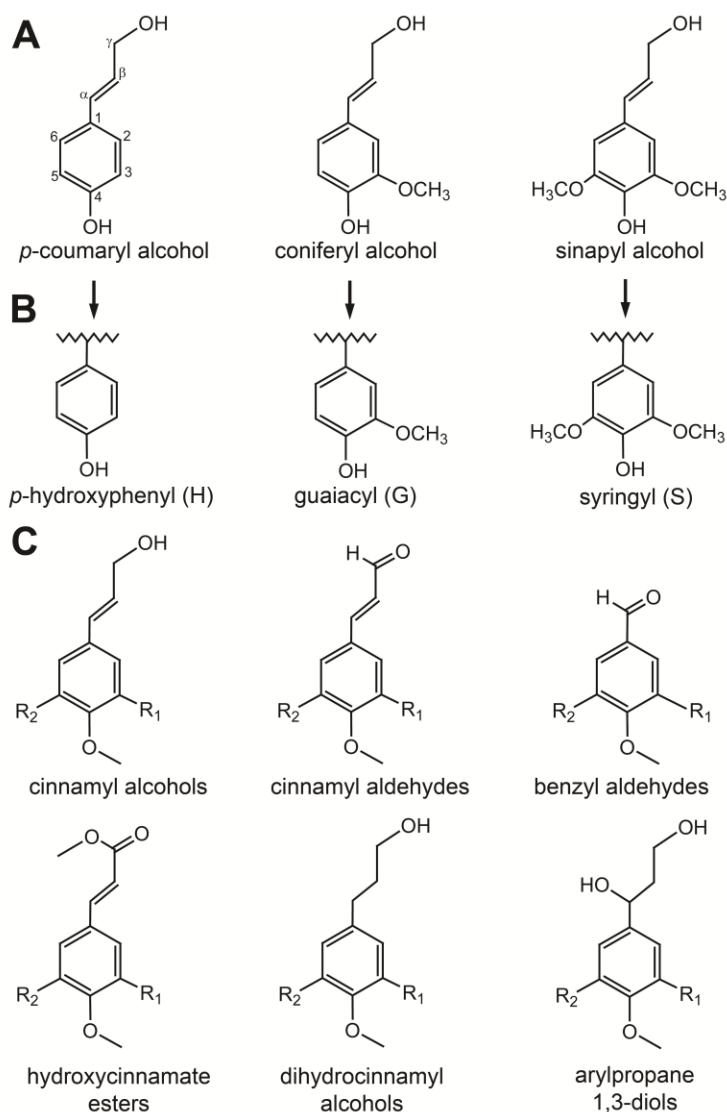


Figure 1.6. Lignin precursors, monomers, and endgroups.

Adapted from Albersheim et al. (2011).

(A) Lignin monomer precursors: *p*-coumaryl, coniferyl, and sinapyl alcohol.

(B) Lignin monomers: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units. Zigzagged line represents lignin linkages.

(C) General structures of lignin monomer endgroups found in lignin. These subunits are derived from *p*-hydroxyphenyl ($R_1 = R_2 = H$), guaiacyl ($R_1 = OCH_3$, $R_2 = H$) or syringyl ($R_1 = R_2 = OCH_3$) units, and are linked to the lignin polymer at the *O*-4 position.

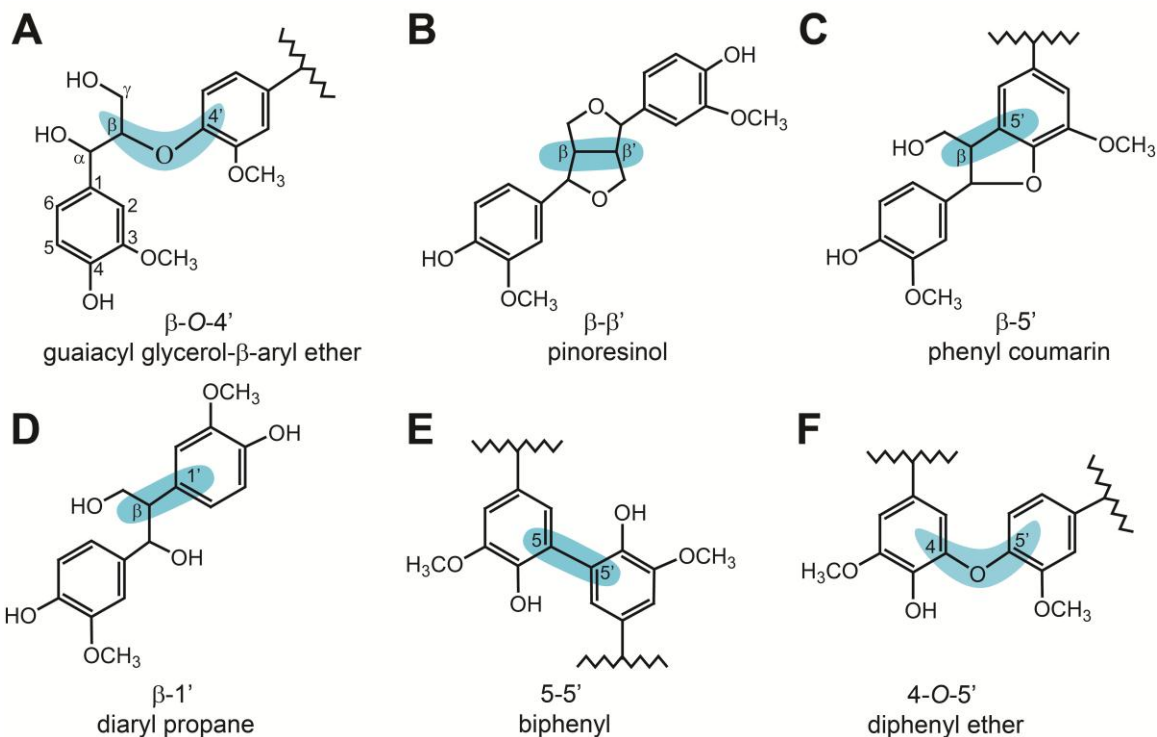


Figure 1.7. Major ether and C-C linkages in lignin.

Adapted from Albersheim et al. (2011).

Linkages are described by the numbered carbons 1-6 of the phenolic rings and the α , β , γ carbons of the propane side chain shown in (A). Structures are shown using guaiacyl units as an example and may be substituted with syringyl units or mixed guaiacyl-syringyl units. The β -O-4' linkage (A) is the most common substructure in lignin. (B) and (D) are shown as dimers, while they can also be connected to the lignin polymer through bonds at the O-4 or 5 positions. Linkages present in (A)-(C) are thought to be more thermodynamically favorable than those in (D)-(F) (Sangha et al., 2012). Zigzagged line represents lignin linkages.

endgroups are found including alcohols, aldehydes, esters, and diols (**Figure 1.6C**). Lignin is a key component of secondary walls, along with cellulose and xylan, and may constitute up to 30% of the wall (Scheller and Ulvskov, 2010). The process of lignification, methods to detect and quantify lignin content, as well as the general phenylpropanoid pathway and genes known to be involved in lignin biosynthesis are reviewed with emphasis on published studies of lignin biosynthetic mutants.

Lignification and lignin measurement

Lignification is a process in which lignin is deposited into the interlamellar voids between cell wall polymers and has been viewed as a glue that forms chemical bonds between non-cellulosic carbohydrates, during specific stages of cell differentiation (Donaldson, 2001). However, lignification is not a completely random event to fill the space in or between the cell walls. Rather, it is a temporally- and spatially-regulated process (Donaldson, 2001; Albersheim et al., 2011). Lignification first starts at the middle lamella and primary wall at the tricellular junction after the formation of secondary wall begins. When secondary wall formation has completed, lignification of the secondary wall begins from the S1 layer and eventually spreads inwards across the S2 and S3 layers of secondary wall towards the lumen (Donaldson, 2001).

Lignin monomers are believed to be made in the cell (Golgi or endoplasmic reticulum) and released into the wall as monolignols or in the form of monolignol 4-*O*- β -D-glucosides (Donaldson, 2001; Boerjan et al., 2003). Whether these lignin building blocks are transported via Golgi-derived vesicles or are pumped out directly through the plasma membrane via ABC transporters remains elusive (Boerjan et al., 2003). At the cell wall, various free- or bound-state peroxidases and oxidases (including laccases) have been shown to have a role in random

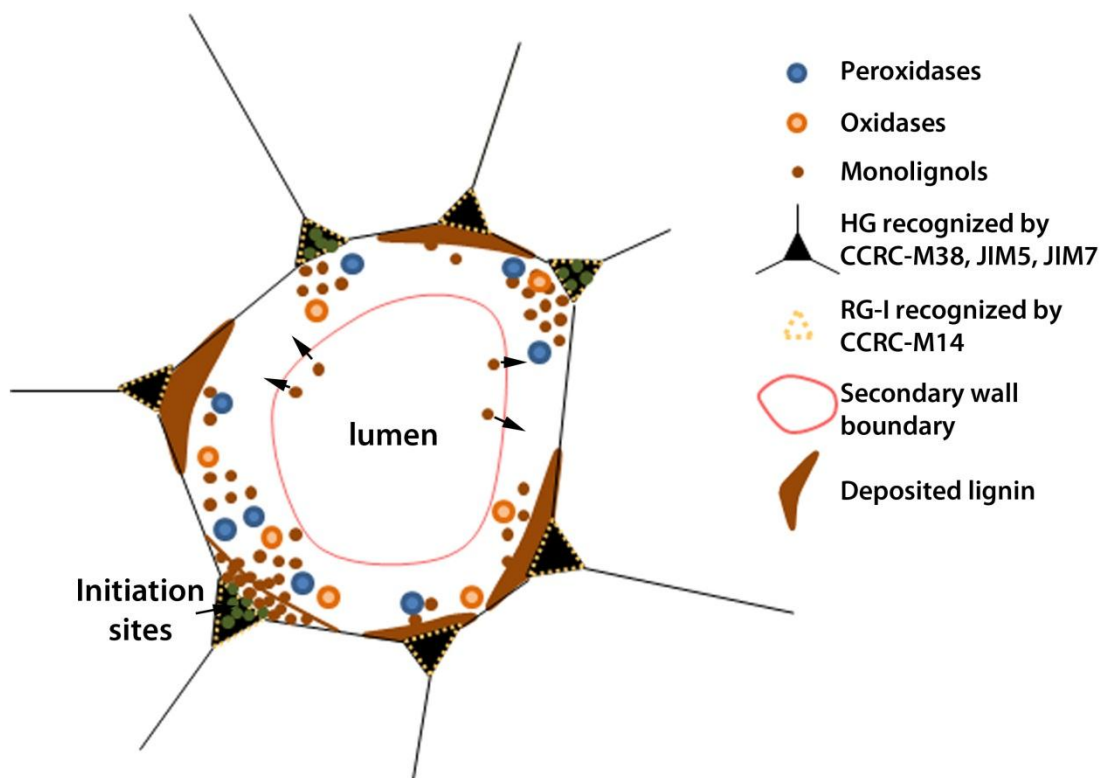


Figure 1.8. A tentative model for lignification in xylem cell walls.

Adapted with modifications from Donaldson, 2001. Monolignols are made in the cell and released into the cell wall where they are coupled onto the existing lignin via free and bound peroxidases and oxidases. Research in this thesis reveals that the tri-cellular junction regions are filled with HG with different degrees of methylesterification (recognized by CCRC-M38, JIM5, and JIM7) and outlined with RG-I (recognized by CCRC-M14). Lignification of primary walls (the middle lamella and tri-cellular junctions) starts after deposition of secondary wall begins; and lignification of secondary walls starts after the formation of secondary walls is completed. Initiation sites may exist for lignification that is ideally built-in during wall carbohydrate deposition. However, the nature of the initiation sites and whether lignin expansion is template-guided or not remains poorly understood.

polymerization of monolignols (Davin et al., 1997; Donaldson, 2001). It is suggested that certain built-in initiation sites may exist for lignification (**Figure 1.8**). Lignin initiation site candidates include bound enzymes such as peroxidases and oxidases, arrays of dirigent sites (proline-rich proteins, extensins), and cell wall polysaccharides (Donaldson, 2001). Currently available evidence does not support the hypothesis that peroxidases and oxidases are the initiation sites for lignification. The specific localization of these enzymes and/or their substrates associated with lignification has not been demonstrated (Donaldson, 2001). In the absence of these oxidizing enzymes, the initiation sites may be calcium-rich since calcium is thought to be needed for the polymerization of coniferyl alcohol (Westermarck, 1982). The 78-kDa non-catalytic dirigent protein was shown to bind and orientate coniferyl alcohol-derived free radicals leading to stereoselective coupling of monolignols to form lignan (Davin et al., 1997) but its role in directing lignification remains controversial (Davin and Lewis, 2000). It has also been suggested that lignin macromolecules themselves may serve as a template for further lignification once the process starts (Sarkanen, 1998).

Alternatively, it has been suggested that the middle lamella pectin may serve as initiation sites for lignin deposition (Donaldson, 2001). TEM examination of stained alfalfa stem sections suggested a non-homogeneous distribution of both pectin and lignin in middle lamella-regions and at tricellular junctions. The irregular pattern was also found in other hardwood species including birch and rubberwood (Wi et al., 2005). These observations suggest the co-localization of the two polymers. To mimic the process of lignification and to address the question of whether pectin serves as initiation sites for lignin deposition, coniferyl alcohol (as the lignin monomer) was mixed slowly in a solution supplemented with pectin and peroxidases (Lairez et al., 2005). The synthetic lignin (also known as dehydrogenated polymers, DHP) was

polymerized with the pectin into a colloidal suspension that contains heterogeneous hydrophobic clusters of the two polymers. These results support a positive correlation between pectin aggregation and lignin polymerization (Lairez et al., 2005). Interestingly, the results from this study indicate that the pectin and lignin interaction may be in part due to their physical properties, as no wall structure is required for lignification in the presence of pectin and peroxidases. Also, the pectin may provide a stabilized microenvironment for lignin polymerization since the lignin polymers formed will precipitate in the absence of pectin (Lairez et al., 2005). Other experiments suggest that pectins, in particular calcium cross-linked HG, aid lignin polymerization via binding to peroxidases (Carpin et al., 2001; Dunand et al., 2002), which presumably catalyze the on-site coupling of lignin monomers at the wall.

Lignin is covalently linked to hemicelluloses and pectins in the wall (Jeffries, 1990), and cell wall carbohydrates influence the expanding of the lignin lamellae during lignification (Donaldson, 2001). Lignin in gymnosperms is mainly composed of G lignin (Donaldson, 2001). In conifers such as pine (Terashima and Fukushima, 1988) and Ginkgo (Fukushima and Terashima, 1991), H lignin is formed in the middle lamella and in tricellular junction regions at early stages of lignification. G lignin is first deposited at the middle lamella and later in the secondary walls, while S lignin is deposited in trace amounts during late stages in secondary wall lignification. Conversely, angiosperm lignin is composed of both G and S lignin in which G lignin is predominant in vessel cell walls and in the middle lamella and S lignin is predominant in fiber cell walls (Donaldson, 2001). In angiosperms such as magnolia (*Magnolia kobus* DC.) (Terashima et al., 1986a) and poplar (*Populus maximowiczii* A. Henry $\times$ *berolinensis* C. Koch) (Terashima et al., 1986b), H lignin is found in the middle lamella as well as in fiber cell walls during early stages of lignification (Fukushima and Terashima, 1990). G lignin is first deposited

in the intercellular junctions and in the middle lamella of vessels walls and later in the fiber walls together with S lignin. In terms of the order and location of lignin deposition in general, it appears that in wood species H and G lignins are deposited at early stages of lignification to fill in the middle lamella and tricellular junctions, and that G lignin is deposited in vessels and fibers earlier than the deposition of S lignin which is mainly deposited in fibers (Donaldson, 2001). In addition, deposition of G lignin appears to be a faster process than the deposition of S lignin.

Lignin can be detected qualitatively *in situ* using histochemical staining techniques. Phloroglucinol-HCl staining (Wiesner test) has been used to detect mainly G lignin, as the reagent reacts with coniferaldehyde endgroups (Lewis and Yamamoto, 1990; Guo et al., 2001). Mäule staining ($\text{KMnO}_4/\text{HCl}/\text{NH}_3$) has been reported to react with syringylpropane moieties of S lignin to produce a rose red color (Lewis and Yamamoto, 1990; Chapple et al., 1992). Other common reagents include the Cross & Bevan staining (chlorine water-sodium sulphite) and the Safranin O reagent (Lewis and Yamamoto, 1990). The Cross & Bevan staining generates a brown color for guaiacyl lignin and a red color for syringyl lignin (Li et al., 2001), while the Safranin O stains all phenol contents red and is the least specific among the four stains. However as a fluorescent dye, Safranin O is able to distinguish the amount of lignin present in a tissue, generating red/orange fluorescence for high lignin regions and yellow fluorescence for low lignin regions (Ramirez et al., 2011).

To quantitatively determine lignin content and composition, several methods are commonly used. Klason lignin refers to the total lignin measured by treating the biomass with 72% sulfuric acid to remove cell wall polysaccharides and obtaining the residual solid weight (washed and dried), which is traditionally used to estimate the lignin content (% lignin) in a given sample (Albersheim et al., 2011). The acetyl bromide assay has also been used to determine total lignin

content, in which the samples are treated with 25% (v/v) acetyl bromide in glacial acetic acid and heated to 50-70 °C for 2-4 hours (Iiyama and Wallis, 1990; Hatfield et al., 1999). The reaction product is mixed with NaOH and hydroxylamine in acetic acid to a final concentration of 0.4 M and 17.5 mM for NaOH and hydroxylamine, respectively, and the absorption at 280 nm is measured to quantitate the lignin content (Hatfield et al., 1999). Compared to the Klason lignin measurement, the acetyl bromide assay may be applied to samples that are limited in quantity (Hatfield et al., 1999). Lignin composition can be determined by alkaline nitrobenzene oxidation, thioacidolysis, and derivatization followed by reductive cleavage (DFRC) (Albersheim et al., 2011). For the alkaline nitrobenzene oxidation, the biomass samples are treated with 2 M NaOH in the presence of nitrobenzene at 160 °C for several hours, generating vanillin as the major product derived from guaiacyl lignin, as well as syringaldehyde from syringyl lignin and 4-hydroxybenzaldehyde from *p*-hydroxyphenyl lignin. The amount of vanillin is quantified in terms of Klason lignin (Kavanagh and Pepper, 1955), or further modified and quantified by gas chromatography and ¹H-NMR (Katahira and Nakatsubo, 2001). In the thioacidolysis method, the solvolysis step using 9:1 dioxane:ethanethiol (v/v) in a 0.2 M boron trifluoride etherate attacks preferentially labile β-O-4 linkages in lignin polymers. Released lignin monomers are then identified by gas chromatography-mass spectrometry as trimethylsilyl (TMS) derivatives (Lapierre et al., 1985). Two key steps for the DFRC method are solubilization of the lignin by bromination and acetylation with acetyl bromide, and reductive cleavage of α- and β-aryl ethers in lignins with zinc dust (Lu and Ralph, 1997, 1998). In brief, cell wall material is completely dissolved in acetyl bromide/acetic acid (1:4, v/v). The solvent is removed by nitrogen gas/rotary evaporation, an internal standard of tetracosane is added, and the soluble lignin is cleaved by the

addition of dioxane/acetic acid/water (5:4:1, v/v/v) and zinc dust. The reaction products are then acetylated by pyridine/acetic anhydride and analyzed by gas chromatography.

Recently, a pyrolysis molecular beam mass spectrometry (pyMBMS) method was developed and applied for a fast and high throughput screening of biomass for lignin content without extraction procedures (Sykes et al., 2009). In this method, biomass samples (3-4 mg for one reaction) are heated at 500 °C in a quartz reactor under an inert helium atmosphere for 90 s. The pyrolysis vapors are sampled through a sampling orifice to cool down while avoiding condensation, and then passed through a skimmer to form a molecular beam, which is detected by mass spectrometry for real-time analysis (Magrini et al., 2002; Sykes et al., 2008). Lignin peaks with m/z 120, 124, 137, 138, 150, 152, 154, 164, 167, 178, 180, 181, 182, 194, and 210 are summed and averaged as the total lignin. The value is corrected by multiplying a correction factor to obtain the percentage of lignin. The correction factor is obtained using a National Institute of Standards and Technology (NIST) sample by dividing the Klason lignin value by the lignin value determined by pyMBMS (Sykes et al., 2008). The S/G ratio is calculated by dividing the sum of syringyl peaks with m/z 154, 167, 168, 182, 194, 208, and 210 by the sum of guaiacol peaks with m/z 124, 137, 138, 150, 164, and 178. Peaks associated with both S and G lignins are eliminated for the S/G ratio calculation (Sykes et al., 2008). Therefore, pyMBMS is able to determine the percentage of lignin as well as H, S, and G monomer composition in a given sample.

The general phenylpropanoid pathway and lignin biosynthesis mutants

The biosynthesis of monolignols starts with deamination of phenylalanine to form cinnamic acid by phenylalanine ammonia-lyase (**PAL**). Cinnamic acid further goes through

successive hydroxylation of the aromatic ring, phenolic *O*-methylation and conversion of the side-chain carboxyl to an alcohol group, and gives rise to *p*-coumaryl, coniferyl, and sinapyl alcohols (Boerjan et al., 2003) (**Figure 1.6A**). Trans-cinnamate 4-hydroxylase (**C4H**) catalyzes the first hydroxylation of trans-cinnamate, immediately after the deamination step catalyzed by PAL. Down-regulation of *PAL* and *C4H* in tobacco significantly reduces lignin content, as they are the key entry steps into the phenylpropanoid pathway (Elkind et al., 1990; Bate et al., 1994; Sewalt et al., 1997). However, it is interesting that individual down-regulation of *PAL* and *C4H* in transgenic tobacco plants increased and decreased S/G ratio, respectively (Sewalt et al., 1997), suggesting that PAL and C4H affect G and S lignin biosynthesis separately, which cannot be explained by the pathway presented in **Figure 1.9**. One possible explanation is that G lignin biosynthesis can bypass C4H, and C4H is committed to S lignin biosynthesis. Tobacco PAL1 and bean PAL have been found in both soluble and microsomal fractions while tobacco PAL2 was only identified in the soluble protein fraction (Rasmussen and Dixon, 1999; Achnine et al., 2004). In addition, the colocalization of a portion of PAL1 and C4H to the ER suggests that metabolic channeling may occur for cinnamic acid during the first two steps of the phenylpropanoid pathway (Rasmussen and Dixon, 1999), although the channeling does not form a close and tight physical interaction between the two enzymes (Achnine et al., 2004). The different expression patterns of PAL isoforms provide some flexibility for the partnership between PAL and C4H, which may explain the discrepancy in S/G ratio change in *PAL* and *C4H* down-regulated transgenic tobacco plants. PAL activity was reduced in leaves and stems of *C4H* down-regulated plants, but not vice versa, suggesting that cinnamic acid serves as a feedback modulator for the expression and activity of PAL (Blount et al., 2000). Arabidopsis C4H mutants (*reduced epidermal fluorescence3, ref3*) carrying mis-sense mutations in the *C4H* gene result in

pleiotropic phenotypes including reduced lignin deposition and accumulation of cinnamoyl malate that is not found in wild type plants (Schilmiller et al., 2009). These results indicate a significant role for C4H for normal plant growth and development.

In Arabidopsis, *p*-coumaric acid is first converted to *p*-coumaroyl CoA by 4-coumarate:CoA ligase (4CL), with subsequent conversion by hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyltransferase (HCT) to *p*-coumaroyl-shikimate/quinate, the substrate for *p*-coumarate 3-hydroxylase (C3H). C3H generates caffeoyl shikimate/quinate, which is converted to caffeoyl CoA again by the reversible enzyme HCT. Caffeoyl CoA is then processed to give rise to G and S lignin (**Figure 1.9**).

4CL activates hydroxycinnamates to produce *p*-coumaryl CoA that leads to the biosynthesis of both lignin and flavonoids. Down-regulation of *4CL* leads to a reduction in lignin content in tobacco (Kajita et al., 1996; Kajita et al., 1997), Arabidopsis (Lee et al., 1997), aspen (Hu et al., 1999), and hybrid poplars (Voelker et al., 2010), and increased amounts of hydroxycinnamic acids including *p*-coumaric, ferulic, and sinapic acids in tobacco (Kajita et al., 1997) and aspen (Hu et al., 1999). The composition of lignin is affected in different ways in these species. In *4CL* down-regulated Arabidopsis plants, G lignin is greatly decreased while S lignin remains unchanged (Lee et al., 1997). Down-regulation of *4CL* in tobacco resulted in brownish xylem that contained lignin low in S units (Kajita et al., 1996; Kajita et al., 1997). It appears that blockage of 4CL activity only modestly affects total lignin content and has little effect on the development of the vascular system in Arabidopsis and tobacco plants (Lee et al., 1997). However, down-regulation of *4CL* caused enhanced growth in aspen possibly due to prolonged duration of epidermal cell expansion that was accompanied by a reduction of 3-45% of total lignin, unchanged S/G ratio, and a 15% increase in cellulose (Hu et al., 1999). The

differing effects on the S/G ratio in these plants may be explained by multiple 4CL isoforms that have different substrate specificities and functions in the phenylpropanoid pathway (Allina et al., 1998; Ehrling et al., 1999; Hu et al., 1999; Harding et al., 2002). Based on enzymatic properties, expression, and evolutionary relationships, 4CL3 is likely to be involved in flavonoid biosynthesis while 4CL1 and 4CL2 are more likely to be involved in lignin formation (Ehrling et al., 1999).

HCT catalyzes the two steps immediately prior and after the addition of the hydroxyl group at C3 of the phenylpropanoid compounds. Silencing *HCT* in *Arabidopsis* resulted in dwarfed plants and changes in lignin composition, while silencing *HCT* in tobacco decreased S units and increased H units (Hoffmann et al., 2004). Particularly, silencing *HCT* in *Arabidopsis* redirected lignin metabolic flux into flavonoid synthesis via activation of chalcone synthase (CHS), and resulting defects in plant development were due to the inhibition of auxin transport by flavonoid accumulation (Besseau et al., 2007). However, simultaneous silencing CHS in the *HCT*-RNAi silenced *Arabidopsis* plants did not rescue the developmental defects, suggesting that down-regulation of *HCT* itself causes these defects by altering lignin biosynthesis (Li et al., 2010). Additionally, silencing *HCT* in alfalfa (*Medicago sativa* L.) under the regulation by the *PAL2* promoter resulted in a strong reduction in lignin content and a predominant deposition of H units in the residual lignin (Shadle et al., 2007). Indeed, HCT holds a critical position in the phenylpropanoid pathway which determines whether *p*-coumaroyl CoA is utilized for lignin or flavonoid biosynthesis (Li et al., 2010).

C3H catalyzes the addition of the second hydroxyl group at the C3 position of the aromatic ring of lignin precursors. Forward genetic approaches led to the discovery of a *reduced epidermal fluorescence8 (ref8)* mutant that is dwarfed and defective in caffeic acid synthesis.

The defective gene was cloned and identified to encode C3H (Franke et al., 2002b). The *ref8* mutant accumulates *p*-coumarate esters instead of sinapoyl malate and predominantly deposits H units (Franke et al., 2002a), resembling *HCT* defective mutants, as can be readily understood by the pathway shown in **Figure 1.9**. Enzymatic activity assays have shown that *p*-coumaryl shikimate/quinate esters are preferred substrates for C3H rather than *p*-coumaryl alcohol or *p*-coumaraldehyde (Schoch et al., 2001; Franke et al., 2002b; Nair et al., 2002). These results suggest that C3H is a key control point leading to the production of guaiacyl and syringyl units in the phenylpropanoid pathway.

Caffeoyl-CoA 3-*O*-methyltransferase (**CCoAOMT**) and caffeic acid *O*-methyltransferase (**COMT**) catalyze the two *O*-methylation steps of the C3 and C5 hydroxyl groups of lignin precursors in both G and S lignin biosynthesis. CCoAOMT expression is closely associated with lignification in dicot plants including zinnia (*Zinnia elegans*), forsythia (*Forsythia suspensa*), tobacco (*Nicotiana tabacum*), alfalfa (*Medicago sativa*), and soybean (*Glycine max*) (Ye, 1997), and can probably be used as a marker for secondary wall formation. In zinnia, *O*-methylation is catalyzed predominantly by CCoAOMT (Ye et al., 1994), and CCoAOMT affects both G and S lignin in poplar (Zhong et al., 2000). However, in alfalfa down-regulation of CCoAOMT leads to reduced G units but has no effect on S units, suggesting that CCoAOMT is not essential for the 3-*O*-methylation step in S lignin biosynthesis and is redundant with COMT for 3-*O*-methylation of G lignin (Guo et al., 2001). Moreover, down-regulation of CCoAOMT in alfalfa also leads to the accumulation of soluble caffeic acid β -D-glucoside (Guo et al., 2001) and soluble *O*- β -D-glucosides of vanillic, caffeic, and sinapic acid in transgenic poplar (Meyermans et al., 2000), all of which are possible derivatives of caffeic acid upon de-esterification of caffeoyl CoA by a thioesterase (Boerjan et al., 2003). Down-regulation of CCoAOMT in *Pinus radiata* leads to

reduced G lignin and the production of catechyl units by the incorporation of caffeoyl alcohol into the lignin polymer (Wagner et al., 2011).

Consistent with the preferential role of COMT in catalyzing the 5-*O*-methylation step, down-regulation of *COMT* results in a reduction in G lignin and a near depletion of S lignin in poplar (Jouanin et al., 2000), alfalfa (Guo et al., 2001), maize (Piquemal et al., 2002), and perennial ryegrass (*Lolium perenne*) (Tu et al., 2010); along with the incorporation of 5-hydroxyconiferyl alcohol into lignin polymers (Atanassova et al., 1995; Jouanin et al., 2000; Guo et al., 2001; Morreel et al., 2004). *E.coli*-expressed recombinant alfalfa COMT was shown to methylate caffeoyl- and 5-hydroxyconiferyl-containing acids, aldehydes, and alcohols with a kinetic preference for the aldehydes and alcohols over the corresponding cinnamic acids (Parvathi et al., 2001; Chen et al., 2004), consistent with the finding that CCoAOMT may not be the key enzyme catalyzing the 3-*O*-methylation step for S lignin, as mentioned above. X-ray crystallographic structural analyses of alfalfa and perennial ryegrass (*Lolium perenne*) COMT demonstrated that the preference of the enzyme for these substrates may be due to hydrogen bonding partners provided by Asp-270, Asn-324, and His-166, and to the COMT methoxy binding pocket provided by Leu-136, Phe-172, and Ala-162, as well as to the spacious active site of COMT which allows facile accommodation of both 3- and 5- substituted phenolic substrates (Zubieta et al., 2002; Louie et al., 2010).

As COMT is essential for S lignin production, it has become a target for improving forage digestibility, pulping efficiency, and utility of plant biomass for biofuel production. Co-down-regulation of *COMT* and *CCoAOMT* leads to very severe growth phenotypes in both alfalfa and *Arabidopsis* (Do et al., 2007; Zhou et al., 2010). Down-regulation of *COMT* in switchgrass by RNAi resulted in transgenic switchgrass plants having normal growth phenotypes

with a modest reduction of lignin content and reduced S/G lignin ratio. More importantly, *COMT* down-regulated transgenic switchgrass plants require less severe pretreatment conditions and provide up to 38% increased ethanol yields using conventional biomass fermentation process (Fu et al., 2011).

Ferulic acid/coniferaldehyde/coniferyl alcohol 5-hydroxylase (**F5H**) adds the third and last hydroxyl group of the aromatic ring to give rise to sinapyl alcohol that forms syringyl units. Forward genetic screening of EMS-mutagenized *Arabidopsis* seeds revealed that *fah1* mutants fail to accumulate sinapoyl malate, a major secondary metabolite for *Arabidopsis* (Chapple et al., 1992). T-DNA tagging cloning of F5H revealed that it defined a new family of P450-dependent monooxygenases that was previously designated CYP84 (Meyer et al., 1996). NMR characterization of the *fah1-2* mutant revealed a near absence of syringyl units, confirming that *F5H* is required for S lignin biosynthesis (Marita et al., 1999). Overexpression of *F5H* under the *C4H* promoter but not the CaMV 35S promoter led to transgenic *Arabidopsis* plants almost entirely composed of syringyl lignin units, suggesting that the *C4H* promoter is more efficient than a general constitutive promoter in inducing lignification (Meyer et al., 1998; Marita et al., 1999). *Arabidopsis F5H* expressed in yeast prefers coniferaldehyde and coniferyl alcohol to ferulic acid as substrates.

The 5-hydroxyferulate, 5-hydroxyconiferaldehyde, and 5-hydroxyconiferyl alcohol produced by F5H can be 5-*O*-methylated by *E.coli*-expressed *Arabidopsis COMT* to generate sinapyl aldehyde/alcohol that forms S lignin (Humphreys et al., 1999). However, F5H cloned from sweetgum xylem displayed a much greater K_{cat}/K_m for coniferaldehyde than that for ferulate (Osakabe et al., 1999), suggesting a re-routed F5H/COMT-mediated pathway for S lignin production. In addition, in the presence of coniferaldehyde, the 5-hydroxylation of ferulic

acid does not occur. Therefore, the production of S monolignol can only be achieved using coniferaldehyde as a substrate (Osakabe et al., 1999), as shown in the current phenylpropanoid pathway (**Figure 1.9**).

Cinnamoyl-CoA reductase (**CCR**) converts *p*-coumaryl-, caffeoyl-, and feruloyl-CoA esters to their corresponding cinnamyl aldehydes. Down-regulation of *CCR* via antisense expression dramatically reduces lignin contents in alfalfa (Jackson et al., 2008), poplar (Leple et al., 2007), and tobacco (Piquemal et al., 1998). The Arabidopsis *irx4* mutant defective in *CCR1* has 50% less lignin than wild-type plants (Jones et al., 2001). Arabidopsis CCR1 is expressed in tissues undergoing lignification and more efficiently uses feruloyl and sinapoyl CoAs as substrates than CCR2, which is not normally expressed during development but is involved in pathogen defense (Lauvergeat et al., 2001). Similarly in *Medicago truncatula*, CCR1 also exhibits high preference for feruloyl and sinapoyl CoAs, although these two substrates strongly inhibit CCR2 activity at high concentration (Zhou et al., 2010). CCR2 is involved in constitutive lignin production with a preference for caffeoyl and *p*-coumaroyl CoAs, suggesting that CCR2 produces the caffeoyl aldehyde and therefore can bypass the requirement for CCoAOMT in the generation of coniferaldehyde (Zhou et al., 2010). *M. truncatula* retrotransposon insertion lines that are disrupted in *CCR1* rather than *CCR2* caused more severe growth phenotypes and lignin content reduction, and *CCR1* fully-, and *CCR2* partially-, complemented the *irx4* mutant phenotype (Zhou et al., 2010). CCoAOMT is up-regulated in *CCR2* knockout lines, and reciprocally *CCR2* is up-regulated in CCoAOMT knockout lines, suggesting that CCR2 and COMT may work together to perform 3-O-methylation of monolignol precursors when CCoAOMT/CCR1 is not functional (Zhou et al., 2010).

Cinnamyl alcohol dehydrogenase (**CAD**) catalyzes the final reduction of cinnamaldehydes to their corresponding alcohols, which are then oxidized to form lignin polymers. Both CAD4 (CAD-C) and CAD5 (CAD-D) are essential CADs that produce guaiacyl and sinapyl alcohol from corresponding cinnamyl aldehydes (Sibout et al., 2003). The *cad5* mutant is reported to have a modest reduction in lignin while the *cad4* mutant has no reduction in Klason lignin overall (Sibout et al., 2003). The double *cad-c cad-d* mutant has 40% of the total lignin and a 94% reduction of β -O-4-linked guaiacyl and syringyl units compared with wild-type plants. As a result, the incorporation of coniferyl and sinapyl aldehydes into the wall is reduced (Sibout et al., 2005). In other plant species, *CAD* down-regulated plants compensate for the loss of monolignol synthesis by direct incorporation of cinnamaldehydes into the lignin polymer (Halpin et al., 1994; Yahiaoui et al., 1998; Kim et al., 2000). Sinapyl alcohol dehydrogenase (SAD), a CAD ortholog from aspen, has been reported to specifically produce sinapyl alcohol during syringyl lignin synthesis in poplar. However, no SAD homolog was identified in Arabidopsis (Li et al., 2001). Interestingly, simultaneous down-regulation of *CCR* and *CAD* by crossing tobacco plants carrying single antisense constructs for either *CCR* or *CAD* resulted in hybrid tobacco plants with significantly reduced lignin but with an overall normal growth phenotype and only slight alterations of vessel shapes. The unexpected phenotype suggests a synergistic effect of down-regulation of both enzymes and that a specific transgene may have a different effect in different genetic backgrounds (Chabannes et al., 2001). A triple *cad c cad d ccr1* mutant in Arabidopsis, named *ccc*, was severely dwarfed and male sterile, and accumulated higher amounts of flavonol glycosides, sinapoyl malate and feruloyl malate in its stems, suggesting that knocking out the major *CCR* and *CAD*s caused a redirection of phenolic compounds in the phenylpropanoid pathway (Thevenin et al., 2011). In addition, *CCR2* and

CAD1 could not compensate for the loss of *CCR1* and *CAD4/5*, suggesting that they do not have the main CCR and CAD functions in *Arabidopsis* (Thevenin et al., 2011).

Besides the *ref8* mutant mentioned above that is defective in *C3H*, functional characterization of other *ref* mutants with reduced leaf sinapoyl malate content have identified important players in the phenylpropanoid pathway (Ruegger and Chapple, 2001). Specifically, rather than having a role in the synthesis of lignin monomers, *REF1* encodes a functional aldehyde dehydrogenase (ALDH) that is capable of oxidizing both sinapaldehyde and coniferaldehyde to sinapic acid and ferulic acid, respectively (Nair et al., 2004) (**Figure 1.9**). *REF2* encodes CYP83A1, a cytochrome P450 closely related to CYP83B1, an enzyme involved in glucosinolate biosynthesis (Hemm et al., 2003). *REF4* encodes a large integral membrane protein unique to plants, the mutation of which causes reductions in sinapoylmalate and lignin content, and a dwarf phenotype (Stout et al., 2008). Sinapoylmalate is one kind of hydroxycinnamic acid ester produced by *Arabidopsis* leaves and fluoresces under UV light. A heterozygous *ref4* mutant plant displays an intermediate phenotype suggesting that the *ref4* mutation is semidominant (Stout et al., 2008). It appears that *REF4* and its homolog *RFR1* (*REF4*-related 1) are part of the Mediator complex that interferes with *PAP1/MYB75* function resulting in the induction of anthocyanin production and the suppression of the lignin branch in the phenylpropanoid pathway (Bonawitz et al., 2011). The *bright trichomes1* (*brt1*) mutant was originally identified as a *ref* mutant defective in sinapoylmalate biosynthesis, but in addition the *brt1* mutant exhibits a hyper-fluorescent trichome phenotype. *BRT1* encodes UGT84A2, an enzyme previously shown to have sinapic acid:uridine diphosphoglucose glucosyltransferase activity. The residual sinapoylmalate and the residual glucosyltransferase activity in the *brt1*

mutant leaves suggest that genes of the *BRT1* family may carry out redundant functions in Arabidopsis sinapate ester biosynthesis (Sinlapadech et al., 2007).

Lignification is a crucial step in the adaption of plants to land habitats. However, lignified biomass is recalcitrant which hinders the utilization of plant materials as animal feed, in the papermaking industry, and during biofuel production. It is important to understand to what extent lignin composition and amount can be altered and yet maintain normal plant development. As monolignol biosynthesis is viewed as a metabolic grid (Dixon et al., 2001), down-regulation of one step in lignin biosynthesis usually causes re-distribution of the phenylpropanoid compounds into other branches of the pathway. In addition, lignin does not exist alone in the wall. Rather, it serves as a space-filler, deposited in a controlled manner, which glues the wall together by random coupling and crosslinking with other wall carbohydrates and proteins *in muro*. It is almost impossible to obtain an intact and pure lignin fraction, as the isolation procedures are often destructive but may not disrupt covalent linkages between the lignin and other macromolecules in the wall. For the same reason, lignin content may be directly or indirectly affected when other wall polymers are defective. Therefore, it is crucial to understand the interactions between lignin and other wall polymers.

Lignin-carbohydrate complexes (LCCs)

Lignin is covalently linked to carbohydrates of the wall and isolation of lignin from milled wood samples using dioxane/water mixtures often results in the isolation of materials containing carbohydrate covalently linked to lignin. The residues that remain after the dioxane extraction of milled wood powder are commonly referred to as milled wood lignin (MWL), in which the inseparable mixture of lignin and carbohydrate is named lignin-carbohydrate

complexes (LCCs) (Björkman, 1956; Lawoko, 2005; Albersheim et al., 2011). The ball-milling procedure, however, has been reported to reduce the degree of polymerization of lignin polymers and increase the α -carbonyl and the phenolic-hydroxyl content of lignin (Hu et al., 2006). To improve the yield and preserve lignin structure, the milled wood can be treated with cellulolytic enzymes (crude cellulases) that remove most of the polysaccharides before or after the dioxane extraction (Chang et al., 1975; Ikeda et al., 2002; Holtman et al., 2004). The residues are called the cellulolytic enzyme lignin (CEL). The lignin structures in MWL and CEL were shown to be identical, given the wood powder is produced by vibratory ball milling in toluene (Ikeda et al., 2002). Nevertheless, CEL is obtained in higher yield with less lignin degradation, and thus is preferred (Ikeda et al., 2002). Improved CEL isolation has been applied to analyze Arabidopsis cell wall materials (Urbanowicz et al., 2012).

Common lignin-carbohydrate (LC) bonds in wood include glycosidic bonds between glycosyl residues and hydroxyls of lignin side chains (phenyl glucosides, **Figure 1.10A**), ether bonds between glycosyl residues and phenolic residues or lignin side chains (benzyl ethers, **Figure 1.10B**), and ester bonds between uronic acids (GlcA or GalA) of polysaccharides and hydroxyls of lignin monomers (benzyl esters, **Figure 1.10D**). In grasses, lignin is often found linked to xylan chains via ferulic acids, forming ferulate- (**Figure 1.10C**) or diferulate esters (**Figure 1.10E, F**). Similar to the cross-linking between two xylan chains via feruloyl esters at the O-5 of arabinosyl residues of grass arabinoxylans, xylan can be incorporated into the lignin via ferulate and diferulate side chains (Grabber et al., 2002).

Whole biomass and isolated LCC structures can be analyzed using infrared (IR), solid-state NMR, and solution-state NMR. Among these, solution-state NMR generates the most information (Yuan et al., 2011). A prerequisite for solution-state NMR analysis is obtaining a

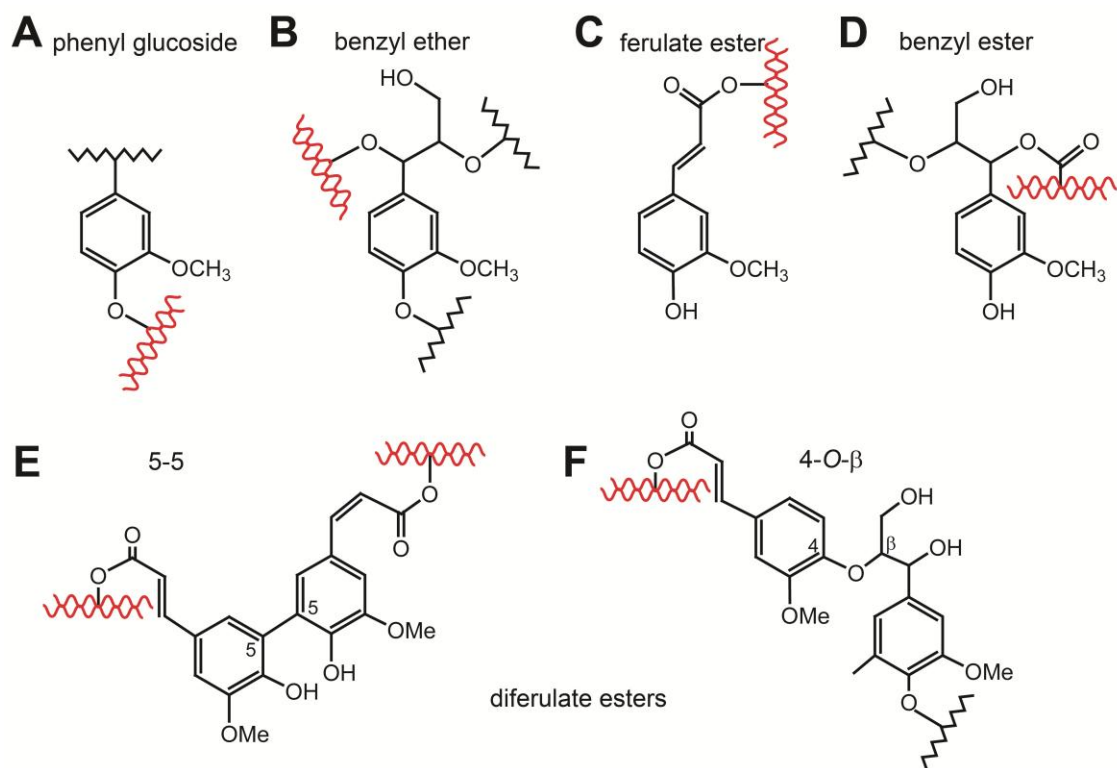


Figure 1.10. Proposed structures for lignin-carbohydrate complexes (LCCs).

Adapted from (Albersheim et al., 2011).

(A) The phenyl glucoside is linked by the propane side chain to lignin and by an O-4 linkage to the carbohydrate.

(B) The benzyl ether is linked to the carbohydrate by an α -O linkage and to the lignin by a β -O- and an O-4 linkage.

(C) The ferulate ester is linked to the carbohydrate by a γ -O linkage and potentially to lignin at the O-4 and 5 position of the ring.

(D) The benzyl ester is linked to lignin by β -O-4 linkage and to carbohydrate by an α -O linkage.

(E) and (F) Diferulate esters with γ -O linkages to carbohydrates and potential 4-O linkages to lignin. Zigzagged line represents lignin linkages, and double wiggled lines in red represent carbohydrates.

homogeneous solution of the lignin in a perdeuterated solvent system. To dissolve the lignin-containing material without any extraction steps, two major types of ionic liquid solvents are used. Ionic liquids are largely composed of ions and short-lived ion pairs from salts with low melting point. One is the imidazolium type ionic liquids (Kilpelainen et al., 2007; Pu et al., 2007; Xie et al., 2007; Li et al., 2008) that requires multi-step reactions to synthesize. The other is the recently developed perdeuterated pyridinium ionic liquid/DMSO- d_6 (1:2, v/v) bi-solvent system (Jiang et al., 2009). One gram of the pyridinium/DMSO- d_6 (1:2, v/v) was able to dissolve 35 mg of ball-milled poplar when heated at 60 °C for 1 h, resulting in a homogeneous solution with increased viscosity and coloration (Jiang et al., 2009). In addition, gel-state NMR has been used to study the structures of lignin (or whole cell wall) material swelled in DMSO- d_6 or a pyridine- d_5 -DMSO- d_6 mixture (1:4, v/v), from which NMR spectra can be obtained (Kim et al., 2008; Kim and Ralph, 2010).

Information on lignin structures and the linkages between lignin and the associated carbohydrates can be obtained through ^{13}C NMR and two-dimensional heteronuclear single-quantum coherence (2D HSQC) NMR spectroscopy. The 2D HSQC NMR analysis provides crucial structural information and separates resonances that are otherwise overlapping in the ^1H or ^{13}C NMR spectra. With a carefully selected internal standard, the amount of lignin and LC-bonds can be quantified (Zhang and Gellerstedt, 2007). The internal standard can be a low molecular weight reference compound added to the polymeric sample for absolute quantification, or a well-resolved cluster of signals chosen within the same sample that has similar structural features, serving as a secondary internal standard (Zhang and Gellerstedt, 2007). With 2D HSQC NMR spectra, data analysis focuses mainly on three regions: the aromatic region, the aliphatic (side chain) region, and the carbohydrate anomeric region. The aromatic region provides

information about the aromatic rings of the H, S, and G lignin and some lignin subunits, while the aliphatic region provides information on the intermolecular linkages within the lignin polymer. The carbohydrate anomeric region provides information about the carbohydrate structures, part of whose signals can also be identified in the aliphatic region (Yuan et al., 2011). The linkages between lignin and carbohydrates can be confirmed using other types of NMR spectroscopy such as the nuclear overhauser effect spectroscopy (NOESY) and total correlation spectroscopy (TOCSY), which elucidate through-space ^1H - ^1H interactions between sugars and lignin components (Byrne et al., 2010).

By a combination of ^{13}C and 2D HSQC NMR analyses, three different types of lignin (MWL, acetic acid-extracted MWL, and CEL) from loblolly pine and white birch were studied (Balakshin et al., 2011). This study showed that while phenyl glycoside and esters are abundant in the MWL and acetic acid-extracted MWL, benzyl ethers are more abundant in the CEL in both soft and hard woods. In addition, pinewood also contains higher amounts of benzyl ether but lower amounts of phenyl glucosides and γ -esters than birch wood (Balakshin et al., 2011). Using the same method, it was shown that acetylated 4-*O*-methylglucuronoxylan is the main carbohydrate associated with lignins in poplar, and the xylan is frequently acetylated at the *O*-2 and *O*-3 positions (Yuan et al., 2011). Besides the large amount of xylose, small amounts of glucose, galactose, rhamnose, and arabinose are also identified in the wood LCC (Yuan et al., 2011). The application of these techniques to lignin and/or xylan mutant biomass samples has the potential to identify mutated gene function associated with the formation of LCC.

PECTIN BIOSYNTHESIS

The reduction of a small fraction of pectin in *irx8* led to a hypothesis that GAUT12 may be involved in synthesizing a specific stretch of pectin that is somehow linked to xylan (Persson et al., 2007). GAUT12 has 60% amino acid sequence similarity and 40% sequence identity to GAUT1, an α -D-galacturonosyltransferase that catalyzes homogalacturonan biosynthesis (Sterling et al., 2006). Pectin structure and its biosynthesis are reviewed below, and the literature that suggests a connection between pectin and xylan are summarized.

Pectin structure and function

Pectin is a family of matrix polysaccharides that contain $\alpha(1\rightarrow4)$ -linked GalA residues and that are abundant in plant primary cell walls. The pectic polysaccharides are classified into five groups based on their structure: homogalacturonan (HG), xylogalacturonan (XG), apiogalacturonan (AGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Ridley et al., 2001).

HG (**Figure 1.11A**) accounts for 55-70% of total pectin with an $\alpha(1\rightarrow4)$ -GalA-linked backbone in which the GalA carboxyl groups are partially methylesterified (Mohnen, 2008). Depending on the plant species, the GalA residues may be O-acetylated at O-2 or O-3. HG isolated from apple, beet, citrus and citrus peel pectins has a degree of polymerization (DP) of 72-117 GalA residues (Thibault et al., 1993; Yapo et al., 2007) while shorter chains have been reported to be interspersed within RG-I (Nakamura et al., 2002). A recent study of HG using atomic force microscopy (AFM) to examine pectin extracted from unripe tomato pericarp identified HG polymers that are three- or four-fold larger in size, ranging from 160~320 GalA

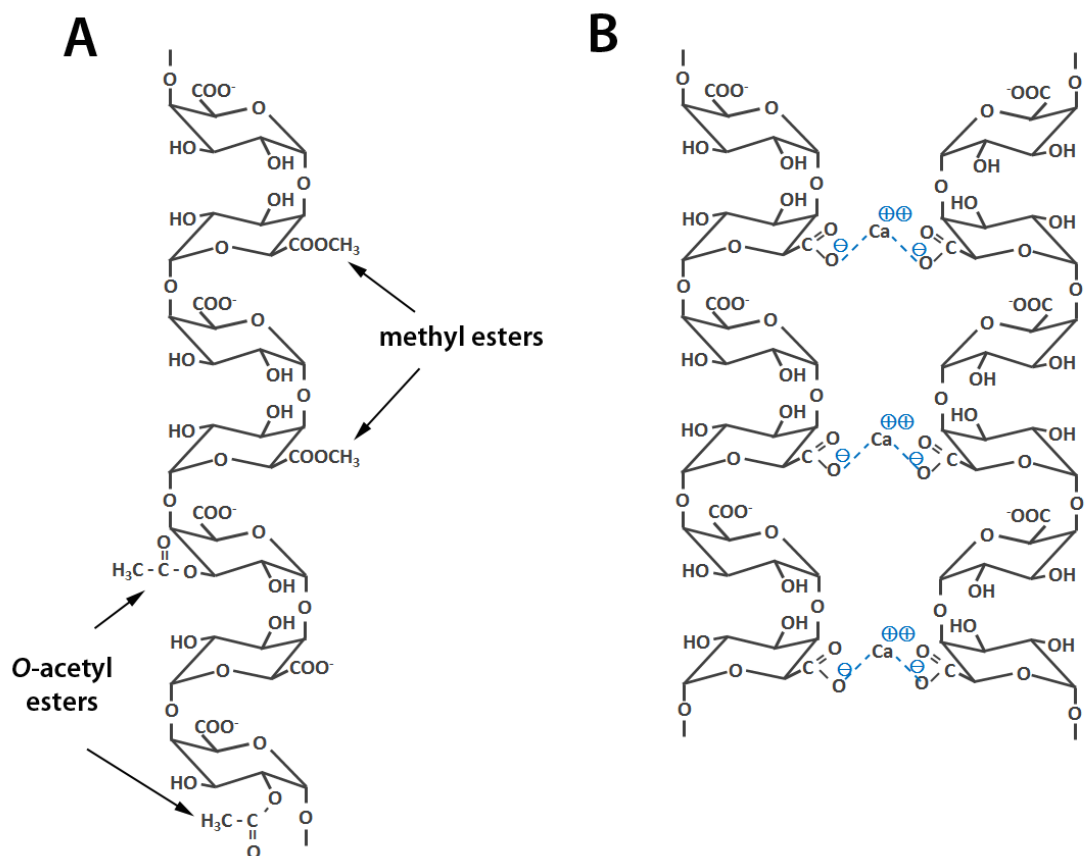


Figure 1.11. Homogalacturonan structure and modification.

(A) HG has an $\alpha(1\rightarrow4)$ -GalA-linked backbone in which some of the carboxyl groups are methylesterified. The GalA residues may also be O-acetylated at O-2 or O-3 depending on the plant species. Adapted from Ridley et al. (2001).

(B) The "egg-box" model of Ca^{2+} crosslinking of non-esterified HG. Adapted from Caffall and Mohnen (2009).

residues as indicated following isolation after acid hydrolysis for 72 h and 24 h (Round et al., 2010).

Non-esterified carboxyl groups of HG (no shorter than 10 GalA residues) are negatively charged and may interact with Ca^{2+} to form a tightly packed gel as shown in **Figure 1.11B**. The structure model is referred to as an “egg-box”. This gel stiffens the wall, affects wall porosity and cell adhesion (Willats et al., 2001; Mohnen, 2008) during e.g. pollen tube growth (Dardelle et al., 2010; Lehner et al., 2010) and hypocotyl elongation (Lieberman et al., 1999). On the other hand, in cells in which wall flexibility is required, such as in the pollen tube tip region (Dardelle et al., 2010; Lehner et al., 2010) and in guard cells (Majewska-Sawka et al., 2002), HG is often found to be highly methylesterified to reduce cross-linking, which is reflected in predominant JIM7 labeling (recognizes high-methylesterified HG) and reduced/eliminated JIM5 labeling (recognizes low-methylesterified HG) in the corresponding tissues/cells.

XGA and AGA (**Figure 1.12**) are both substituted galacturonans that are abundant in some plant species. XGA has β -D-Xylp (and/or 2-linked xylobiose) attached to O-3 of GalA in an HG backbone, which has been detected in storage tissues and reproductive organs in various plant species and in leaves and stems of Arabidopsis (Zandleven et al., 2006). AGA is HG substituted with β -D-Apif or the disaccharide D-Apif- $\beta(1\rightarrow3')$ -D-Apif at the O-2 and O-3 of GalA (Ridley et al., 2001), and has been found in aquatic plants such as duckweeds and marine seagrasses (Caffall and Mohnen, 2009).

RG-II (**Figure 1.13**) is another branched galacturonan with a short $\alpha(1\rightarrow4)$ -GalA backbone (7-9 GalA residues) that is substituted at O-2 and O-3 of the GalA residues with four complex side chains designated A through D (O'Neill et al., 2004). RG-II constitutes ~10% of total pectin, and is a highly conserved and complex polymer (**Figure 1.13**) present in all vascular

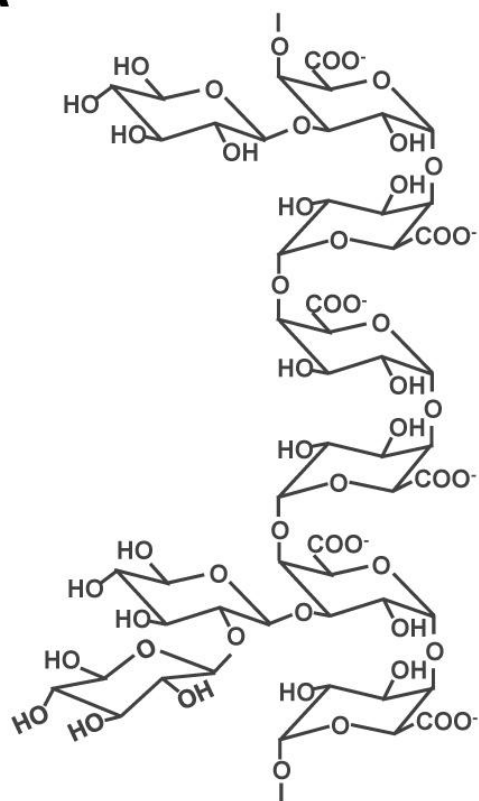
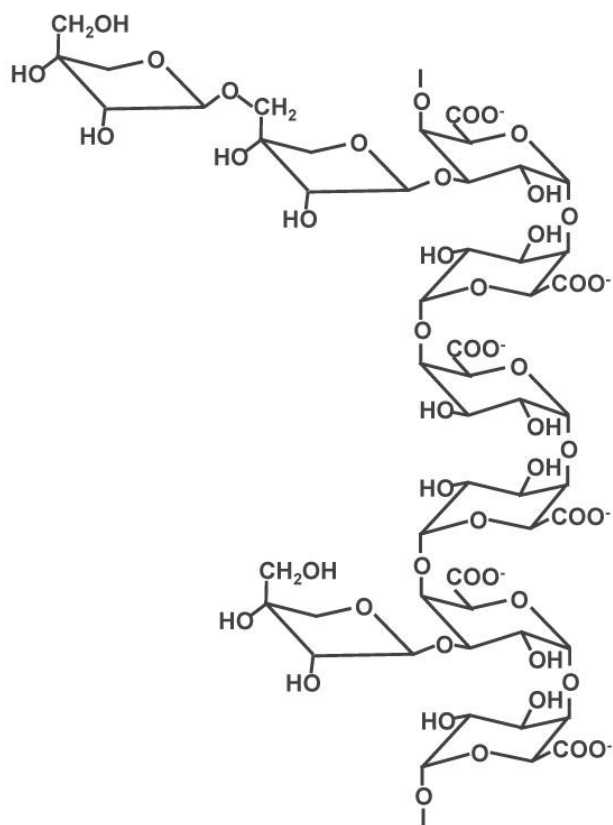
A**B**

Figure 1.12. Structure of xylogalacturonan (XGA) and apiogalacturonan (AGA).

Adapted from Caffall and Mohnen (2009).

plants. EPG-released RG-II has a low molecular weight of 5-10 kDa, and is composed of at least 12 different glycosyl residues that are linked through more than 20 different glycosidic linkages (O'Neill et al., 2004). In higher plants, RG-II predominantly exists as a dimer due to the formation of a borate-diol ester via the *Apif* residues of side chain A in each RG-II molecule (Ishii et al., 2001). The RG-II dimer provides mechanical strength for plant development, as shown by the *Arabidopsis* mutant, *mur1*, which has reduced borate cross-linking due to the absence of fucose residues in RG-II side chain A and has a resulting dwarfed stature and brittle tissues (O'Neill et al., 2001).

Distinct from these four pectic polysaccharides, RG-I (**Figure 1.14**) contributes ~25-35% of pectin and is composed of a repeating disaccharide [$\rightarrow 4$)- α -D-GalpA-($1 \rightarrow 2$)- α -L-Rhap-($1 \rightarrow$] backbone in which 20-80% of the Rhap residues are substituted at O-4 with side chains of arabinan, galactan, and/or arabinogalactan (O'Neill et al., 1990). The arabinan domain is composed of an $\alpha(1 \rightarrow 5)$ -L-arabinan substituted with α -L-Ara at O-2 and O-3, while the galactan domain is mostly $\beta(1 \rightarrow 4)$ -D-galactan. The $\beta(1 \rightarrow 4)$ -galactan side chains isolated from soybean soluble polysaccharides (SSPS) were estimated to contain 43-47 residues (Nakamura et al., 2002). Arabinogalactan side chains are more complex and include $\beta(1 \rightarrow 4)$ -D-galactan backbones substituted at O-3 with α -L-Ara or with arabinan branching, i.e. type I arabinogalactan. RG-I is also substituted with the so-called type II arabinogalactan which are $\beta(1 \rightarrow 3)$ -D-galactan with $\beta(1 \rightarrow 6)$ -D-galactan or arabinogalactan branching (Mohnen, 2008). These side chains of RG-I have also been referred to as the “hairy regions” and their precise structure and abundance vary among different plant species (Vincken et al., 2003). The $\alpha(1 \rightarrow 5)$ -L-arabinan side chains of RG-I have been implicated in the opening and closing of guard cells, where it is thought that they disrupt the tight cross-linking of HG (Jones et al., 2003).

The backbone of HG is covalently linked to the RG-I and RG-II backbone, as evidenced by their co-extraction and the inability to separate them by size exclusion chromatography prior to either endopolygalacturonase (EPG) digestion of the HG backbone or harsh chemical treatment (Caffall and Mohnen, 2009). Direct covalent linkages have been demonstrated between the backbones of HG and RG-I (Nakamura et al., 2002), HG and RG-II (Ishii and Matsunaga, 2001), and HG/XGA and RG-I (Coenen et al., 2007). Analyses of apple MHR (modified hairy regions) by controlled acid hydrolysis and purification revealed pectic oligomers with linkages that indicate a connection of HG/XGA to RG-I, and that the HG/XGA domain is located at the non-reducing end of RG-I (Coenen et al., 2007). Possible representative structures envisioned by such a model are depicted in **Figure 1.15**. Other arrangements of these pectic polymers may exist and await future structural verification.

Pectin has been viewed as a matrix that the cellulose-xyloglucan framework, the major load-bearing component of primary walls, is embedded within (Carpita and Gibeau, 1993). However, the interactions between pectin and cellulose, in the presence of reduced xyloglucan (XG), have been recently emphasized. A solid-state NMR study of unfractionated and insoluble primary cell wall suggests that there are limited xyloglucan-cellulose interactions but extensive interactions between pectins and cellulose (Dick-Perez et al., 2011). The findings by Dick-Perez et al. (2011) suggest an integral role of pectins in the load-bearing properties of primary cell walls. Competitive binding analyses of pectin and XG to cellulose suggest that the association of pectin to cellulose depends on the amount of XG present in the wall. That is, the less XG present, the more tightly pectin binds to cellulose (Zykwiniska et al., 2008). The double *xxt1 xxt2* mutant has no detectable XG but no gross morphological phenotype, suggesting that pectin may to some extent be compensatory to the loss of XG (Cavalier et al., 2008).

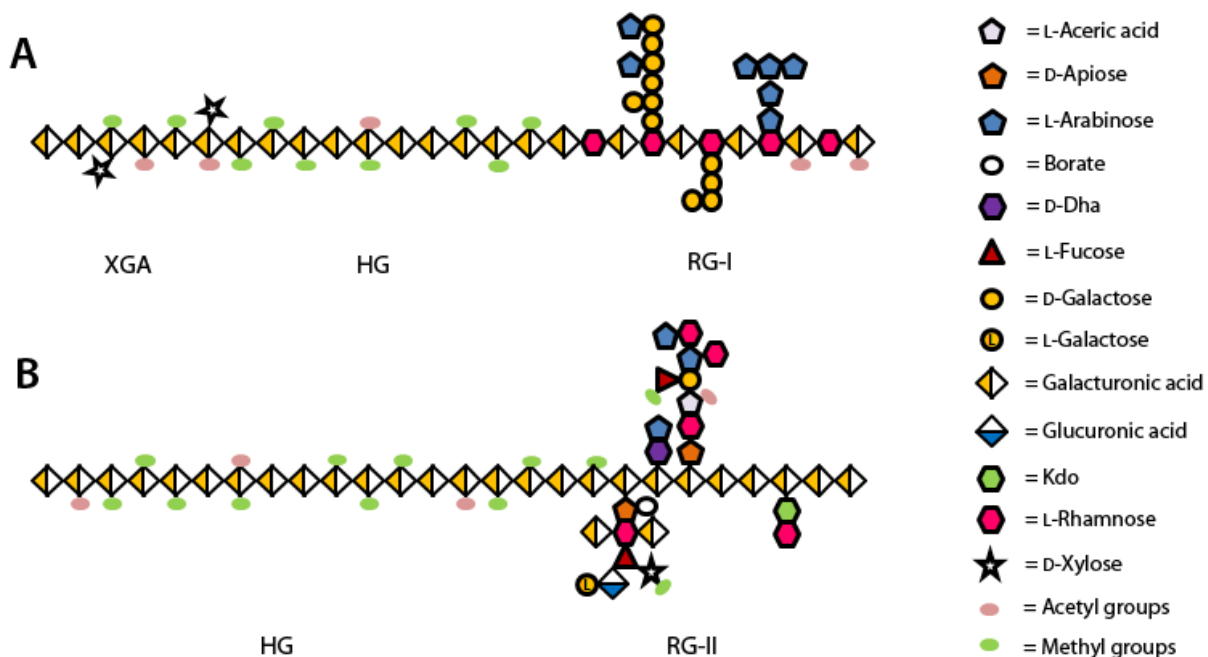


Figure 1.15. Schematic structure of pectic polysaccharides homogalacturonan (HG), rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II), and xylogalacturonan (XGA) linked via their galacturonic acid backbones.

The structures in (A) and (B) represent possible representative connections between the pectic polymers based on reported structures. HG and RG-I should be increased ~12.5-fold and ~2.5-fold, respectively, to approximate the amounts of these polymers *in muro*. Adapted from Mohnen (2008).

HG is also reported to be linked to XG since XG fragments are not readily solubilized unless treated with EPG in suspension-cultured sycamore cells (Talmadge et al., 1973). The presence of a pectin-xyloglucan complex was also suggested in etiolated pea stem tissues, in which an apparent pectin-xyloglucan complex served as an endogenous acceptor for galactose incorporation (Abdel-Massih et al., 2003; Cumming et al., 2005). Pulse-labeling *Arabidopsis* cell-cultures with [³H]arabinose and analysis of the pectin-bound xyloglucan indicated that the proposed XG-pectin complexes could be labeled very rapidly. This result indicates that the XG-pectin complex forms intra-protoplasmically (Popper and Fry, 2008). The amount of the apparent pectin-bound XG remained constant *in vivo*. In the sloughed XG present in the media surrounding the cultured *Arabidopsis* suspension cells, only ~30% was pectin-bound, suggesting that the pectin-xyloglucan complex was rather stable and that pectin may associate with XG in the wall (Popper and Fry, 2008). These results support a close interaction between pectin and XG in primary cell walls.

Pectin biosynthetic enzymes and genes

The biosynthesis of pectic polysaccharides is estimated to require 67 different enzyme activities including glycosyltransferases, methyltransferases, and acetyltransferases (Ridley et al., 2001; Mohnen, 2008). Multiple enzymatic activities involved in pectin biosynthesis have been identified in microsomes from multiple plant species, including HG:GalAT activities in mung bean (Villemeze et al., 1965), tomato (Lin et al., 1966), sycamore (Bolwell et al., 1985), tobacco (Doong et al., 1995), pea (Sterling et al., 2001), azuki bean (Takeuchi and Tsumuraya, 2001), pumpkin (Ishii, 2002), petunia (Akita et al., 2002), and *Arabidopsis* (Sterling et al., 2006); RG-I β -(1,4)-galactan:GalT activities in azuki bean shoots (McNab et al., 1968) and in potato (Geshi

et al., 2000); HG-methyltransferase (HG-MT) activities in mung bean (Kauss et al., 1967), flax hypocotyls (Vannier et al., 1992), tobacco (Goubet et al., 1998), and soybean hypocotyls (Ishikawa et al., 2000); both a β -(1,3)-arabinopyranosyltransferase activity involved in α -(1,5)-arabinan biosynthesis (Ishii et al., 2005b) and an α -L-arabinopyranosyltransferase activity that recognizes β -(1,4)-galactan as acceptors in mung bean hypocotyls (Ishii et al., 2005a). Some of these enzyme activities have been solubilized from membrane preparations (Doong and Mohnen, 1998; Goubet and Mohnen, 1999; Geshi et al., 2002; Nunan and Scheller, 2003).

The identification of genes encoding such enzymes has been challenging. Conclusive verification of gene function requires the demonstration of enzymatic activities from the proteins encoded by the putative glycosyltransferase genes (see **Appendix B**). The sequencing of multiple plant genomes combined with reverse genetics studies of cell wall mutants has led to the identification of many putative pectin biosynthetic genes. These genes include *QUASIMODO1* (*QUA1/GAUT8*) encoding a putative GalAT affecting cell adhesion (Bouton et al., 2002; Leboeuf et al., 2005), *ARABINAN DEFICIENT1* (*ARAD1*) encoding a putative arabinosyltransferase (AraT) affecting RG-I arabinan side chain biosynthesis (Harholt et al., 2006), *Nicotiana plumbaginifolia* *GLUCURONOSYLTRANSFERASE1* (*NpGUT1*) claimed to encode a RG-II glucuronosyltransferase (GlcAT) in RG-II side chain A that affects cell adhesion and adventitious shoot formation in tobacco callus (Iwai et al., 2002), *MALE GAMETEPHYTE DEFECTIVE4* (*MGP4*) encoding a putative RG-II xylosyltransferases (RGXT) affecting pollen tube and root growth (Liu et al., 2011), *QUA2* encoding a putative HG-MT affecting cell adhesion (Mouille et al., 2007), *QUA3* encoding another putative HG-MT affecting pectin methylation in *Arabidopsis* suspension cells (Miao et al., 2011), and Cotton-Golgi-Related-3 (*CGR3*) involved in HG methylesterification (Held et al., 2011).

Genes identified as encoding functionally proven enzymes include *GAUT1* that encodes an HG:GalAT (Sterling et al., 2006), *RGXT1* and *RGXT2* that encode two RG-II:α-1,3-XylTs that catalyze the addition of D-xylose from UDP-α-D-xylose onto L-fucose in an α-(1,3) linkage specific to the RG-II structure (Egelund et al., 2006), and *XYLOGALACTURONAN DEFICIENT1* (*XGD1*) that encodes an XGA:XylT that catalyzes the transfer of D-xylose from UDP-D-Xyl onto OGA (HG oligosaccharide) and endogenous acceptors in microsomes, likely in a β-(1,3)-linkage consistent with the structure of XGA (Jensen et al., 2008).

Table 1.4 summarizes the list of glycosyltransferases and methyltransferases encoded by putative and confirmed pectin biosynthetic genes and their known mutant phenotypes. Lists of the specific enzymatic activities required to synthesize each individual linkage present in the pectic polysaccharides can be found in the following reviews (Ridley et al., 2001; Mohnen, 2008; Caffall and Mohnen, 2009).

Pectin biosynthetic protein complexes

Recent studies of *GAUT1* have shown that protein complexes are involved in pectin biosynthesis. Following detergent-solubilization and sequential purification, the active enzyme fractions containing the HG:GalAT activity in *Arabidopsis* suspension culture cells were sequenced and BLASTed against the *Arabidopsis* genome. Two homologous proteins were identified, namely *GAUT1* and *GAUT7*, respectively. Transient expression in human embryonic kidney (HEK) 293 cells of *GAUT1*, but not *GAUT7*, yielded HG:GalAT activity, demonstrating that *GAUT1* encodes an active HG:GalAT (Sterling et al., 2006). A recent study showed that *GAUT1* and *GAUT7* form a core- HG:GalAT enzyme complex via both covalent and non-covalent interactions (Atmodjo et al., 2011). This study also showed that the N-terminal

Table 1.4. Arabidopsis genes encoding confirmed and putative GTs and HG-MTs involved in pectin biosynthesis.

Pectic polymer	Activity	Gene name ¹	CAZy	Linkages	Acceptors	Mutant phenotype	Reference
HG							
Backbone	GalAT	GAUT1	GT8	α -(1,4)-GalA	OGA ² , and endogenous acceptors	Homozygous <i>gaut1</i> mutants are not viable.	Sterling et al., 2006; Caffall et al., 2009; Atmodjo et al., 2011
		GAUT7	GT8	α -(1,4)-GalA	N/A	The <i>gaut7</i> mutant has no observable phenotype; GAUT7 alone has no HG:GalAT activity, it is a proven membrane-anchor for GAUT1.	Sterling et al., 2006; Atmodjo et al., 2011
		QUA1/GAUT8	GT8	Putative	N/A	Dwarf, reduced cell adhesion, reduced HG synthase and xylan synthase activity in <i>qua1</i> .	Bouton et al., 2002; LeBouef et al., 2005; Orfila et al., 2005
Methyl ester	HG-MT	QUA2	N/A	Putative	N/A	Dwarf, reduced cell adhesion, 50% reduction in HG in <i>qua2</i> .	Mouille et al., 2007
		QUA3	N/A	Putative	N/A	No growth phenotype in <i>qua3</i> plants, reduced pectin methylation in QUA3 RNAi suspension cells.	Miao et al., 2011
		CGR3	N/A	Putative	N/A	CGR3-overexpression increased petiole length and HG methyl esters, while <i>cgr3</i> contained less HG methylesters in etiolated hypocotyls.	Held et al., 2011
RG-I							
α -(1,5)-arabinan	AraT	ARAD1	GT47	Putative	N/A	No growth phenotype in <i>arad1</i> ; 25% and 54% reduction of Ara in leaf and stems; 70% reduction of Ara in RG-I.	Harholt et al., 2006
		ARAD2	GT47	Putative	N/A	No growth or wall phenotype; double <i>arad1 arad2</i> has <i>arad1</i> phenotype.	Harholt et al., 2012
RG-II							
Side chain A	XylT	RGXT1	GT77	α -(1,3)-Xyl	Me L-Fuc, (Me) α -L-Fuc-(1,4)- α / β -L-Rha, L-fucose	No growth phenotype in <i>rgxt1</i> and <i>rgxt2</i> ; residual 2-O-methyl D-Xyl due to gene redundancy.	Egelund et al., 2006
		RGXT2	GT77	α -(1,3)-Xyl			
		RGXT3	GT77	α -(1,3)-Xyl		The <i>rgxt3</i> mutant is not available.	Egelund et al., 2008
		MGP4	GT77	α -(1,3)-Xyl	Free L-fucose	Weaker allele <i>mgp4-1</i> has severely defective pollen tube growth; homozygous <i>mgp4-2</i> mutants were not viable; pollen-rescued <i>prmgp4</i> has impaired root growth.	Liu et al., 2011
XGA							
Xyl substitution	XylT	XGD1	GT47	β -(1,3)-Xyl	OGA, and endogenous acceptors	Reduced pectic XGA, no xylose defect in other polymers	Jacob et al., 2008

1. The enzymes encoded by genes with names shown in bold have confirmed enzyme activity. 2. OGA: oligogalacturonides (HG oligosaccharide).

sequence of GAUT1 containing the transmembrane domain is posttranslationally cleaved *in vivo*, and that GAUT7 serves as a membrane anchor to hold GAUT1 in the Golgi apparatus. The GAUT1:GAUT7 complex shows high specificity for HG oligomers (DP 7-23) compared to RG-II monomers or RG-I oligomers (DP 6-26) containing either GalA or Rha at the non-reducing end (Atmodjo et al., 2011). These results indicate that the GAUT1:GAUT7 complex is unlikely to be involved in the elongation of either the RG-I backbone or the RG-II backbone with HG. It remains unclear whether GAUT1 is involved in the initiation of pectin biosynthesis or only in the elongation of the HG backbone. The identification and verification of the GAUT1:GAUT7 complex indicate that glycosyltransferase complexes function in pectin biosynthesis (Atmodjo et al., 2011).

Another example of protein complexes in pectin biosynthesis is the recently reported ARAD1 and ARAD2 complex (Harholt et al., 2012b). Although *arad1*, *arad2*, and the *arad1 arad2* double mutant show no apparent growth phenotypes, the available data indicate both genes encode putative RG-I arabinan: α -(1,5)-AraTs that are genetically non-redundant (Harholt et al., 2006; Harholt et al., 2012b). BiFC and Förster resonance energy transfer (FRET) showed that upon transient expression in *N. benthamiana*, these putative arabinan:AraTs form both homo- and heterodimers. When the differentially tagged, co-expressed ARAD1 and ARAD2 were separated on reducing versus non-reducing SDS-PAGE and analyzed by western blotting, the results indicated the occurrence of three kinds of dimers, i.e., ARAD1/ARAD1, ARAD2/ARAD2, and ARAD1/ARAD2, each mediated by disulfide bridges. Whether such dimers are biologically relevant or are artifacts of protein overexpression remains to be determined. Nevertheless, each type of dimer may serve a somewhat distinct function because, as mentioned earlier, ARAD1 and ARAD2 are not redundant (Harholt et al., 2012b).

Nucleotide sugars and pectin biosynthesis

Nucleotide-sugar biosynthesis (**Figure 1.16**) provides sugar substrates for pectin biosynthesis. However, two issues arise in associating nucleotide-sugar biosynthesis defects with pectin biosynthesis.

First, since many nucleotide-sugars are substrates for the synthesis of diverse polymers including hemicelluloses, mutants defective in nucleotide-sugar biosynthetic genes often manifest alterations in many carbohydrate structures. For example, a mutation of *MURUS4* (*MUR4*), a protein that catalyzes the conversion of UDP-D-xylose to UDP-L-arabinose, leads to a 50% reduction of arabinose residues in all arabinose-containing wall polymers, e.g. arabinan and arabinogalactan (Burget and Reiter, 1999; Burget et al., 2003). The *UGE* genes are essential for the *de novo* synthesis of UDP-D-Gal, which affects galactose metabolism and provides precursors for multiple polysaccharides, glycolipids, and glycosides (Barber et al., 2006). Mutant analyses suggest that *UGE2* and *-4* function in overall plant growth and development, *UGE3* is specific for pollen development, and *UGE1* and *-5* may function during stress (Dormann and Benning, 1998; Seifert et al., 2002; Nguema-Ona et al., 2006; Rosti et al., 2007). Interestingly, *E.coli*-expressed Arabidopsis *UGE1* and *UGE-3* also have high UDP-Xyl 4-epimerase activity in addition to their UDP-Glc 4-epimerase activity (Rosti et al., 2007), indicating the complexity of these enzymes and the broad effects their mutation brings about.

Second, due to the presence of gene families and homologous genes in plant genomes, mutants defective in a single nucleotide-sugar synthase gene may show no obvious growth phenotypes. No mutant studies have been reported for UDP-D-glucuronate 4-epimerase (*GAE*)/*UGlcAE* genes, which belong to a gene family of six homologs, *GAE1-6*, in Arabidopsis.

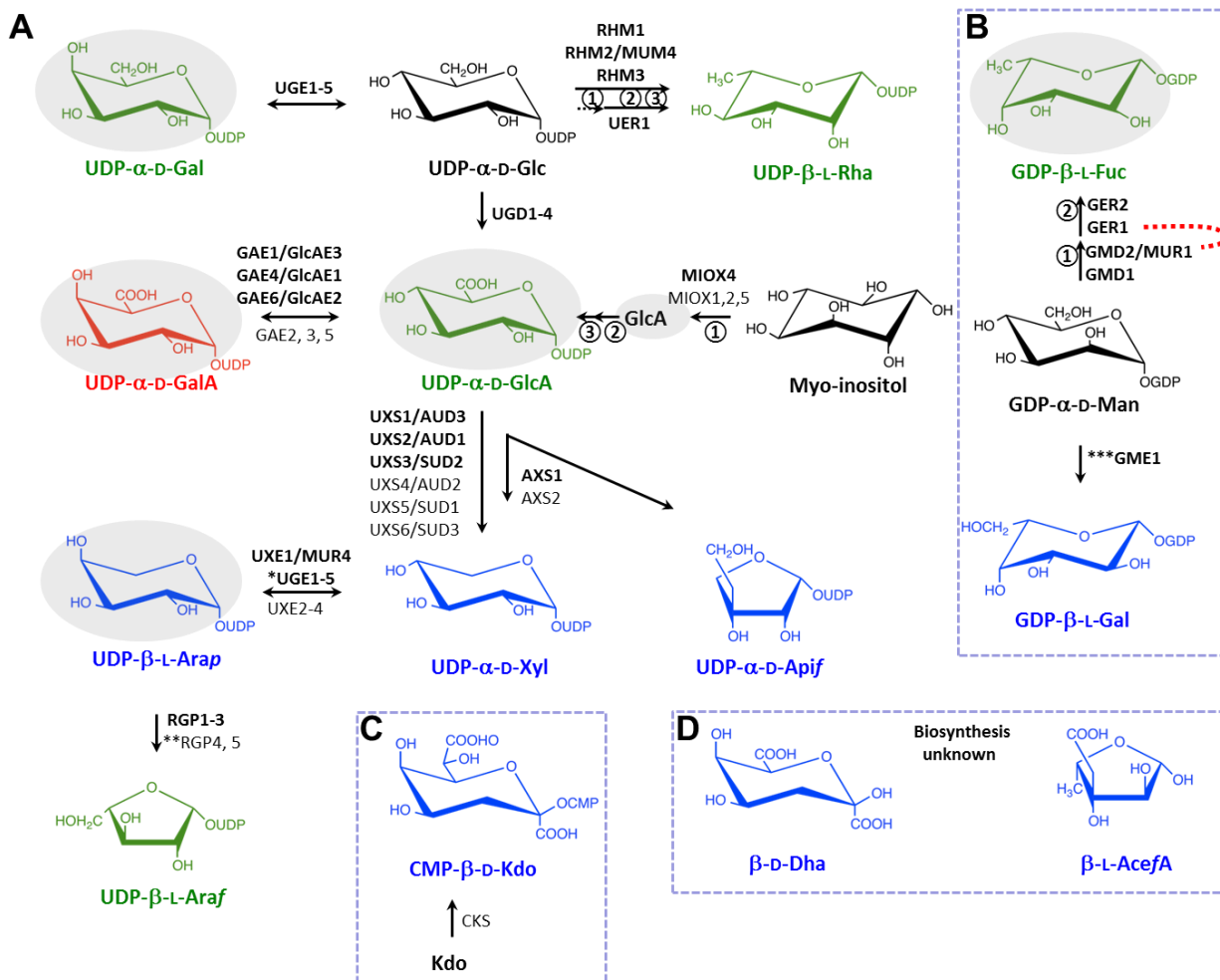


Figure 1.16. Nucleotide-sugar interconversion pathway providing substrates for pectin biosynthesis.

Proven (in bold) and putative nucleotide-sugar biosynthesis enzymes have been identified in *Arabidopsis*. Nucleotide sugars in red are present in all pectins, those in green are present in rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II), and those in blue are specific to RG-II. Nucleotide-sugars highlighted with grey oval backgrounds are also produced via salvage pathways, i.e. conversion of nucleotide-sugars from free sugars through the sequential action of sugar-1-P kinases and pyrophosphorylases. Genes with names in bold encode enzymes with

proven *in vitro* enzymatic activities. Adapted from Atmodjo, Hao, and Mohnen, Annual Review Plant Biology Vol.64, 2013 (in press).

(A) Partial nucleotide sugar metabolic pathway involving UDP- α -D-Glc. UGEs, UDP-Glc 4-epimerase/UDP-Gal 4-epimerases; RHMs, tri-activity enzymes including a UDP-Glc 4,6-dehydratase, a UDP-4-keto-6-deoxy-D-Glc 3,5-epimerase, and a UDP-4-keto-L-Rha 4-keto-reductase; UER1, a bi-activity enzyme with UDP-4-keto-6-deoxy-D-Glc 3,5-epimerase and UDP-4-keto-L-Rha 4-keto-reductase activities; UGDs, UDP-Glc dehydrogenases; GAEs/UGlAEs, UDP-Glucuronate 4-epimerases; UXs, UDP-GlcA decarboxylases/UDP-Xyl synthases; AXSs, UDP-D-Apif/UDP-D-Xyl synthases; AUDs, membrane-anchored UDP-D-GlcA decarboxylases; SUDs, soluble UDP-D-GlcA decarboxylases; MIOXs, myo-inositol oxygenases that catalyze the conversion of myo-inositol to GlcA, which is subsequently converted to UDP- α -D-GlcA via the salvage pathway; UXEs, UDP-Xyl 4-epimerase/UDP-Ara 4-epimerase; RGPs, UDP-L-Arabinopyranose mutase; * UGE1 and UGE3 also have UDP-Xyl 4-epimerase activity, while UGE2, -4, -5 have only weak UDP-Xyl 4-epimerase activity; ** *E.coli*-expressed recombinant RGP4 and -5 were found to be inactive.

(B) Partial nucleotide sugar metabolic pathway involving GDP- α -D-Man. GMDs, GDP-Man 4,6-dehydratases; GERs, GDP-4-keto-6-deoxy-Man 3,5-epimerase/4-reductase; GME1, GDP-Man 3',5'-epimerase. Red dotted line between GER1 and GMD2/MUR1 indicates protein complex formation between the two enzymes. *** GME1 also catalyzes the conversion of GDP- α -D-Man to GDP- β -L-Gul, which is 5-epimer of D-Man and 3-epimer of L-Gal (not included in figure).

(C) CMP-Kdo is synthesized from Kdo by CKS (CMP-Kdo synthetase).

(D) No activated forms of β -D-Dha and β -L-AcefA have been identified.

GAE1/UGlcAE3, *GAE4/UGlcAE1*, *GAE6/UGlcAE2* have been shown to encode UDP-D-GlcA 4-epimerases that catalyze the interconversion of UDP-D-GlcA and UDP-D-GalA, favoring the formation of UDP-D-GalA with an equilibrium constant of ~1.9 (Gu and Bar-Peled, 2004), thereby providing UDP-D-GalA for the biosynthesis of possibly all pectic polymers (Gu and Bar-Peled, 2004; Molhoj et al., 2004; Usadel et al., 2004b). Similarly, single *ugd2* and *ugd3* mutants defective in *UGD2* and *UGD3* that encode UDP-Glc dehydrogenases have no obvious growth phenotypes, while the *ugd2 ugd3* double mutant displays abnormal seedling development, dwarfed stature, and lower seed set (Reboul et al., 2011).

Contrary to the issues discussed above, genes that synthesize nucleotide-sugars that are more or less specific for pectin structures have been shown to affect the pectin structure and content. For example, *RHAMNOSE BIOSYNTHESIS2 (RHM2)/MUCILAGE MODIFIED4 (MUM4)* encodes a protein with UDP-D-glucose 4,6-dehydratase, UDP-4-keto-6-deoxy-D-glucose 3,5-epimerase, and UDP-4-keto-L-rhamnose 4-keto-reductase activities, which converts UDP-D-glucose to UDP-L-rhamnose (Oka et al., 2007). The Arabidopsis *mum4* mutants produce seeds with reduced seed mucilage and contain RG-I with a reduced molecular weight (Usadel et al., 2004a; Western et al., 2004).

Another example is *MUR1* that encodes an isoform of GDP-D-mannose-4,6-dehydratase that converts GDP-D-mannose to GDP-L-fucose (O'Neill et al., 2001; Pauly et al., 2001). The *mur1* mutant contains almost no L-fucose, which reduces the borate cross-linking of RG-II and leads to a dwarf plant with brittle tissues (O'Neill et al., 2001). Due to the presence of fucose in xyloglucan, the *mur1* mutant also has defects in xyloglucan in the root elongation zone, which will not be discussed in detail here (Bonin et al., 2003; Freshour et al., 2003).

A third example is *UDP-D-APIOSE/UDP-D-XYLOSE SYNTHASE1* (*AXS1*) that encodes a UDP-D-apiose/UDP-D-xylose synthase that synthesizes UDP-D-apiose and UDP-D-xylose from UDP-D-glucuronate (Molhoj et al., 2003). Virus-induced gene silencing (VIGS) of *NbAXS1* in *Nicotiana benthamiana* leads to arrested growth and yellow leaves with reduced levels of D-apiose, 2-O-methyl-L-fucose and 2-O-methyl-D-xylose in the wall, which are signature residues in RG-II side chains A and B, respectively (**Figure 1.13**) (Ahn et al., 2006). These results suggest that the synthesis of nucleotide-sugars is essential and may become a limiting factor for pectin biosynthesis.

Besides the interconversion pathway, nucleotide-sugars can also be produced via the less understood “salvage pathway”, in which nucleotide-sugars are synthesized from free sugars by the sequential action of sugar-1-P kinases and a pyrophosphorylase (Bar-Peled and O'Neill, 2011). Mutation in the Arabidopsis UDP-SUGAR PYROPHOSPHORYLASE (*AtUSP*), also known as *SLOPPY* (Bar-Peled and O'Neill, 2011), results in a male gametophytic mutant due to defective pollen formation (Schnurr et al., 2006).

Nucleotide-sugars produced in the cytosol may be transported into the Golgi, where the biosynthesis of pectin takes place. It is believed that the Golgi-mediated assembly of wall matrix polysaccharides including pectin requires concerted efforts of glycosyltransferases, nucleotide-sugar transporters, and nucleotide-sugar interconversion enzymes (Driouch et al., 2012). In addition to the aforementioned protein complex formation between GAUT1 and GAUT7 (Atmodjo et al., 2011), and between ARAD1 and ARAD2 to produce homo- and heterodimers (Harholt et al., 2012b), the co-immunoprecipitation of RGP1, RGP2 and RGP5 from Arabidopsis seedlings suggests that the UDP-Ara mutase activity-containing proteins form a heteroprotein complex. However, no other Golgi-associated proteins have been identified in the same fraction

(Rautengarten et al., 2011). Thus, it remains largely unknown whether glycosyltransferases interact with other proteins such as nucleotide-sugar interconversion enzymes and transporters during pectin biosynthesis (Driouich et al., 2012).

Location of pectin biosynthesis

Immunocytochemical labeling of several pectic epitopes suggests that HG and RG-I backbones are synthesized in the *cis*- and *medial*-Golgi, HG is methylesterified in the *medial*-Golgi, and substitution of arabinose-containing side chains takes place in the *trans*-Golgi network (Zhang and Staehelin, 1992; Young et al., 2008). The pectic polymers are delivered to the wall via vesicle trafficking from the trans-Golgi network, possibly along actin filaments powered by myosin motors (Nebenfuhr et al., 1999; Nebenfuhr and Staehelin, 2001).

Indeed, Golgi-enriched membrane fractions contain enzymatic activities required for pectin biosynthesis, and several members of the GAUT family have been identified in the Golgi (Mohnen, 2008). A recent proteomics study by Parsons et al. (2012) demonstrated the presence of GAUT1, -4, -7, -8, -9, and -10 in triplicate Golgi membrane preparations isolated from *Arabidopsis* suspension culture cells as well as the presence of GAUTs 3 and -11 in two out of the three Golgi preparations (Parsons et al., 2012). GAUT1, -3, -7, -9 were also identified in an earlier proteome analysis of *Arabidopsis* callus culture Golgi membranes (Dunkley et al., 2006).

The topology of pectin synthesis was demonstrated in pea (*Pisum sativum* L. var. Alaska) when an α -1,4-galacturonosyltransferase (GalAT) was shown to be Golgi-localized with its catalytic domain facing the luminal side of Golgi (Sterling et al., 2001), confirming type II topology for HG:GalAT and indicating that pectin biosynthesis takes place in the lumen of Golgi. Type II membrane proteins in general have a short N-terminal sequence, followed by a

single transmembrane domain, and a larger C-terminal region which presumably contains the catalytic domain for glycosyltransferases involved in matrix polysaccharide biosynthesis (Keegstra and Raikhel, 2001). Such domain structure is consistent with the predicted topology and domain structure of the GAUTs (Yin et al., 2010).

Pectin in secondary cell walls

Besides being a major matrix polysaccharide in primary walls, pectin has also been detected in cells/tissues with secondary walls (Albersheim et al., 2011) and there is evidence that pectin affects secondary wall formation (Roach et al., 2011; Hongo et al., 2012). During cotton fiber elongation, a cotton fiber middle lamella (CFML) forms that consolidates the individual fibers into bundles, thus fostering maximum growth of cotton fibers in the confined space within a boll (Singh et al., 2009). The adhesive CFML, composed mainly of low-esterified HG and partially fucosylated XG, is eventually degraded to release individual cotton fibers. The unique existence and degradation of the pectin-enriched CFML in developing cotton fibers highlights the significance of pectin structures as possible primers or scaffolds for secondary growth.

In addition to pectin degradation, dynamic post-synthetic modification of pectin structure is catalyzed by the action of pectin methylesterases (PMEs). PMEs remove methyl esters from the C-6 position of the GalA residues in the HG backbone, thus generating negatively charged carboxyl groups for possible Ca^{2+} -dependent HG cross-linking. Decreased demethylesterification of pectin through down-regulation of *PttPME1* promotes sympastic growth (i.e. diameter expansion) of both vessel and fiber cells and intrusive growth (i.e. length elongation) of fiber cells in poplar plants (Siedlecka et al., 2008). Conversely, up-regulation of *PttPME1* resulted in a reversed phenotype. These results suggest that regulation of pectin de-

methylesterification facilitates symplastic and intrusive growth of fiber and vessel cells through control of wall flexibility.

The dynamic modification of pectin has also been visualized in eucalyptus cambium cells in tissues undergoing secondary growth using the antibody JIM5, which recognizes low-methylesterified HG (Goulao et al., 2011). Despite abundant JIM5 epitopes being present in the newly formed xylem cells and later remaining in the protoxylem region, no JIM5 epitopes were found in cambium cells in apical tissues, thereby suggesting active pectin modification and/or biosynthesis during xylem and phloem differentiation. Recent analysis of an *Arabidopsis* mutant carrying a defect in *PME35* indicates that a reduction in PME activity and accompanying suppression of HG de-methylesterification in cortex cell primary walls and in the middle lamella of interfascicular fiber cells leads to a pendant stem phenotype and impaired mechanical strength of the middle and basal stem (Hongo et al., 2012). A direct or indirect involvement of pectin in secondary wall formation and thickening in interfascicular fibers has been proposed.

There is no direct evidence to date that pectin is synthesized as a component of the secondary wall itself. However, the expression of many members of the GAUT and GATL gene families has been documented in tissues undergoing secondary thickening (Caffall et al., 2009a; Minic et al., 2009; Kong et al., 2011), thus supporting a role for multiple GT8s in pectin biosynthesis in such tissues. For example, *GAUT1* and *GAUT7* expression has been demonstrated via promoter-GUS analysis in vascular tissues as well as in meristematic and reproductive tissues (Atmodjo et al., 2011), and the expression of both genes remains constant throughout *Arabidopsis* stem development (**Figure 1.17**). In addition, other representatives of each sub-clade of the GAUT family that we tested showed increased expression in the mid and bottom stems (**Figure 1.17**). Assuming that primary wall synthesis occurs in the elongating

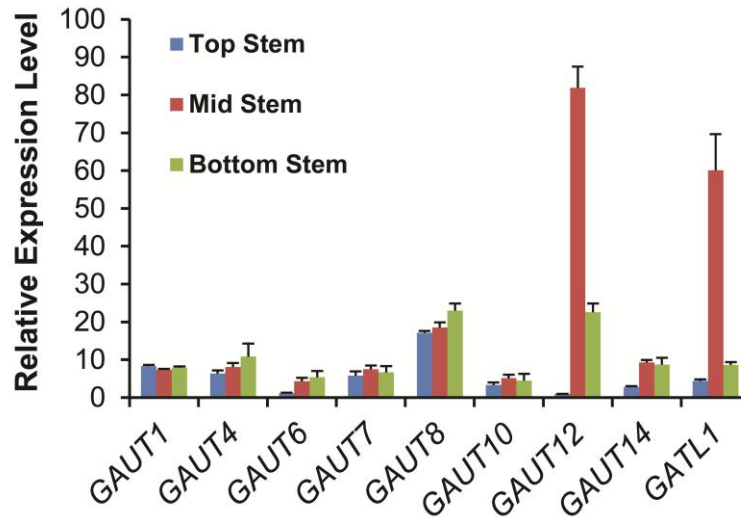


Figure 1.17. Selected *GAUT* and *GATL* gene expression in *Arabidopsis* stems.

Adapted from Atmodjo, Hao, and Mohnen, Annual Review Plant Biology Vol. 64, 2013 (in press).

Total RNAs were isolated from 1-inch stem segments of 8-week-old wild type *Arabidopsis*: the top stem (immediately below the inflorescence), mid stem, and bottom stem (immediately above the soil). Total RNA (1 μ g) was used to synthesize the 1st strand cDNA, and *GAUT* and *GATL* gene expression was determined via Real-time PCR analyses. Relative gene expression was normalized using *ACTIN2* as the reference gene and the expression of *GAUT12* in the top stem was set to 1. Value = mean $\pm$ standard deviation of three independent experiments.

young/top stem while secondary wall synthesis starts in mid stem (where elongation ceases) and decreases in the mature/bottom stem, these results suggest roles for many of the GAUTs not only in primary wall synthesis, but also during secondary wall formation. However, because stem tissue is composed of multiple cell types, it will be necessary to isolate specific cells such as developing vessels and fiber cells by laser microdissection (Nelson et al., 2006) or to use a xylogenic suspension culture system (Kubo et al., 2005) to establish direct correlations between GAUT gene expression and secondary wall thickening.

Connection between pectin and xylan biosynthesis

In dicots, pectin is particularly abundant in primary walls whereas xylan is a hemicellulose abundant in secondary walls. It is therefore surprising that increasing evidence indicates a connection between pectin and xylan biosynthesis. A priori, it would be expected that most pectin is laid down prior to secondary cell wall xylan deposition. Thus, it is possible that preexisting pectin influences xylan biosynthesis and deposition. It is also possible that regulation of pectin biosynthesis or post-biosynthetic modifications such as HG de-methylesterification generates pectin structures that could affect xylan deposition during secondary growth.

Mutant studies revealed that pectin defects sometimes lead to a reduction in xylan biosynthesis and a subsequent reduction of xylan in the walls. Based on wall chemical analyses, immunolabeling with JIM5 and JIM7, and cell-cell adhesion phenotypes, *qua1* plants (Bouton et al., 2002) and suspension culture cells (Leboeuf et al., 2005) have reductions in HG, suggesting that QUA1/GAUT8 has a role in HG biosynthesis. However, studies of *qua1* stem microsomes revealed a reduction not only in HG:GalAT activity but also in xylan synthase activity (Orfila et al., 2005), indicating that the mutation also affects xylan biosynthesis. Similarly, the *atcsld5*

mutant has reduced xylan synthase and HG:GalAT activities (Bernal et al., 2007). However, sequence similarity between *ATCSLD5* and *CESAs* and the abundant presence of xylan in *atcsld5* suggest that *ATCSLD5* may be involved in synthesizing noncrystalline cellulose or some other β -(1,4)-glucan rather pectin or xylan biosynthesis *per se*. Hence, the reduction in HG:GalAT and xylan synthase activities in *atcsld5* is likely a secondary effect due to the reduction of a wall polymer that remains to be discovered (Bernal et al., 2007).

Another class of xylan defective mutants, *irx8* and *parvus/gatl1*, has reduced wall xylan content that causes a collapsed xylem phenotype (Brown et al., 2005; Persson et al., 2005; Brown et al., 2007; Pena et al., 2007; Persson et al., 2007) but normal or even apparently elevated levels of xylan synthase activity in mutant stem microsomes (Brown et al., 2007; Lee et al., 2007a; Lee et al., 2007b). These results indicate that the amount of xylan *in muro* can be downregulated, owing not to defects in xylan backbone elongation but rather to xylan deposition, possibly due to the lack of an attachment point. Detailed characterization of xylan in both mutants revealed a reduced signal for the XRES, D-Xylp- β (1 $\rightarrow$ 4)-D-Xylp- β (1 $\rightarrow$ 3)-L-Rhap- α (1 $\rightarrow$ 2)-D-GalpA- α (1 $\rightarrow$ 4)-D-Xylp, which is abundant in wild-type xylan (Pena et al., 2007) and has been proposed to act as either a primer or terminator for xylan elongation (York and O'Neill, 2008). This xylan reducing-end sequence has a free reducing end when isolated in xylan preparations; however, whether it is free or anchored during biosynthesis is not known (Pena et al., 2007). Both *GAUT12* and *PARVUS/GLZ1/GATL1* encode GTs that may be involved in the synthesis of this glycosyl sequence (Brown et al., 2007; Pena et al., 2007), although no enzymatic activity has been demonstrated. Complications arise as both mutants also have defects in specific pectin fractions. Detailed wall fractionation of *irx8/gaut12* stem walls revealed a significant loss of GalA in an EPG/PME-released wall fraction, likely an HG fraction, as

demonstrated by linkage analyses (Persson et al., 2007). The *parvus/glz1/gatl1* mutant has a low-humidity-dependent dwarf phenotype with indehiscent anthers as well as a reduction in RG-I branching (Lao et al., 2003). The data do not rule out the possibility that both genes encode GTs that synthesize a specific pectic polymer or oligomer domain that provides a substructure for xylan deposition. This hypothesis is particularly interesting considering the sequence similarity of both GAUT12 and GATL1 to GAUT1, a confirmed pectin biosynthetic HG:GalAT (Sterling et al., 2006).

Based on the data to date, we hypothesize that xylan chains are deposited onto attachment points in the wall via a primer such as the xylan reducing-end sequence, a pectic substructure, or a structure that contains both. Challenges in testing these hypotheses lie in the lack of availability of, and knowledge about, the putative pectic substructure/domain and in preserving during chemical isolation, the moiety (if any) onto which the xylan reducing-end sequence is attached.

CHAPTER 2

ARABIDOPSIS *GAUT12/IRX8* IS ESSENTIAL FOR LIGNIN DEPOSITION AND ANTHER DEHISCENCE ¹

¹ Zhangying Hao, Utku Avci, Li Tan, Xiang Zhu, Yingzhen Kong, John Glushka, Stefan Eberhard, Tipton Sholes, Grace Rothstein, Wolfgang Lukowitz, Ron Orlando, Michael G. Hahn, Debra Mohnen. To be submitted to The Plant Journal or PLOS ONE.

ABSTRACT

GAUT12 (GAlactUronosylTransferase12)/IRX8 (IRregular Xylem8) is a putative glycosyltransferase involved in Arabidopsis secondary cell wall biosynthesis. Previous work has shown that the Arabidopsis *irregular xylem8* (*irx8*) mutant has collapsed xylem due to a reduction in xylan and a lesser reduction of a subfraction of homogalacturonan. We generated an anti-GAUT12 polyclonal antibody and used available acceptors to test for GAUT12 activity. We found that GAUT12 is not a homogalacturonan galacturonosyltransferase (HG:GalAT) and does not have the activity comparable to GAUT1. Characterization of *irx8* mutants revealed that *irx8* has indehiscent anthers due to reduced deposition of both xylan and lignin in the endothecium cell layer, leading to the male sterility of *irx8*. The lignin reduction was also manifested in *irx8* stems, as demonstrated by histochemical lignin staining, pyrolysis Molecular Beam Mass Spectrometry (pyMBMS), and 2D ^{13}C - ^1H Heteronuclear Single-Quantum Correlation (HSQC) NMR spectroscopy. The lignin reduction is likely due to reduced lignin biosynthesis as indicated by changes in lignin biosynthetic gene expression levels. A reduction in immunolabeled xylan and an increase in RG-I epitopes in *irx8* mutant fiber cells are consistent with the reduction in xylose and increased mole percent rhamnose in *irx8* walls. The results lead us to hypothesize that GAUT12 synthesizes a primer-like structure required for xylan and lignin deposition during secondary cell wall formation in which a structure of RG-I may be involved.

INTRODUCTION

The complex and interacting polymers in plant cell walls implies that an alteration in any one polymer will likely affect the other components of the composite. Accordingly, mutations in presumptive glycosyltransferase (GT) genes sometimes result in dwarfing and are associated

with alterations in multiple cell wall polymers. For example, the Arabidopsis mutant *irx7* (Zhong et al., 2005) is defective in both xylan and cellulose deposition, whereas *qua1* (Bouton et al., 2002; Leboeuf et al., 2005; Orfila et al., 2005), *parvus-3* (Shao et al., 2004; Brown et al., 2007; Lee et al., 2007b; Kong et al., 2009), and *irx8/gaut12* mutants (Pena et al., 2007; Persson et al., 2007) are affected in pectin as well as in xylan synthesis. These complex changes in cell wall composition make it difficult to infer primary gene functions on the basis of mutant phenotypes alone.

At the same time, it remains challenging to biochemically define enzymatic activities of predicted gene products. Glycosyltransferases are often low in abundance, sometimes act as part of larger protein complexes, and may require unknown and/or unavailable acceptor substrates for *in vitro* assays. Heterologous expression of plant GTs in yeast or insect cells has rarely been successful (Pena et al., 2007; Oikawa et al., 2012). An alternative approach is to compare the activity of crude microsomal membranes (or microsomes) isolated from wild type and mutant tissues. For example, stem microsomes prepared from *irx9* and *irx14* contained reduced xylan xylosyltransferases (XylT) activity—in combination with reduced xylan content in both mutants, supporting the view that *IRX9* and *IRX14* may encode xylan backbone synthases (Brown et al., 2007; Lee et al., 2007a; Pena et al., 2007). Indeed, Lee et al. (2012) recently demonstrated significantly greater microsome xylan:XylT activity upon co-expression of Arabidopsis *IRX9* and *IRX14* in tobacco suspension culture cells, suggesting that xylan backbone GTs may function cooperatively. A putative xylan biosynthetic complex has been isolated from etiolated wheat hypocotyl microsomes, where XylT, glucuronosyltransferase (GlcAT), and arabinosyltransferase (AraT) activities were found to co-immunoprecipitate (Zeng et al., 2010). Hetero-oligomeric GT complexes have also been shown in the syntheses of cellulose (Gardiner et al., 2003; Desprez et

al., 2007) and pectin homogalacturonan (Atmodjo et al., 2011) and implicated in the syntheses of arabinan (Harholt et al., 2012), and xyloglucan (Chou et al., 2012).

The gene mutated in the xylan- and pectin-deficient *irx8* mutants is *At5g54690* or *GAUT12*, a member of the CAZy family 8 GTs related to homogalacturonan (HG): α -1,4-galacturonosyltransferase (GalAT) GAUT1 (Sterling et al., 2006). We will herein refer to the gene as *GAUT12*, its protein as GAUT12, and its mutant as *irx8*. GAUT12 is predicted to be a type II transmembrane protein with its C-terminal catalytic domain facing the Golgi lumen. Transient expression of YFP-tagged GAUT12 protein co-localized with CFP-tagged MUR4, consistent with localization in the Golgi apparatus (Pena et al., 2007). Transcription of *GAUT12* is strongest in xylem vessels and interfascicular fiber cells, and *irx8* mutant cell walls show a substantial reduction in glucuronoxylan as well as a modest reduction in a sub-fraction of α -1,4-linked GalA (Pena et al., 2007; Persson et al., 2007). Since stem microsomes isolated from *irx8* plants contain comparable amounts of xylan:XylT and xylan:GlcAT activity as their wild type counterparts (Brown et al., 2007; Lee et al., 2007a), it seems unlikely that GAUT12 is involved in the elongation or substitution of the xylan backbone (York and O'Neill, 2008; Scheller and Ulvskov, 2010).

How may the complex phenotype of *irx8* mutants be rationalized? The reduction in α -1,4-linked GalA in the endopolygalacturonase/pectin methylesterase (EPG/PME)-accessible wall fraction of *irx8* mutants raises the possibility that GAUT12 is involved in the synthesis of an as-yet-undetermined HG species. Such a GAUT12-dependent HG may be tightly associated with xylan and, when missing, disrupt xylan biogenesis and/or deposition indirectly (Persson et al., 2007). Alternatively, the reduction in *irx8* mutant xylan fractions of xylan reducing end tetrasaccharide sequence (XRES; β -D-Xylp-(1 $\rightarrow$ 3)- α -L-Rhap-(1 $\rightarrow$ 2)- α -D-GalpA-(1 $\rightarrow$ 4)-D-Xylp)

led to speculations that GAUT12 may catalyze the addition of GalA into the nascent XRES (Pena et al., 2007). However, it remains unknown how XRES synthesis is initialized and how its production relates to xylan biosynthesis (York and O'Neill, 2008).

The GAUT protein family contains three ancient clades, named A-C, all of which are supported by orthologs in basal land plants such as *Physcomitrella patens* and *Sellaginella moelendorffii* (Yin et al., 2010). GAUT1 is a member of the A clade, while GAUT12, along with GAUT13, -14 and -15, is part of the C clade (Sterling et al., 2006). Arabidopsis GAUT12 has two orthologs in *Populus trichocarpa* (poplar), PtrGT8D1 and PtrGT8D2. Transgenic poplar plants in which both PtrGT8D1 and PtrGT8D2 function has been impaired by RNA-interference display brittle stems and reduced wall thickness in vessels and fibers, although the collapsed xylem and dwarfed phenotype of Arabidopsis *irx8* mutants are not re-capitulated (Li et al., 2011). The rice genome contains orthologs of the C class genes GAUT13 and -14 (Caffall et al., 2009) but not of GAUT12, indicating that any specific functions associated with GAUT12 are either missing in grasses or carried out by other class C genes. Similarly, XRES has thus far only been identified in eudicots and gymnosperms (Johansson and Samuelson, 1977; Andersson et al., 1983; Pena et al., 2007) but not in basal land plants, including *P. patens*, *Sellaginella kraussiana*, or in grasses such as rice (Kulkarni et al., 2012). Thus, the currently available phylogenetic data suggests a possible correlation between the presence of GAUT12 orthologs and the presence of XRES.

Besides being severely dwarfed and slow growing, Arabidopsis *irx8* mutants are sterile (Persson et al., 2007). Consistent with a potential role in reproduction, *GAUT12* transcripts are not only regulated by transcription factors that regulate vessel and fiber formation, such as MYB46 (Ko et al., 2009), MYB83 (McCarthy et al., 2009), VND6 and VND7 (Yamaguchi et al.,

2010), but also by transcription factors that act in anther development, such as MYB26/MALE STERILE35 (Steiner-Lange et al., 2003; Yang et al., 2007), NST1/NST2 (Mitsuda et al., 2005), and AHP4 (Jung et al., 2008). Within the anthers, secondary wall thickenings are found in the endothelium cell layer and provide part of the biophysical force that enables dehiscence, the programmed rupture of the anthers to release mature pollen (Wilson et al., 2011). Several lignin-defective mutants have recently been shown to be indehiscent and to generate defective pollen grains (Schilmiller et al., 2009; Weng et al., 2010; Thevenin et al., 2011).

In this study, we report the characterization of an anti-GAUT12 polyclonal antibody and demonstrate that immunoabsorbed-GAUT12 fractions do not contain significant HG:GalAT activity. Furthermore, we uncover a specific role for GAUT12 in anther dehiscence that underlies the sterility of *irx8* mutants. We show that while pollen development is not affected, reduced deposition of both xylan and lignin in the endothecium cell layer prevents rupture of the anthers. A similar reduction in lignin content was also observed in *irx8* stems and is correlated with reduced expression of lignin biosynthetic genes in the mutants. We showed by histochemical staining, pyrolysis molecular beam mass spectrometry (pyMBMS) and 2D HSQC NMR spectroscopy that a reduction in guaiacyl lignin contributes to total lignin reduction in the *irx8* mutant. In addition, an increased RG-I epitope in *irx8* fiber cell walls was identified, indicating a potential connection between RG-I and the *GAUT12*-dependent wall product. Our results provide further support for the hypothesis that GAUT12 may participate in the synthesis of a primer-like structure required for xylan and lignin deposition during the formation of the secondary cell wall.

RESULTS

irx8 mutant alleles and complementation by a G12 overexpression construct

All reported *irx8* mutant alleles are comparable dwarfs with collapsed xylem phenotypes (Pena et al., 2007; Persson et al., 2007). We initially selected two *GAUT12* T-DNA insertion mutants, *irx8-2* and *irx8-5*, for this study (**Figure 2.1**). Both alleles are associated with phenotypes similar to those previously described (Pena et al., 2007; Persson et al., 2007). Specifically, both mutants have a dwarfed growth habit (**Figure 2.1C**), reduced secondary cell wall thickness in xylem and fiber cells, and collapsed xylem vessels (**Figure 2.1 E, F**). The T-DNA insertion of *irx8-2* and *irx8-5* are in the promoter region and the fourth intron of *GAUT12*, respectively (**Figure 2.1A**). Semi-quantitative RT-PCR analyses revealed that *irx8-2* plants contain some residual full-length *GAUT12* transcript, whereas *irx8-5* plants contain transcripts that are truncated at the insertion site (**Figure 2.1I**). No GAUT12 protein was detectable in *irx8-5* stem microsomes (**Figure 2.2A**), suggesting that the allele is a protein null. Since both *irx8-2* and *irx8-5* mutants are similar overall, we used them interchangeably in the present study.

The *irx8* phenotypes are complemented (**Figure 2.1H, Figure 2.S1**) by the constitutive expression of an EGFP-tagged GAUT12 construct (*G12*) in both mutants. The EGFP was connected to GAUT12 via a Val-Pro linker (Pattathil et al., 2005) which may cause the EGFP-tag to bend away and therefore allow access of substrates to the predicted C-terminal catalytic domain of GAUT12 (**Figure 2.1B**). The construct produced a Golgi-localized protein when transiently expressed in tobacco epidermal cells (**Figure 2.S2**). This *G12* construct restored the *irx8* phenotype significantly as evidenced by the restoration of plant stature (**Figure 2.S1I**) and cell wall thickness to wild-type levels (**Figure 2.S3, C and G**). Cell wall neutral sugar

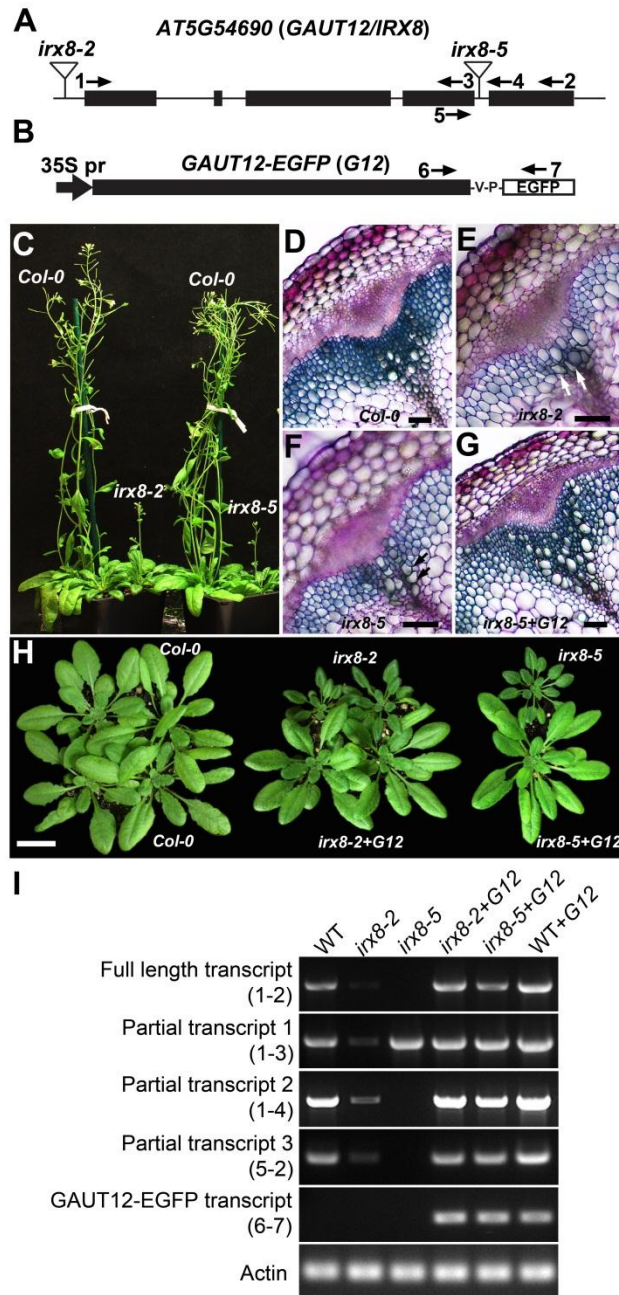


Figure 2.1. *irx8* mutant alleles are complemented by a GAUT12-EGFP construct.

(A) Relative position of T-DNA insertions in *irx8-2* and *irx8-5* and position of primers used for transcript expression levels as indicated by arrows.

(B) The *GAUT12-EGFP (G12)* construct is driven by the CaMV 35S promoter and contains the full *GAUT12* coding sequence followed by enhanced GFP via a valine-proline (-V-P-) linker.

(C) Seven-week-old *irx8-2* and *irx8-5* mutants have similar dwarf phenotypes. *Col-0*, wild-type Columbia-0.

(D) to (G) Toluidine blue-O stained free-hand transverse sections of WT (D), *irx8-2* (E), *irx8-5* (F), and *irx8-5+G12* (G). Arrows indicate collapsed xylem vessels due to reduced wall thickness, which was complemented by the *G12* construct. Bar = 50 μ m.

(H) Constitutive expression of the *G12* construct in both *irx8-2* and *irx8-5* homozygote mutants rescued the rosette leaf size of 5-week-old mutants to wild-type levels.

(I) Full length *GAUT12* transcript (by primer 1 and 2 indicated in A) was absent in *irx8-5* but reduced in *irx8-2*, while a partial N-terminal transcript (by primer 1 and 3) was also detected in *irx8-5*. Partial transcript 2 (by primer 1 and 4) and 3 (by primer 5 and 2) both spanning the T-DNA site were absent in *irx8-5*. The *G12* expression was only detected in the transgenic plants (by primers 6 and 7). Total RNAs were harvested from bottom stems (1 inch from soil) of corresponding plants.

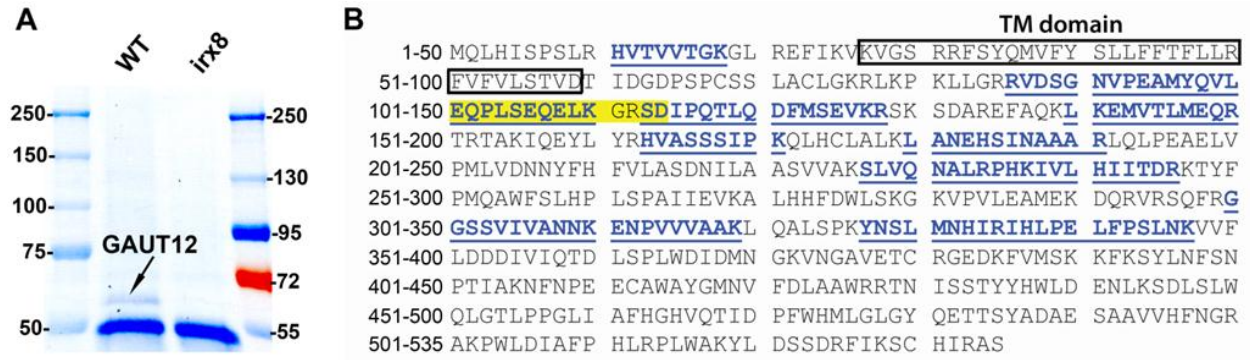


Figure 2.2. Characterization of the anti-GAUT12 polyclonal antibody.

(A) Western blot analysis of immunoprecipitated (IP) GAUT12 fractions from wild type (WT) and *irx8-5* seven-week-old stem microsomes (500 µg total protein), using 30 µl anti-GAUT12 antibody-conjugated magnetic beads, respectively. GAUT12 protein band as indicated (~58-60 kDa) is present in WT but absent in *irx8-5* microsomes.

(B) GAUT12 protein sequence (535-a.a.). The antigenic-peptide used to generate the anti-GAUT12 antibody is highlighted yellow. Peptides identified in GAUT12-IP fractions by LC-MS/MS are labeled blue with underlines. The predicted GAUT12 transmembrane (TM) domain is shown as labeled.

composition analyses showed that *irx8-5+G12* and *irx8-2+G12* plants have significantly increased xylose content compared with the *irx8-5* mutant (**Figure 2.S1K**).

Anti-GAUT12 antibody specifically immunoprecipitates GAUT12 from WT Arabidopsis stem microsomes

To enrich for GAUT12 and study its enzyme activity, a rabbit polyclonal antibody was generated against an antigenic synthetic-peptide of GAUT12 (**Figure 2.2B**) and affinity-purified against the antigen peptide. Due to the low abundance of GAUT12 protein present in stem microsomes, the anti-GAUT12 antibody was not able to detect a clear GAUT12 signal in WT stem microsomes (~50 µg total proteins) via western blot analysis (**Figure 2.S4**, lane 5). The immunoabsorbed-GAUT12 from WT stem microsomes (~500 µg total proteins), on the other hand, appeared as a ~58-60 kDa band on western blots, and was absent in immunoabsorbed fractions incubated with a comparable amount of *irx8-5* stem microsomes (**Figure 2.2A**). To confirm that the detected ~58-60 kDa protein band was GAUT12, tryptic peptides were prepared from the protein band resolved from a scale-up immunoprecipitation preparation and sequenced by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Fifteen peptides were recovered, representing ~25% of the GAUT12 protein sequence (**Figure 2.2B**). The purified anti-GAUT12 antibody did not react with either GAUT1 or GAUT7 (**Figure 2.S4**), and LC-MS/MS sequencing of the entire IP fraction did not recover any other GAUT proteins besides GAUT12, confirming the specificity of the anti-GAUT12 antibody (see **APPENDIX C**).

Enzymatic activity of GAUT12

The separation of the three major GAUT clades already existed in basal land plants including *P. patens* and *S. moellendorffii* because there are corresponding orthologs for each clade from these species (Yin et al., 2010). This suggests that the potential functional divergence of GAUT12 in clade-C from GAUT1 in clade-A occurred already in lower land plants. However, due to the small reduction in α -1,4-linked GalA found in *irx8* stems (Persson et al., 2007), and to the high level of GAUT12 protein sequence identity (40%) and similarity (61%) to GAUT1 (Sterling et al., 2006), we tested whether GAUT12 is functionally similar to GAUT1, by determining whether it synthesized homogalacturonan.

We first compared stem microsomes prepared from *irx8* mutants versus WT for HG:GalAT activity and found that equal amounts of *irx8* stem microsomes contained 55% less HG:GalAT activity compared to WT (**Figure 2.3A**). However, we reasoned that GAUT1 may contribute the majority of this activity due to GAUT1 expression in Arabidopsis stems (Atmodjo et al., 2011). Moreover, we considered that GAUT1 HG:GalAT activity may have been reduced in *irx8* stems due to their retarded growth. We therefore tested whether the immunoabsorbed-GAUT12 fraction had HG:GalAT activity. We found that the immunoabsorbed-GAUT12 from WT stem microsomes had no significant HG:GalAT activity (**Figure 2.3B**). The GAUT1:GAUT7 complex immunoabsorbed from WT Arabidopsis suspension culture- and WT stem microsomes were used as positive controls, and the HG:GalAT activity of GAUT1:GAUT7 immunoabsorbed from WT stem total microsomes was ~20% of that immunoabsorbed from WT suspension cell cultures.

Since GAUT12 protein was not detected in WT suspension culture cell microsomes by immunoprecipitation (Chapter 3, Figure 3.S7), we tested the HG:GalAT activity in the

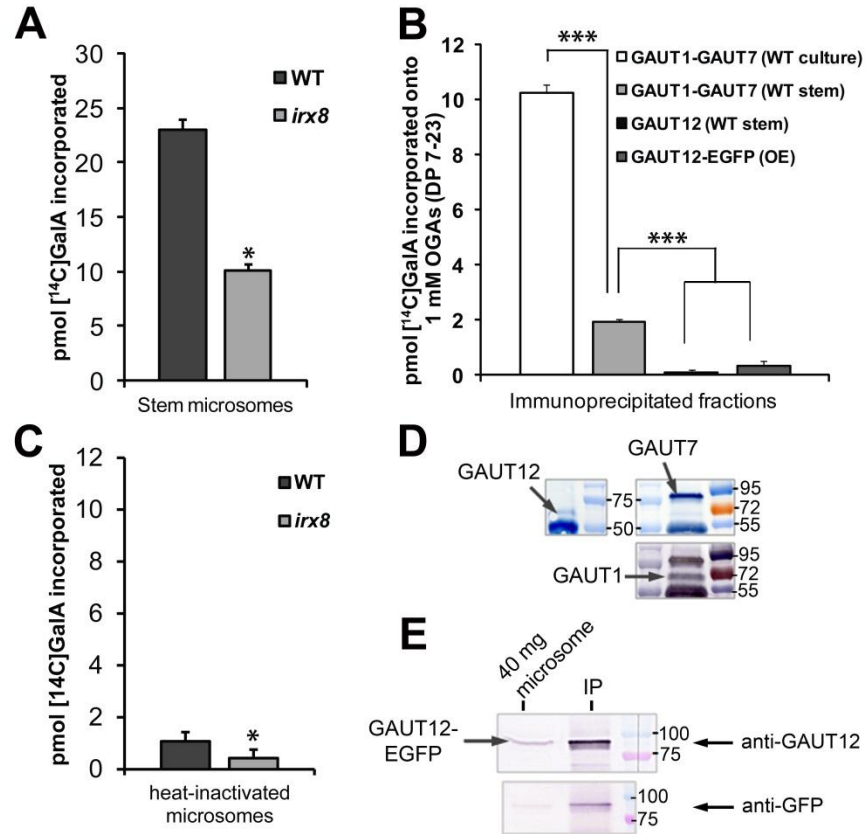


Figure 2.3. Enzymatic activity assays for GAUT12.

(A) HG:GalAT activity of total microsomes (50 μ g) from WT and *irx8-5* stems. Value = mean $\pm$ standard deviation (n=3), * indicates significant reduction of HG:GalAT activity in *irx8* stem microsomes at P (*t*-test) $\leq$ 0.05.

(B) HG:GalAT activity of immunoabsorbed-GAUT12 from WT stem microsomes and from GAUT12-EGFP fusion protein expressed in Arabidopsis suspension culture cells (OE). Immunoabsorbed-GAUT1:GAUT7 complex from WT suspension cell cultures and from WT stem microsomes were used as positive controls. Equal amounts of the corresponding antibody-conjugated beads (30 μ l) were incubated with equal amounts of TX-100 permeabilized microsomes from different tissues (500 μ g total protein). Value = mean $\pm$ standard deviation (n=3), *** indicates significant reduction of HG:GalAT activity at P (*t*-test) $\leq$ 0.001.

Immunoabsorbed-GAUT12 and GAUT12-EGFP activity are similar to background with P (*t*-test) values = 0.44 and 0.22, respectively.

(C) Immunoabsorbed-GAUT12 fractions were incubated with UDP-[¹⁴C]GalA and heat-inactivated microsomes (94 µg) from WT and *irx8* stems for 1.5 h at 29°C. Value = mean ± standard deviation (n=3). The *irx8* heat-inactivated microsomes provide less acceptor substrates for [¹⁴C]GalA incorporation ($P_{t\text{-test}} = 0.02$).

(D) Western blots showing the presence of GAUT12, GAUT1, and GAUT7 in the corresponding immunoabsorbed fractions. GAUT12 was immunoabsorbed by the anti-GAUT12 antibody, and the GAUT1:GAUT7 complex was immunoabsorbed by the anti-GAUT7 antibody.

(E) GAUT12-EGFP (~86 KDa) fusion protein in the anti-GAUT12 immunoabsorbed fractions used in reactions in (B).

All enzyme activity assays were repeated at least twice. One set of representative results is shown.

GAUT12-EGFP fusion protein immunoabsorbed from microsomes prepared from WT Arabidopsis suspension cell cultures that stably expressed the *G12* construct (**Figure 2.1B**). The GAUT12-EGFP protein also had little HG:GalAT activity, insignificant from background levels ($P_{t\text{-test}} = 0.22$) (**Figure 2.3B**).

Most glycosyltransferases produce a unique glycosidic linkage using specific nucleotide-sugar and acceptor substrates, which are critical for enzymatic activity and for *in vitro* assays. We reasoned that the GAUT12 acceptor substrate would be enriched in the *irx8* stem microsomes due to the absence of GAUT12 activity. We therefore used heat-inactivated *irx8* and WT stem microsomes as acceptor substrates to test for GalAT activity of the immunoabsorbed-GAUT12 fraction, in the presence of UDP-[¹⁴C]GalA (**Figure 2.3C**). A low level of GalAT activity was obtained for both acceptors, and less GalA incorporation was observed using *irx8* heat-treated microsomes compared with wild-type microsomes. These results suggest that either the heat-inactivated microsomes do not contain any or insufficient amounts of GAUT12 acceptors, or that UDP-D-GalA is not the correct nucleotide-sugar substrate for GAUT12.

It is highly speculated that GAUT12 is involved in the synthesis of the XRES β -D-Xylp-(1 $\rightarrow$ 3)- α -L-Rhap-(1 $\rightarrow$ 2)- α -D-GalpA-(1 $\rightarrow$ 4)-D-Xylp (Pena et al., 2007) found in birch, spruce, and Arabidopsis, presumably adding the GalA onto the xylose due to GAUT12 homology to GAUT1 (Pena et al., 2007). We therefore tested WT stem microsomes for the ability to catalyze the addition of GalA from UDP-D-GalA onto 4-methylumbelliferyl- β -D-xylopyranoside and on 4-nitrophenyl- β -D-xylopyranoside, substrates that mimic the reducing-end xylose. We observed no addition of GalA onto either of these acceptor substrates based on HPLC analyses of the products under our assay conditions (data not shown). Currently, it remains a major challenge to further test this speculation, since it is not known whether the reducing-end xylose is free or

attached to some moiety during xylan synthesis *in vivo* (Pena et al., 2007; York and O'Neill, 2008).

The irx8 phenotypes cannot be complemented by GAUT1

To confirm our *in vitro* HG:GalAT assay results suggesting that GAUT12 has a divergent function from GAUT1, we tested if GAUT1 could replace GAUT12 function *in vivo*. We transformed heterozygous *irx8* plants with two GAUT1 constructs and selected for homozygous *irx8* plants with the transgenes. The GAUT1 constructs were: (1) the full-length *GAUT1* cDNA driven by a constitutive CaMV 35S promoter, and (2) a truncated-*GAUT1* sequence encoding the Arabidopsis processed-form of GAUT1 (Atmodjo et al., 2011), placed behind a partial GAUT12 5'-sequence driven by the *GAUT12* promoter (**Figure 2.4A**). We did not recover any complemented *irx8* plants upon analyzing >80 transgenic plants for each construct. The homozygous *irx8* plants carrying either construct remained similar in size and stature to non-transformed *irx8* mutants (**Figure 2.4B**). These results confirm that GAUT12 has a function different from GAUT1 and establish that GAUT12 is not an HG:GalAT comparable to GAUT1.

Reduction in lignin and xylan leads to indehiscent anthers in irx8

While growing the *irx8* mutants for stem microsome preparations, we noticed that both *irx8-2* and *irx8-5* mutants produced small and empty siliques with no seeds. Comparably, prior studies have reported various alleles of homozygous *irx8* mutants to be “semi-sterile” (Brown et al., 2005; Persson et al., 2007). Thus, homozygous *irx8* mutants are always selected as segregants produced by heterozygous parents, and heterozygous *irx8* mutants are used for transformation to obtain transgenic plants. We decided to investigate the reasons causing *irx8* to



Figure 2.4. *GAUT1* constructs and *GAUT1*-transformed *irx8* plants.

(A) The *G12+1* construct is driven by the *GAUT12* promoter (pr, ~2 Kbp upstream the ATG site) and contains the first 903 bp of the *GAUT12* 5' gDNA sequence connected directly to a partial *GAUT1* cDNA sequence encoding the processed-*GAUT1*. The construct encodes a chimeric protein containing the 1-114 a.a. of *GAUT12* and the 169-673 a.a. of *GAUT1*. The *GAUT1* construct is driven by the CaMV 35S promoter and contains the full-length *GAUT1* coding sequence (1-673 a.a.). TM, predicted transmembrane domain.

(B) The homozygous *irx8* mutant has smaller rosette size with dark green color, while the *irx8* heterozygote (HT) mutant appears WT-like. Expression of either construct in *irx8-5* did not complement the mutant phenotype. Bar = 3 cm.

be sterile. First, we observed open flowers of *irx8* and WT at stage 13 and 14 (anthesis and fertilization) (Bowman, 1994), respectively under a dissecting microscope (**Figure 2.5**). An open flower of *irx8-5* contains reproductive organs of the correct shape but smaller in size than wild type (**Figure 2.5A**), with shorter stamens as previously described (Persson et al., 2007). At this stage, wild-type anthers have already dehisced and pollen grains are released onto the stigma and style (**Figure 2.5B**, arrowheads), while *irx8-5* has smooth intact anthers with no pollen release (**Figure 2.5C**). The same phenotype was also observed in *irx8-2* (**Figure 2.S5**).

Secondary wall thickening in endothecium cells of pollen sacs provides the mechanical strength for anther dehiscence. After anther dehydration and pollen swelling, the stomium breaks open to release pollen at anthesis, followed by anther filament extension to achieve fertilization (Wilson et al., 2011). Phloroglucinol-HCl staining (Wiesner test) has been used to colorimetrically identify coniferaldehyde end-groups in G lignin, and Mäule reagent has been reported to react with syringylpropane moieties of S lignin to produce a rose red color (Lewis and Yamamoto, 1990). The lignin of the secondary thickening in wild type mature anther stains red using phloroglucinol-HCl along the stomium furrow (**Figure 2.5D**, **F** arrowhead). In contrast, the stomium furrow was not stained at all in *irx8* anthers at the same stage (**Figure 2.5E**, **G**), indicating indehiscent anthers and likely a reduction of lignin in the *irx8* endothecium cell layer. In addition, wild type pollen stains brown-red with Mäule reagent when released from dehiscent anthers or at the stomium opening (**Figure 2.5H**). The pollen sacs also partially stain brown-red since the stain was able to access the endothecium layer due to the open stomium (**Figure 2.5J**). However, at the same developmental stage (anthesis), such staining is absent in *irx8* (**Figure 2.5I**, **K**). We also observed that in *irx8* flowers, anthers appear to be turgid (**Figure 2.5C**, **Figure 2.S5**) and enzymatic lysis of the stomium and septum breakage appear to have occurred (**Figure 2.5M**,

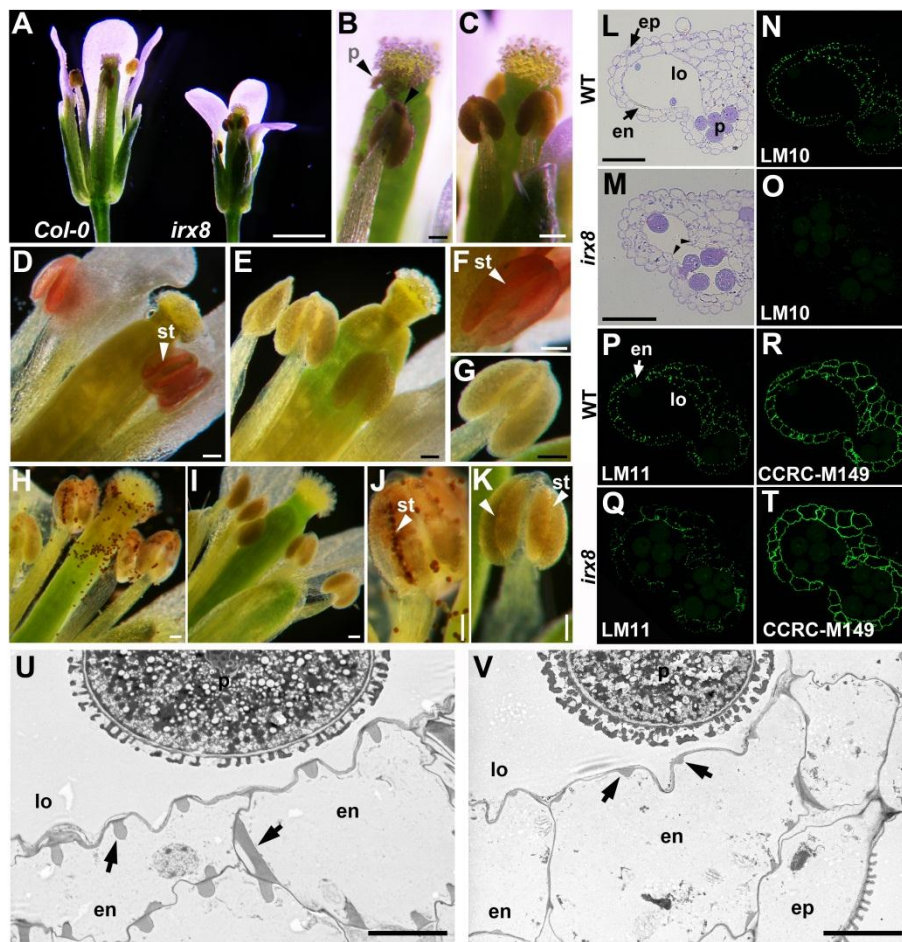


Figure 2.5. Loss of lignin and reduction in xylan deposition in anther endothecium cells results in failure of anther dehiscence in *irx8* mutants.

(A) Light microscope images of open flowers (stage 13, anthesis) from *Col-0* (B) and *irx8-5* (C) stained with phloroglucinol-HCl (D to G) and Mäule reagents (H to K). Bar in (A) = 1 mm; bar in (B) to (K) = 0.1 mm. p, pollen; st, stomium.

(D) to (G) Red phloroglucinol-HCl staining of flower anther indicates presence of lignin. Endothecium layer is stained red in wild type (D), lack of staining in *irx8-5* (E), close-up of a wild type anther (F, stomium indicated by arrow), and close-up of an *irx8* anther with no staining (G).

(H) to (K) Mäule staining of flowers of the wild type: pollen and dehiscent anther walls stained red (H); lack of staining in *irx8-5* (I); close-up of wild type anther stomium (J, arrowhead), released pollen stained dark red; close-up of *irx8-5* stomium (K, arrowhead), no lignin staining and no pollen released.

LR White-embedded (L) WT and (M) *irx8-5* anther transverse sections (250 nm-thick) were stained with toluidine blue O, arrowheads point to septum breakage. WT (N, P, R) and *irx8* (O, Q, T) anthers were immunolabeled with xylan antibodies LM10 (N, O), LM11 (P, Q), and CCRC-M149 (R, T). Bar = 50 μm for (L) to (T).

(U) TEM of WT and (V) *irx8-5* anther. Arrows point to the secondary wall thickenings in the endothecium cell layer, which is reduced in *irx8-5*. ep, epidermis; en, endothecium; p, pollen; lo, locule. Bar = 5 μm for (U) and (V).

black arrowheads). The indehiscent anther phenotype was confirmed in both *irx8-2* and *irx8-5* mutants by SEM. This phenotype was complemented by the GAUT12-EGFP (*G12*) construct (**Figure 2.6A, Figure 2.S5**).

The secondary wall thickening of the endothecium cells contains not only lignin, but also xylan and cellulose (Wilson et al., 2011). Since *irx8* has a known xylan defect, we embedded open flowers of *irx8* and analyzed anther cell walls using plant cell wall glycan-directed monoclonal antibodies against xylan and other major polysaccharides (Pattathil et al., 2010). In wild type endothecium cell walls, LM10 which binds low-substituted xylan and LM11 which binds both low- and high-substituted xylan (McCartney et al., 2005) showed almost identical punctate labeling patterns (**Figure 2.5N, P**), demonstrating a common location of the two xylan epitopes recognized by these two antibodies. Both epitopes are reduced in *irx8* (**Figure 2.5O, Q**), with LM10 labeling almost completely absent in *irx8*, indicating a pronounced reduction in low-substituted xylan deposition. Transmission electron microscopy (TEM) showed that the secondary wall thickening in WT endothecium cells, in the shape of teeth- and ribbon-like structures (**Figure 2.5U**, arrows), was significantly reduced in *irx8* endothecium cells (**Figure 2.5V**, arrows). Residual xylan present in the *irx8* endothecium was detected using CCRC-M149 (**Figure 2.5T**), CCRC-M137, CCRC-M138, and CCRC-M160 (**Figure 2.S6, O, S, T**), suggesting that *GAUT12* affects only partial xylan synthesis and that residual xylan with unchanged epitopes is present. No apparent difference is obtained in *irx8* endothecium walls using pectin antibodies JIM5, JIM7, and CCRC-M38 (**Figure 2.S6, U-W**) and little change is observed using CCRC-M14, JIM13, and CCRC-M1 in this tissue, which recognize RG-I backbone, AGP, and fucosylated xyloglucan, respectively (**Figure 2.S6, X-Z**).

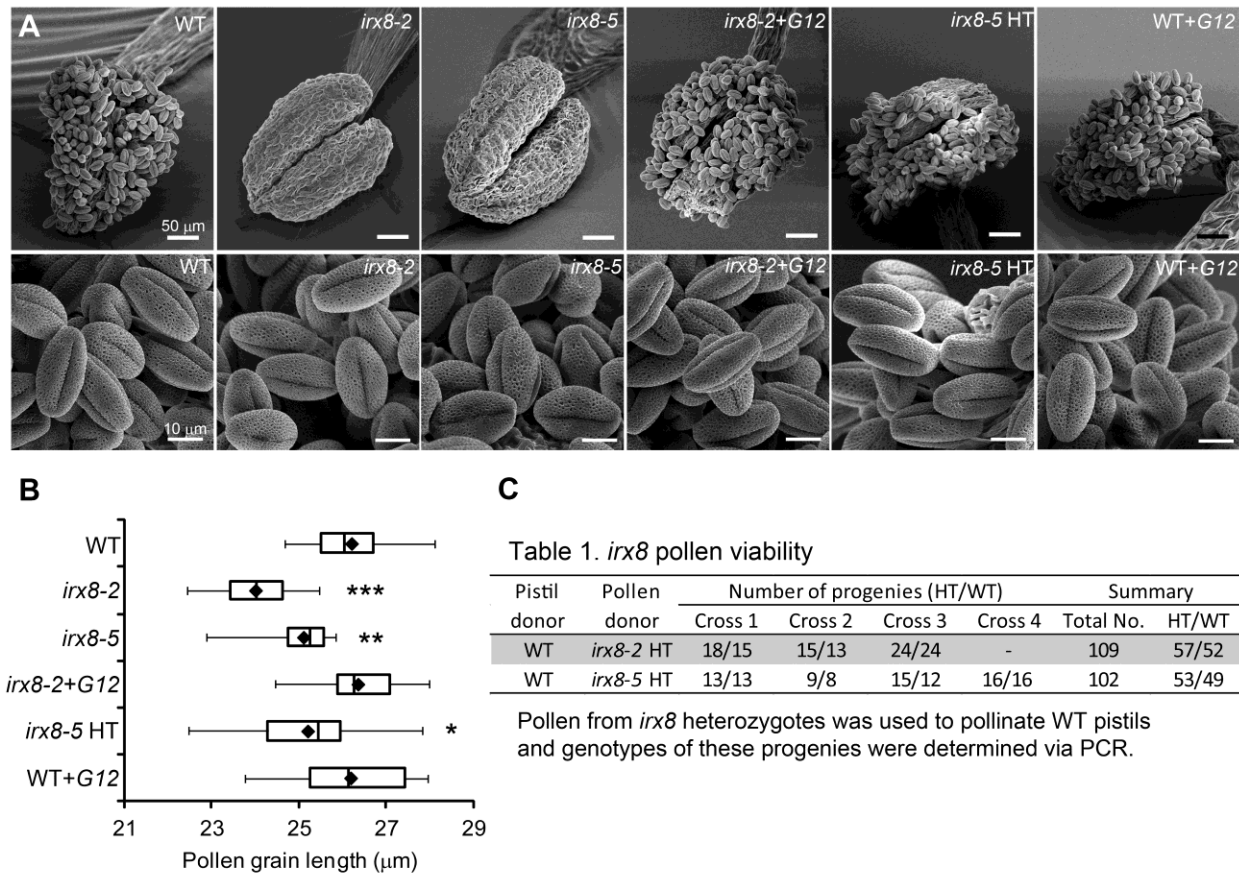


Figure 2.6. SEM of anthers and pollen grains (PG) of *irx8* and *G12*-transgenic plants.

(A) SEM images of open flower anthers and PG of WT, *irx8-2*, *irx8-5*, *irx8-2+G12*, *irx8-5* heterozygote (HT), and WT+*G12*. Bar = 50 μ m in upper panel and = 10 μ m in lower panel.

(B) Box plot diagram of PG length measured under SEM of WT (n=10), *irx8-2* (n=19), *irx8-5* (n=21), *irx8-2+G12* (n=20), *irx8-5* HT (n=26), and WT+*G12* (n=25). n = number of PG measured. PG length is significantly smaller in *irx8-2* and *irx8-5* and is complemented by *G12*. $P_{t\text{-test}} \leq 0.05$ (*), ≤ 0.01 (**), and ≤ 0.001 (***).

(C) The *irx8* pollen viability test. Pollen from heterozygous *irx8* plants was used to pollinate WT pistils and their progenies were genotyped by PCR.

TEM revealed smaller pollen size in *irx8-5* (**Figure 2.5V**), which was confirmed by SEM (**Figure 2.6A**). While the wild type pollen grains are uniform in size and shape, both mutant alleles of *irx8* had smaller pollen grains when manually released from pollen sacs (**Figure 2.6B**). Occasionally we observed defective *irx8* pollen grains under both SEM and TEM (data not shown). However, since pollen development is easily affected by growth conditions, and pollen formation involves both sporophytic and gametophytic factors (Braam, 1999), we asked whether *GAUT12* affects pollen fertility. We found that while manually released *irx8* pollen was able to pollinate both WT and heterozygous *irx8* pistils to produce viable seeds, manual fertilization of the *irx8* pistil with its own pollen has not been successful. It appears that *irx8* inflorescence is often dried soon after the stem stops elongation, which is likely due to the poor water conduction of this mutant. To further analyze whether *GAUT12* affects pollen tube formation and viability, we manually fertilized WT pistils with *irx8* heterozygote pollen, a population of which contains both *irx8* and WT pollen formed in the same anther locule. The number of heterozygote vs. WT progeny from these crosses were 57:52 and 53:49 for *irx8-2* and *irx8-5* heterozygotes, respectively (**Figure 2.6C**), suggesting that *irx8* pollen has normal fertility. Therefore, we conclude that the sterility of *irx8* mutants is due to indehiscent anthers not able to release viable pollen for fertilization.

The irx8 mutant has reduced lignin content in basal stems due to reduced expression of major lignin biosynthetic genes

Reduction of lignin in the *irx8* endothecium cell layer led us to examine the lignin in *irx8* stems by histochemical staining. We found an expected reduction in total lignin in *irx8* stems (**Figure 2.7**). Wild-type xylem vessels and fibers had thicker walls with evenly distributed lignin

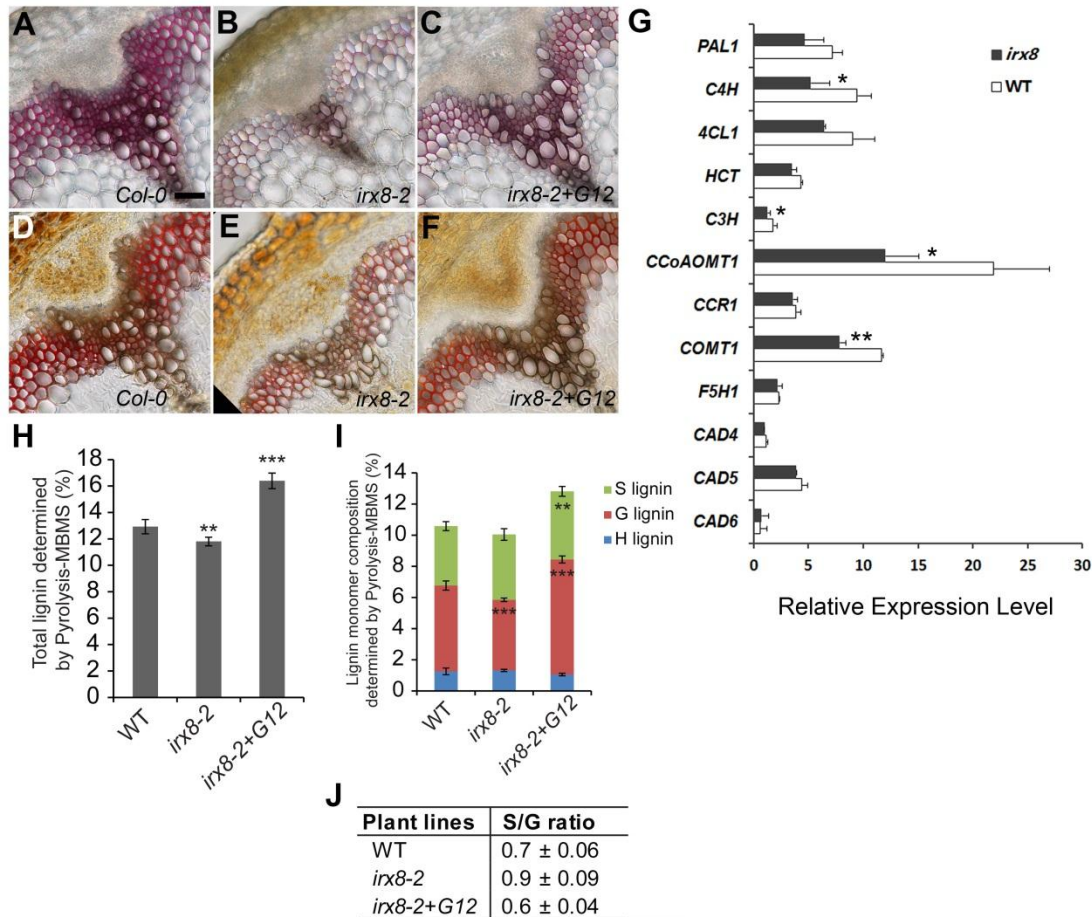


Figure 2.7. Lignin analysis and lignin biosynthetic gene expression in the *irx8* mutant.

Phloroglucinol-HCl staining of free-hand basal stem cross-sections of (A) WT, (B) *irx8-2* and (C) *irx8-2+G12*. Mäule staining of free-hand basal stem cross-sections of (D) WT, (E) *irx8-2* and (F) *irx8-2+G12*. Bar = 50 μ m.

(G) Expression analyses in Arabidopsis WT and *irx8* lower stems of lignin biosynthetic genes using Real-Time PCR. Genes labeled with asterisks have significantly lower expression in *irx8-5* compared to WT (*, $p < 0.05$; **, $p < 0.01$). Relative expression level of each gene was normalized using *Actin2* as the reference gene and the expression of *C3H* in wild type basal stem was set to 1. Values = mean $\pm$ standard deviation ($n=3$). Lower-stem refers to the lower half of the inflorescence. PAL, phenylalanine ammonia lyase; C4H, trans-cinnamate 4-hydroxylase; 4CL,

4-coumarate: CoA ligase; HCT, hydroxycinnamoyl-CoA: shikimate/quinic acid hydroxycinnamoyltransferase; C3H, *p*-coumarate 3-hydroxylase; CCoAOMT1, caffeoyl-CoA 3-*O*-methyltransferase; CCR, cinnamoyl-CoA reductase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid/5-hydroxyferulic acid *O*-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; PER/LAC, peroxidases/laccases.

(H) Total lignin (%) determined by pyrolysis molecular beam mass spectrometry (pyMBMS) of stem alcohol insoluble residues.

(I) Lignin monomer composition determined by pyMBMS. The *irx8* and *irx8+G12* values were compared to WT (**, $p < 0.01$; ***, $p < 0.001$) for (H) and (I).

(J) S/G ratio determined by pyMBMS.

while *irx8* had reduced lignin deposition, as shown in interfascicular fiber cells by phloroglucinol-HCl staining (**Figure 2.7B**), indicating a loss of G lignin. The brown color stained with Mäule reagent suggests a lower S lignin content (Guo et al., 2001). The brown color of the *irx8* xylem and the reduced red color in the interfascicular fiber cells with Mäule reagent (**Figure 2.7E**) indicate a reduction of S lignin in *irx8* stems.

To determine whether the reduced lignin content was due to reduced expression of specific lignin biosynthetic genes, we evaluated in the bottom half of *irx8* stems the expression of major genes encoding ten enzymes (**Figure 2.7G**) involved in the lignin biosynthesis pathway (Raes et al., 2003). A significant reduction of expression was observed in key biosynthetic enzymes including trans-cinnamate 4-hydroxylase (C4H), *p*-coumarate 3-hydroxylase (C3H), caffeoyl CoA *O*-methyltransferase (CCoAOMT1), and caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (COMT1) that are likely to affect lignin precursor generation in the *irx8* mutant (**Figure 2.7G**).

To evaluate the lignin structure and composition changes in *irx8*, alcohol insoluble residue (AIR) prepared from stem tissues was analyzed using pyrolysis molecular beam mass spectrometry (pyMBMS) (Sykes et al., 2008). While the NIST (National Institute Standards and Technology) standard for Arabidopsis is not available, the lignin percentage is corrected using a standard of eastern cottonwood (*NIST 8492*). There is a significant reduction in total lignin in *irx8* stems, which is complemented in the *irx8+G12* stems (**Figure 2.7H**). The reduction of total lignin in *irx8* stems is largely due to a significant reduction of guaiacyl (G) subunits, while the amount of *p*-hydroxyphenyl (H) and syringyl (S) units in *irx8* were similar to those in wild type (**Figure 7I**), resulting in an S/G ratio increase from 0.7 (WT) to 0.9 (*irx8*) (**Figure 2.7J**).

The stem AIR was sequentially extracted with 50 mM ammonium oxalate, 50 mM sodium carbonate, 1 M and 4 M KOH, acidified sodium chlorite, and post-chlorite 4 M KOH buffers (Pattathil et al., 2012). We first used 2D ^{13}C - ^1H heteronuclear single-quantum correlation (HSQC) NMR spectroscopy to analyze the chlorite fractions extracted with acidified sodium chlorite, which delignifies the biomass. The aromatic region in the HSQC spectrum of the *irx8* chlorite fraction revealed a dramatic loss of G lignin C/H-2,5,6 signals (**Figure 2.8B**), which is consistent with the reduction in G lignin shown by phloroglucinol-HCl staining (**Figure 2.7B**) and pyMBMS (**Figure 2.7I**). The G lignin cross peaks were recovered in the HSQC spectrum of the *irx8*+G12 chlorite fraction (**Figure 2.8C**). Interestingly, only trace amounts of H and S lignin signals were identified in the chlorite fraction across all three samples, indicating that either these lignin monomers (H and S) were lost during the extraction and dialysis procedure or that they were located in other fractions. We then examined both WT and *irx8* 1 M KOH-, 4 M KOH-, post-chlorite 4 M KOH fractions, and residual pellets by HSQC NMR spectroscopy. Surprisingly, we found that both the 1 M and 4 M KOH fractions of WT and *irx8* contained most of the H lignin, with the major H lignin signals located in the 1 M KOH fractions (**Figure 2.9A-D**). The G lignin in the *irx8* mutant was found almost exclusively in the 1 M KOH fraction (**Figure 2.9B**), while the G lignin in the wild type was located in both the chlorite- and post-chlorite 4 M KOH fractions (**Figure 2.9E, G**). These results suggest that the G lignin is easily extracted in the *irx8* mutant, possibly due to the xylan defect in the walls (Pena et al., 2007; Persson et al., 2007). The lignin aliphatic (side-chain) regions in the HSQC NMR spectra of the *irx8* chlorite fractions suggested a reduction in the signals for β -O-4, β -5, and β - β linked lignin (**Figure 2.S7B**), which are the major and thermodynamically favorable linkages within lignin polymers (Sangha et al., 2012). Only trace amounts of S lignin were found across all five

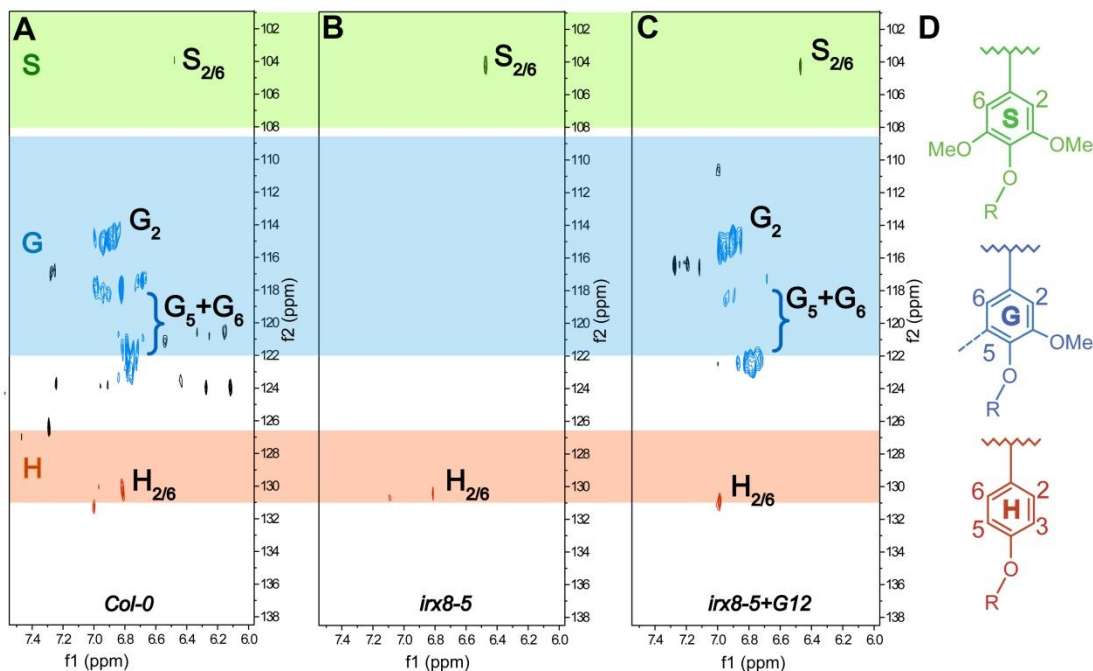


Figure 2.8. Aromatic regions of 2D ^{13}C - ^1H Heteronuclear Single-Quantum Correlation (HSQC) NMR spectra of chlorite fractions extracted from (A) WT, (B) *irx8*, and (C) *irx8+G12* stem alcohol insoluble residues (AIR).

The $^{13}\text{C}/^1\text{H}$ signals for G lignin were absent in the HSQC spectrum of *irx8-5* compared to wild type, while the G lignin signals were recovered in the spectrum of *irx8-5+G12*.

(D) Lignin monomer structures.

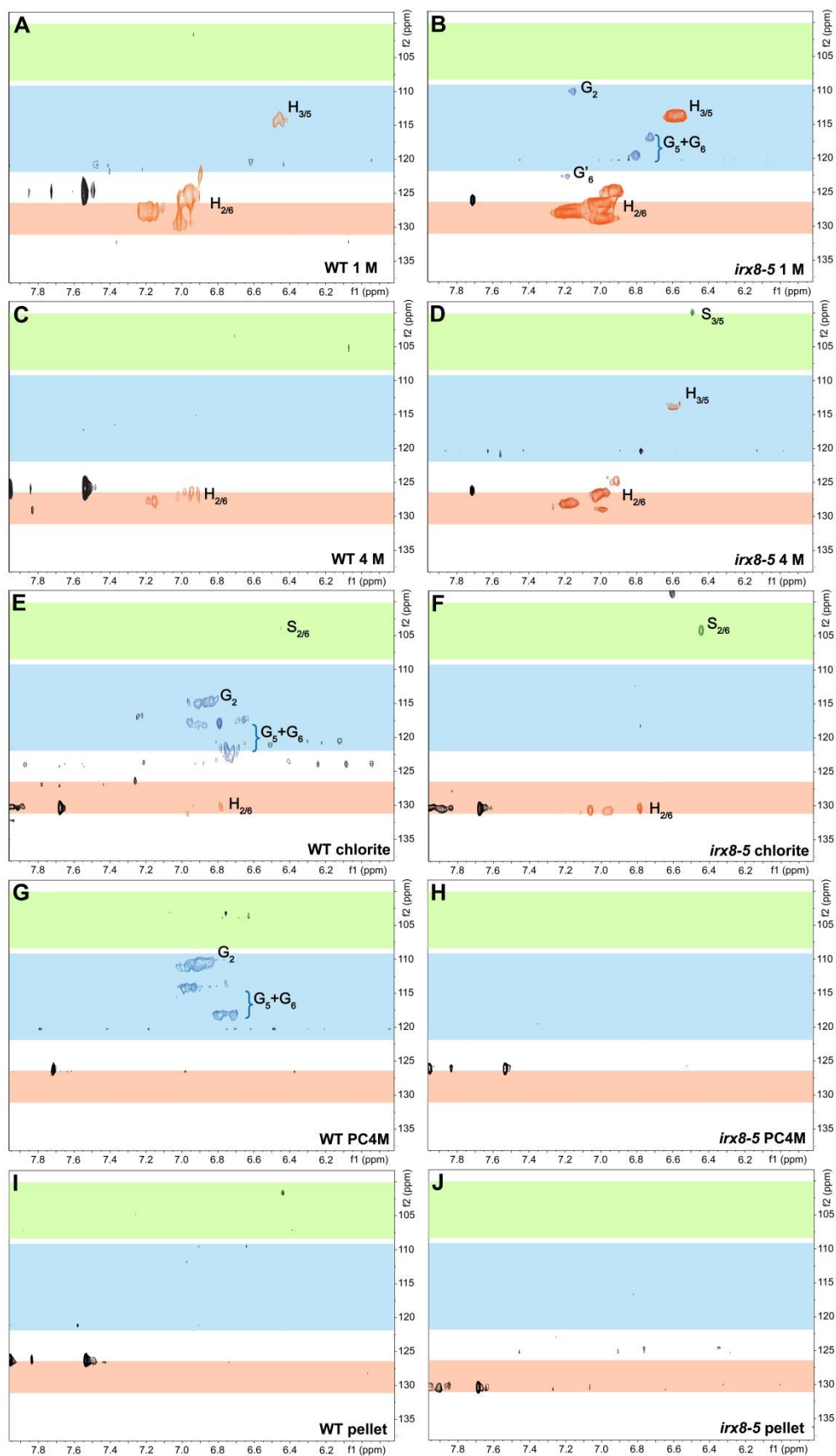


Figure 2.9. Aromatic regions of 2D ^{13}C - ^1H Heteronuclear Single-Quantum Correlation (HSQC) NMR spectra of 1 M KOH, 4 M KOH, chlorite, post-chlorite-4 M KOH, and residual pellets of WT and the *irx8-5* mutant.

(A) WT and (B) *irx8-5* 1 M KOH fractions.

(C) WT and (D) *irx8-5* 4 M KOH fractions.

(E) WT and (F) *irx8-5* chlorite fractions.

(G) WT and (H) *irx8-5* post-chlorite 4 M KOH fractions.

(I) WT and (J) *irx8-5* pellets. The signals of H, S, and G lignin monomers are as labeled.

fractions, indicating that S lignin is likely lost in dialysis during the fractionation procedure. The aromatic regions of both wild-type and *irx8* pellets did not show recognizable signals for major lignin structures (**Figure 2.9I, J**).

*The *irx8* mutant exhibits more RG-I epitopes recognized by CCRC-M14 in fiber cell walls*

In addition to the significant reduction of *irx8* fiber cell wall immunolabeling by the anti-xylan antibodies LM10 (**Figure 2.10**) and LM11 labeling (**Figure 2.S8**), there is also an unexpected increase in immunolabeling by the antibody CCRC-M14, which recognizes RG-I backbones (**Figure 2.10**). CCRC-M14 detects an empty triangle-shaped epitope region in tricellular junctions of wild-type fiber cells (**Figure 2.10**). However, in the *irx8* mutants, this antibody labels the entire fiber cell wall in a dotted and lamellate pattern, suggesting alterations in RG-I-associated cell wall epitopes and/or composition in *irx8* fiber cells. Consistent with this increased CCRC-M14 labeling, the mole percentage of Rha also increased in *irx8* stem cell walls (**Figure 2.S1K**), which may be a result partially due to the large reduction in the Xyl content. The *G12* construct complemented the CCRC-M14 phenotype in *irx8* fiber cells (**Figure 2.10**). However, the over-expression of *GAUT12* in wild type (WT+*G12*) plants did not lead to an obvious phenotype or altered sugar composition of total cell walls (**Figure 2.10**, **Figure 2.S8**, and **Figure 2.S9**).

DISCUSSION

GAUT12 is required for anther dehiscence

Persson *et al.* have described the *irx8* mutants as semi-sterile (*irx8-1* and *irx8-2*) having shorter anther filaments, less pollen, and being seedless (Persson *et al.*, 2007). Indeed, in our

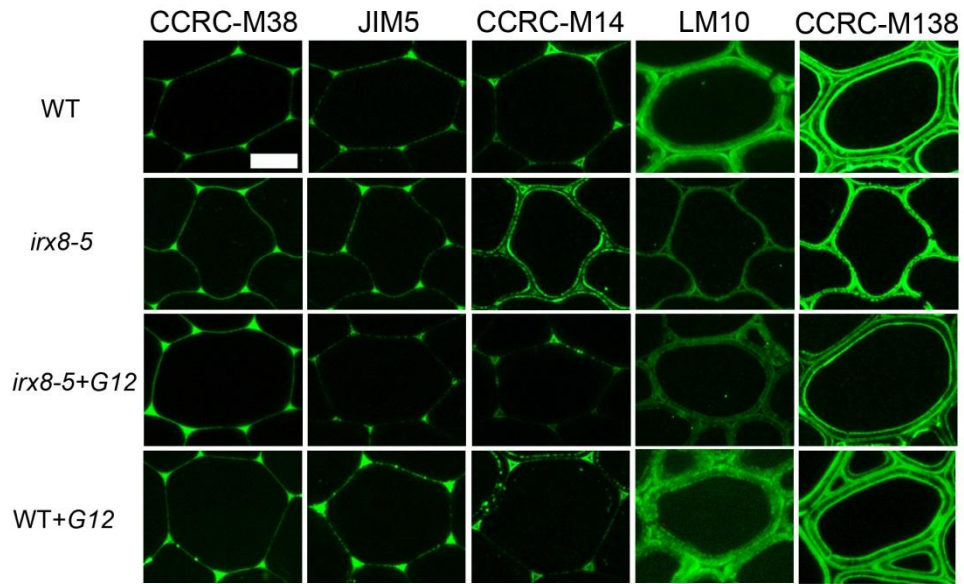


Figure 2.10. Immunolabeling of one interfascicular fiber cell from basal stems of 6-week-old wild type (WT), *irx8-5*, *irx8-5+G12*, and WT+G12 plants with anti-pectin and anti-xylan antibodies.

CCRC-M38 recognizes un-esterified HG, JIM5 recognizes low-esterified HG, CCRC-M14 recognizes RG-I backbone, LM10 (Xylan-6) binds to low-substituted xylan, and CCRC-M138 (Xylan-6) binds to xylohexaose (Pattathil et al., 2010). Bar = 10 μ m.

hands we were not able to recover any seeds from either *irx8-2* or *irx8-5* plants, even under low temperature and elevated humidity conditions. It is interesting that other dwarf mutants with collapsed xylem phenotypes, particularly *irx9* and *parvus-3*, have dehiscent anthers that release pollen (**Figure 2.S5**) and produce seeds under the same growing conditions. These results suggest that the function of *GAUT12* may, at least in the endothecium cell layer that is critical for anther dehiscence, be more important than *IRX9* and *PARVUS/GATL1*.

The reduction of lignin in *irx8* anther cell walls (**Figure 2.5E, I**), together with the reduction in xylan as recognized by LM10 and LM11 in endothecium cell walls (**Figure 2.5O, Q**), contributes to reduced secondary wall thickening in the endothecium layer (**Figure 2.5V**), and therefore results in reduced tension during anther wall dehydration leading to indehiscent anthers of the *irx8* mutants. The pollen grains produced by *irx8*, albeit smaller in size (**Figure 2.6B**), are viable upon manual release, as they are able to fertilize both wild type and *irx8* heterozygote pistils to produce viable seeds. We also demonstrated by manual fertilization of *irx8* heterozygote pollen to WT pistil that *irx8* pollen has similar viability to WT pollen (**Figure 2.6C**).

Reported transcriptomic and proteomic analyses of pollen and pollen tubes have not detected *GAUT12* transcript or protein in these tissues (Wang et al., 2008; Zou et al., 2009). And indeed, the expression of *GAUT12* is quite low in hydrated pollen grains and elongating pollen tubes compared to its expression in elongating inflorescence stems based on qRT-PCR analysis (**Figure 2.S10**). There was, however, no expression of *CESA4* or *IRX9* transcript in either hydrated pollen grains or growing pollen tubes, and very low amounts of *GATL1* transcript was detected compared to the relative high expression of *GAUT1* and *CESA1* (**Figure 2.S10B**). These results suggest that secondary cell wall cellulose and xylan are not actively produced in

germinating pollen grains or during pollen tube growth, a result consistent with the lack of xylan labeling observed in pollen grains using various anti-xylan antibodies (**Figure 2.5** and **Figure 2.S6**).

GAUT12 is required for lignification

We identified a reduction of lignin in a well-known *irregular xylem* mutant, *irx8*, previously shown to have a large reduction of xylan and small reduction in pectin (Pena et al., 2007; Persson et al., 2007). Our data show significantly reduced expression of four key lignin biosynthetic enzymes (C4H, C3H, CCoAOMT1, and COMT1) that may lead to reduced lignin precursor production in *irx8* (**Figure 2.7G**), consistent with a reduction in total lignin in *irx8* walls, in particular through a reduction in G lignin. The lignin reduction was identified using immunohistochemical staining (**Figure 2.7B, E**), pyMBMS (**Figure 2.7H, I**), and 2D ^{13}C - ^1H HSQC NMR spectroscopy (**Figure 2.8**).

Roughly the same amount of dry material (~2 mg) from each fraction was used for semi-quantitative 2D ^{13}C - ^1H HSQC NMR analyses. Only trace amounts of H and S lignin were found in the chlorite fractions across WT, *irx8-5*, and *irx8-5+G12* (**Figure 2.8**). Interestingly, H lignin signals were found in comparable amounts in WT and *irx8* 1 M and 4 M KOH fractions, respectively, with the major signals located in the 1 M KOH fractions (**Figure 2.9A-D**). These results suggest that H lignins are removed during xylan solubilization, suggesting that the H lignin may be connected to the KOH-solubilized xylan in these fractions, possibly through alkaline-labile ester linkages (Balakshin et al., 2011). Considering the total amount of glucuronoxylan (GX) extracted by 1 M and 4 M KOH, the 1 M KOH is able to remove two thirds of the GX in wild type and approximately half of the GX in the *irx8* walls, probably due to

the more heterodisperse xylan chains found in the *irx8* mutants (Pena et al., 2007). Nevertheless, the xylan heterodispersity does not seem to affect the amount of H lignin in *irx8* stem walls, as shown by the similar amounts of H lignin in wild type and *irx8* as determined by pyMBMS (**Figure 2.7I**).

The G lignin signals of wild type were found mostly in the chlorite- and post chlorite 4 M KOH fractions (**Figure 2.8A** and **Figure 2.9E, G**), indicating that the G lignin is potentially linked to wall polysaccharides via alkaline-resistant linkages such as benzyl ethers and phenyl glucosides, and that only harsh conditions like acidic sodium chlorite are able to degrade these linkages. The wild-type chlorite fraction is mainly composed of G lignin (**Figure 2.8A**). The *irx8* chlorite fraction, however, is nearly deplete of the G lignin signal (**Figure 2.8B**), consistent with the reduced phloroglucinol-HCl staining of *irx8* stem cross sections (**Figure 2.7B**) and the reduced G lignin monomer determined by pyMBMS (**Figure 2.7I**). Surprisingly, the bulk of the G lignin signals in *irx8* is found in the 1 M KOH fraction (**Figure 2.9B**), clearly indicating an altered G lignin extractability in the *irx8* mutant. These results suggest that the G lignin linkages in the *irx8* are either alkaline-labile or altered due to the reduction of xylan.

GAUT12 enzyme activity

It is highly speculated that GAUT12 synthesizes the XRES β -D-Xylp-(1-3)- α -L-Rhap-(1-2)- α -D-GalpA-(1-4)-D-Xylp (Pena et al., 2007), presumably by adding the GalA onto the reducing-end xylose due to GAUT12 homology with GAUT1, a confirmed homogalacturonan galacturonosyltransferase (Sterling et al., 2006). Whether the reducing-end xylose is free or attached to some moiety during xylan *in vivo* biosynthesis remains unknown (Pena et al., 2007; York and O'Neill, 2008), posing a major challenge in studying this hypothesis. Our attempts in

using the fluor-labeled xylose derivatives 4-methylumbelliferyl- β -D-xylopyranoside (4-MU-Xyl) and 4-nitrophenyl- β -D-xylopyranoside (pNp-Xyl) as reducing-end xylose acceptor substrates for GAUT12 were not successful. Clearly, demonstration of whether GAUT12 synthesizes this glycosyl sequence will require knowledge about the definitive structure of the intact substrate.

In addition to the ~50-60% reduction in xylan (Pena et al., 2007; Persson et al., 2007), Person et al. identified a small reduction in EPG/PME-accessible pectin in *irx8* stems, indicating that GAUT12 may provide a pectic substructure onto which xylan chains are deposited during secondary cell wall formation. We tested this hypothesis using the HG:GalAT assay optimized to study GAUT1, which is a known HG:GalAT (Sterling et al., 2006). Immunoabsorption of GAUT12 using anti-GAUT12 antibody under conditions suitable for immunoabsorption of the GAUT1:GAUT7 complex did not yield significant HG:GalAT activity using HG oligosaccharide OGA (DP 7-23 mer) acceptors. We confirmed that GAUT12 protein was efficiently immunoabsorbed from WT Arabidopsis stem microsomes, and that the assay conditions used were successful to measure the HG:GalAT activity of immunoabsorbed-GAUT1:GAUT7 from both WT suspension culture cells and WT stem microsomes. The lack of recovered HG:GalAT activity in immunoabsorbed-GAUT12, however, must be interpreted cautiously. It is possible that instead of OGAs (DP 7-23), GAUT12 uses smaller, larger, or alternative pectic substrates, or that the detergent (1% Triton X-100) used for GAUT12 purification by anti-GAUT12 antibody may not be suitable to measure GAUT12 enzymatic activity. However, the two *GAUT1* constructs (**Figure 2.4A**), one containing full-length *GAUT1* cDNA driven by a constitutive CaMV 35S promoter and the other containing the truncated-*GAUT1* that encodes the Arabidopsis processed-form of GAUT1 (Atmodjo et al., 2011) placed behind a partial GAUT12 N-terminal sequence driven by the *GAUT12* gene-specific promoter, failed to complement the

irx8 mutant phenotypes (**Figure 2.4B**). These results corroborate the lack of HG:GalAT activity of the immunoabsorbed-GAUT12 fractions and indicate that GAUT12 is not an HG:GalAT comparable to GAUT1.

Our data show that HG oligosaccharides (OGA, DP 7-23) are not efficient substrates for GAUT12. However, the results do not rule out that GAUT12 synthesizes other linkages in unique stretches of pectic domains during secondary cell wall formation. It is possible that GAUT12 is involved in making a short stretch of HG or RG-I that links to a specific type of xylan. Interestingly, in *irx8* interfascicular fiber cell walls, more RG-I epitopes are exposed for recognition by antibody CCRC-M14 (**Figure 2.10**). One explanation for this observation is that the reduction of xylan in *irx8* causes more CCRC-M14 epitopes to be exposed. Another possible explanation is that increased RG-I epitopes in *irx8* fiber cell walls are due to wall alterations in the *irx8* mutants. Previous glycosyl residue composition analyses of *irx8* stem cell walls revealed more easily extractable pectins in the EPG-, PME/EPG-, and EGTA/Na₂CO₃-released fractions from *irx8* stem cell walls. Specifically *irx8-5* had four times the amount (mol %) of rhamnose compared with the corresponding wild-type fractions, in accordance with the increased Rha identified in total cell walls of *irx8* (Persson et al., 2007). We also demonstrated an increase in Rha in *irx8* stem walls as shown in **Figure 2.S1K**. In addition, although there is 50-60% reduction of xylose in the *irx8* mutants and a large reduction in LM10-labeled xylan (Pena et al., 2007; Persson et al., 2007), residual xylan in *irx8* mutants was detected using LM11, CCRC-M137, CCRC-M138, CCRC-M149 and CCRC-M160 (**Figure 2.S8**), suggesting that GAUT12 affects a subfraction of xylan in Arabidopsis.

Lignin-pectin and lignin-xylan connections

The pleiotropic effects of *irx8* on xylan, lignin, and pectin during secondary wall formation suggest that different cell wall polymers interact and imply an order in which they are deposited *in muro*. As a glue to strengthen the polysaccharide wall, lignification appears at two distinct stages. In the first stage, lignin deposition is known to start as early as during the formation of the middle lamella at the cell plate and then at the cell wall corners, indicating the existence of possible pectin initiation/nucleation sites for the primary wall lignin (Donaldson, 2001). Our data demonstrate that *Arabidopsis* interfascicular fiber tricellular junctions are filled with un-esterified to low-esterified homogalacturonan, as recognized by pectin-antibodies CCRC-M38 and JIM5 (**Figure 2.10**) in a solid triangle shape. This tricellular junction triangle also contains a small amount of high-esterified HG as labeled by JIM7 (**Figure 2.S8**). RG-I backbone as recognized by CCRC-M14 appears to be present towards the outer layer of this HG triangle, whose labeling forms an empty triangle at the tricellular junction (**Figure 2.10**). The lignin in this tricellular junction area was stained solidly for both G and S lignin (**Figure 2.7B, E**), suggesting that this process is unlikely affected in the *irx8* mutant. In the second stage of lignification, secondary cell wall lignin is believed to be deposited after the formation of secondary cell wall (Donaldson, 2001). We showed by colorimetric staining that both G and S lignin are reduced in the *irx8* xylem vessels and fiber cells, apparently due to the reduction in the wall thickness in these cells. The G lignin in the *irx8* mutant is significantly reduced (**Figure 2.7I** and **Figure 2.8B**), and extracted in the 1 M KOH fraction (**Figure 2.9B**) rather than in the chlorite fraction in wild type (**Figure 2.8A**), suggesting a possible correlation between the xylan reduction and lignin alteration in this mutant. Our data support the proposition that lignin formation is down-regulated in a xylan defective mutant, and that *GAUT12* affects both xylan

and lignin deposition. The lignin reduction may be a secondary effect due to the dramatic loss of xylan in *irx8*, since it has been reported that 4-*O*-methylglucuronoxylan is the major carbohydrate linked to lignin in wood (Yuan et al., 2011), and that xylan and lignin are linked via ferulate esters in maize (Grabber et al., 2000). The exact lignin-carbohydrate linkages in the *irx8* mutant are currently under investigation. Such information will likely generate insights into the relationships between lignin and wall polysaccharides.

We show in the present study that GAUT12 is not an HG:GalAT comparable to GAUT1. However, we were not successful in demonstrating that GAUT12 adds the GalA in the xylan reducing-end sequence (XRES). Similarly, many putative type II xylosyltransferases proposed to be involved in xylan biosynthesis have not been proven to possess *in vitro* enzymatic activity (Keegstra and Raikhel, 2001; Mohnen, 2008; Scheller and Ulvskov, 2010). *GAUT12* appears not to have any functional homologs in graminaceous monocots (Caffall et al., 2009a; Kulkarni et al., 2012). The absence of XRES in grass species examined to date would tend to support a function of GAUT12 in the synthesis of this structure. However, further study is required to identify GAUT12 enzymatic function, as well as its role in the carbohydrate-lignin connection.

METHODS

Plant materials

Arabidopsis alleles used are wild type (*Col-0*), *irx8-5* (SALK_044387), *irx8-2* (SAIL_603_G02), *parvus-3* (SALK_045368), and *irx9-1* (SALK_058238). The plants were grown on soil in a controlled-environment chamber under a 14-h-light/10-h-dark cycle at 19 °C during the light period and 15 °C during the dark period. The light intensity was 150 $\mu\text{Em}^{-2}\text{s}^{-1}$, and the relative humidity was maintained at 50%. The plants were harvested after six- to seven-

week for experiments. T-DNA insertions were confirmed using a primer from the genomic regions flanking the T-DNA and the general T-DNA left border primer (**Table 2.S1**). *Arabidopsis Col-0* and *irx8* heterozygote plants were transformed via the floral dip method (Clough and Bent, 1998), and transgenic plants were selected on ½ MS media plates containing 15 mg/L hygromycin. Transgenic plants harboring the construct in *Col-0* and *irx8* homozygote mutant backgrounds were genotyped using PCR (**Table 2.S1**).

Generation of constructs

For the *GAUT12-EGFP* construct, RT-PCR was performed using the SuperScript™ III One-Step RT-PCR System with Platinum Tag High Fidelity (Invitrogen 12574-030) and 7-week-old *Arabidopsis Col-0* stem total RNA (0.5 µg) to amplify the *GAUT12* coding sequence (CDS) (**Table 2.S1**). The *GAUT12* CDS was cloned into the over-expression construct pCambia35tl:egfps2#4 (Pattathil et al., 2005) between the *NcoI* and *KpnI* restriction sites.

For the *GAUT1* construct, the coding region of *GAUT1* was amplified by PCR using a pfx50 DNA polymerase (Invitrogen) with primers *GAUT1-F-KpnI* and *GAUT1-R-XbaI* (**Table 2.S1**). For the *G12+1* construct, the genomic fragment of *GAUT12* that contains the 2002 bp promoter region and the first 903 bp covering the stem region was amplified with *GAUT12-F-PstI* and *GAUT12-R-KpnI*, and ligated into the pCAMBIA vector. The cDNA corresponding to the catalytic region of *GAUT1* was amplified with *GAUT1Tr-F-KpnI* and *GAUT1Tr-R-KpnI*, and inserted downstream of the *GAUT12* stem region sequence. The construct was confirmed using *GAUT12*-specific and vector-specific primers and further confirmed with *PstI* enzyme digestion.

All plasmids were sequenced to verify their constructions. These plasmids were individually electroporated into *Agrobacterium tumefaciens* strain GV3101 competent cells and used for transformation into both wild-type and heterozygous *irx8* Arabidopsis plants.

Histochemical staining

Mäule reagent was prepared as described (Chapple et al., 1992) with slight modification to detect S lignin. Arabidopsis open flowers and hand-cut stem transverse sections were first treated with 0.5% KMnO₄ solution for 10 min and rinsed with water [for the flowers, this solution was supplemented with 0.01% 7X detergent (Linbro, Flow Laboratories) to break surface tension]. The flowers were then treated with 10% HCl for 5 min, rinsed with water and mounted in concentrated ammonia for microscopic observation.

Phloroglucinol-HCl stain was freshly prepared as described (Guo et al., 2001). Two parts of 2% phloroglucinol (w/v) in 95% ethanol were mixed with one part concentrated HCl. Pictures were taken 10 min after the stain was applied to samples. Stained flower samples were viewed using a dissecting scope under dark field. Stained stem transverse sections were viewed in a Nikon Eclipse80i microscope under bright field. Images were captured using a Nikon DS-Ril camera head (Nikon, Melville, NY).

Scanning electron microscopy

Manipulations of anthers were carried out under a dissecting scope. Anthers from wild type and mutant open flowers were removed using dissecting forceps and pollen was released onto specimen stubs topped with double-sided sticky carbon tabs by gently tapping the forceps, or lightly tapping the anthers onto the stubs. The *irx8* mutant anthers were first manually

dissected to open them and the pollen was gently scooped out using the forceps tip and transferred onto the stub surface. The samples were dehydrated and coated with gold particles for 120 sec in a Sputter Coater, and then imaged using a JEOL JSM-5800 (SEM/EDAX).

In vitro pollen tube growth and RNA preparation

Pollen tubes were grown *in vitro* as previously described (Dardelle et al., 2010). In brief, wild type pollen grains were collected from 40 open flowers by vortexing for 3 min in a microcentrifuge tube containing 1 ml pollen germination medium (PGM) composed of 5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01% (w/v) H_3BO_3 , 5 mM KCl, 1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 10% (w/v) sucrose (pH adjusted to 7.5 using KOH). The flowers were carefully removed and pollen grains were pelleted by 3,200 *g* centrifugation for 6 min. The old media was removed and the pollen pellet was re-suspended gently in 250 μL of fresh (PGM). The pollen grains were then transferred into a 13 $\times$ 100 mm glass tube, covered with 3M micropore tape and set in the dark at 22°C for 6 h or 24 h.

For RNA isolation, pollen grains from 200 open WT flowers were collected in PGM (collected in 5 tubes). This pollen from 200 flowers was either directly harvested as hydrated pollen grains (0.5 h) or grown for pollen tubes for 6 h and 24 h. Hydrated pollen grains (0.5 h) were combined and ground in liquid nitrogen in a microcentrifuge tube using a plastic pestle. Pollen tubes grown for 6 h and 24 h for RNA isolation were collected by centrifugation for 6 min at 3,200 *g* and ground in microcentrifuge tubes. RNA isolation was repeated using three batches of independently collected tissues.

Tissue fixation and immunolabeling

Freshly cut plant tissues were fixed immediately in 25 mM sodium phosphate buffer (pH 7.1) with 1.6% (w/v) paraformaldehyde and 0.2% (w/v) glutaraldehyde overnight at 4°C. The following steps were done with a lab-grade microwave (PELCO BioWave Pro, Ted Pella, CA) set at 250 Watt. Tissues were washed three times for 1 min each, with 25 mM sodium phosphate buffer (pH 7.1) followed by two washes with water. The samples then underwent a series of 40-second ethanol gradient incubations (35%, 50%, 75%, 95%, 100%, 100%, and 100% [v/v]) to dehydrate the tissue. The samples were infiltrated with cold LR White embedding resin (Ted Pella) in a gradient (1:3, 1:1, 3:1 resin:ethanol [v/v]) and finally three times with 100% resin. Each step was conducted under vacuum (20" Hg) for 2.5 min. After the last resin change samples were kept at 4°C for 24 h and then transferred into gelatin capsules filled with resin which were polymerized under 365 nm UV light at 4°C for 48 h. Tissue cross sections (250 nm) were cut with a Leica EM UC6 ultramicrotome (Leica Microsystems), mounted on pre-coated slides (Colorfrost/Plus, Fisher Scientific) and stained with 0.05% toluidine blue for light microscopy or used for immunolabeling experiments.

Immunolabeling for fluorescent microscopy was done as follows. Cell sections were treated with freshly made 3% non-fat dry milk (NFDM) dissolved in 10 mM KPBS (pH 7.2) with 500 mM NaCl for 30 min (blocking). The blocking solution was removed and the sections were incubated with 10 μ L of designated CCRC monoclonal antibodies for 1 h. Sections were washed three times with 10 mM KPBS (pH 7.2) containing 500 mM NaCl (wash buffer) by removal of existing droplets and application of fresh buffer followed by a 5 min incubation at room temperature for each wash. The secondary antibody, Alexa fluor 488 goat anti-mouse IgG (Cat#A11001, Invitrogen), was applied to the sections at a dilution factor of 1:100 [v/v] in wash

buffer. The sections were incubated in the dark at room temperature for 1 h. The sections were washed with wash buffer and diH₂O, dried by wicking, a droplet of mounting media (Citifluor mounting medium AF1, Electron Microscopy Sciences) added and a cover slip placed on the section. Sections were viewed under a fluorescent microscope (Nikon Eclipse80i). For LM series and JIM series antibodies, a wash buffer containing 10 mM KPBS (pH 7.2) and 100 mM NaCl was used because we found that the use of 500 mM NaCl adversely affected consistent binding of these antibodies in our hands. The secondary antibody used for the LM and JIM series was Alexa fluor 488 goat anti-rat IgG (Cat#A11006, Invitrogen) which was applied to the sections in the same fashion as described above but diluted in the low salt wash buffer. Images were captured using a Nikon DS-Ril camera head (Nikon, Melville, NY).

Transmission electron microscopy

Ultrathin sections (80 nm) were made by the ultramicrotome and collected on Formvar-coated nickel grids. The grids were stained with 2% (w/v) uranyl acetate for 4 min followed by 10 dips in 3 changes of deionized water and dried by wicking. Micrographs were recorded on film in a JEOL 100S transmission electron microscope. The negatives were developed and scanned in Adobe Photoshop.

For immunogold labeling, ultrathin sections were blocked in TBS (150 mM NaCl, pH 7.5) plus 0.06% (w/v) bovine serum albumin for 30 min at room temperature in a petri-dish containing a folded piece of water-soaked Kimwipe to one side. The sections were transferred onto 10 μ L drops of primary antibody diluted (1:5) in TBS for 1 h. After washing three times by dipping the grids (10 times) in TBS, secondary antibody (goat anti-rat IgG coupled to 15 nm gold,) diluted 1:10 in TB (20 mM Tris buffer) plus 0.06% (w/v) bovine serum albumin were

applied to the sections. The sections were then dipped in TB and distilled water for washing and dried by wicking.

Stem RNA isolation and quantitative PCR analyses

Plant tissue was harvested and flash frozen in liquid nitrogen. Stem tissues were ground to a fine powder in liquid nitrogen in pre-chilled mortars with pestles. Total RNA was isolated from ~100 mg of frozen powder from three individual tissue samples using RNeasy Plant Mini Kit (Qiagen, 74904). First-strand cDNA was synthesized from 1 µg of total RNA using SuperScript III First-Strand Synthesis Super mix (Invitrogen, 18080-400) followed by quantitative PCR analysis using iQTM SYBR Green Supermix (Bio-Rad 170-8882) on a CFX96TM Real-Time PCR Detection System (Bio-Rad), following the manufacturer's instructions. Melt curve analyses were performed after each run to ensure single size amplicon production. Data are the average ± standard deviation of three biological samples. The data were analyzed as described by Livak and Schmittgen (Livak and Schmittgen, 2001). Primer sequences are provided in **Table 2.S1**.

Generation of Arabidopsis suspension cultures

A wild-type Arabidopsis suspension culture was generated from seed callus using a previously described method (Doelling and Pikaard, 1993). An aliquot of 7 ml packed cells were transferred into 100 ml fresh medium every ten days for subculture. The GAUT12-EGFP construct was stably transformed into wild-type suspension culture cells via *Agrobacterium tumefaciens* strain GV3101. Transformed calli were selected on Gamborg B5 medium 0.6% agar plates supplemented with 15 mg/L hygromycin. Surviving calli were genotyped, and RT-PCR

was used to determine the expression level of *GAUT12-EGFP*. The *GAUT12-EGFP* transcript was found to be the highest on day 5 compared to day 2 and day 8, therefore the cells were harvested on day 6 for microsome preparations.

Microsomal membrane preparation

Microsomal membranes used for enzyme activity assays were prepared as described. Liquid nitrogen flash frozen *Arabidopsis* stems (10 grams) were cut into small pieces and homogenized on ice in 20 ml of pre-chilled homogenization buffer containing 50 mM Hepes (pH 7.3), 0.4 M sucrose, 0.1 M sodium ascorbate, 0.25 mM MnCl_2 , 25 mM KCl, 1% (w/v) polyvinylpyrrolidone (PVPP), and EDTA-free protease inhibitor cocktail (Roche) until the tissues were pureed. The homogenate was filtered through 3 layers of miracloth and the filtrate centrifuged at 4°C for 30 min at 4,000 *g* to remove cell debris and intact cells. The supernatant was ultra-centrifuged at 110,000 *g* for 1 h, yielding the microsome pellet which was re-suspended on ice in the pre-chilled storage buffer (homogenization buffer without PVPP, 30 μl buffer/gram fresh weight) using a glass homogenizer. Total protein concentration was measured using the Bradford assay (Bio-Rad Protein Assay 500-0006) and BSA as a standard. Aliquots of the microsomes were used directly or flash frozen in liquid nitrogen and stored at -80°C for later use in enzyme assays and immunoprecipitation experiments.

Generation of anti-GAUT12 polyclonal antibody and immunoprecipitation

The anti-GAUT12 polyclonal antibody was generated against synthetic peptides corresponding to GAUT12 amino acid residues 101-114 (EQPLSEQELKGRSD). The polyclonal antibody was antigen-purified over a column packed with the antigenic-peptide (service via New

England Peptide, <http://www.newenglandpeptide.com/>). The purified antibody (1.14 mg/ml) was incubated with Dynabeads M-280 Sheep anti-Rabbit IgG (Invitrogen, 112-04D) in a ratio of 1:20 (v/v) for two hours at 4°C on a tube rotator and the beads were collected on a magnet stand and washed 3× with PBS (pH 7.4). Pre-immune serum did not contain anti-GAUT12 antibody. Anti-GAUT1 and anti-GAUT7 were generated previously (Sterling et al., 2006; Atmodjo et al., 2011). For western blotting, a dilution factor of 1:5000, 1:10000, 1:3000, and 1:2000 was applied for anti-GAUT12, anti-GAUT7, anti-GAUT1, and anti-GFP (abcam, ab6556), respectively. Either horseradish peroxidase (HRP)- or alkaline phosphatase (AP)-conjugated goat anti-rabbit secondary antibody (Sigma) was used followed by a reaction with corresponding substrates for a blue or purple color precipitation on the target protein band.

For enzymatic activity assays, each reaction contained 30 µl anti-GAUT12-conjugated beads incubated at 4°C for 2 h on the tube rotator with ~500 µg Triton X-100 permeabilized microsomes. The nonionic detergent Triton X-100 (TX-100) has been used to solubilize GalAT activity during GAUT1 and GAUT7 purification (Mohnen and Doong, 1998; Sterling et al., 2006; Atmodjo et al., 2011). A concentration of 4% TX-100 was used to homogenize wild type stem microsomes on ice and immediately diluted to a final concentration of 0.5% for incubation with anti-GAUT12-conjugated magnetic beads. After 2 h end-to-end incubation at 4°C, the beads were washed with pre-chilled storage buffer on ice thrice and the original 30 µl beads were re-suspended in 13 µl pre-chilled storage buffer for each enzyme reaction. Immunoabsorbed-GAUT1 was prepared by incubating Dynabeads M-280 Sheep anti-Rabbit IgG (Invitrogen, 112-04D) with anti-GAUT7 anti-serum in a ratio of 1:3 (v/v) in parallel. Anti-GAUT7 antibody was previously shown to immunoabsorb the HG:GalAT activity-containing GAUT1-GAUT7 core complex (Atmodjo et al., 2011).

Enzyme Activity Assay

UDP-D-[¹⁴C]GalpA (specific activity 180.3 mCi/mmol; 1 Ci = 37 GBq) was synthesized enzymatically from UDP-D-[¹⁴C]GlcA using UDP-D-GlcA 4-epimerase as described (Atmodjo et al., 2011). HG:GalAT activity was assayed in 30- μ L reactions containing enzyme [15 μ L total microsomes (~50 μ g total protein) or 13 μ L immunoabsorbed beads], 50 mM Hepes (pH 7.3), 0.2 M sucrose, 0.05% (w/v) BSA, 25 mM KCl, 1.9 mM MnCl₂, 1 mM homogalacturonan oligosaccharides (referred to as oligogalacturonides, OGA) of a degree of polymerization (DP) of 7-23, and 6.9 μ M UDP-[¹⁴C]GalpA (specific activity 180.3 mCi/mmol; 1 Ci = 37 GBq). The reactions were carried out for 3 h in a 29°C water-bath. The reactions were terminated with 5 μ L 400 mM NaOH, vortexed, and the entire reaction volume spotted onto 1-inch² filter paper squares that had been pre-treated with cetylpyridinium chloride (CPC), dried, and washed 4 $\times$ in 150 mM NaCl to remove access UDP-[¹⁴C]GalpA. The spotted filter paper squares were dried and added to 4 ml ScintiVerse™ BD cocktail for scintillation counting.

The *irx8* heat-inactivated microsomes were prepared as follows. An aliquot of 400 μ L *irx8* stem microsomes (6 μ g/ μ L) was ultracentrifuged at 110,000 *g* for 1 h, re-suspended in 210 μ L diH₂O, and boiled at $\geq$ 95°C for 15 min to inactivate enzymatic activity. The GalAT activity was assayed in 30- μ L reactions containing 13 μ L immunoabsorbed-GAUT12 beads, 50 mM Hepes (pH 7.3), 0.2 M sucrose, 0.05% (w/v) BSA, 25 mM KCl, 1.9 mM MnCl₂, 8 μ L of *irx8* heat-inactivated microsomes (equivalent of 91.4 μ g total protein), and 6.9 μ M UDP-[¹⁴C]GalpA. The reactions were incubated for 90 min in a 29°C water-bath and terminated by adding 700 μ L of chloroform:methanol (3:2, v/v). An aliquot of 90 μ g OGA was added as a carrier, pellets were obtained by centrifugation at 13,200 rpm and washed thrice with 65% ethanol by re-suspension

via sonification and centrifugation. Each reaction pellet was dissolved in 50 μ l H₂O and 2 mL of ScintiVerse™ BD cocktail was added for scintillation counting.

For reactions using *p*-Nitrophenol-xylose (*p*Np-Xyl) and 4-methylumbelliferyl- β -D-xylopyranoside (4-MU-Xyl, Sigma Aldrich), reactions were carried out in a 30- μ l mixture containing 50 mM Hepes (pH 7.3), 200 mM sucrose, 5 mM MnCl₂ (or 25 mM KCl and 1.9 mM MnCl₂), 1 mM *p*Np-Xyl or 0.2 mM 4-MU-Xyl and 1 mM UDP-D-GalA in a 28°C water-bath for 100 min. The reactions were terminated with 300 μ l chloroform:MeOH (5:1, v/v) by vortexing, and soluble products were extracted twice with 200 μ l diH₂O by vortexing and centrifugation at 10,000 rpm for 1 min. The aqueous supernatants from two extractions were pooled (~400 μ l), extracted again with 500 μ l chloroform, and spun at 10,000 rpm for 1 min, the aqueous supernatant was removed and analyzed using a Dionex CarboPac PA1 column.

Liquid Chromatography-tandem mass spectrometry (LC-MS/MS) analysis

For LC-MS/MS spectrometry sequencing, 500 μ l of the anti-GAUT12-conjugated magnetic beads were incubated at 4°C overnight on a tube rotator with microsomes (5 mg total protein) that were homogenized with 4% TX-100 supplemented with 200 mM NaCl, 100 mM NaOAc, and 2 mM EDTA for maximum recovery of GAUT12 protein for mass spectrometry sequencing (final TX-100 concentration for incubation was diluted to 0.5% with PBS buffer). The beads were washed 5 $\times$ with PBS (pH 7.4). The recovered beads were denatured in 3% SDS and reduced in 25 mM DTT, and separated on a 10% SDS-PAGE gel. The entire sample lane was cut into 12 pieces, and in-gel trypsin digestion was carried out as described (Atmodjo et al., 2011). Briefly, each gel piece was washed 2 $\times$ with deionized water and cut into 1 mm³ cubes

with a clean razor blade. The gel cubes were washed sequentially with 100 µl diH₂O, 100 mM ammonium bicarbonate (ambic): acetonitrile (ACN) (1:1, v/v), and ACN. The proteins in the gel cubes were reduced with 10 mM DTT in 100 mM ambic solution at 56°C for 1 h and alkylated with 55 mM iodoacetamide in 100 mM ambic solution. The samples were treated with 20 µg of sequencing grade porcine trypsin (Promega, V511) for 16 h at 37°C and the digested peptides were extracted once with 100 mM ambic: ACN (1:1, v/v) and twice with 5% formic acid: ACN (1:1, v/v). Combined extracts from each sample were dried by vacuum centrifugation to completion and the samples were dissolved in 50 µl 0.1% formic acid for LC-MS/MS analysis. The peptide samples obtained from the proteolytic digestions were analyzed on an Agilent 1100 capillary LC (Palo Alto, CA) interfaced directly to a LTQ linear ion trap mass spectrometer (Thermo Fisher, San Jose, CA). Mobile phases A and B were H₂O-0.1% formic acid and acetonitrile-0.1% formic acid, respectively. Peptides were eluted from the C18 column into the mass spectrometer during an 80 min linear gradient from 5 to 55% mobile phase B at a flow rate of 4 µl/min. The instrument was set to acquire MS/MS spectra on the nine most abundant precursor ions. Generated raw tandem mass spectra were converted into the mzXML format and then into peak lists using ReAdW software followed by mzMXL2Other software (Clough and Bent, 1998). The peak lists were searched using Mascot 2.2 (Matrix Science, Boston, MA).

Database searching and protein identification

A target database was created using the Arabidopsis annotated sequences obtained from the TAIR10_pep_20101214 protein database in TAIR (http://ftp.arabidopsis.org/home/tair/Genes/TAIR10_genome_release/TAIR10_blastsets/TAIR10_pep_20101214). A decoy database (decoy) was then constructed by reversing the sequences in

the normal database. Searches were performed against the normal and decoy databases using the following parameters: full tryptic enzymatic cleavage with two possible missed cleavages, peptide tolerance of 1000 ppm, fragment ion tolerance of 0.6 Da. Fixed modification was set as carbamidomethyl due to carboxyamidomethylation of cysteine residues (+57 Da) and variable modifications were chosen as oxidation of methionine residues (+16 Da) and deamidation of asparagine residues (+1 Da). Statistically significant proteins from both searches were determined at a $\leq 1\%$ protein false discovery rate (FDR) using the ProValT algorithm, as implemented in ProteoIQ (BioInquire, LLC, Athens, GA) (Taylor and Hepler, 1997).

Cell wall extraction and fractionation

Stem tissues were harvested from 7-week-old *Col-0*, *irx8-5*, *irx8-5+G12*, *irx8-2+G12*, and WT+*G12*, ground by mortar and pestle to a fine powder in liquid nitrogen, and re-suspended in 80% (v/v) ethanol and rotated end-to-end for 12 h. The pellet obtained by 4,000 rpm centrifugation was then sequentially washed in 80% (v/v) ethanol, 100% ethanol, chloroform: methanol (1:1, v/v), and acetone, by re-suspension, rotation for 12 h, and re-centrifugation. The resulting alcohol insoluble residue (AIR) was dried for 24 h at room temperature. The cell wall fractionation was conducted as described (Pattathil et al., 2012).

Pyrolysis molecular beam mass spectrometry (pyMBMS)

About 4 mg of sample was weighed and transferred into 80- μ l stainless steel sample cups of a commercially available auto sampler of a double shot pyrolyzer (PY-2020iD, Frontier Ltd). The samples were pyrolyzed at 500°C, and the residues analyzed using a custom built Super Sonic Molecular Beam Mass Spectrometer (Extrel Model MAX-1000).

Mass spectral data from m/z 30-450 were acquired on a Merlin Automation Data System version 3.3. Multivariate analysis was performed using Unscrambler software version 10.1 (CAMO). The intensities of the lignin peaks were summed and averaged in order to estimate the lignin contents in the sample (Evans and Milne, 1987). Total lignin peaks correspond to m/z 120, 124, 137, 138, 150, 152, 154, 164, 167, 178, 180, 181, 182, 194, and 210, the syringyl peaks correspond to m/z 154, 167, 168, 182, 194, 208, and 210, and the guaiacol peaks correspond to m/z 124, 137, 138, 150, 164, and 178. Syringyl to Guaiacol (S/G) ratios were also determined by summing syringyl peaks and dividing by the sum of guaiacol peaks. The lignin values thus generated and calculated were compared with the WT. The uncorrected lignin values were generated and corrected to standard NIST 8492 (eastern cottonwood) to obtain the lignin percentage value equivalent to the Klason lignin.

Determination of lignin monomer composition by HSQC NMR Spectroscopy

Perdeuterated pyridinium molten salt (ionic liquid) was synthesized as described (Jiang et al., 2009). Approximately 2 mg of cell wall extract fractions were weighed and dissolved in 180 μ l of the ionic liquid [DMSO- d_6 /pyridine- d_5 (2:1, v/v)] at 65 °C. Data were collected at 60°C on Agilent 600 MHz Direct Drive spectrometers equipped with either a 5 mm or 3 mm cold probe. A standard Agilent pulse program (“HSQCAD”) was used to acquire the ^{13}C - ^1H heteronuclear correlated spectra. The proton dimension of 1200 complex data points covered 20 ppm centered at 6 ppm, and the carbon dimension of 48 or 64 complex points was centered at 92 ppm with a width of either 100 ppm or 184 ppm, respectively. In the former case, some resonances in the alkyl or aromatic regions were folded along the F1 axis. Total data acquisition times ranged from 7 to 16 hours, with the number of transients between 240 and 400 per t_1 increment. Data were

processed with NMRPipe (NIH) and visualized with NMRViewJ (One Moon Scientific) or with MNova (Mestrelab Research). Typically, squared cosine window functions were applied in both dimensions after zero filling and linear prediction in t_1 . Data were referenced to DMSO at 2.50 ppm in proton and 39.51 ppm in carbon. HSQC cross-peak assignments were determined using Kim and Ralph (2010) as a reference.

SUPPORTING INFORMATION

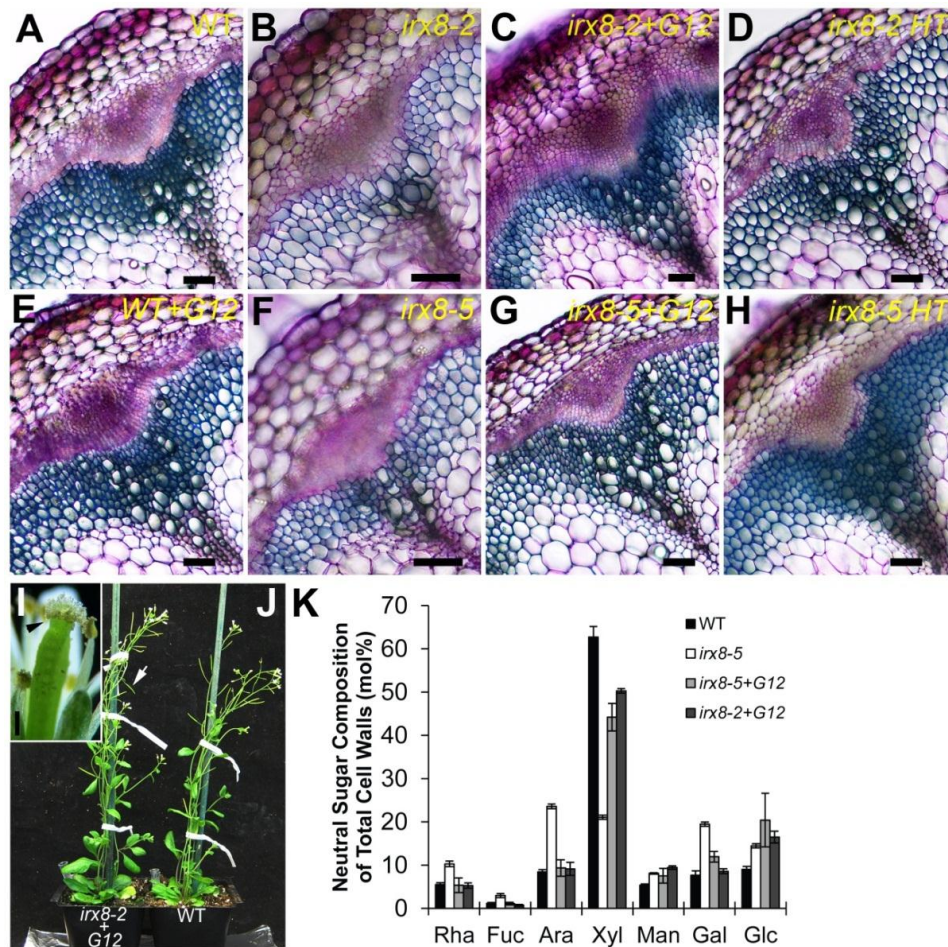


Figure 2.S1. G12-complementation of the *irx8* mutant alleles.

Basal stem hand-cut transverse-sections of (A) WT, (B) *irx8-2*, (C) *irx8-2 + G12*, (D) *irx8-2* heterozygote (HT), (E) WT+G12, (F) *irx8-5*, (G) *irx8-5 + G12*, and (H) *irx8-5* HT stained with Toluidine blue O.

(I) *irx8-5+G12* plant flower, the black arrowhead indicates pollen shed onto stigma.

(J) *irx8-2+G12* plant restored stature to WT-like, white arrow indicates formation of siliques.

(K) Neutral sugar composition of total cell walls (AIR) from 7-week-old plant stems determined by gas chromatography-mass spectrometry of alditol acetates. Value = mol% of individual sugar $\pm$ standard error.

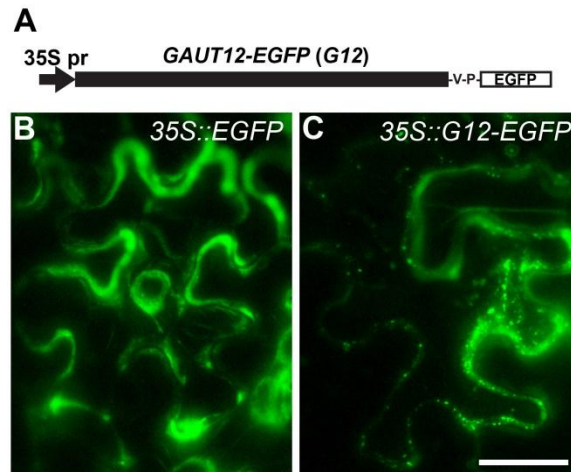


Figure 2.S2. Transient-expression of the *GAUT12-EGFP (G12)* construct in tobacco epidermal cells.

(A) The *GAUT12-EGFP* construct.

(B) The 35S:EGFP empty vector (Pattathil et al., 2005) which has a ubiquitous cytosol expression was used as a control.

(C) The *G12* construct produced punctated fluorescent signals, consistent with Golgi localization as reported previously (Pena et al., 2007). Bar = 50 μ m.

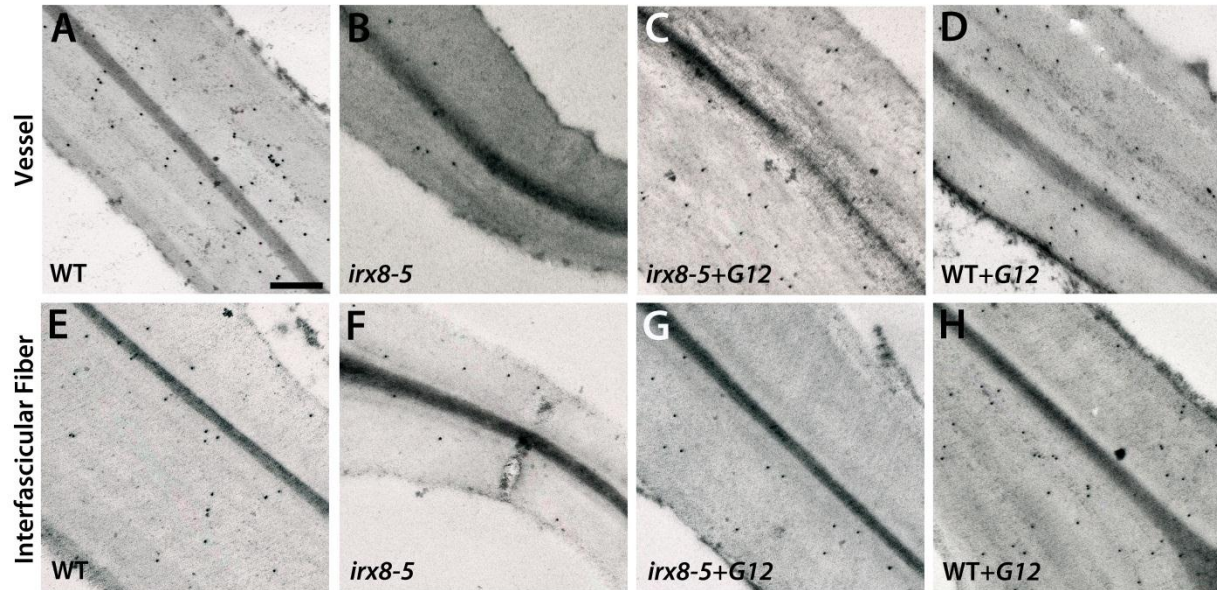


Figure 2.S3. TEM of LM10 immunogold-labeled xylem vessel and interfascicular fiber cell walls of WT, *irx8*, *irx8-5+G12* and WT+G12.

Xylem vessel cell walls labeled with the anti-xylan antibody LM10 in (A) WT (B) *irx8-5* (C) *irx8+GAUT12* and (D) WT+*GAUT12*.

Interfascicular fiber cell walls labeled with LM10 in (E) WT (F) *irx8-5* (G) *irx8+GAUT12* and (H) WT+*GAUT12*. Bar = 0.3 μm .

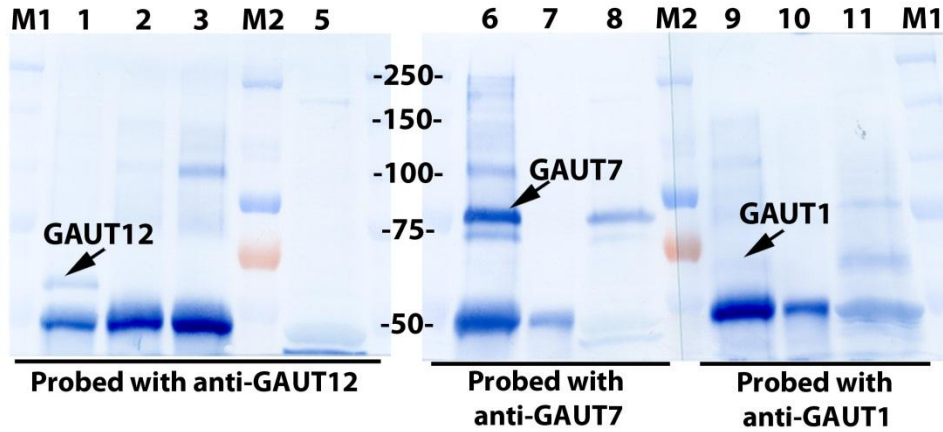


Figure 2.S4. Specificity of the anti-GAUT12 antibody.

Immunoprecipitation (IP) using anti-GAUT1 and anti-GAUT7 antibodies were used as controls to verify the specificity of the anti-GAUT12 antibody. Entire IP products were resolved by 10% SDS-PAGE and analyzed in Western blots. A ~58-60 kDa protein was detected in IP of Arabidopsis stem TX-100-treated microsomes using anti-GAUT12 antibody (lane 1) but not in IPs using anti-GAUT1 (lane 2) or anti-GAUT7 (lane 3) antibodies. Reciprocally, no GAUT1 (lane 10) or GAUT7 (lane 7) protein was detected in the anti-GAUT12 immunoabsorbed fractions. Samples are GAUT12-IP (lane 1, 7, 10), GAUT1-IP (lane 2, 9), GAUT7-IP (lane 3, 6), total WT stem microsomes (50 μ g protein) (lane 5, 8, 11), and protein ladders: M1 (Bio-Rad, #161-0373) of sizes 250, 150, 100, 75, 50 kDa, and M2 (Fermentas, SM1811) of size 250, 130, 95, 72, 55 kDa.

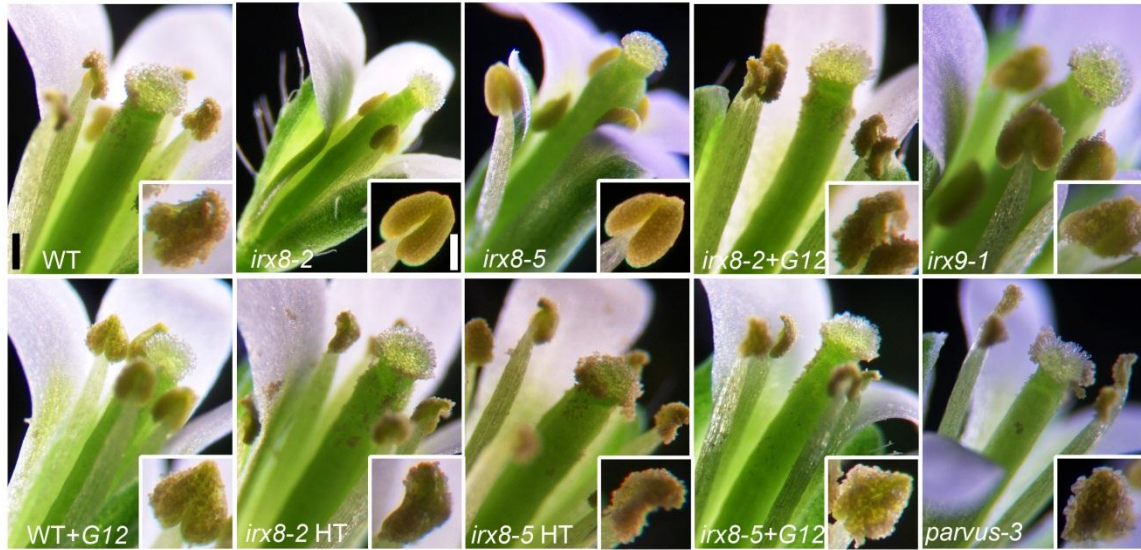


Figure 2.S5. Light microscope images of anther and pistil in open flowers of WT, *irx8-2*, *irx8-5*, *irx8-2* and *irx8-5* heterozygote (HT), *irx8-2+G12*, *irx8-5+G12*, WT+G12, *irx9-1*, and *parvus-3*.

Both *irx8-5* and *irx8-2* have short anther filaments and indehiscent anthers with no pollen release. The insets show close-up images of an anther from the corresponding flowers. Bar = 250 μm for flowers and = 100 μm for inset anthers.

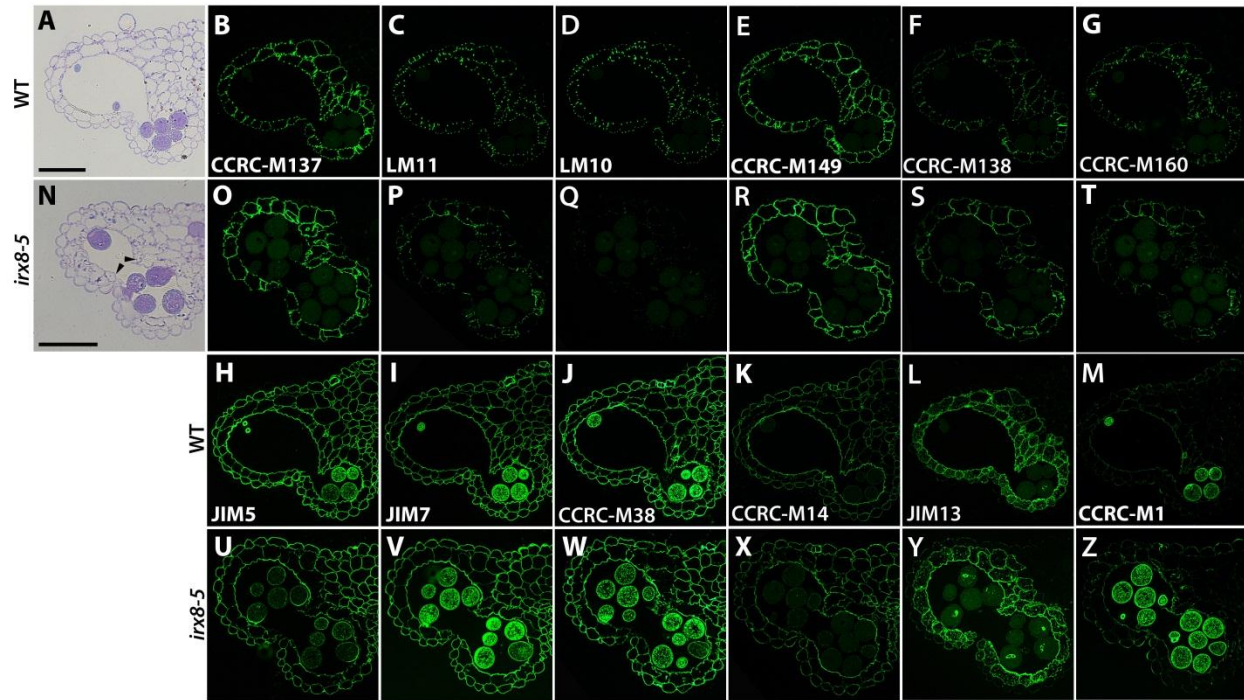


Figure 2.S6. Immunofluorescent labeling of transverse-sections of LR White-embedded *irx8* and wild type (WT) anthers in open flowers.

(A) WT and (N) *irx8-5* anther sections (250-nm-thick) were stained with toluidine blue, arrowheads point to septum breakage. WT and *irx8-5* anthers labeled with xylan antibodies (B, O) CCRC-M137, (C, P) LM11, (D, Q) LM10, (E, R) CCRC-M149, (F, S) CCRC-M138, and (G, T) CCRC-M160; pectin antibodies (H, U) JIM5, (I, V) JIM7, (J, W) CCRC-M38, and (K, X) CCRC-M14; AGP antibody (L, Y) JIM13; and fucosylated xyloglucan antibody (M, Z) CCRC-M1; Bar = 50 μ m. CCRC-M38 recognizes un-esterified HG, CCRC-M14 recognizes RG-I backbone, JIM5 recognizes low-esterified HG, JIM7 recognizes high-esterified HG, LM10 (Xylan-6) binds to low-substituted xylan, LM11 (Xylan-6) binds to both low- and high-substituted xylan, CCRC-M137 (xylan-7), CCRC-M149 (Xylan-7) binds strongly to xylotetraose and less to xylohexaose, CCRC-M138 (Xylan-6) binds to xylohexaose, and CCRC-M160 (Xylan-7) (Pattathil et al., 2010; Pattathil et al., 2012).

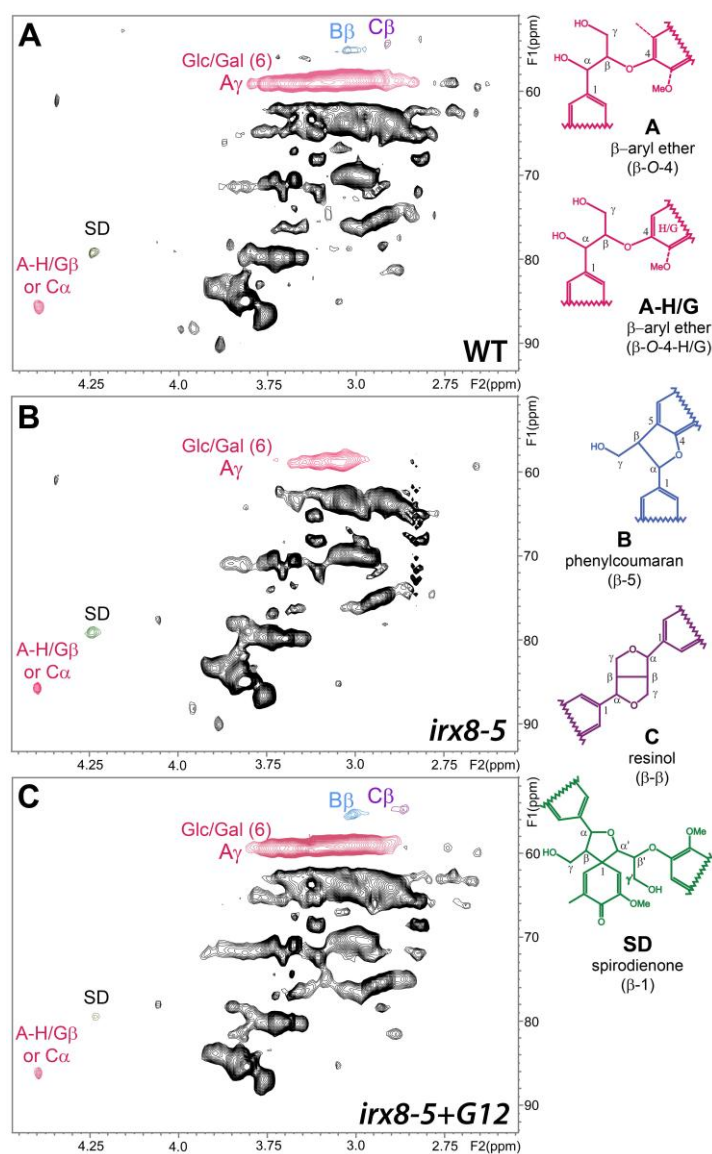


Figure 2.S7. Aliphatic sidechain region of 2D ^{13}C - ^1H Heteronuclear Single-Quantum Correlation (HSQC) spectra of chlorite fractions extracted from (A) WT, (B) *irx8-5*, and (C) *irx8-5+G12* stem alcohol insoluble residues (AIR).

Cross peaks representing lignin aliphatic side chains are labeled. Major changes in the *irx8* mutant from the WT were colored according to the lignin structures provided on right. There is a reduction of Glc/Gal (6)/A γ signal as well as a complete loss of B β and C β signals in *irx8*, which are recovered in *irx8+G12*.

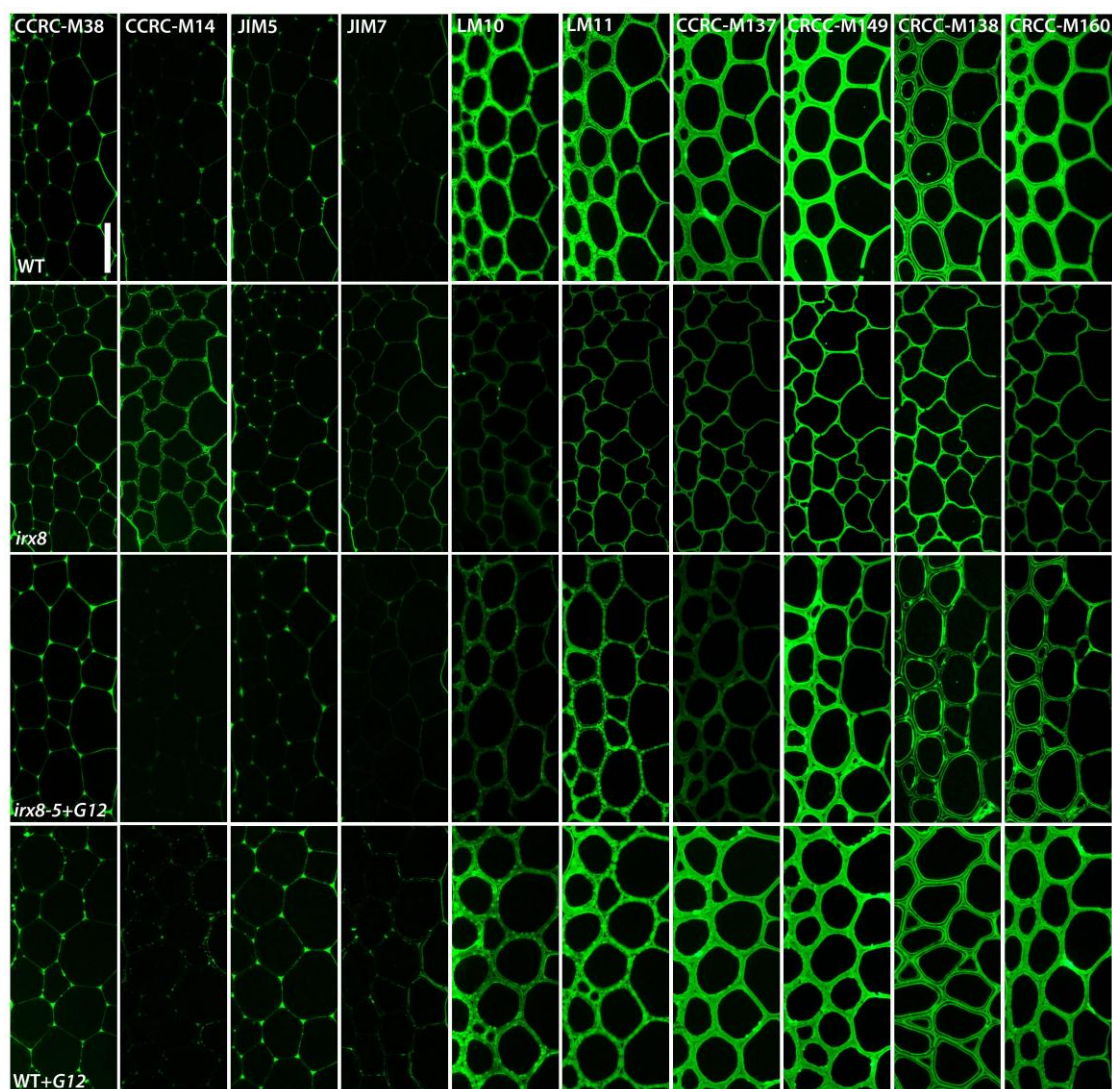


Figure 2.S8. Immunolabeling of interfascicular fibers from basal stems of 6-week-old wild type (WT), *irx8-5*, *irx8-5+G12*, and WT+G12 plants.

CCRC-M38 recognizes un-esterified HG, CCRC-M14 recognizes RG-I backbone, JIM5 recognizes low-esterified HG, JIM7 recognizes high-esterified HG, LM10 (Xylan-6) binds to low-substituted xylan, LM11 (Xylan-6) binds to both low- and high-substituted xylan, CCRC-M137 (xylan-7), CCRC-M149 (Xylan-7) binds strongly to xylotetraose and less to xylohexaose, CCRC-M138 (Xylan-6) binds to xylohexaose, and CCRC-M160 (Xylan-7) Pattathil et al. (2010).

Bar = 25 μ m.

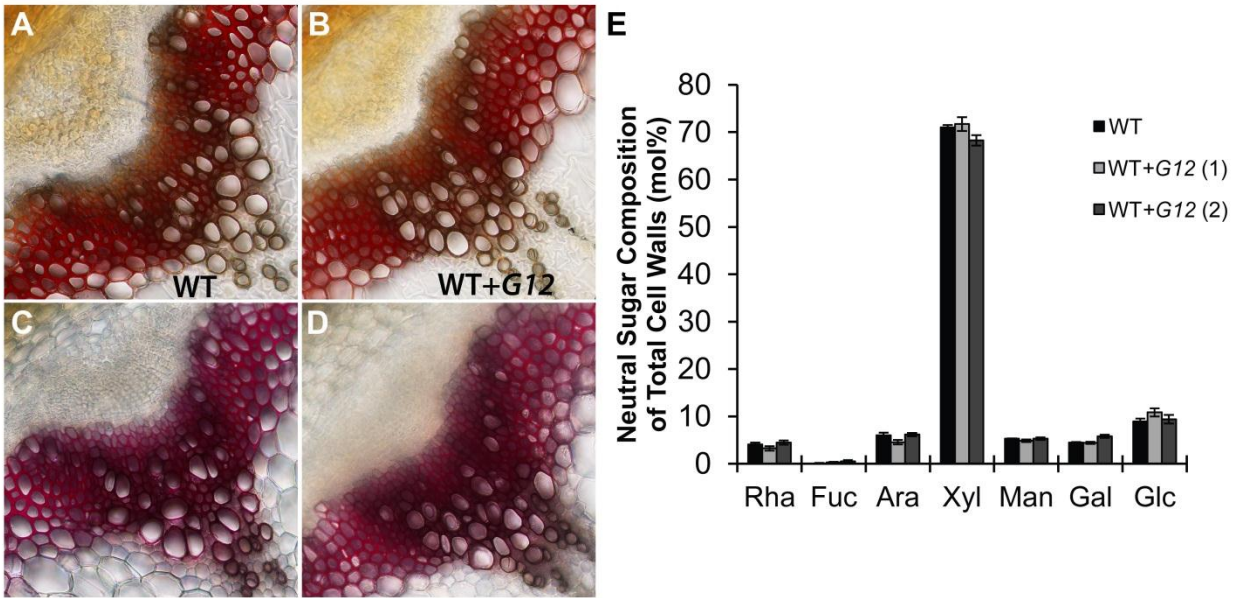


Figure 2.S9. Lignin staining and cell wall composition of 7-week-old WT+G12 (*GAUT12*-overexpression) plants.

(A) WT and (B) WT+G12 basal stem transverse sections stained with Mäule reagents.

(C) WT and (B) WT+G12 basal stem transverse sections stained with phloroglucinol-HCl.

(E) Neutral sugar composition of total cell walls (AIR) of 7-week-old plants determined by alditol acetate method. Value = mol% of individual sugar $\pm$ SE (n=5).

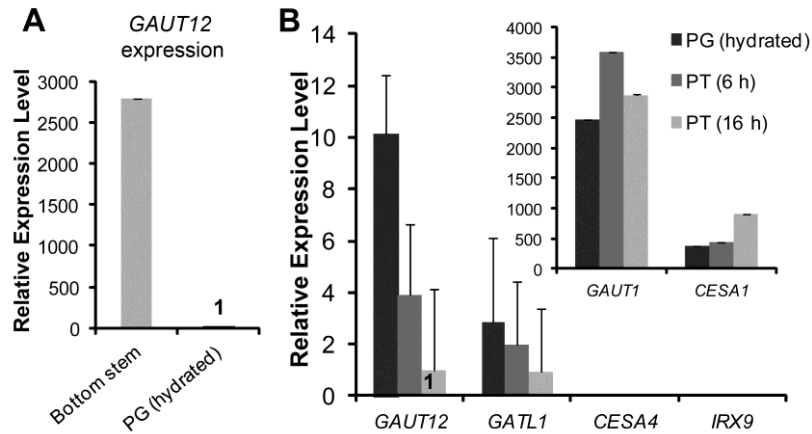


Figure 2.S10. *GAUT12* expression in pollen grains (PG) and pollen tubes (PT).

(A) *GAUT12* expression in Arabidopsis bottom stems and hydrated pollen grains (use *GAUT12* expression in PG as 1).

(B) *GAUT12* expression in hydrated pollen grains (0.5 h) and *in vitro* grown pollen tubes (*GAUT12* expression in 16 h PT was used as 1). *CESA4* and *IRX9* showed no expression in both pollen grains and growing pollen tubes. Values = average relative expression level $\pm$ standard deviation of three independent experiments.

Table 2.S1. List of primer sequences.

Genotyping primers	Forward (5'-3')	Reverse (5'-3')
<i>irx8-5</i>	GGTTTGCTTCTTGCTTCCGCT	TTTGGGACATTGACATGAATGGA
<i>irx8-2</i>	TTGTAACCACCAAACAGCTCC	TGAGAATCGAATGTTTTGTCTG
<i>irx9</i>	CCAAAAGTGTCAATTTATAACATTGG	ATGTTCAATGTGCCTCAAAGC
<i>gat11</i>	GTTGAAGTAGCATGCTTTCCG	TATGCACAGACAAACATAGCG
Lb1	GCGTGGACCGCTTGCTGCAACT (for <i>gat11</i>)	
Lbc	GGTGATGGTTCACGTAGTGGGCCATCGC (for <i>irx8-5</i>)	
Lb3	TAGCATCTGAATTTTATAACCAATCTCGATACAC (for <i>irx8-2</i>)	
<i>G12</i> transgenics	TCTGGCATATGCTTGGTCTC	CGTTTACGTCGCCGTCCAGCTC
Semi-qPCR primers	Forward (5'-3')	Reverse (5'-3')
For <i>GAUT12</i> transcript analysis (Figure 2.11)	CCATGGCACAGTTACATATATCTCCGAGCTTGAG (1)	GGTACCGCTGATGCTCTAATGTGACAGCTCTTG (2)
	CATGGGCTTATGGAATGAATG (5)	CCGCTAGGTCGAAAACATTC (3)
	TCTGGCATATGCTTGGTCTC (6)	TTGGACATGACCGTGGAAG (4)
		CGTTTACGTCGCCGTCCAGCTC (7)
Construct primers	Forward (5'-3')	Reverse (5'-3')
GAUT12-EGFP	CCATGGCACAGTTACATATATCTCCGAGCTTGAG	GGTACCGCTGATGCTCTAATGTGACAGCTCTTG
GAUT1	ggtaccAATGGCGCTAAAGCGAGGGCT (GAUT1-F-KpnI)	tctagaTTATTCATGAAGGTTGCAACGAC (GAUT1-R-XbaI)
GAUT12	ctgcagGGTTAGTGATGCAGGGCAAC (GAUT12-F-PstI)	ggtaccATCTGATCTTCCTTTGAGTTCTTG (GAUT12-R-KpnI)
GAUT12+1	ggtaccGCAAATGAGTTAGTTCAGCACAATG (GAUT1Tr-F-KpnI)	ggtaccTTATTCATGAAGGTTGCAACGACG (GAUT1Tr-R-KpnI)
qPCR primers	Forward (5'-3')	Reverse (5'-3')
<i>ACTIN2</i>	GGTAACATTGTGCTCAGTGGTGG	AACGACCTTAATCTTCATGCTGC
<i>GAUT12</i>	CATGGGCTTATGGAATGAATG	AGCTGCCACAACTCAGGTC
<i>IRX9</i>	TACTTTGGGACCCTGAGAGATG	ACAATCTTGTGCCGGAAGTC
<i>GATL1</i>	CTGCAGATTGCCCTTAATC	TCGACGTGAGCAGAAGATTG
<i>CESA1</i>	GGCAGTTAAGGTGATTCCA	TTGGGTCCACATCTTCTTCC
<i>GAUT1</i>	CCACAAGTGGCAAAACATGA	CGCCTTGTTTAAGGGATGTG
<i>PAL1</i>	TATCCCGAACAGGATCAAGG	TCTCCGGTCAAAAGCTCTGT
<i>C4H</i>	CAGGTAGTGAAGGATTGAAATGTG	CCCCTCGATAGACCACAATG
<i>4CL1</i>	TTTGCTCATCGGTCATCCTG	CGACACGAATTGCTTCACATC
<i>HCT</i>	GCTCTTAAGGCGAAATCCAAG	CTTCCCACTGATCTCCACAC
<i>C3H</i>	GTTGCTAACGTTGAAGGATCAG	TTCCGCTGTTATCGCTGTC
<i>CCOAOMT1</i>	CTCACAAGATCGACTTCAGGG	ACGCTTGTGGTAGTTGATGTAG
<i>CCR1</i>	ACCAAGTGCAAGGACGAGAA	GTCGTAGAGGCTTTGCTTGG
<i>COMT1</i>	TTGATCTCCACATGTCATCG	ATGTTTCGTCACCTCCAGTCATG
<i>F5H1</i>	TCCATCAAACCTACCCGTGAC	TGTTGGACCCGTTTATAGATCC
<i>CAD4</i>	ATGTCTAATTATCCTATGGTTCTTGG	ACTCCGACTACATCTCCTACG
<i>CAD5</i>	CATCAATGGTCAACCTACACAAG	TCAACCGCCATTCCTTCTG
<i>CAD6</i>	TTGGGACGAAAATCGATAGC	TGCTTTTATGCCATGCTCTG

CHAPTER 3

CHARACTERIZATION OF XYLOGENIC *irx8* SUSPENSION CELL CULTURES THAT PRODUCE REDUCED LIGNIN AND HAVE ALTERED XYLAN BIOSYNTHESIS²

² Zhangying Hao, Utku Avci, Sivakumar Pattathil, Huanzhong Wang, Lisa A. Jackson, Haibao Tang, K. David Hucheson, Grace Rothstein, Carolyn Bandu Moore, Rick A. Dixon, Michael G. Hahn, Debra Mohnen. To be submitted to Molecular Plant.

ABSTRACT

Cellulose, xylan, and lignin are the main components of lignocellulosic biomass that is the potential feedstock for biofuel production. Mutation in the Arabidopsis gene *GA*lac*U*ronosyl*T*ransferase12 (*GAUT12*)/*IR*regular *X*ylem8 (*IRX8*) was previously reported to cause collapsed xylem along with a reduction in cell wall xylan and in a specific pectic fraction. Phylogenetic analysis shows that *GAUT12/IRX8* belongs to the *GAUT1*-related protein family. In this study, we generated Arabidopsis seed-derived *irx8* and wild type suspension cell cultures and induced tracheary element (TE) formation using 2,4-epibrassinolide (BL). The induced TEs in *irx8* cells cultured under xylogenic conditions had slightly bloated shapes and reduced cell wall thickness compared to TEs formed in wild type cells cultured under the same condition. Immunolabeling experiments revealed that *irx8* TEs lack a xylan epitope recognized by LM10, a monoclonal antibody that recognizes low esterified xylan. Both phloroglucinol-HCl staining and lignin composition analyses revealed that lignin production was reduced in *irx8* cells grown under xylogenic conditions. Real-time PCR indicated that the reduction in lignin correlated with a reduction in lignin biosynthetic gene expression. We investigated gene expression patterns of putative xylan biosynthesis-related genes via real-time PCR using RNA from *irx8*, wild type, and *GAUT12*-overexpression lines grown on xylogenic and control media. The results led to the identification of genes tightly correlated with TE formation including those that may correlate with *GAUT12*-associated xylan biosynthesis. These data suggest that *GAUT12* affects lignin and xylan biosynthesis during Arabidopsis secondary cell wall formation.

INTRODUCTION

The plant cell wall is a highly complex yet dynamic structure composed of polysaccharides and proteins. Primary cell walls retain their ability to expand and re-shape as plant cells grow, whereas secondary cell walls laid down by some differentiated cells are rigid and add mechanical strength. Tracheary elements (TEs) consisting of vessels and tracheids are unique types of plant cells that are dead and hollow at maturity and have annular, spiral, reticulate or pitted secondary cell wall thickening patterns. *In vivo*, plants conduct water and nutrients through vessels that form end-to-end tubing with connective perforation plates and by tracheids that transport contents through bordered pits between adjacent cells (Northcote, 1995). To study the process of xylogenesis, i.e. the irreversible cell differentiation of TEs, various types of plant cells, such as isolated Zinnia mesophyll cells and Arabidopsis suspension cell cultures, have been used by inducing TEs *in vitro* with phytohormones (Fukuda and Komamine, 1980; Kubo et al., 2005). *In vitro* differentiated TEs may appear in clusters, nested bands or single cells (Fukuda, 1996).

Suspension cell cultures of various species are useful systems to study cell wall synthesis (Doong et al., 1995; Sterling et al., 2005; Sterling et al., 2006). Arabidopsis suspension cultures generated from cell wall mutants have also been used to study gene functions in cell wall synthesis (Pauly et al., 2001; Leboeuf et al., 2005). In addition, both chemical-treated and hormone-induced suspension cultures have been used to study cell wall biosynthetic genes (Manfield et al., 2004) and new transcription factors involved in TE formation (Kubo et al., 2005). Due to the mixed-cell nature of stem tissues, the *in vitro* TE formation system provides a useful tool to study genes involved in secondary wall formation because of the ease of induction and the ability to control experimental parameters (Fukuda, 1996). The fact that various

irregular xylem mutants have retarded growth and reduced stature (Brown et al., 2005; Persson et al., 2005) makes it difficult to compare mutant tissues with wild type counterparts at the same developmental stage. Therefore, the generation of such mutant suspension cell cultures and the induction of TE formation *in vitro* provide a powerful system to investigate gene function and interactions during secondary cell wall formation.

The secondary cell walls of TEs are composed mainly of cellulose, xylan, and lignin in dicot woody plants. Being the most abundant biopolymer on earth, cellulose contribute nearly 30% of the total plant biomass (Somerville, 2006a), while xylan (notably glucuronoxylan) and phenolic compounds (particularly lignin) contribute 20-30% and up to 30% of the dicot secondary wall biomass, respectively (Scheller and Ulvskov, 2010). While cellulose and lignin have been studied extensively (Boerjan et al., 2003; Somerville, 2006b; Mutwil et al., 2008; Zhao and Dixon, 2011), much remains unknown about xylan biosynthesis as well as the interactions between xylan and other cell wall polymers. The xylan in wood is mainly glucuronoxylan, which is composed of a linear backbone of β -1,4-xylosyl residues with substitutions of α -1,2-GlcA or 4-O-methyl GlcA at every 6 to 12 xylose residues. The xylose can also be arabinosylated or acetylated at the O2 or O3 positions (Ebringerova and Heinze, 2000). A xylan reducing-end sequence (XRES) β -D-Xylp-(1 $\rightarrow$ 4)- β -D-Xylp-(1 $\rightarrow$ 3)- α -L-Rhap-(1 $\rightarrow$ 2)- α -D-GalpA-(1 $\rightarrow$ 4)-D-Xylp was found in xylans of birch, spruce and Arabidopsis (Shimizu, 1976; Johansson and Samuelson, 1977; Andersson et al., 1983; Pena et al., 2007) but is absent in mosses and grass species (Kulkarni et al., 2012). Xylan mutant analyses have revealed genes that affect xylan biosynthesis, including xylan backbone biosynthesis-related genes *IRX9* and *IRX9-L/I9H*, *IRX10* and *IRX10-L*, *IRX14* and *IRX14-L/I14H*, *IRX15* and *IRX15-L* (Brown et al., 2007; Lee et al., 2007a; Pena et al., 2007; Brown et al., 2009; Wu et al., 2009a; Lee et al., 2010; Wu et al., 2010;

Brown et al., 2011b; Jensen et al., 2011), XRES biosynthesis-related genes *FRA8/IRX7*, *GATL1*, *GAUT12/IRX8*, and the *GUX* genes encoding xylan:4-*O*-methyl-glucuronosyltransferases (GlcAT) (Mortimer et al., 2010; Lee et al., 2012b; Rennie et al., 2012).

GAUT12/IRX8 belongs to CAZy family GT8 and has been identified as a gene involved in TE formation because its knock-out mutant *irx8* has reduced cell wall thickness in xylem vessels and fibers. Cell wall analyses confirmed a ~25% reduction in cellulose (Brown et al., 2005; Persson et al., 2005), a 50-60% reduction in xylose (Pena et al., 2007; Persson et al., 2007), a reduction of XRES (Pena et al., 2007), and a reduction of 4-linked and 3,4-linked GalA in endopolygalacturonase (EPG)/pectin methylesterase (PME)-released wall fractions (Persson et al., 2007) in *irx8* walls. These results indicate a role for *GAUT12/IRX8* in xylan formation, or perhaps in the synthesis of the XRES, or a specific pectic structure associated with xylan (Pena et al., 2007; Persson et al., 2007).

Several other glycosyltransferases in the GT8 family have been characterized to have diverse functions in plant cell wall biosynthesis, including *GAUT1*, *GAUT7*, *GAUT8/QUA1*, and *GATL1/PARVUS*, which belong to the *GAUT1*-related protein family (Sterling et al., 2006; Caffall et al., 2009b). *GAUT1* is a homogalacturonan (HG): α -1,4-galacturonosyltransferase (GalAT) that functions in a *GAUT1:GAUT7* complex (Atmodjo et al., 2011) to catalyze the addition of D-GalA from UDP-D-GalA onto oligogalacturonides (Sterling et al., 2006). The mutation in *GAUT8/QUA1* causes a dwarfed plant with reduced cell adhesion, a 25% reduction of GalA (Bouton et al., 2002), and a reduction of both HG:GalAT and xylan synthase activity (Orfila et al., 2005). The mutation in *GATL1/PARVUS* causes a dwarfed plant phenotype similar to *irx8* that is humidity-sensitive (Lao et al., 2003; Shao et al., 2004). The *parvus/gatl1* mutants have reduced rhamnogalacturonan I branching (Lao et al., 2003) and ~50% reduction in xylose,

and they also lack the XRES (Lee et al., 2007b). The phenotypes of *qua1* and *parvus/gatl1* suggest potential connections between pectin and xylan in the walls. The recently identified xylan:GlcAT encoded by GUX1 and GUX2 from the CAZy GT8 family revealed further diversified functions of GT8 family members (Mortimer et al., 2010; Lee et al., 2012b; Rennie et al., 2012).

Using an Arabidopsis brassinolide (BL)-induced xylogenic suspension cell culture system, Kubo et al. (2005) conducted a microarray analysis to identify genes up- and down-regulated during TE formation (Kubo et al., 2005). Among the up-regulated genes in Set 3 were secondary cell wall biosynthesis-related genes including *GAUT12/IRX8*, *CESA4*, *CESA7*, *CESA8*, *GATL1/PARVUS*, *IRX10/GUT2*, and lignin biosynthetic genes *CCoAOMT* and *4CL1*, as well as TFs such as KNAT7, four NAC-domain proteins VND3, VND4, VND5, VND7, and three MYB-domain proteins MYB52, MYB85, and MYB43. These results identified the co-expression of *GAUT12/IRX8* with these genes during TE differentiation. Since no other *GAUT* genes were identified in this study, the data indicate a specific role for *GAUT12/IRX8* in Arabidopsis secondary cell wall formation.

To study the role of *GAUT12* in secondary cell wall formation, we generated Arabidopsis suspension cell cultures from seed-derived calli of wild type and *irx8*, and over-expressed *GAUT12* (OE) in WT culture cells. Upon analyzing these cell lines grown on xylogenic (+BL) and control media, we identified a reduction in lignin biosynthesis and deposition in xylogenic *irx8* cells during TE formation, suggesting a role for *GAUT12/IRX8* in lignin production. To understand how *GAUT12/IRX8* affects xylan biosynthesis and to explore its interaction with other xylan biosynthesis-related genes, we compared the expression of selected xylan biosynthesis-related genes and *GAUT* genes in xylogenic *irx8* and *GAUT12*-overexpression

(OE) suspension cell cultures to the WT counterpart. Evaluation of the expression patterns of xylan biosynthesis-related genes led to the identification of a subset of genes that may function with *GAUT12/IRX8* in the same biosynthetic pathway during xylan biosynthesis.

RESULTS

*Generation and characterization of WT, *irx8*, and G12-OE suspension cell culture lines*

The *irx8-5* allele has a T-DNA insertion disrupting the fourth intron of *GAUT12* (**Figure 3.1A**) and expresses no full length *GAUT12* transcript (**Figure 3.2E**). WT and *irx8* suspension cell cultures were generated from seeds of heterozygous *irx8* plants. Both WT and homozygous *irx8* calli generated from individual seeds were confirmed by genotyping (**Figure 3.1D**). The segregated WT line provided the same genetic background as the *irx8* line except for the T-DNA insertion within the *GAUT12* gene.

WT and *irx8* calli appeared to be similar when grown on solid medium (**Figure 3.S1**), however, after transfer into liquid medium and sub-culture for approximately four years, the *irx8* suspension culture cells became fluffier and slightly lighter in color (**Figure 3.1C**) in comparison with WT cells (**Figure 3.1B**). Through several cycles of sieving followed by re-inoculation of cultures using the sieved-through cells, we managed to create relatively fine cell cultures of WT and *irx8* containing single cells and proliferating clumps.

A *GAUT12*-EGFP fusion protein driven by a cauliflower mosaic virus (CaMV) 35S promoter (**Chapter 2**) was stably over-expressed in wild type *Arabidopsis* suspension culture cells. This cell line appeared similar to the WT line, and was named *G12*-OE.

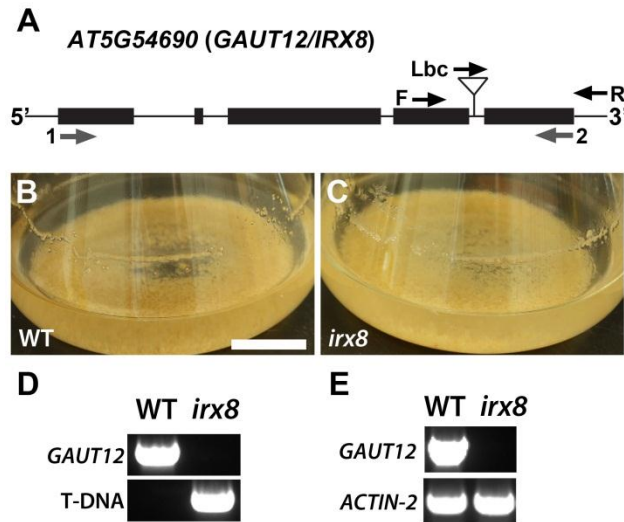


Figure 3.1. Characterization of wild-type and *irx8* suspension culture cells.

(A) *GAUT12* gene model. The empty triangle indicates the location of the *irx8*-5 T-DNA insertion site. Arrows indicate the primers used for genotyping (F, Lbc, and R) and for *GAUT12* transcript analysis (1 and 2).

(B) WT and (C) *irx8* day 2 suspension cell cultures.

(D) Genotyping results confirming T-DNA insertion in *irx8* suspension cell culture line. The *GAUT12* band was amplified by primers F+R, and the T-DNA band was amplified by primers Lbc+R.

(E) Full-length *GAUT12* transcript (amplified by primer 1+2) is absent in the *irx8* cells.

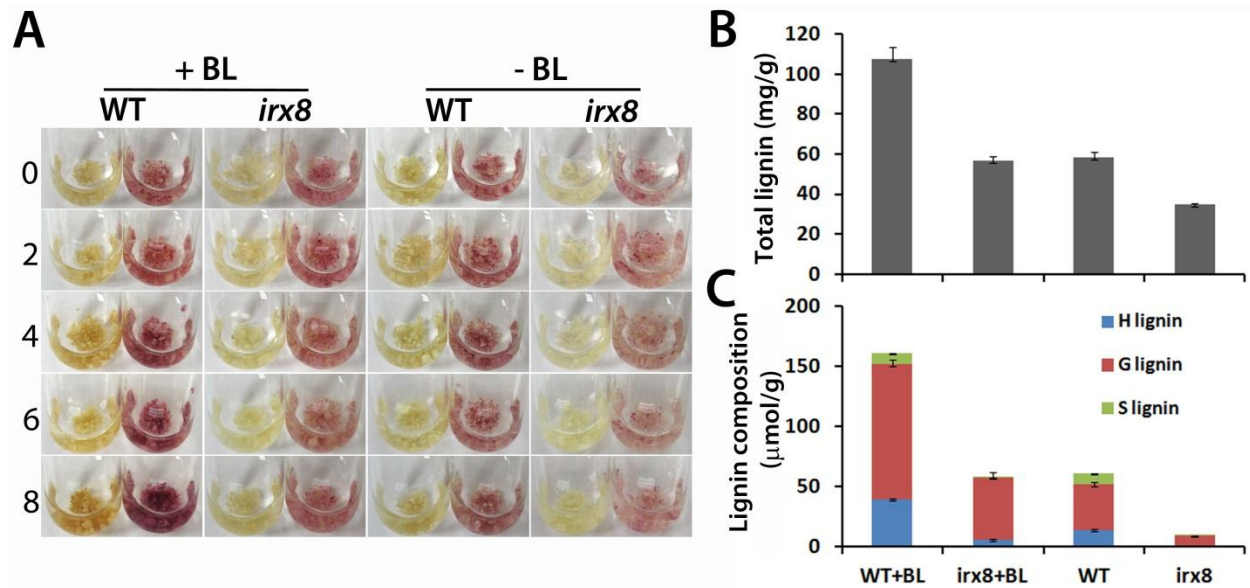


Figure 3.2. Lignin analyses of xylogenic wild type and *irx8* suspension culture cells.

(A) Phloroglucinol-HCl staining of wild type and *irx8* suspension culture cells grown on xylogenic (+BL) or control (-BL) medium on days 0, 2, 4, 6, and 8. Phloroglucinol-HCl reagent reacts with hydroxycinnamaldehyde endgroups in G lignin and creates a red color (Norman & Yamamoto, 1990).

(B) Total lignin measured by acetyl bromide method as milligram per gram of dry wall mass. Y-value = average $\pm$ standard deviation (n=9).

(C) Lignin composition measured by thioacidolysis to determine the amounts of H, G, and S lignin as μ mol per gram of dry wall mass. Y-value = average $\pm$ standard deviation (n=9).

Comparison of walls of irx8 and WT suspension culture cells grown on control medium that supports primary wall formation

Suspension culture cells undergo rapid cell division and growth and have long been used as a source of actively growing cells for studies of primary cell wall synthesis. Thus, it was of interest to determine if the reduced xylose and xylan phenotype observed in vascular tissues of *irx8* plants was also observed in *irx8* suspension culture cells grown on control medium that supported primary wall formation. Cell wall sugar composition analysis of 9-day-old stationary-phase WT and *irx8* culture cells grown on control medium revealed that the *irx8* and WT cells had similar amounts of arabinose, rhamnose, fucose, xylose, and mannose residues (**Table 3.1**). There was an apparent ~23% reduction of galactose ($P_{t\text{-test}} = 0.056$) and a 19% increase in glucuronic acid ($P_{t\text{-test}} = 0.08$) in *irx8* cells compared to WT cells. However, the only significant difference was an ~52% reduction in glucose in *irx8* cells ($P_{t\text{-test}} = 0.006$). The reduced xylose associated with the *irx8* phenotype in vascular tissues of plants was not observed in the suspension culture cells. To study this further we examined LR White-embedded 9-day-old WT and *irx8* cells by immunolabeling with a variety of monoclonal antibodies directed against diverse cell wall carbohydrate epitopes. No major differences were observed in the labeling of WT versus *irx8* cells grown on control medium with a series of antibodies directed against HG epitopes with differing levels of methylesterification (JIM5, JIM7, CCRC-M38 LM-20 and JIM7) or RG-I backbone antibody CCRC-M14 (**Figure 3.S2**). There was also no difference in labeling with the antibody CCRC-M1 that recognizes xyloglucan, or with antibodies LM5, LM6, and CCRC-M7 that recognize RG-I side chain β -1,4-galactan and α -1,5-arabinan, and AGPs 6-linked β -D-galactose oligomers that contain arabinose, respectively (**Figure 3.S3**).

Table 3.1. Glycosyl residue composition of cell walls (AIR) isolated from 9-day-old WT and *irx8* suspension culture cells grown on xylogenic (+BL) and control media as determined by gas chromatography-mass spectrometry (GC-MS) of trimethylsilyl (TMS) derivatives.

	Ara	Rha	Fuc	Xyl	Man	Gal	GalA	Glc
WT + BL	42.7 ± 16.9	12.0 ± 5.1	2.7 ± 1.4	22.2 ± 8.8	3.8 ± 2.0	25.4 ± 10.7	9.0 ± 4.1	9.9 ± 3.9
<i>irx8</i> + BL	44.6 ± 29.2	15.8 ± 10.4	2.8 ± 1.7	27.2 ± 14.2	4.0 ± 1.1	29.6 ± 7.3	14.7 ± 6.0	15.6 ± 3.6
WT	32.1 ± 20.1	10.8 ± 6.5	1.8 ± 0.8	17.2 ± 9.6	3.6 ± 0.6	29.2 ± 2.6	13.2 ± 2.1	13.2 ± 2.0
<i>irx8</i>	28.7 ± 20.2	10.1 ± 6.1	1.9 ± 1.2	15.4 ± 7.3	3.6 ± 1.5	22.6 ± 6.5	15.7 ± 2.4	6.4 ± 3.8

Data = mean (μg of sugar per mg of dry AIR mass) $\pm$ standard deviation (n=6). Samples were collected on day 9 after inoculation in three independent induction trials. TMS GC-MS reactions were done in duplicate for each sample.

Student T-test analyses show that GalA in WT+BL cells is significantly reduced compared with WT cells ($P_{\text{t-test}} = 0.025$); *irx8*+BL cells have significantly more Glc than WT+BL cells ($P_{\text{t-test}} = 0.034$) and *irx8* ($P_{\text{t-test}} = 0.0014$); *irx8* cells have less Glc than WT cells ($P_{\text{t-test}} = 0.0055$).

Xylogenic irx8 cell suspension culture cells have reduced lignin

Kubo *et al.* reported the induction of metaxylem- and protoxylem-like vessel elements in wild type *Arabidopsis* suspension cells cultured with 1 μ M brassinolide (BL), 1 μ g/ml 2,4-dichlorophenoxyacetic acid (2,4-D), and 10 mM H_3BO_3 (Kubo *et al.*, 2005). Using the same phytohormone conditions (which we refer to as xylogenic medium or +BL), we induced TE formation in both WT and *irx8* suspension culture cells. Initially, we conducted a time-course study by phloroglucinol-HCl staining to monitor the production of phenolic compounds in cells grown on xylogenic medium. Phloroglucinol reacts with the coniferaldehyde end-groups in G lignin (Lewis and Yamamoto, 1990). Starting on day 4 of BL induction, WT culture cells showed increase red staining with phloroglucinol-HCl, compared to cells grown on control medium (**Figure 3.2A**). The intensity of the red color increased on subsequent days in cells grown on xylogenic medium as lignin and monolignol components accumulated. We thus used the red coloration upon phloroglucinol-HCl staining as an indicator of secondary wall formation. In contrast, *irx8* cells grown on the xylogenic medium did not show such an intense increase in red color upon phloroglucinol-HCl staining (**Figure 3.2A**). This result suggests that *irx8* cells accumulated less lignin than WT cells grown on xylogenic medium.

To study the apparent reduction in lignin in *irx8* cells grown on xylogenic medium, we measured total lignin in three independent trials of WT and *irx8* suspension culture cells grown for 9 days on xylogenic (+BL) and control media using the acetyl bromide method (Iiyama and Wallis, 1990). On both media, *irx8* cells had lower total lignin in cell walls (alcohol insoluble residue, AIR) than WT counterparts (**Figure 3.2B** and **Figure 3.S4**). Both WT and *irx8* cells grown on xylogenic medium produced more total lignin than those grown on control medium (**Figure 3.2B**). WT cells produced 84% more lignin while *irx8* cells produced 64% more lignin

when grown on xylogenic medium, **Figure 3.S4B**), showing that the deposition of lignin is induced in cells grown on xylogenic medium. The lignin composition in these cultures was measured using thioacidolysis which degrades lignin polymer to monomers (Lapierre et al., 1985). Day 9 xylogenic *irx8* culture cells produced less H, G, and S lignin compared to WT cells, corroborating the total lignin reduction determined by the acetyl bromide method (**Figure 3.2C**). Upon growth on xylogenic medium, H and G lignin were induced in both WT and *irx8* cells, while S lignin was not induced in WT cells. Interestingly, there was almost no or little S lignin detected in *irx8* cells grown on xylogenic or control medium (**Figure 3.2C** and **Figure 3.S4B**). These results indicated that the *irx8* mutation affects S lignin production as well as the ability to produce H and G lignin upon growth on xylogenic medium.

Xylogenic irx8 suspension culture cells produce vessel elements with reduced xylan

To ensure that xylem vessels were formed in the suspension cultured cells grown on xylogenic medium, we fixed 9-day-old wild type and *irx8* culture cells and made sections through resin-embedded cell blocks. The sections were examined by immunolabeling (**Figure 3.3**). Xylogenic WT cells formed vessels of elongated shape with sharp cell corners that often existed in clusters and the xylan in the walls was labeled with xylan antibodies (**Figure 3.3A-D**). TEs also formed in *irx8* cells grown on BL-induction medium (+BL), however *irx8* TEs lack xylan epitopes recognized by LM10 (**Figure 3.3E**) which binds low-substituted xylan. The *irx8* TEs also display reduced xylan epitopes recognized by LM11 (**Figure 3.3F**) which binds to both low- and high-substituted xylan. Nevertheless, the TEs formed in *irx8* retain xylan epitopes recognized by CCRC-M138 and CCRC-M160 (**Figure 3.3G, H**). Contrary to the collapsed xylem vessel phenotype in *irx8* stems, the vessel elements formed in *irx8* suspension

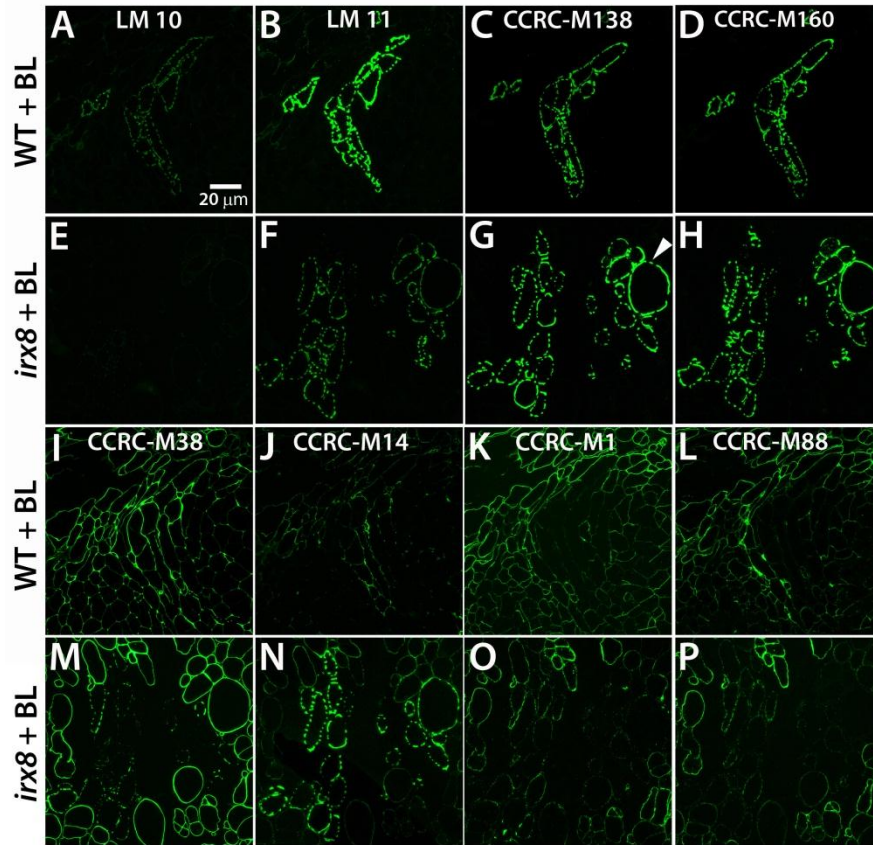


Figure 3.3. Immunolabeling of TEs in xylogenic WT and *irx8* suspension cell cultures.

Vessel elements formed in 9-day-old wild-type (WT + BL) and *irx8* (*irx8* + BL) cells grown on xylogenic medium. White arrowhead in (G) indicates larger diameter of *irx8* vessels suggesting less rigid cell walls due to defects in secondary wall thickening.

Wild-type and *irx8* cells grown on xylogenic medium labeled for xylan epitopes with LM10 (A, E), LM11 (B, F), CCRC-M138 (C, G), and CCRC-M160 (D, H), un-esterified HG with CCRC-M38 (I, M), RG-I backbone with CCRC-M14 (J, N), fucosylated xyloglucan with CCRC-M1 (K, O), and non-fucosylated xyloglucan with CCRC-M88 (L, P). Bar = 20 μ m.

cells appeared to be larger in diameter and rounder than WT TEs (**Figure 3.3G** and **Figure 3.S5**, white arrowheads). The *irx8* TEs appear to have thinner cell walls and the cells are more loosely packed than those formed in WT cultures under xylogenic conditions (**Figure 3.4**).

Cell wall composition analyses of the cultures revealed an apparent increase in xylose in both WT and *irx8* cells grown on xylogenic medium compared with control medium, by ~29% ($P_{t\text{-test}} = 0.18$) and ~77% ($P_{t\text{-test}} = 0.055$), respectively (**Table 3.1**), indicating a potential increase in cell wall xylan. Interestingly, despite the reduction in xylan epitopes in *irx8* TEs, the amount of xylose present in *irx8*+BL cells is comparable to, if not more than, wild type. The amount of glucose increased specifically in *irx8* cells by ~144% ($P_{t\text{-test}} = 0.0014$) but decreased by an apparent 25% ($P_{t\text{-test}} = 0.051$) in WT cells. Interestingly, the amount of GalA present in WT cell walls decreased ~32% ($P_{t\text{-test}} = 0.025$) upon growth on xylogenic medium, whereas *irx8* cells showed little change in wall GalA content when grown on either medium.

The increased xylose observed in *irx8* cells grown on xylogenic medium was accompanied by an increase in xylan labeling as shown by glycome profiling, a method developed to study global cell wall epitope changes using a selected array of wall-glycan directed monoclonal antibodies (Pattathil et al., 2012). Cell wall samples (AIR) prepared from three independent sets of WT and *irx8* cells grown for 9 days on xylogenic (WTX, MX) and control (WTC, MC) media were sequentially extracted to generate six cell wall fractions, namely oxalate, carbonate, 1 M KOH, 4 M KOH, chlorite, and 4 M KOH post-chlorite fractions (**Figure 3.S6**). There are significant increases in the presence of xylan epitopes in 1 M and 4 M KOH-extracted fractions of both WT and *irx8* cells grown on xylogenic medium compared with the control medium (**Figure 3.S6**, yellow boxes). Furthermore, the *irx8* cells grown on control medium showed less xylan labeling than the wild type, suggesting a reduced amount of

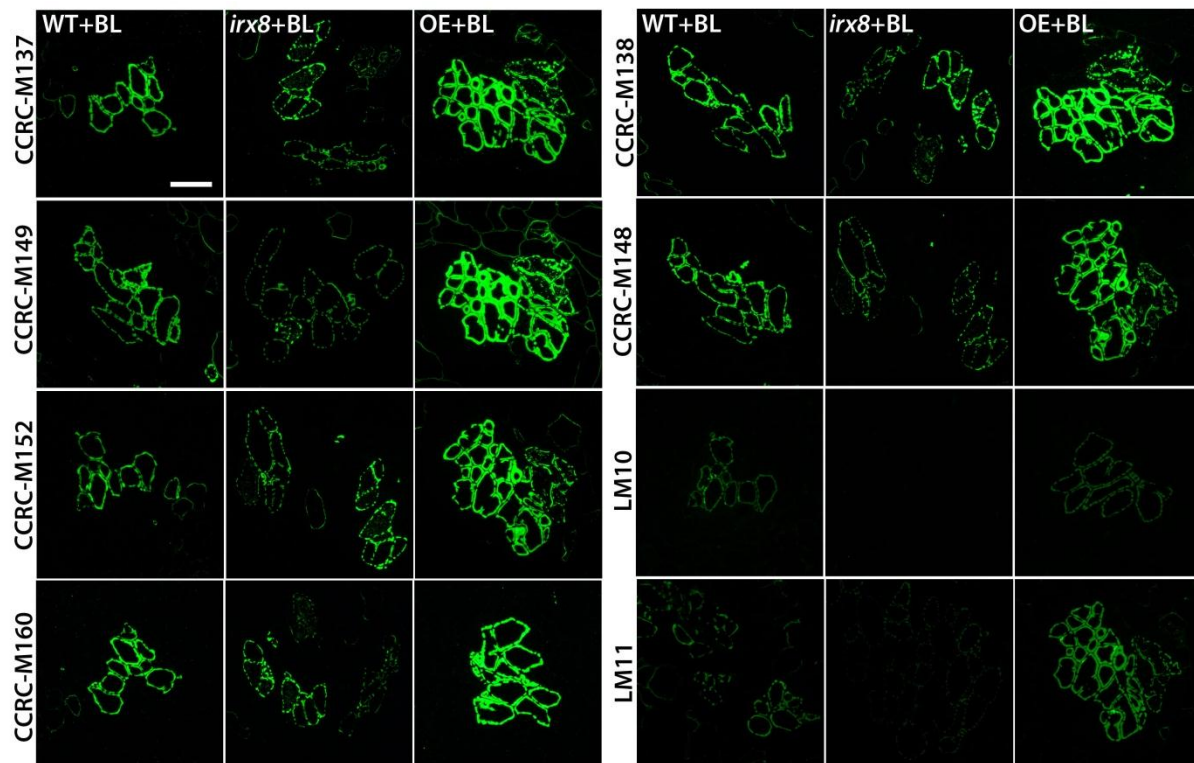


Figure 3.4. Vessel elements formed in 9-day-old wild type (WT + BL), *irx8* (*irx8* + BL), and *GAUT12*-overexpression (OE + BL) cells grown on xylogenic medium immunolabeled with xylan antibodies CCRC-M137, CCRC-M149, CCRC-M152, CCRC-M160, CCRC-M138, CCRC-M148, LM10, and LM11.

CCRC-M138 and CCRC-M148 (Xylan-6 antibodies), CCRC-M137, CCRC-M149, CCRC-M152, and CCRC-M160 (Xylan-7 antibodies) (Pattathil et al., 2012). LM10 recognizes low-substituted xylan, and LM11 recognizes both low- and high-substituted xylan.

extractable xylan in the KOH fractions (**Figure 3.S6**, white boxes). Interestingly, the BL-induction appeared to cause a reduction in xyloglucan epitopes in both WT and *irx8* cells in the carbonate and chlorite fractions (**Figure 3.S6**, green boxes), and *irx8* cells has less xyloglucan epitopes in the oxalate fraction grown on xylogenic medium (**Figure 3.S6**, aqua-blue box).

Interestingly, TEs formed in the *G12*-OE line appeared to have thicker cell walls, as shown by immunolabeling with CCRC-M138 and CCRC-M148 (Xylan-6 antibodies), CCRC-M137, CCRC-M149, CCRC-M152, and CCRC-M160, and LM10, LM11 (Xylan-7 antibodies, **Figure 3.4**). These xylan antibodies label the entire xylem tissues and interfascicular fibers with different intensities due to different epitopes present and/or recognized in the wall (Pattathil et al., 2010; Pattathil et al., 2012). The reduced cell wall thickness in *irx8* TEs is consistent with a role for *GAUT12* in xylan biosynthesis, and the increased xylan labeling in OE line suggests that *GAUT12*-overexpression results in increased xylan deposition during secondary wall formation.

Xylogenic irx8 suspension cultures have reduced expression of lignin biosynthetic genes

The lignin reduction in xylogenic *irx8* suspension culture cells led us to investigate how lignin biosynthesis was affected in this mutant. We analyzed the transcript levels of ten major lignin biosynthetic genes using real-time PCR. Total RNA was prepared from WT, *irx8*, and *G12*-OE cells grown on xylogenic (+BL) and control media on days 0, 3, 6, and 9 (**Figure 3.5**). Compared with cells grown on control medium, the WT cells grown on xylogenic medium had increased expression of all lignin biosynthetic genes examined, with peak expression on day 3 after BL induction. Conversely, the gene expression pattern in WT control cells remained relatively stable, except for *CCoAOMT1* and *C4H* which had the highest expression out of the

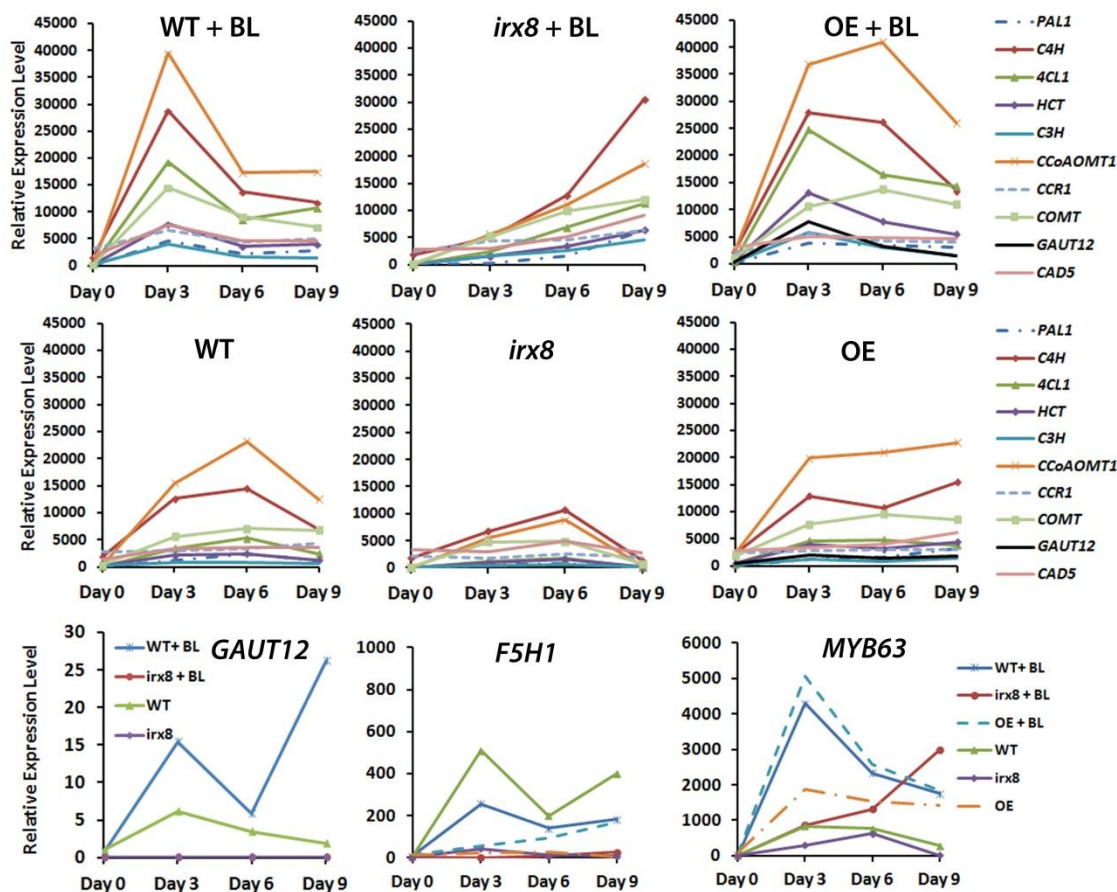


Figure 3.5. Expression of lignin biosynthetic genes in wild-type, *irx8*, and *GAUT12*-overexpression cells grown on xylogenic (WT + BL, *irx8* + BL, OE + BL) and control (WT, *irx8*, OE) media.

Relative expression level represents fold-change of transcript expression relative to *GAUT12* expression in day 0 WT control cells, which was set as 1. *PAL1*, Phe ammonia lyase; *C4H*, trans-cinnamate 4-hydroxylase; *4CL1*, 4-Coumarate: CoA ligase; *HCT*, hydroxycinnamoyl-CoA: shikimate/quinate hydroxycinnamoyltransferase; *C3H*, *p*-Coumarate 3-hydroxylase, *CCoAOMT1*, Caffeoyl-CoA 3-*O*-methyltransferase, *CCR1*, cinnamoyl-CoA reductase, *F5H1*, Ferulate 5-hydroxylase; *COMT*, Caffeic acid *O*-methyltransferase; *CAD*, Cinnamyl alcohol dehydrogenase. *MYB63*, a lignin-specific transcription factor gene (Zhou et al., 2009).

ten genes tested and showed highest expression on day 6. The *irx8* cells grown on xylogenic medium (*irx8*+BL) displayed a slow increase in the expression of these lignin genes, suggesting a slow start in the lignin production process. The expression levels of *PAL1*, *C4H*, *4CL1*, *HCT*, *C3H*, *CCoAOMT1*, *CCR1* and *COMT* did increase in day 9 compared to day 6 in *irx8* cells grown on xylogenic medium, but apparently the lignin content did not increase on day 8 as measured by phloroglucinol-HCl staining (**Figure 3.2A**). *CCoAOMT1* had relatively low expression compared to *C4H* in *irx8* cells grown on both media, whereas the *CCoAOMT1* expression was higher than *C4H* in WT and *G12*-OE cells grown on both media. The SND1-regulated *F5H1* which controls S lignin formation (Zhao et al., 2010) had very low expression in *irx8* cells grown on xylogenic medium, which is consistent with the low S lignin content in this mutant as measured by thioacidolysis (**Figure 3.2C** and **Figure 3.S4**). The expression changes in the lignin-specific transcription factor MYB63 (Zhou et al., 2009) was congruent with the changes in the lignin biosynthetic genes in WT and *irx8* cells, respectively (**Figure 3.4**). *MYB63* expression was up-regulated in the *G12*-OE cells compared to its expression in WT cells grown on xylogenic medium, which may explain the continued high expression of lignin biosynthetic genes on day 6 in the *G12*-OE cell line.

We also monitored the expression pattern of *GAUT12* in WT cells grown on xylogenic and control media on days 0, 3, 6, and 9 (**Figure 3.5**). The expression of *GAUT12* on day 0 in control medium was set to 1. In cells grown on control medium, *GAUT12* expression reached the maximum on day 3 (5-fold compared to day 0), decreased on day 6 and decreased further on day 9. In cells grown on xylogenic medium, *GAUT12* was up-regulated on day 3 reaching 15-fold compared to day 0, decreased to 6-fold on day 6, and then dramatically increased to 27-fold on day 9. The *irx8* culture cells showed no *GAUT12* expression during the 40 cycle amplification of

the real-time PCR as expected for this knock-out mutant (**Figure 3.1E**). The *G12*-OE cells had dramatically increased *GAUT12* transcript levels as high as >1000-fold when grown on control medium and >5000-fold when grown on xylogenic medium, compared with *GAUT12* expression in day 0 WT control cells (**Figure 3.5**). Despite the increase in *GAUT12* transcript in wild-type cells grown on xylogenic medium, GAUT12 protein was not detected in either wild-type or *irx8* cells by immunoprecipitation using the anti-GAUT12 antibody (**Figure 3.S7A**). However, the abundant *GAUT12* transcript produced GAUT12-EGFP fusion protein in *G12*-OE cells grown on control medium, as detected by the anti-GAUT12 antibody (**Figure 3.S7B**).

Xylan epitope changes and expression analyses of putative xylan biosynthesis genes

As shown in **Figure 3.3** and **Figure 3.4**, the TEs formed in xylogenic *irx8* culture cells lacked the xylan LM10 epitope but retained to some degree the xylan epitopes recognized by antibodies LM11, CCRC-M137, CCRC-M138, CCRC-M148, CCRC-M149, CCRC-M152 and CCRC-M160. The TEs formed in xylogenic *irx8* cells also had thinner cell walls and were slightly bloated in shape compared to TEs formed in WT cells (**Figure 3.3G** and **Figure 3.S5**). The LM10 and LM11 epitope reduction and the reduced cell wall thickness is consistent with reported defects in *irx8* mutant plants, which have reduced wall thickness in vessel and fiber cells (Pena et al., 2007; Persson et al., 2007). Based on these results, we hypothesize that GAUT12 has a role in the biosynthesis and/or deposition of xylan: either it affects a xylan sub-fraction present in TE secondary walls, or the *irx8* mutation alters wall architecture in such a way that xylan is not properly laid down.

To understand whether the partial xylan defect is due to altered expression of some xylan biosynthesis-related genes in *irx8*, we measured the transcript levels of 13 genes related to xylan

biosynthesis in the xylogenic *irx8* cells. The expression values for each gene were calculated relative to *GAUT12* expression in day 0 WT cells grown on control medium, which was set as 1. Such relative expression values are displayed as a heat map and grouped based on the overall expression patterns in **Figure 3.6** (line charts are provided in **Figure 3.S8**). These gene expression patterns fall into four groups. **Group (I)** genes were tightly up-regulated upon BL-induction: *CESA4*, *IRX9*, *IRX15*, and *GUX1*. These genes had almost no expression in cells grown on control medium in which primary cell walls are produced. However, their expression was induced in cells grown on xylogenic medium in which TE formation was induced. **Group (II)** genes had some basal expression in cells grown on control medium and were slightly up-regulated upon growth on xylogenic medium: *I14H/IRX14-L*, *IRX15-L*, *GATL1*, and *IRX10*. This group of genes showed almost no change in WT cells grown on control versus xylogenic medium but was induced highly in xylogenic *irx8* and *G12*-OE cells. **Group (III)** genes had relatively high expression in control cells and were slightly induced upon growth on xylogenic medium: *I9H/IRX9-L*, *IRX10-L*, and *IRX14*. **Group (IV)** genes had low expression in xylogenic *irx8* cells but were induced in both xylogenic WT and *G12*-OE cells: *GUX2*, *IRX7* and its homolog *F8H*. This result suggests that *IRX7* and *GUX2* may work cooperatively with *GAUT12*. *F8H* had lower expression in control medium compared to xylogenic medium for each culture cell line, and its overall expression was very low compared to other genes we examined.

The secondary cell wall-related transcription factor (TF) *MYB46* (Zhong et al., 2007) showed expression patterns similar to genes in Groups (I) and (II) across all three cell lines examined on both media, suggesting the correlation of *MYB46* with these target genes.

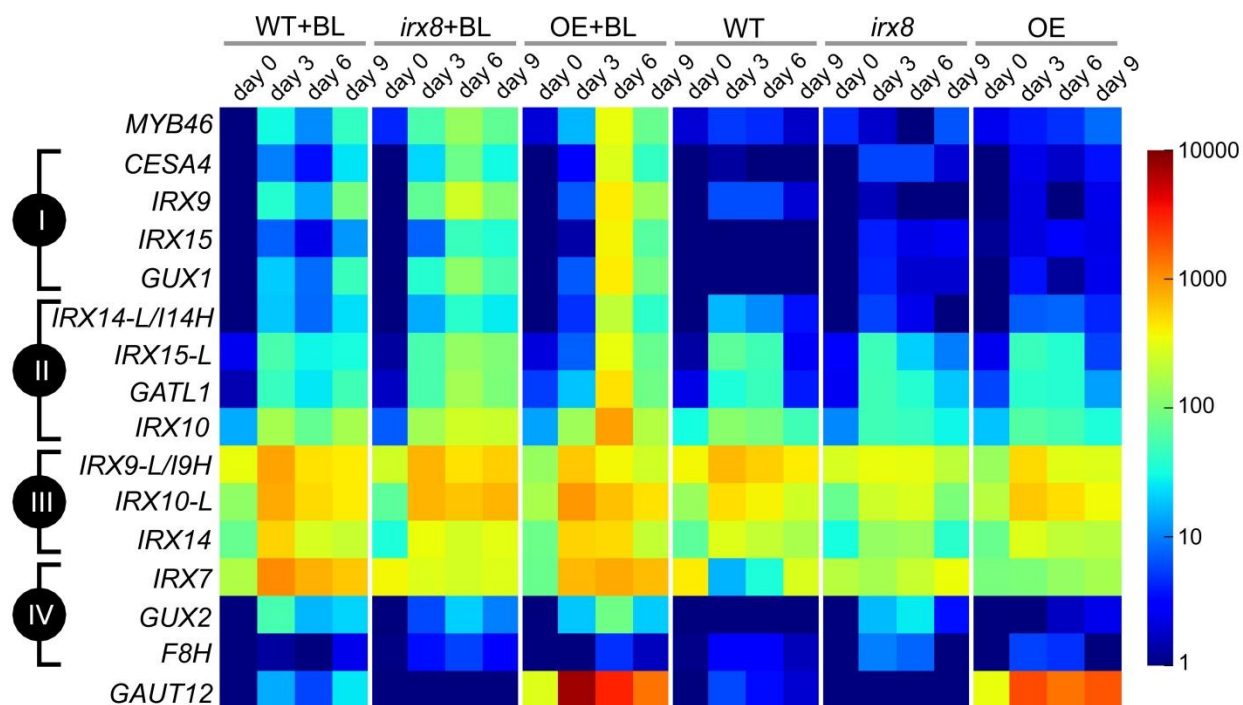


Figure 3.6. Expression of genes involved in secondary cell wall and xylan biosynthesis in wild-type, *irx8*, and *GAUT12*-overexpression cells grown on xylogenic (WT + BL, *irx8* + BL, OE + BL) and control (WT, *irx8*, OE) media.

The heat map represents fold-change of gene expression relative to *GAUT12* expression in WT Day 0 control cells, which was set as 1. *MYB46*, encodes a secondary wall transcription factor; *CESA4*, secondary cellulose synthase gene; *IRX9*, *I9H/IRX9-L*, *IRX10*, *IRX10-L*, *IRX14*, *I14H/IRX14-L*, *IRX15*, *IRX15-L*, putative xylan backbone biosynthetic genes; *GUX1*, *GUX2*, xylan GlcA transferase genes; *GATL1*, *IRX7*, *F8H*, genes putatively involved in the biosynthesis of the xylan reducing-end sequence (XRES).

Expression patterns of primary cell wall biosynthesis-related genes in xylogenic irx8 cells

There were some apparent changes in GalA and Gal residues in *irx8* cells grown on both control and xylogenic media compared to wild-type cells (**Table 3.1**), however, pectin epitopes as detected by immunolabeling using selected pectin antibodies appeared to be unaltered in *irx8* cells (**Figure 3.3** and **Figure 3.S2**). To study this further, we selected several GAUT genes and the primary cell wall cellulose synthase gene *CESA1* and analyzed their expression levels in the culture system. *CESA1* had similar expression patterns in WT cells grown in xylogenic and control media, while its expression was induced in *irx8* cells and repressed in GAUT12-OE cells grown on xylogenic medium (**Figure 3.7** and **Figure 3.S9**), suggesting that the WT, *irx8*, and GAUT12-OE cells may have different primary wall synthesis rates in response to BL-induction. Interestingly, *GAUT1*, which is involved in primary cell wall homogalacturonan biosynthesis, had higher expression in *irx8* cells grown on xylogenic medium. Likewise, *GAUT7* which encodes a subunit of the GAUT1:GAUT7 HG:GalAT complex (Atmodjo et al., 2011) had almost identical expression patterns to that of *GAUT1* across all three cell lines. Surprisingly, *GAUT8*, a gene thought to be involved in pectin biosynthesis and affecting xylan biosynthesis (Bouton et al., 2002; Orfila et al., 2005) had dramatically increased expression in xylogenic *irx8* cells. The expression of *GAUT8* in xylogenic WT and *G12*-OE cells, however, was only slightly induced upon growth on xylogenic medium (**Figure 3.7** and **Figure 3.S9**). *GAUT14* expression was also induced in cells grown on xylogenic medium and had a higher expression in xylogenic *irx8* cells, with a pattern resembling that of the *IRX9-L/I9H* and *IRX10-L* in Group (III) (**Figure 3.S9**).

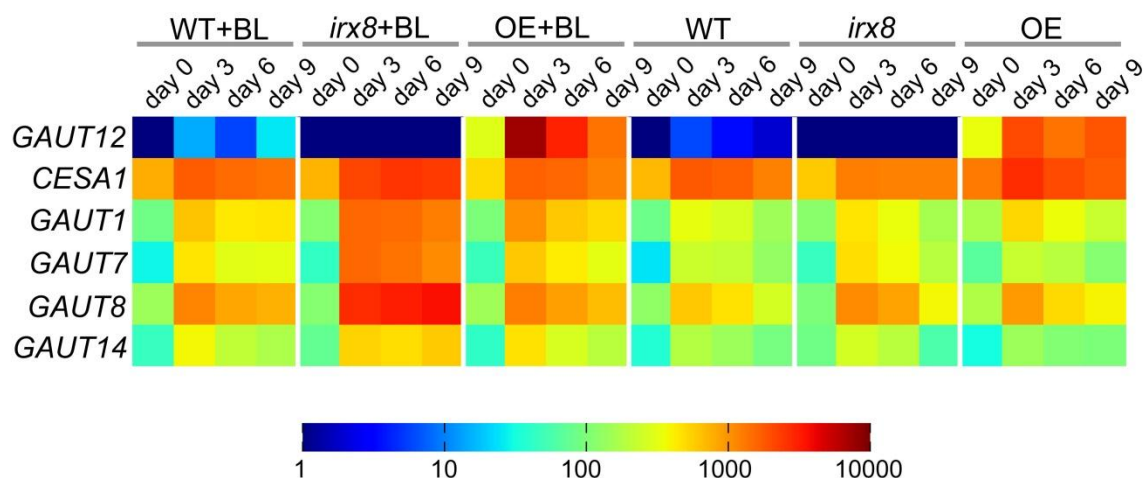


Figure 3.7. Expression of genes involved in primary cell wall biosynthesis in wild-type, *irx8*, and *GAUT12*-overexpression cells grown on xylogenic (WT + BL, *irx8* + BL, OE + BL) and control (WT, *irx8*, OE) media.

The heat map represents fold-change of gene expression relative to *GAUT12* expression in WT day 0 control cells, which was set as 1. *CESA1*, primary cellulose synthase gene; *GAUT1*, HG backbone GalA transferase gene (Sterling et al., 2006); *GAUT7* encodes a protein that forms a core complex with *GAUT1* (Atmodjo et al., 2011); *GAUT8* affects HG and xylan biosynthesis (Bouton et al., 2002; Orfila et al., 2005); and *GAUT14* affects wall GalA content (Caffall et al., 2009a).

DISCUSSION

GAUT12/IRX8 T-DNA insertion mutant lines (*irx8*) are severely dwarf plants that are slow growing and sterile (Pena et al., 2007; Persson et al., 2007). The *irx8* cell walls contain reduced amounts of xylan, a sub-fraction of pectin (Pena et al., 2007; Persson et al., 2007), and lignin (**Chapter 2**). We have generated seed-derived WT, *irx8*, and *GAUT12*-overexpression (*G12-OE*) suspension culture cells to study the function of *GAUT12* during TE differentiation, and the effect of the *irx8* mutation on the expression of other genes related to secondary cell wall formation. *In vitro* TE induction in Arabidopsis suspension culture cells provides a reproducible system to study such *irregular xylem* mutants, overcoming the difficulty of comparing *irx8* mutant and wild-type tissues of comparable developmental stages, a particular problem with *irx8* plants which have slow growth rates and are dwarfed.

Lignin down-regulation in irx8 suggests a role for GAUT12 in lignin formation

Secondary wall thickening is accompanied by the deposition of lignin, yet the mechanism controlling secondary wall formation is not strictly coupled to lignification since blocking lignification does not prevent secondary wall thickening (Karkonen et al., 2011; Sato et al. 1993). We demonstrated in this study that despite the reduced wall thickness of individual TEs formed in *irx8* cells grown on xylogenic medium (**Figure 3.4**), 9-day-old xylogenic *irx8* cells produced comparable amounts of xylan (**Table 3.1**) but less lignin detected by phloroglucinol-HCl staining (**Figure 3.2A**). The reduced lignin was confirmed by total lignin and lignin composition analyses (**Figure 3.2B, C**). These results indicate a role for *GAUT12* in lignin biosynthesis and/or deposition.

Our data showed that the major lignin induced during TE formation in xylogenic suspension culture cells are G and H lignin, in both WT and *irx8* cells (**Figure 3.2C**). This is consistent with G lignin being the major lignin in vessel elements in typical hardwood species and in *Arabidopsis* and indicates that vessels are being formed in cells grown on xylogenic medium. H lignin is the major lignin in the middle lamella and at the cell wall corners, and its deposition happens in early stages of lignification (Donaldson 2001). The induction of H lignin suggests that H lignin is required to be laid down prior to, or during, vessel formation, since many TEs appeared to form in clusters. The increased lignin production in both WT and *irx8* cells grown on xylogenic medium indicates successful induction of TE formation in both cell lines. This conclusion was confirmed by immunolabeling of 9-day-old cell sections (**Figure 3.3** and **Figure 3.4**). However, S lignin was barely induced in the xylogenic suspension culture system, consistent with the finding that S lignin is deposited primarily in fiber cells of woody plants (Musha and Goring, 1975). It is interesting that very little H and S lignin were detected in the *irx8* suspension cells grown on control medium, as shown by the thioacidolysis analysis. Furthermore, the amount of total lignin induced in the wild-type cells, as well as the H, S, and G lignin monomer content, was more than in *irx8* cells grown on xylogenic medium (**Figure 3.S4**). These results show that *irx8* has a lignin defect.

The expression of ten major lignin synthetic genes, except for *F5H1*, was similar in each cell line grown on xylogenic medium. The lignin biosynthetic genes had expression patterns similar to the lignin-specific transcription factor MYB63, consistent with a role for MYB63 in regulating the expression of these genes, as previously reported (Zhou et al., 2009). The expression patterns of the lignin biosynthetic genes in xylogenic cultures were also quite consistent with their expression in wild-type and *irx8* basal stems (**Chapter 2, Figure 2.7G**). In

wild-type *Arabidopsis* suspension cells grown on xylogenic medium, lignin biosynthetic genes had peak expression on day 3, which was concomitant with lignin deposition starting on day 4 as indicated by increased red staining by phloroglucinol-HCl (**Figure 3.2A**). However in xylogenic *irx8* cells, expression of these genes gradually increased and did not peak until day 9, indicating reduced and slow lignin biosynthesis in xylogenic *irx8* cells, which may contribute to the lack of lignin deposition detected by phloroglucinol-HCl staining (**Figure 3.2A**) and by quantitative lignin measurements of this mutant (**Figure 3.2B, C**).

Several lignin biosynthetic genes have been shown to function in TE differentiation and have been used as markers for xylogenesis, including genes encoding phenylalanine ammonia-lyase (PAL), cinnamate hydroxylase (C4H), *O*-methyltransferase (OMT), and 4-coumarate:CoA ligase (4CL) (Northcote, 1995; Fukuda, 1996). Except for *PAL1*, *CCoAOMT1*, *C4H*, and *4CL* had relatively high expression in xylogenic WT and *G12*-OE cells on day 3, and retained high expression in *G12*-OE cells on day 6 (**Figure 3.4**), which may have contributed to the increased wall thickness of TEs formed in xylogenic *G12*-OE cells. Moreover, *CCoAOMT1* had the highest expression out of all lignin biosynthetic genes examined in xylogenic WT and *G12*-OE cells, consistent with its high expression in *Arabidopsis* stem tissues (**Chapter 2, Figure 2.7G**). PAL catalyzes the first step of the general phenylpropanoid pathway by producing cinnamic acid from phenylalanine. Apparently, over-expressing *GAUT12* did not affect the expression level of *PAL1*.

F5H1 had a distinct expression pattern among the genes examined, since *F5H1* is directly regulated by *SND1* and controls S lignin formation in *Arabidopsis* (Meyer et al., 1996; Zhao et al., 2010). *F5H1* had relatively low expression in *irx8* and *G12*-OE cells grown on both control and xylogenic media, but its expression was higher in WT cells. Furthermore, *F5H1* expression

was greater in cells grown on control medium than on xylogenic medium, suggesting that S lignin is not being induced in cells grown on xylogenic medium. Although it is unclear why *F5H1* expression was much higher in WT cells than that in *irx8* mutant and *GAUT12*-OE cells (**Figure 3.5**), its expression level is concomitant with the presence of S lignin in the WT cells. Likewise, the low expression of *F5H1* in *irx8* cells parallels the extremely low level of S lignin in *irx8* cells (**Figure 3.2C**), as does the lack of *F5H1* induction in *irx8* cells grown on xylogenic medium (**Table 3.1**).

TE formation in xylogenic irx8 cells and alterations in xylan epitopes

The TEs formed in xylogenic *irx8* cells have thinner cell walls with reductions in xylan epitopes consistent with changes found in *irx8* stems (Pena et al., 2007; Persson et al., 2007). The *irx8* TEs lack epitopes recognized by LM10 and have reduced labeling by LM11, but retain xylan epitopes recognized by CCRC-M137, CCRC-M138, CCRC-M149, and CCRC-M160 (**Figure 3.3** and **Figure 3.4**). CCRC-M138 belongs to the Xylan-6 clade, and CCRC-M137, CCRC-M149, and CCRC-M160 belong to the Xylan-7 clade (Pattathil et al., 2012), all of which label the entire xylem tissues and interfascicular fibers (Pattathil et al., 2010). TEs formed in xylogenic *irx8* cells display rather normal structure and appear slightly expanded in shape compared with those of the WT cultures (**Figure 3.3G** and **Figure 3.S5**), while TEs formed in the *irx8* stem are collapsed (Pena et al., 2007; Persson et al., 2007). The lack of the collapsed xylem phenotype in the *irx8* suspension culture cells is likely due to the absence of negative pressure associated with water transport. Despite the loss of the LM10 epitope, cell wall sugar composition analysis and glycome profiling revealed similar amounts of, if not more, xylose (**Table 3.1**) and xylan epitopes (**Figure 3.S6**) in *irx8* cells compared to wild-type cells grown on

xylogenic medium. These results suggest that the ability to produce xylan may indeed be unaffected in this mutant, consistent with the unchanged xylan:xylosyltransferase activity in *irx8* stem microsomes reported previously (Brown et al., 2007; Lee et al., 2007a). More interestingly, the TEs formed in xylogenic *G12*-OE cells have increased wall thickness shown by xylan immunolabeling (**Figure 3.4**). The increased xylan production in the xylogenic *G12*-OE cells could be due to the increased expression of xylan biosynthesis-related genes (**Figure 3.6** and **Figure 3.S8**). These results suggest that *GAUT12* expression is tightly correlated with xylan deposition, and that *GAUT12* affects only a part of xylan biosynthesis, perhaps by providing a primer-like structure for xylan elongation, polymerization, or architecture in the wall.

Xylogenic irx8 and GAUT12-OE lines reveal new insights into xylan biosynthesis

It has been suggested that there may exist at least two “types” of xylan (Persson et al., 2007), although the structures of these xylan “types” have not been defined. Both *irx8* and *irx9* single mutants contain nearly no glucuronoxytan but have residual 4-*O*-methyl glucuronoxytan, and the double *irx8 irx9* mutant has additive phenotypes in that it has a greater than 80% reduction of xylose compared to the 40-50% reduction in the single mutants (Persson et al., 2007). This notion is also supported by the evidence that no xylan mutants exhibit a complete loss of xylan in their walls. Although the exact biochemical function of *GAUT12* remains to be defined, our data suggest that in *irx8* cells grown on xylogenic medium, certain xylan biosynthesis-related gene expression has changed, and these genes are likely involved in producing the same type of xylan cooperatively with *GAUT12*.

Multiple studies showed that xylan biosynthesis-related genes have functional orthologs in *Arabidopsis*, including *IRX9* and *IRX9-L/I9H*, *IRX10* and *IRX10-L*, *IRX14* and *IRX14-L/I14H*,

IRX15 and *IRX15-L*, *FRA8/IRX7* and *F8H*, and *GUX1* and *GUX2* (Brown et al., 2009; Lee et al., 2009; Wu et al., 2009b; Keppler and Showalter, 2010; Lee et al., 2010; Mortimer et al., 2010; Wu et al., 2010; Brown et al., 2011a; Jensen et al., 2011; Lee et al., 2012b; Rennie et al., 2012). By analyzing the expression of these genes, we noticed that the two homolog genes have different expression patterns and fall into separate groups (**Figure 3.6**). For example, *IRX9-L/I9H* and *IRX14* with similar expression are in group (III), while *IRX9* and *IRX14-L/I14H* belong to Group (I) and (II) respectively, the two groups of genes which are more tightly-correlated with TE secondary cell wall formation. This is consistent with the more significant role for *IRX9* in maintaining normal plant stature and the xylem-specific expression of *I14H* in stems, respectively (Lee et al., 2010). The four different expression patterns of these xylan genes (**Figure 3.6**) may suggest that they have different functions in xylan biosynthesis, consistent with the report that the *irx9 irx9L* double mutant could not be complemented by the over-expression of *IRX10*, *IRX14*, or *FRA8*, the result of which indicates that these genes are functionally non-redundant (Wu et al., 2010).

Expression levels of Group (I) and (II) genes were up-regulated in *irx8* cells grown on xylogenic medium, suggesting that these genes may work in a different pathway from *GAUT12*, since they may play compensatory roles for the loss of xylan due to the *GAUT12* mutation. LM10 is the only xylan antibody that we have studied so far that showed a complete loss of labeling in *irx8* secondary walls. Hence, we hypothesize that the *GAUT12* mutation caused the loss of xylan epitopes recognized by LM10 (low-substituted xylan). Interestingly, LM10-labeling is present in *irx9* (Pena et al., 2007), *irx10* and *irx10-L* single mutants (Brown et al., 2008), and *irx14* and *irx14-L* single mutants (Keppler and Showalter, 2010). Group (III) genes including *I9H/IRX9-L*, *IRX10-L*, and *IRX14* had relatively high expression in control cells and were

slightly induced in xylogenic medium. The high expression levels of these genes in control cells suggest their possible roles in primary cell wall formation. It should be noted that glucuronoarabinoxylan accounts 5% of dicot primary walls (Scheller and Ulvskov, 2010). Group (IV) contains only three genes. Both *IRX7* and *GUX2* were not induced in *irx8* mutant cells during secondary cell wall formation, suggesting that they are likely involved in the same pathway with *GAUT12* in xylan biosynthesis. *F8H* had barely any expression in any of the culture cells, which is consistent with the finding that the *f8h* mutant was not distinguishable from the WT (Lee et al., 2009). Surprisingly, the very low expression of *F8H* seems to respond negatively to BL-induction. Nevertheless, constitutive over-expression of *F8H* was able to complement the *fra8* mutant (Lee et al., 2009) and the *fra8 f8h* double mutant (Wu et al., 2010). It is possible that *F8H* serves as a functional redundant copy of *FRA8* that is not expressed under normal conditions in the xylogenic suspension culture system.

Why would a biosynthetic gene affect other gene expression?

It has been proposed that *GAUT12*, together with *IRX7/FRA8* and *GATL1/PARVUS*, encodes a putative glycosyltransferase that synthesizes the xylan reducing-end sequence (XRES) in Arabidopsis (York and O'Neill, 2008; Scheller and Ulvskov, 2010). *IRX7/FRA8* and *GATL1/PARVUS* had different expression patterns in *irx8* and *GAUT12*-OE cells grown on xylogenic medium (**Figure 3.S6**). *GATL1/PARVUS* had an expression pattern that is relatively tightly-correlated with secondary wall biosynthesis. In fact, it was more up-regulated in *irx8* and *G12*-OE cells than in WT cells grown on xylogenic medium. Conversely, *IRX7/FRA8* had increased expression in WT and OE cells upon growth on xylogenic medium, but its expression in *irx8* cells was not induced upon growth on xylogenic medium, suggesting that it was not up-

regulated when *GAUT12* was absent. Another gene that has similar expression with *IRX7/FRA8* in *irx8* cells is *GUX2* (**Figure 3.S6**), which has previously been determined to be a redundant homolog of *GUX1*, both of which transfer the GlcA and methyl GlcA residues onto the xylan backbone (Mortimer et al., 2010; Lee et al., 2012b; Rennie et al., 2012). *GUX2* had lower expression than *GUX1*, however it had a similar expression pattern to *GUX1* in *irx8* and *G12*-OE cells grown on xylogenic medium. Interestingly, it also had high expression in *irx8* control cells and relatively high expression in WT cells grown on xylogenic medium. These results suggest that *IRX7/FRA8* and *GUX2* likely work cooperatively with *GAUT12* to produce a specific type of xylan, or a structure required for xylan biosynthesis.

In the microarray study conducted by Kubo et al. (2005), three sets of genes, namely Set 3, Set 8, and Set 22, had up-regulated expression patterns correlating with tracheary element formation starting on day 6 in *Arabidopsis* cells grown on xylogenic medium (Kubo et al., 2005). Among these genes, *GAUT12* was in Set 3, *IRX7/FRA8* appeared in Set 8, and *GUX2* was not identified. Lignin-related TF MYB63 was clustered into Set 4, a group of genes whose transcript levels display slight increases. Besides *GAUT12/IRX8*, Set 3 also included *CESA4*, *GUX1*, *IRX10*, *IRX15-L*, and *GATL1*. *IRX14-L/I14H* was labeled as unclassified, and the genes including *IRX15*, *IRX10-L*, *IRX14*, *F8H*, and *GUX2* were not identified (Kubo et al., 2005). Different from the microarray analysis, which relies on the natural expression abundance of a given gene, our analyses of gene expression by real-time PCR focuses on specific genes and identifies expression pattern of genes with lower expression profiles.

The high expression of *GAUT1* and *GAUT7* in xylogenic *irx8* cells (**Figure 3.7** and **Figure 3.S9**) may suggest that primary cell wall pectin biosynthesis was up-regulated when secondary cell wall xylan biosynthesis was impaired. Alternatively, the fact that both genes have

higher expression in cells grown on xylogenic medium compared to control medium is congruent with a evident role for the GAUT1:GAUT7 complex in both primary and secondary wall formation (Atmodjo et al., 2011). The dramatic up-regulation of *GAUT8*, due to its proposed role in both pectin and xylan biosynthesis, suggests that *GAUT8* may compensate for the loss of *GAUT12* in xylogenic *irx8* cells, although TEs in *irx8* still have reduced wall thickness. Although it is a close homolog to *GAUT12* and previously was suggested to share a similar function as *GAUT12* (Caffall et al., 2009a), the similar expression pattern of *GAUT14* to that of *IRX9-L/19H* and *IRX10-L* in Group (III) reported here suggests that it is unlikely that *GAUT14* compensates for the loss of *GAUT12*. Rather *GAUT14* may have a role in secondary cell wall biosynthesis. Complementation of the *irx8* mutant phenotype by *GAUT14* has not been reported, however, the *irx8 gaut14* double mutant was reported to have no additional phenotypes (Wu et al., 2010). Revealing the biochemical functions of these genes will help to elucidate their potential interaction.

METHODS

Generation of Arabidopsis suspension cell cultures

The Arabidopsis suspension cell cultures were generated as described with slight modifications (Doelling and Pikaard, 1993). All media used were autoclaved and antibiotics were filter (0.22 μ M)-sterilized. All procedures were carried out in a laminar flow hood. Seeds of heterozygous *irx8-5* Arabidopsis plants were sterilized with 70% ethanol (v/v) for 1 min, 10% bleach (v/v) for 10 min, rinsed with sterilized ddH₂O 6-8 times, and plated individually on callus germination media plates containing 0.6% agar, 4.4 g/L MS salt with Gamborg's vitamins (Sigma M0404), 2 mg/L 2,4-D, 0.05 mg/L kinetin, and 20 g/L sucrose (pH 5.7). The sterilized

seeds on plates were grown under a 24-h photoperiod at 23°C. Friable calli from each seed was generated after 8 weeks. The calli were maintained on callus inducing medium (CIM) plates containing 0.6% agar, 3.2 g/L Gamborg's B-5 basal medium with minimal organics (Sigma G5893 or Phytochemistry), 2 mg/L 2,4-D, 0.05 mg/L kinetin (Sigma K3253), and 20 g/L sucrose (pH 5.7). Genomic DNA was isolated from callus germinated from individual seeds using PowerPlant™ DNA Isolation Kit (MO BIO 13200-50) following the manufacturer's instruction. PCR was used to genotype the callus (primer sequences see **Table 3.S1**). The homozygous *irx8-5* callus was identified to contain no *GAUT12* gene product by semi-quantitative RT-PCR. The wild-type callus segregated from the *irx8* heterozygote parent was confirmed to contain no T-DNA insertions within the genome (data not shown) and was thereby used as the WT since it has the most similar genetic background to the *irx8-5* homozygous callus. The homozygous *irx8-5* and WT calli were transferred into 25 ml of CIM in 125 ml flasks and cultured on gyrotory shakers in the dark for 10-12 days. Aliquots of six to seven milliliter compact cultured cells were transferred using serological pipettes (with tip removed) into 100 ml of CIM in a 500 ml flask and cultured on gyrotory shakers at 130 rpm in the dark for 10 days. All sub-cultures were done in the same manner.

Induction of tracheary element

An aliquot (8-9 ml) of 10-day-old WT or *irx8* compact suspension culture cells were inoculated into a 500 ml flask containing 100 ml xylogenic medium. The xylogenic medium contains 3.2 g/L Gamborg's B-5 basal medium with minimal organics (Sigma G5893 or Phytochemistry), 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 1 µM brassinolide (24-epibrassinolide, E244, PhytoTechnology Laboratories), 10 mM H₃BO₃ (Sigma B6768), and 20

g/L sucrose (pH 5.7). Corresponding control cells grown on control medium (CIM) were inoculated in parallel from the same parent culture lines. These cells were cultured on gyrotory shakers at 130 rpm in the dark, and cell samples were taken on different days for analyses.

Stable transformation of Arabidopsis cultures

An aliquot of 1 ml packed cells from 4-day-old culture was co-cultured in 8 ml of fresh CIM with 100 μ l of Agrobacterium harboring the *GAUT12-EGFP* vector (**Chapter 2**) re-suspended in CIM to an OD₆₀₀ of 0.8. The agrobacterium was previously seed-cultured overnight in 3 ml YEP supplemented with rifampicin 50 mg/L, kanamycin 30 mg/L, and gentamycin 50 mg/L and re-cultured in 25 ml of the above YEP. The co-culture was done in a 1-inch-deep petri-dish in the dark on a gyrotory shaker at 120 rpm for 24 h. Cells were removed from co-cultivation and washed 3x with CIM and then with CIM supplemented with cefotaxime 500 mg/L. In each wash, the old medium was completely removed and 20 ml fresh medium introduced and vortexed for 30 s. The washed cells were then plated onto CIM 0.6% agar plates containing 300 mg/L Cefotaxime and 15 mg/L hygromycin for selection. Hygromycin-resistant transgenic calli emerged in 2-3 weeks and were further transferred onto fresh media plates supplemented with the corresponding antibiotics and grown for up to 4 weeks. After two to three transfer cycles, the calli were free of agrobacterium and used to initiate suspension cultures. The suspension cultures were maintained as described above, with supplements of 10 mg/L hygromycin in the medium.

Fixation of suspension culture cells and immunolabeling of tracheary elements

Cells were taken from 9-day-old suspension cultures grown on xylogenic or control media, removed from the media and fixed immediately overnight at 4°C with fixative solution containing 50 mM sodium phosphate buffer (pH 5.8) with 1.6% (w/v) paraformaldehyde and 0.2% (w/v) glutaraldehyde. The following steps were done using a lab-grade microwave (PELCO BioWave Pro, Ted Pella, CA) set at 250 watts. Cells were washed 3× with 50 mM sodium phosphate buffer (pH 5.8) and twice with diH₂O, with each step for 1 min in the microwave. The cells were then dehydrated using an ethanol gradient (35%, 50%, 75%, 95%, 100%, 100%, and 100% [v/v]), with each step done for 40 s. The cells were then infiltrated with cold LR White embedding resin (Ted Pella) in a gradient (1:3, 1:1, 3:1 resin:ethanol [v/v]) and then infiltrated thrice with 100% resin. Each infiltration step was conducted under vacuum (20" Hg) for 2.5 min. The cells were then transferred to gelatin capsules filled with fresh resin, and polymerized under 365-nm UV light at 4°C for 48 h. Cell sections of 250 nm thickness were cut with a Leica EM UC6 ultramicrotome (Leica Microsystems), mounted on pre-coated slides (Colorfrost/Plus, Fisher Scientific) and stained with 0.05% (w/v) toluidine blue for light microscopy or used for immunolabeling experiments.

Immunolabeling for fluorescent microscopy was done as follows. Cell sections were treated for 30 min with freshly made 3% (w/v) non-fat dry milk (NFDM) dissolved in 10 mM KPBS (pH 7.2) containing 500 mM NaCl (blocking). This blocking solution was removed and the sections were incubated with 10 µL of designated CCRC monoclonal antibodies for 1 h. Sections were washed three times with 10 mM KPBS (pH 7.2) containing 500 mM NaCl (wash buffer) by removal of existing droplets and application of fresh buffer followed by a 5 min incubation at room temperature for each wash. The secondary antibody, Alexa fluor 488 goat

anti-mouse IgG (Cat#A11001, Invitrogen), was applied to the sections at a dilution factor of 1:100 (v/v) in the wash buffer. The sections were incubated in the dark at room temperature for 1 h. The sections were washed with the wash buffer and diH₂O, dried by wicking, and a droplet of mounting medium (Citifluor mounting medium AF1, Electron Microscopy Sciences) was added and a cover slip placed on the section. Sections were viewed under a fluorescent microscope (Nikon Eclipse80i). For LM series and JIM series antibodies, a wash buffer containing 10 mM KPBS (pH 7.2) and 100 mM NaCl was used because we found that the use of 500 mM NaCl adversely affected consistent binding of these antibodies. The secondary antibody used for the LM and JIM series was Alexa fluor 488 goat anti-rat IgG (Cat#A11006, Invitrogen) which was applied to the sections in the same fashion as described above but diluted in the low salt wash buffer. Images were captured using a Nikon DS-R1 camera head (Nikon, Melville, NY).

Phloroglucinol-HCl staining

Phloroglucinol-HCl stain for suspension cell cultures was prepared by dissolving 1.25 gram of phloroglucinol in 5 ml 95% ethanol and adding 6 N HCl to 50 ml. This stain was wrapped with aluminum foil and stored in the dark. The stain was made freshly and used for one culture period (10 days). At each time point, 250 µl of cell cultures were taken into glass tubes (13 x 100 mm) and swirled gently with 250 µl of the stain. Pictures were taken after 30 min.

RNA isolation and 1st strand cDNA synthesis of suspension cell cultures

Four and a half ml of medium containing 1 ml of settled cells was taken from each cell culture line on the corresponding day and washed twice with sterilized diH₂O using a Buchner funnel under suction. The cells were transferred into a clean mortar (autoclaved), liquid nitrogen

was added and the cells were ground to powder with a pre-chilled pestle. The cell powder (~100 mg) was transferred to pre-chilled microcentrifuge tubes and frozen at -80°C for RNA extraction. RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, 74904) and first-strand cDNA was synthesized using SuperScript III First-Strand Synthesis Super mix (Invitrogen, 18080-400).

Real-time PCR analyses (primer) and heat map generation

Real-time (quantitative) PCR analysis (Bio-Rad 170-8882) was done using the CFX96™ Real-Time PCR Detection System (Bio-Rad), following the manufacturer's instructions. Melt curve analyses were performed after each run to ensure single-size amplicon production. Data are the average ± standard deviation of three biological samples. The data were analyzed as described by Livak and Schmittgen. Transcript heat maps were created using Python plotting library matplotlib (<http://matplotlib.org/>).

AIR extraction and cell wall glycosyl residue composition analyses

The suspension cells were collected on day 9 grown on either control or xylogenic medium, rinsed thrice with sterilized water and freeze dried. The dried cells were ground to a powder with liquid nitrogen, and extracted for 1-1.5 h per extraction twice with methanol, twice with chloroform: methanol (2:1, v/v), twice with methanol, and thrice with H₂O. The samples were freeze-dried for later use.

Determination of lignin content and composition

The total lignin content of the *Arabidopsis* cell cultures was determined by the acetyl bromide method using 30 mg of extractive-free material (Hatfield et al., 1999). The same molar

extinction coefficient of 17.2 was used for all samples. Lignin composition was determined by thioacidolysis (Lapierre et al., 1985). In brief, 15 mg of extractive-free samples was reacted with thioacidolysis reagents. Lignin-derived monomers were identified by gas chromatography mass spectrometry (GC/MS) and quantified by GC as their trimethylsilyl (TMS) derivatives.

Glycome profiling

Glycome profiling analyses were carried out by enzyme-linked immunosorbent assays of cell wall extracts using a large and diverse collection of plant glycan-directed monoclonal antibodies (<http://glycomics.ccruc.uga.edu/wall2/jsp/abIndex.jsp>) as previously described (Pattathil et al., 2010; Pattathil et al., 2012).

SUPPORTING INFORMATION

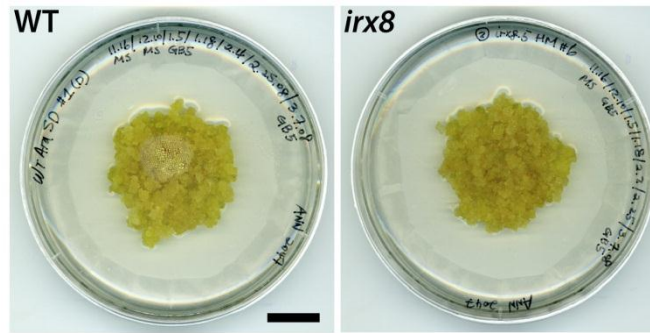


Figure 3.S1. WT and *irx8* calli generated from individual *irx8-5* heterozygote plant seeds.

Both calli are similar in appearance and are friable. The calli were maintained on Gamborg B5 medium (0.6% agar). Bar = 15 mm.

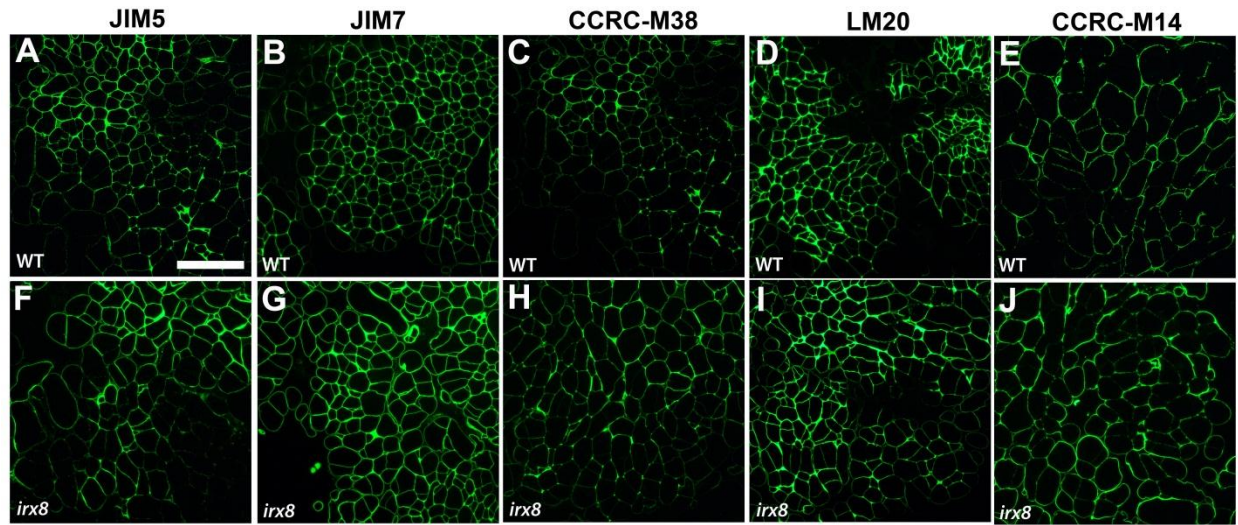


Figure 3.S2. Pectin epitopes in 9-day-old cells of wild-type and *irx8* cells grown on control medium.

(A) WT and (F) *irx8* cells labeled with JIM5 that recognizes low-methylesterified HG.

(B) WT and (G) *irx8* cells labeled with JIM7 that recognizes highly-methylesterified HG.

(C) WT and (H) *irx8* cells labeled with CCRC-M38 that recognizes un-esterified HG.

(D) WT and (I) *irx8* cells labeled with LM20 that recognizes HG similar to JIM7 but that requires some esters for recognition.

(E) WT and (J) *irx8* cells labeled with CCRC-M14 that recognizes RG-I backbone. Bar = 50 μm .

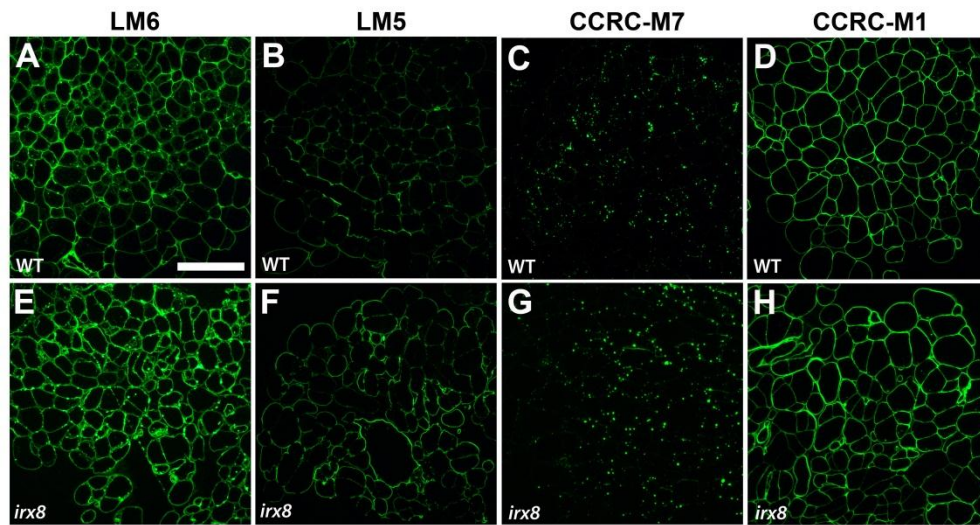


Figure 3.S3. Immunolabeling of 9-day-old cells of wild-type and *irx8* cells grown on control medium.

(A) WT and (E) *irx8* cells labeled with LM6 that recognizes RG-I α -1,5-arabinan side-chains.

(B) WT and (F) *irx8* cells labeled with LM5 that recognizes RG-I β -1,4-galactan side-chains.

(C) WT and (G) *irx8* cells labeled with CCRC-M7 that recognizes 6-linked β -D-galactose oligomers that contain arabinose from AGPs.

(D) WT and (H) *irx8* cells labeled with CCRC-M1 that recognizes fucosylated xyloglucan. Bar = 50 μ m.

A

Cell line	Total lignin (mg/g)	H lignin (umol/g)	G lignin (umol/g)	S lignin (umol/g)
WT + BL (A)	108.95	38.57	114.83	9.10
WT + BL (B)	100.68	39.84	115.55	9.09
WT + BL (C)	113.32	37.42	108.34	9.33
<i>irx8</i> + BL (A)	57.45	5.47	45.86	0.16
<i>irx8</i> + BL (B)	58.43	4.41	53.30	0.18
<i>irx8</i> + BL (C)	54.77	6.33	55.58	0.23
WT (A)	56.9	13.96	40.71	9.94
WT (B)	58.6	13.11	37.11	9.60
WT (C)	59.75	13.20	35.65	9.44
<i>irx8</i> (A)	34.8	0.62	7.34	0.10
<i>irx8</i> (B)	34.68	0.89	7.88	0.15
<i>irx8</i> (C)	34.45	0.80	8.23	0.14

B

Cell line	Total lignin (%)	H lignin (%)	G lignin (%)	S lignin (%)
WT + BL	184 ± 10.2	287.6 ± 9.82	298.5 ± 11.28	95.0 ± 1.89
<i>irx8</i> + BL	97 ± 3.1	40.3 ± 6.44	136.4 ± 12.08	2.0 ± 0.06
WT	100 ± 4.6	100 ± 3.95	100 ± 6.35	100 ± 0.77
<i>irx8</i>	59 ± 1.6	5.7 ± 0.95	20.7 ± 1.27	1.3 ± 0.05

Figure 3.S4. Lignin content and composition determined by acetyl bromide and thioacidolysis analyses.

(A) Nine-day-old cells grown on xylogenic (+BL) medium and control medium. Data are the amount of lignin presented in mg or μmol per gram dry weight of cell walls. A, B, and C are three individual culture trials.

(B) The amount of total lignin and H, S, and G monomers in WT control cells are set to 100%, and the corresponding values in WT+BL, *irx8*+BL, and *irx8* cells were calculated as a percentage ratio, accordingly.

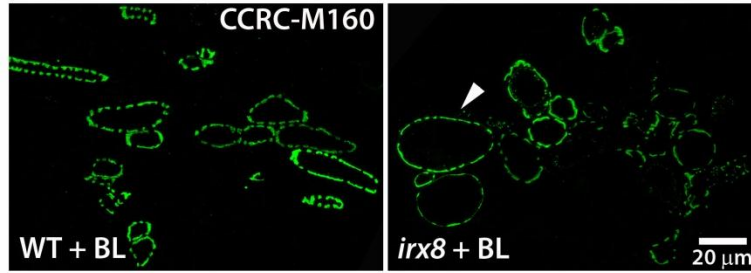


Figure 3.S5. Immunolabeling of TEs in wild-type and *irx8* cells grown on xylogenic medium using CCRC-M160 (Xylan-7).

Vessel elements formed in 9-day-old wild-type (WT + BL) and *irx8* (*irx8* + BL) cells grown on xylogenic medium. White arrowhead indicates larger diameter of *irx8* vessels suggesting less rigid cell walls due to defects in secondary thickening. Bar = 20 μm.

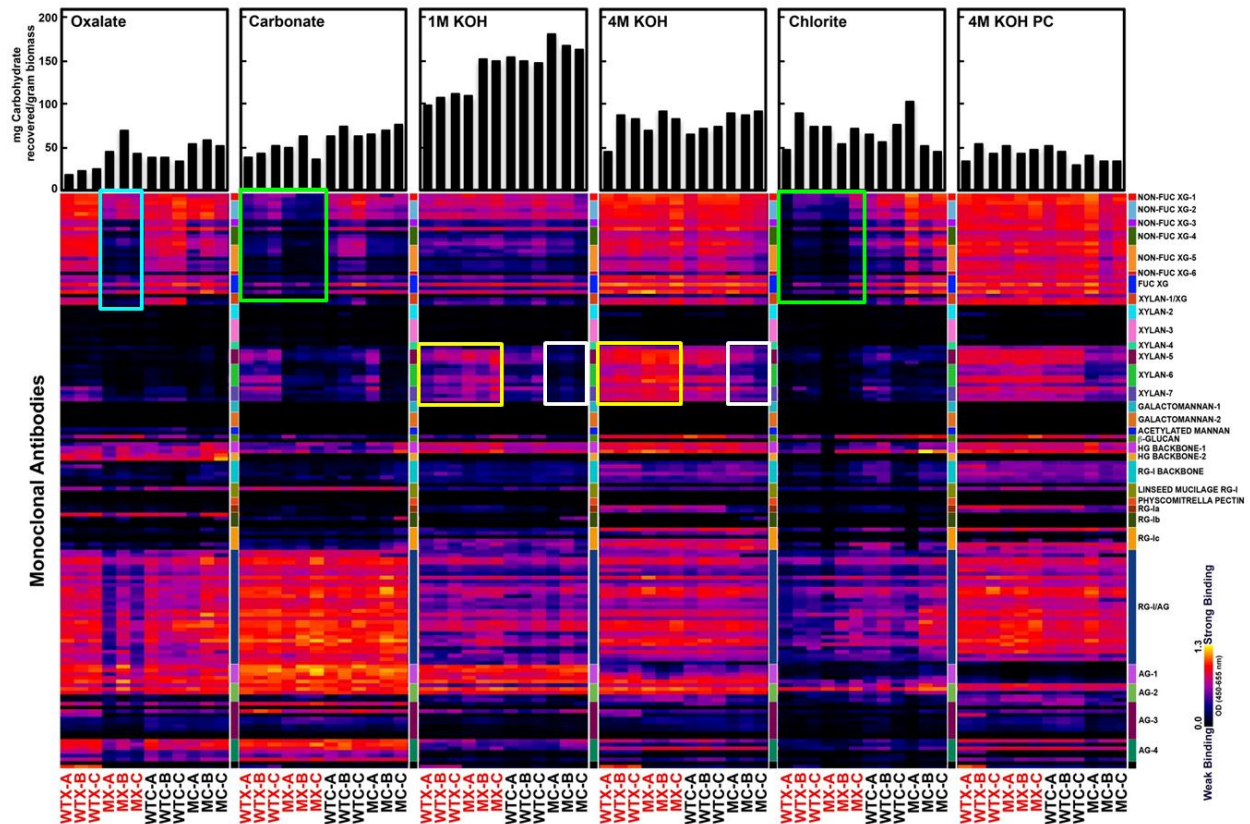


Figure 3.S6. Glycome profiling of cell wall extracts from 9-day-old wild-type and *irx8* suspension culture cells grown on control and xylogenic media.

Sequential extracts were prepared from the cell walls (AIR) isolated from 9-day-old wild-type and *irx8* suspension culture cells grown on control and xylogenic media, using a set of reagents with increasing harshness (as indicated in the top panel). The bars in the top panels depict the amount of carbohydrate masses released by the sequential extractions. The black, blue, red, and yellow colors indicate the binding intensities of plant glycan-directed monoclonal antibodies (black color represents the least/no binding and bright yellow represents the strongest binding). The antibodies used are specific to the different cell wall carbohydrates denoted on the right hand side of the figure. The yellow boxes indicate increased xylan labeling in the 1 M and 4 M KOH fractions from both WT and *irx8* cells grown on xylogenic media. The white boxes indicate less xylan epitopes in *irx8* cells grown on control medium. Green boxes indicate the

reduced xyloglucan epitopes in both WT and *irx8* cells grown on xylogenic medium. And aqua-blue box indicates the reduced xyloglucan epitopes in *irx8* cells grown on xylogenic medium. WTX and MX, WT and *irx8* cells grown on xylogenic medium; WTC and MC, WT and *irx8* cells grown on control medium. The list of plant cell wall glycan-directed monoclonal antibodies (mAbs) used for the glycome profiling analysis are as previously described (Pattathil et al., 2012).

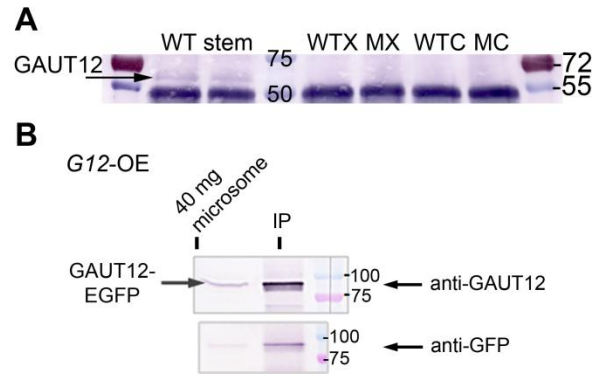


Figure 3.S7. Immunoprecipitation of GAUT12 and GAUT12-EGFP protein from suspension culture cell microsomes and detection by western blotting.

(A) GAUT12 was present in immunoprecipitates from microsomes prepared from WT stems but not from microsomes from WT or *irx8* suspension culture cells grown on xylogenic (WTX, MX) or control (WTC, MC) media.

(B) GAUT12-EGFP (~86 KDa) fusion protein was detected in microsomes (40 µg total protein) prepared from *G12*-OE cells grown on control medium by western blotting using the anti-GAUT12 antibody. GAUT12-EGFP protein was enriched in the immunoabsorbed-GAUT12 fractions.

Preparation of microsomes and immunoprecipitation were done as described in Chapter 2.

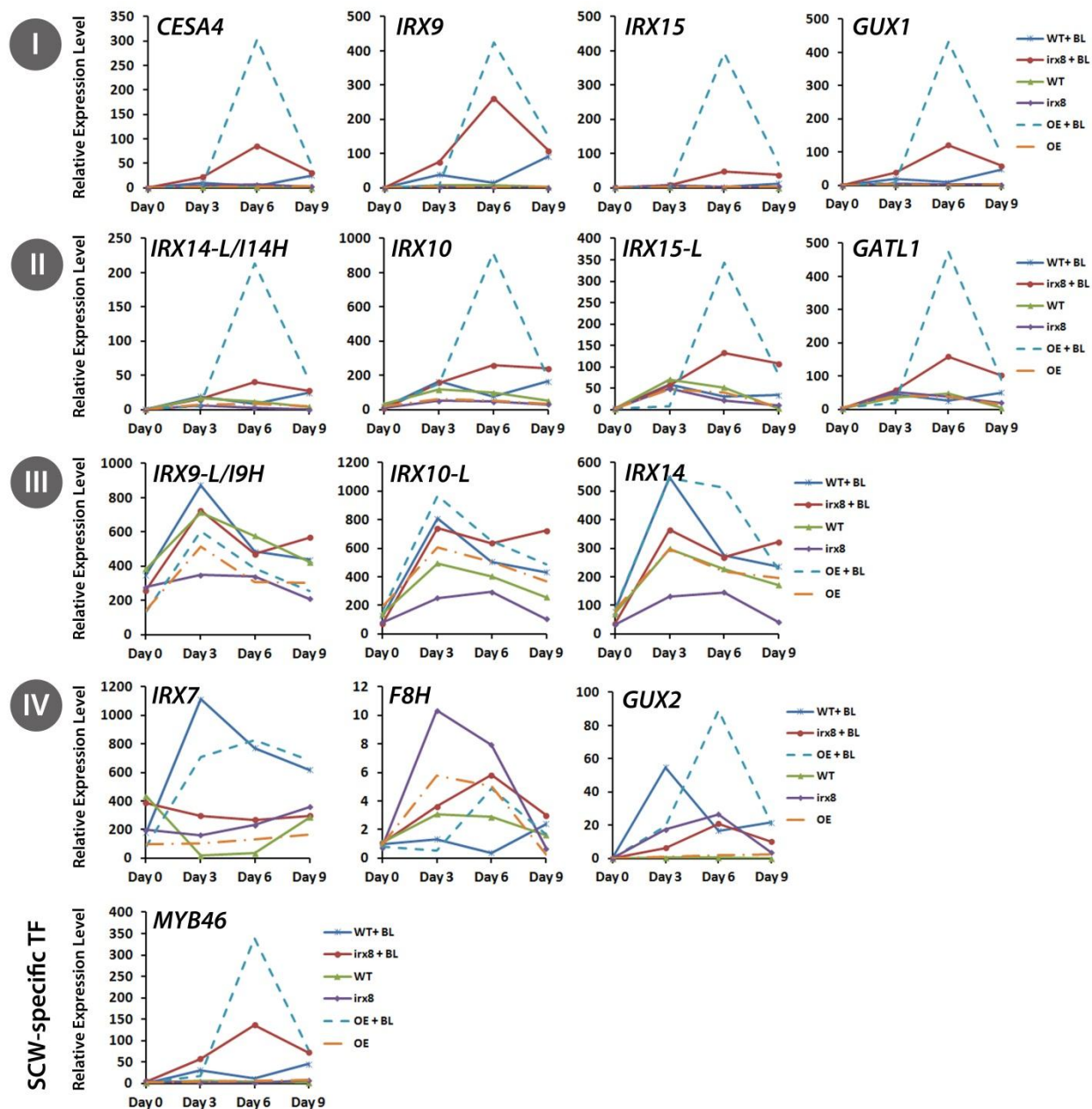


Figure 3.S8. Expression of genes involved in secondary cell wall and xylan biosynthesis in wild-type, *irx8*, and *GAUT12*-overexpression cells grown on xylogenic (WT + BL, *irx8* + BL, OE + BL) and control (WT, *irx8*, OE) media.

This is the same data as shown in Figure 3.6 but is here displayed in line charts with relative expression level values given. *GAUT12* expression in day 0 WT control cells was set to 1. *MYB46*, encodes a secondary wall transcription factor; *CESA4*, secondary cellulose synthase

gene; *IRX9*, *I9H/IRX9-L*, *IRX10*, *IRX10-L*, *IRX14*, *I14H /IRX14-L*, *IRX15*, *IRX15-L*, putative xylan backbone biosynthetic genes; *GUX1*, *GUX2*, xylan GlcA transferase genes; *GATL1*, *IRX7*, *F8H*, genes putatively involved in the xylan XRES biosynthesis.

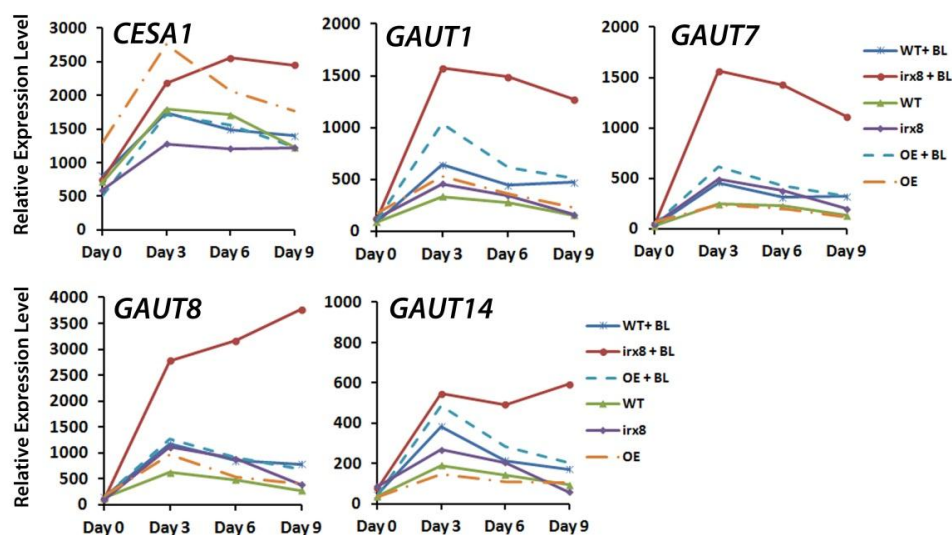


Figure 3.S9. Expression of genes involved in primary cell wall biosynthesis in wild-type, *irx8*, and *GAUT12*-overexpression cells grown on xylogenic (WT + BL, *irx8* + BL, OE + BL) and control (WT, *irx8*, OE) media.

This is the same data as shown in Figure 3.7 but is here displayed in line charts with relative expression level values given. *GAUT12* expression in WT day 0 control cells was set to 1. *CESA1*, primary cellulose synthase gene; *GAUT1*, HG backbone GalA transferase gene (Sterling et al., 2006); *GAUT7* encodes a protein that forms a core complex with *GAUT1* (Atmodjo et al., 2011); *GAUT8* affects HG and xylan biosynthesis (Bouton et al., 2002; Orfila et al., 2005); and *GAUT14* affects wall GalA content (Caffall et al., 2009a).

Table 3.S1. List of primer sequences used in this study.

Genotyping primers	Forward (5'-3')	Reverse (5'-3')
<i>irx8-5</i>	GGTTTGCTTCTTGCTTCCGCT (F)	TTTGGGACATTGACATGAATGGA (R)
<i>Lbc</i>	GGTGATGGTTCACGTAGTGGGCCATCGC (for <i>irx8-5</i>)	
<i>G12</i> transgenics	TCTGGCATATGCTTGGTCTC	CGTTTACGTCGCCGTCCAGCTC
Semi-qPCR primers	Forward (5'-3')	Reverse (5'-3')
	CCATGGCACAGTTACATATATCTCCGAGCTTGAG (1)	GGTACCGCTGATGCTCTAATGTGACAGCTCTTG (2)
Construct primers	Forward (5'-3')	Reverse (5'-3')
GAUT12-EGFP	CCATGGCACAGTTACATATATCTCCGAGCTTGAG	GGTACCGCTGATGCTCTAATGTGACAGCTCTTG
qPCR primers	Forward (5'-3')	Reverse (5'-3')
<i>ACTIN2</i>	GGTAACATTGTGCTCAGTGGTGG	AACGACCTTAATCTTCATGCTGC
<i>GAUT12</i>	CATGGGCTTATGGAATGAATG	AGCTGCCACAAACTCAGGTC
<i>CESA4</i>	GTCTTCTCACCGGCAAGTTC	CCACTCCACCGGAGTTCTAA
<i>IRX9</i>	TACTTTGGGACCCTGAGAGATG	ACAATCTTGTGCCGGAAGTC
<i>IRX9-L/19H</i>	ACAGCACTATACTCTGGGACC	ACACCTTCCATCTCGCTTTC
<i>IRX10</i>	CGCTCTATCTTTGTCTACTTCCG	TTGTCGGGTGATCTGTTGAG
<i>IRX10-L</i>	AAGTGCCATTACAGCTTATTGC	CCTCTTCTATTGCCTTCTCTTC
<i>IRX14</i>	TGGAAGCTGTGGAAGACAAG	TTAGCCGCAACTGTGATCTC
<i>IRX14-L/114H</i>	TGAGAGTCCTTTGTCTTTGGTG	AGGTGACTTTATGATCCAGCC
<i>IRX15</i>	GCTTCTCGCTTTCGTCTCTTTC	TGATGCTGAAGGTGTGGATC
<i>IRX15-L</i>	CAATGCTTCACTACGCTTCAAG	TTCCAGAGAAGGGTTTCGTG
<i>GUX1</i>	CGACTGTAACITCAACTCCGAC	CATATTCCAGCTGTGCCTTTTG
<i>GUX2</i>	TCTTGCTCTTCCTTTGTGGG	CCACAGACATAAGACTCGGAAG
<i>GATL1</i>	CTGCAGATTGCCCCTTAATC	TCGACGTGAGCAGAAGATTG
<i>IRX7/FRA8</i>	GCAaGCGTGTAAGGACGAAT	CTGACTGGTAACCGGCAAAT
<i>F8H</i>	AGTCAACCCTAAGAATATTAGCGG	TCTGATCGGTATCCAGCAAAC
<i>MYB46</i>	GAAGATGTCCGATACCTCCAAC	GTGACGAAGCCTTGTTCTTG
<i>MYB63</i>	AGATCTGAAGCGTGGCAACT	GCGATTTTCGACCACtGTT
<i>PAL1</i>	TATCCCGAACAGGATCAAGG	TCTCCGGTCAAAAAGCTCTGT
<i>C4H</i>	CAGGTAGTGAAGGATTGAAATGTG	CCCACTCGATAGACCACAATG
<i>4CL1</i>	TTTGCTCATCGGTCATCCTG	CGACACGAATTGCTTCACATC
<i>HCT</i>	GCTCTTAAGGCGAAATCCAAG	CTTCCCACTGATCTCCACAC
<i>C3H</i>	GTGCTAACGTGAAGGATCAG	TTCCGCTGTTATCGCTGTC
<i>CCOAOMT1</i>	CTCACAAGATCGACTTCAGGG	ACGCTTGTTGGTAGTTGATGTAG
<i>CCR1</i>	ACCAAGTGCAAGGACGAGAA	GTGCTAGAGGCTTGCTTGG
<i>COMT1</i>	TTGATCTCCACATGTCATCG	ATGTTTCGCTCACTCCAGTCATG
<i>F5H1</i>	TCCATCAAACCTTACCCGTGAC	TGTTGGACCCGTTTTAGATCC
<i>CAD5</i>	CATCAATGGTCAACCTACACAAG	TCAACCGCCATTCTCTCTG
<i>GAUT1</i>	CCACAAGTGGCAAAACATGA	CGCCTTGTTTAAAGGGATGTG
<i>GAUT7</i>	ATGCTTGTCTCTGGATGCTG	GCTTCGCTCGACTCATCTC
<i>GAUT8</i>	TCAAAGAGAAGTTTAATCCCAAAGC	GTCCCCAGTTTCCATAGAGC
<i>GAUT14</i>	AGAAACGTATCACTCTTGGCTTAG	CTCTGGTAGCCTAATCCTAGC
<i>CESA1</i>	GGGCAGTTAAGGTGATTCCA	TTGGGTCCACATCTTCTTCC

CHAPTER 4

CONCLUSIONS AND FUTURE PROSPECTS

Secondary cell walls are composed of cellulose, xylan and lignin which contribute to the major plant biomass on earth. There is increasing evidence that pectin, in addition to its major role in dicot primary walls, may also have a role during secondary wall formation. The *Arabidopsis GAUT12* knock-out mutant *irx8* is severely dwarfed and has collapsed xylem due to reduced wall thickness of vessel and fiber cells (Pena et al., 2007; Persson et al., 2007). Cell wall composition and linkage analyses of *irx8* mutants showed largely reduced glucuronoxylan, a near absence of the xylan reducing-end glycosyl sequence (XRES), and a loss of a small fraction of GalA (Pena et al., 2007; Persson et al., 2007), indicating an interesting role for GAUT12 during secondary cell wall formation. To understand how GAUT12 contributes to this process, as well as to understand the biological and biochemical role(s) it has in *Arabidopsis*, my thesis research focused on the further characterization of the *irx8* mutant in an attempt to identify novel phenotypes that indicate its function, and to establish an *in vitro* biochemical assay to determine GAUT12 enzymatic activity.

To study the biological function of GAUT12, I further characterized the *irx8* mutant. I identified a role for *GAUT12* in lignin biosynthesis and deposition, as both lignin biosynthesis and deposition is reduced in *irx8* stems (**Chapter 2**) and in xylogenic suspension culture cells (**Chapter 3**). The major lignin monomer reduced in the *irx8* mutant is G lignin, which indicates

potential linkages between xylan and G lignin and an alteration in such linkages in the *irx8* mutant. In addition, I showed that *GAUT12* is indispensable for both lignin and xylan deposition through the characterization of a double *irx8 wrky12* mutant (**Appendix E**), in which the ectopic lignin formed in pith cells due to the *WRKY12* mutation was reduced further due to the absence of *GAUT12*. The reduced lignin and xylan in the *irx8* mutant also lead to indehiscent anthers that result in male sterility (**Chapter 2**). Although the pollen grains appear to be smaller in the *irx8* mutant, the pollen is viable upon manual release from the anther for fertilization. Pollen formed in the heterozygous *irx8* mutants was used to pollinate WT pistils, and the progenies displayed a ~1:1 ratio for WT (+/+) and *irx8* heterozygote (+/-) genotypes as determined by PCR analyses (**Chapter 2**). These results show that *GAUT12* does not affect pollen fertility, and that the *irx8* sterility is solely due to indehiscent anthers.

To determine the biochemical function of *GAUT12* and to elucidate the mechanism beyond the *irx8* phenotypes, I designed research to test two hypotheses regarding *GAUT12* enzymatic function (**Chapter 2** and **Appendix D**). Based on the *irx8* wall defects identified in previous studies (Pena et al., 2007; Persson et al., 2007), I hypothesized that *GAUT12* catalyzes either the addition of GalA in the xylan reducing-end sequence (XRES) D-Xylp- β (1 $\rightarrow$ 4)-D-Xylp- β (1 $\rightarrow$ 3)-L-Rhap- α (1 $\rightarrow$ 2)-D-GalpA- α (1 $\rightarrow$ 4)-D-Xylitol (*hypothesis 1*) or the synthesis of a pectic structure (likely to be HG) that is somehow connected to xylan (*hypothesis 2*). Proving *hypothesis 1* remains challenging, as the moiety onto which the reducing-end glycosyl sequence is attached is unknown. Fluor-labeled xylose substrates failed to act as acceptors for the addition of GalA from UDP-GalA using WT stem microsomes as a source of enzymes. Testing *hypothesis 2* was practical, since standard assays for HG:GalAT activity have been established (Doong et al., 1995; Sterling et al., 2005). Compared with wild-type stem microsomes,

microsomes prepared from *irx8* stems indeed had reduced HG:GalAT activity. However, microsomes contain a mixture of enzymes and endogenous acceptors in which GAUT1 may contribute the majority of such HG:GalAT activity, UDP-GalA may undergo conversion to other substrates such as UDP-GlcA or UDP-Xyl, and the GalA may be incorporated onto endogenous acceptors. Being a severely dwarfed plant, the *irx8* mutant is likely to contain less GAUT1 activity in total stem microsomes. Therefore, I used immunoabsorbed-GAUT12 fractions from wild-type stem microsomes and heterologously expressed-GAUT12 to test *hypothesis 2*. I generated an anti-GAUT12 polyclonal antibody that specifically recognizes GAUT12, and immunoabsorbed the native GAUT12 from detergent-permeabilized wild-type stem microsomes. Such immunoabsorbed-GAUT12 fractions contained little HG:GalAT activity, compared to immunoabsorbed-GAUT1:GAUT7 from the same wild-type stem microsomes or from wild-type suspension culture microsomes. Heterologously expressed GAUT12 protein in Arabidopsis suspension cultures also contained little HG:GalAT activity. To investigate whether addition of an HG:GalAT can rescue the *irx8* phenotype, two *GAUT1* constructs were designed and transformed into heterozygous *irx8* plants. Neither *GAUT1* construct complemented the *irx8* phenotype, suggesting that GAUT12 is unlikely to have the same HG:GalAT activity as GAUT1.

To determine whether RG-I or RG-II are possible acceptors for GAUT12, I tested the ability of immunoabsorbed-GAUT12 to incorporate GalA from UDP-GalA onto RG-I oligomers and RG-II monomer. Both forms of RG-I oligomers (with Rha or GalA at the non-reducing end) and RG-II monomers were slightly better acceptors than HG oligosaccharides (OGAs), however, the total incorporation of GalA was low and the specificity was weak (**Appendix D**). Careful characterization of the pectic epitopes in the tricellular junction area of interfascicular fiber cells revealed more abundant labeling of CCRC-M14 in secondary walls in *irx8* stems compared to

wild type. These results indicate that these RG-I epitopes/domains may provide a foundation for xylan deposition, and that GAUT12 function may produce a linker that connects xylan to the RG-I or to a so-called “primer” sequence such as the XRES.

There is a near absence of LM10-recognized xylan and a reduction of LM11-recognized xylan in *irx8* vessels and fiber cells, and conversely, the presence of xylan recognized by other xylan antibodies such as CCRC-M137, CCRC-M138, CCRC-M149, and CCRC-M160 in both *irx8* stems and suspension culture cells grown on xylogenic medium. This suggests that GAUT12 is likely to affect the biosynthesis of either a subfraction of xylan or a wall architecture/structure required for xylan biosynthesis. To investigate the effect of the *irx8* mutation on xylan biosynthesis, Arabidopsis suspension culture cell lines of wild type, *irx8*, and GAUT12-overexpression (*G12-OE*) were established and expression level changes of genes involved in xylan biosynthesis were analyzed in these cells grown on control and xylogenic media (**Chapter 3**). Expression patterns of these genes fell into four distinctive groups, and particularly, *IRX7* and *GUX2* were not induced in *irx8* cells grown on xylogenic medium, indicating that these two genes may encode proteins that work cooperatively with GAUT12 in xylan biosynthesis.

To conclude, although the exact biochemical function of GAUT12 remains elusive, I have shown that it is not an HG:GalAT similar to GAUT1, using anti-GAUT12 polyclonal antibody that I generated and well-characterized. Besides being defective in xylan and a subfraction of pectin as previously reported, the *irx8* mutant has a lignin defect, particularly a reduction in G lignin. The reduced xylan and lignin contents caused the *irx8* mutant to have indehiscent anthers resulting in male sterility. In addition, the study of xylan biosynthesis-related genes in xylogenic *irx8* suspension culture cells revealed that *IRX7* and *GUX2* may function

cooperatively with *GAUT12* in xylan biosynthesis. Further work will focus on the elucidation of the connection between xylan and lignin that is defective in the *irx8* mutant, and testing the potential interactions of GAUT12 with other GTs during xylan biosynthesis.

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APPENDIX A

ACRONYMS AND ABBREVIATIONS

a.a.	amino acid
AHP	Arabidopsis histidine phosphotransfer proteins
AIR	alcohol insoluble residue
ALDH	aldehyde dehydrogenase
<i>Apif</i>	apiofuranose
<i>Araf</i>	arabinofuranose
<i>Arap</i>	arabinopyranose
ASL	ASYMMETRIC LEAVES2-LIKE
ATHB	ARABIDOPSIS THALIANA HOMEODOMAIN GENE
AX	arabinoxylan
AXS	UDP-D-APIOSE/UDP-D-XYLOSE SYNTHASE
AXY	ALTERED XYLOGLUCAN
BiFC	bi-molecular fluorescence complementation
BL	brassinolide
C4H	trans-cinnamate 4-hydroxylase
CAZy	Carbohydrate Active enZymes
CEL	cellulosic enzyme lignin

CESA	cellulose synthase
CHS	chalcone synthase
CIM	callus inducing medium
CPS	capsular polysaccharide
DEX	dexamethasone
DFRC	derivatization followed by reductive cleavage
Dha	3-deoxy-D-lyxo-2-heptulosaric acid
DR	dominant repression
DUF	domain of unknown function
EMS	ethyl methanesulfonate
EPG	endopolygalacturonase, EPGase
FRET	Förster resonance energy transfer
Fuc	fucose
GAE	UDP-D-GLUCURONATE 4-EPIMERASE
Gal	galactose
GalA	galacturonic acid
GalAT	galacturonosyltransferase
GAUT	GALACTURONOSYLTRANSFERASE
GAX	glucuronoarabinoxylan
GC	gas chromatography
Glc	glucose
GlcA	glucuronic acid
GlcAT	glucuronosyltransferase

GR	glucocorticoid
GT	glycosyltransferase
GUS	beta-glucuronidase
HCT	hydroxycinnamoyl CoA:shikimate/quinic acid hydroxycinnamoyltransferase
HEK	human embryonic kidney
HG	homogalacturonan
HK	heat-killed, heat-inactivated
HM	homozygous
HSQC	heteronuclear single-quantum correlation spectroscopy
IF	interfascicular fiber
IFL1	INTERFASCICULAR FIBERLESS1
IP	immunoprecipitation
IR	infrared
IRX	irregular xylem
KD	knockdown
Kdo	3-deoxy-D-manno-octulosonic acid
KNAT	KNOTTED1-LIKE HOMEODOMAIN PROTEIN
KO	knock-out
KOR	KORRIGAN
LBD	LATERAL ORGAN BOUNDARIES DOMAIN
LCC	lignin-carbohydrate complex
LC-MS/MS	liquid chromatography tandem mass spectrometry
LPS	lipopolysaccharide

Man	mannose
meGlcA	methyl glucuronic acid
MGP4	MALE GAMETEPHYTE DEFECTIVE4
MT	methyltransferase
MUM	MUCILAGE MODIFIED
MUR	MURUS
MW	molecular weight
MWL	milled wood lignin
NMR	nuclear magnetic resonance
NOESY	nuclear overhauser effect spectroscopy
NST	NAC SECONDARY WALL THICKENING PROMOTING FACTOR
OGA	oligogalacturonide
PAL	phenylalanine ammonia-lyase
PAP1	PRODUCTION OF ANTHOCYANIN PIGMENT1
PCD	programmed cell death
PGSIP	plant glycogenin-like starch initiation protein
PME	pectin methylesterase
pyMBMS	pyrolysis molecular beam mass spectrometry
QUA	QUASIMODO
REF	REDUCED EPIDERMAL FLUORESCENCE
RG-I	rhamnogalacturonan-I
RG-II	rhamnogalacturonan-II
RGP	Reversibly Glycosylated Protein

RGXT	RG-II:α-1,3-xylosyltransferase
Rha	rhamnose
RHM	RHAMNOSE BIOSYNTHESIS
RNAi	RNA interference
RT	real-time, room temperature
RWA	REDUCED WALL ACETYLATION
SAD	sinapyl alcohol dehydrogenase
SD	standard deviation
SE	standard error
SEM	scanning electron microscopy
SND	SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN
TBL	Trichome Birefringence-Like
T-DNA	transfer DNA
TE	tracheary element
TEM	transmission electron microscopy
TF	transcription factor
TM	transmembrane (domain)
TMS	trimethylsilyl
TOCSY	total correlation spectroscopy
TX-100	Triton X-100
UGD	UDP-GLC DEHYDROGENASE
USP	UDP-SUGAR PYROPHOSPHORYLASE
UV	ultraviolet

VIGS	Virus-induced gene silencing
VND	VASCULAR-RELATED NAC-DOMAIN
XAT	xylan arabinosyltransferase
XCP	xylem-specific papain-like cysteine peptidase
XG	xyloglucan
XGD	XYLOGALACTURONAN DEFICIENT
XRES	xylan reducing-end sequence
Xyl	xylose
XylT	xylosyltransferase
4CL	4-coumarate:CoA ligase

APPENDIX B

RECOMMENDED CRITERIA FOR DETERMINING WHETHER A GENE ENCODES A CONFIRMED OR PUTATIVE PLANT CELL WALL BIOSYNTHETIC ENZYME

Confirmed (confirmed/proven) Enzyme Function

Obtaining desired enzyme activity upon expression of the gene of interest in a heterologous system that does not itself produce the product of interest or contain the enzyme activity. Data must include a negative control that proves that the heterologous system by itself does not have the enzyme activity or produce the product of interest. Related data that show that the product is produced in the cell wall of the heterologous host upon expression of the gene of interest would also apply, providing that the negative controls show no presence of the product in untransformed/transfected host walls.

Putative (strong, medium, weak) function

Strong:

Obtaining higher levels of desired enzyme activity and/or product upon expression of the gene of interest in a heterologous system that does itself contain the enzyme activity and produce the product of interest, albeit at lower levels than the transformed/transfected host. In this case, rigorous controls must make clear that the activity/product present in the untransformed

heterologous host is significantly and reproducibly lower than in the transformed/transfected heterologous host cells.

Medium:

Microsomal membranes or soluble/solubilized enzyme preparations isolated from mutants of the gene of interest have lower enzyme activity/fewer products than wild type. Rigorous determination of equivalent developmental state for the tissue(s) compared in the mutant versus control must be presented.

Walls from mutants of the gene of interest have structural changes and/or antibody/carbohydrate binding domain reactive epitope changes compared to wild type that are consistent with a change of structure that is predicted to occur upon a reduction in the amount of the enzyme activity of interest. Rigorous determination of equivalent developmental state for the tissue(s) compared in mutant versus control must be presented.

Weak:

Gene of interest is a member of a gene family with a member that has been shown to have specified enzyme activity. This is a very weak indication of possible function as there are many examples of members of gene families that have disparate enzyme activities.

For example, GAUT7 and GAUT1 belong to the 15 member Arabidopsis GAUT gene family and have 36% identity/59% similarity. Yet, available evidence indicates that while GAUT1 has homogalacturonan:α-1,4-galacturonosyltransferase activity, GAUT7 does not have this activity when expressed in a comparable heterologous systems. They do, however, belong to a GAUT1:GAUT7 complex and GAUT7 serves as an anchor to hold GAUT1 in the Golgi.

APPENDIX C

IDENTIFICATION OF PROTEINS THAT CO-IMMUNOABSORB WITH GAUT12 FROM WILD-TYPE ARABIDOPSIS STEM MICROSOMES

To help elucidate GAUT12 enzymatic activity, we developed a polyclonal anti-GAUT12 antibody (a service through New England Peptide, NEP, <http://www.newenglandpeptide.com/>). The characterization of this antibody and the immunoprecipitation methods were described in **Chapter 2**. This appendix presents the preliminary work to identify other proteins besides GAUT12 in the immunoabsorbed-GAUT12 fraction from detergent-treated wild-type Arabidopsis stem microsomes using liquid chromatography tandem mass spectrometry (LC-MS/MS) and the GAUT12-specific antibody. The LC-MS/MS was done by Xiang Zhu from Ron Orlando's lab, CCRC.

The immunoabsorbed-GAUT12 fraction was denatured and reduced with the sample loading buffer (3% SDS and reduced by 25 mM DTT), and then separated by SDS-PAGE. The protein band of interest was cut from the un-stained gel and processed for LC-MS/MS by in-gel trypsin digestion. The detailed method was described in **Chapter 2** and the procedure is recapitulated in **Figure C-1**.

While the anti-GAUT12 antibody was antigen-purified (a service through NEP), a small aliquot of antibody was biotinylated. The reason to generate such a biotinylated anti-GAUT12

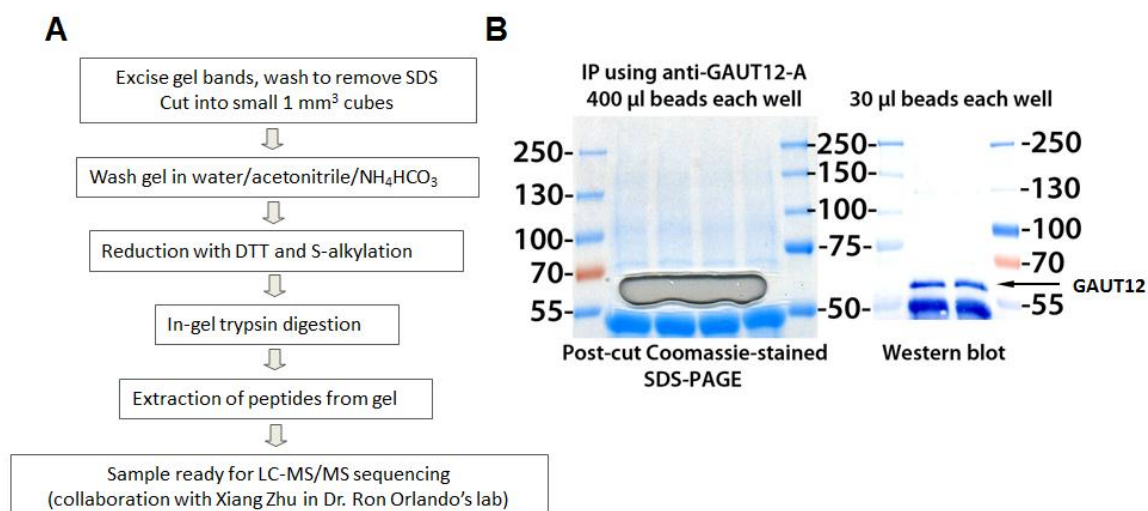


Figure C-1. Preparation for LC-MS/MS to determine the specificity of the anti-GAUT12 antibody.

(A) Schematic diagram of in-gel trypsin digestion. The estimated GAUT12 band (between 55 and 70 KDa marker) immunoabsorbed from detergent-treated wild-type Arabidopsis stem microsomes was cut from the un-stained gel and in-gel trypsin digestion was carried out as described (**Chapter 2**).

(B) Post-cut gel was stained with Coomassie blue, and a small aliquot of the same sample was examined on a separate gel followed by western blot analysis to verify the presence of the GAUT12 protein.

antibody was to efficiently separate the GAUT12 protein from its antibody so that GAUT12 and its associated proteins could be studied. The biotinylated anti-GAUT12 antibody was incubated with streptavidin-conjugated magnetic beads (Invitrogen), and the immunocomplex was subsequently incubated with detergent-permeabilized microsomes to immunoabsorb GAUT12. In theory, the non-covalent interactions between the biotinylated antibody and the streptavidin beads is stronger than the interaction between GAUT12 and its antibody, and therefore, GAUT12 can be eluted using 0.1 M glycine (pH 2.5). The eluted fraction would then be neutralized immediately with one volume of 1 M Tris (pH 9.0), and analyzed by western blot to verify the efficiency of the immunoprecipitation and to check for presence of the anti-GAUT12 antibody. The results were somewhat unexpected. As shown in **Figure C-2**, a small amount of anti-GAUT12 antibody always eluted together with GAUT12 (**Figure C-2A**). Although the amount of anti-GAUT12 eluted was reduced after the beads were “recycled” (**Figure C-2B**), it was not possible to completely eliminate the co-elution of GAUT12 and anti-GAUT12 antibody. “Recycle” means that the beads have been recovered by the magnet after incubation with detergent-permeabilized microsomes, treated with 0.1 M glycine (pH 2.5) to elute GAUT12, immediately washed and stored in 1×PBS (pH 7.4) buffer, and reused in the next immunoprecipitation experiment.

In a separate experiment, to remove loosely attached IgG, 500 µl streptavidin beads were incubated with biotinylated anti-GAUT12 antibody and treated four times with 0.1 M glycine (pH 2.5) before incubation with detergent-permeabilized microsomes. The eluted fraction was divided in half. One half of the elution product was denatured and reduced (**Figure C-2, E7a**), and the other half was denatured but kept non-reduced (**Figure C-2, E7b**). Interestingly, a larger sized protein band (~140 kDa, red arrow) was identified in the non-reduced fraction

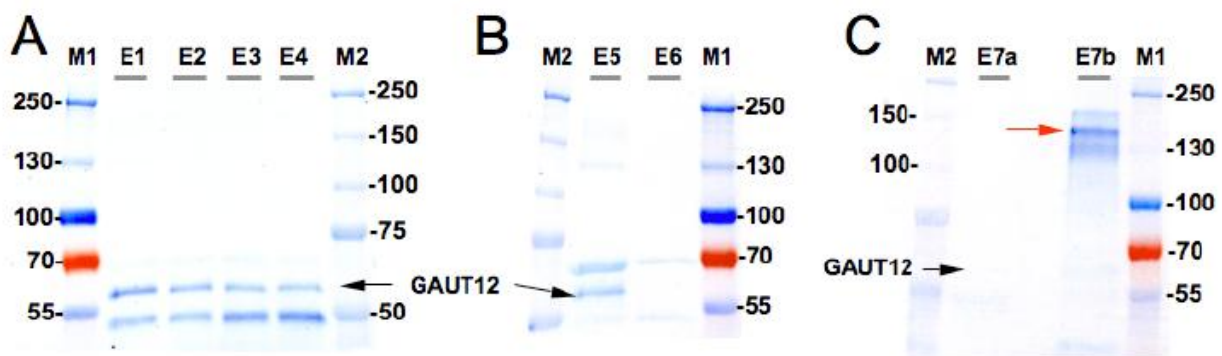


Figure C-2. Optimization of immunoprecipitation (IP) using biotinylated anti-GAUT12 from wild type Arabidopsis stem microsomes (2% Triton X-100 treated).

Biotinylated anti-GAUT12 was bound to magnetic streptavidin beads (Invitrogen) via biotin-streptavidin association and then used to pull down GAUT12 from detergent-permeabilized wild-type Arabidopsis stem microsomes. Elution of GAUT12 was achieved using 9 volumes of 0.1 M glycine (pH 2.5). The supernatant was immediately neutralized with 1 volume of 1 M Tris (pH 9.0).

(A) Time-course of elution of GAUT12 from 250 µl of beads. E1-E4: 1, 2, 5, 10 min of 0.1 M glycine (pH 2.5) incubation. Samples were denatured, reduced, and separated on 7.5% SDS-PAGE gel and probed with anti-GAUT12 antibody by western analysis. GAUT12 is indicated by the arrow. The band of ~50 kDa was the released antibody (IgG). An incubation time of 1 min was sufficient to release GAUT12.

(B) Beads (130 µl) were recovered from the experiments in (A) and reused to immunoprecipitate GAUT12 from a new batch of detergent-permeabilized microsomes. E5, presence of GAUT12 as indicated (arrow), the amount of IgG released was reduced. However, an additional band of ~67 kDa (BSA) was introduced by wash buffer (1×PBS, 0.1% BSA). To avoid BSA, subsequent IP beads were washed additionally with 1×PBS before elution. E6, Beads (100 µl) from (A) were

eluted without further incubation with microsomes, and there was no detectable GAUT12 released. This suggested elution in (A) was sufficient and complete.

(C) Streptavidin beads (500 μ l) were incubated with biotinylated anti-GAUT12 antibody and treated four times with 0.1 M glycine (pH 2.5) before incubation with microsomes to remove the loosely attached IgG. Following incubation with 300 μ l detergent-permeabilized microsomes and proper elution, half of the elution product was denatured and reduced (E7a), and the other half of was denatured but kept non-reduced (E7b). GAUT12 protein band (black arrow) had faded (E7a) due to possible loss in elongated transfer (for larger proteins) from the gel to the PVDF membrane. However, a larger sized protein band ($\sim$ 140 kDa, red arrow) was identified (E7b). This result was reproducible, but the band/smear of higher molecular weight also appeared to be present in the absence of primary antibody (anti-GAUT12) in western blot analyses (not shown), indicating that this band was likely the non-reduced IgG but not the GAUT12-containing protein complex.

(**Figure C-2, E7b**). This result was reproducible, but the band/smear of higher molecular weight also appeared to be present in the absence of the primary antibody (anti-GAUT12) in western blot analysis (not shown), indicating that this band was likely the non-reduced IgG but not a GAUT12-containing protein complex. Therefore, I did not continue to use this biotinylated anti-GAUT12 antibody but switched to the purified anti-GAUT12 antibody for the study of GAUT12 and its associated proteins.

A large scale immunoprecipitation was done using 550 μ l of the anti-GAUT12 antibody-conjugated beads for incubation with wild-type stem microsomes (5 mg total protein) that were homogenized with 4% TX-100 supplemented with 200 mM NaCl, 100 mM NaOAc, and 2 mM EDTA for maximum recovery of GAUT12 protein (final TX-100 concentration for incubation was diluted to 0.5% with PBS buffer). The negative control was done in parallel using pre-immune IgG purified from the pre-immune serum using a HiTrap Protein-A HP 1 ml column (GE Healthcare Life Sciences) as described (Atmodjo et al., 2011). The purified total IgG was examined on SDS-PAGE for purity (**Figure C-3A**). Two aliquots of 25 μ l immunoabsorbed-GAUT12 fraction were analyzed by western blot that serves as a standard for gel-cutting (**Figure C-3B**), and the remaining 500 μ l immunoabsorbed-GAUT12 fraction was separated on a different SDS-PAGE gel in parallel (in the same gel tank). The un-stained gel was cut into 12 pieces according to the marks in the western blot (**Figure C-3B**) based on the protein marker positions. The gel pieces were individually processed as described in Chapter 2, and sent for LC-MS/MS sequencing.

The LC-MS/MS was done as described in Chapter 2. The immunoprecipitation was performed thrice with anti-GAUT12 antibody and twice with the pre-immune IgG, and the

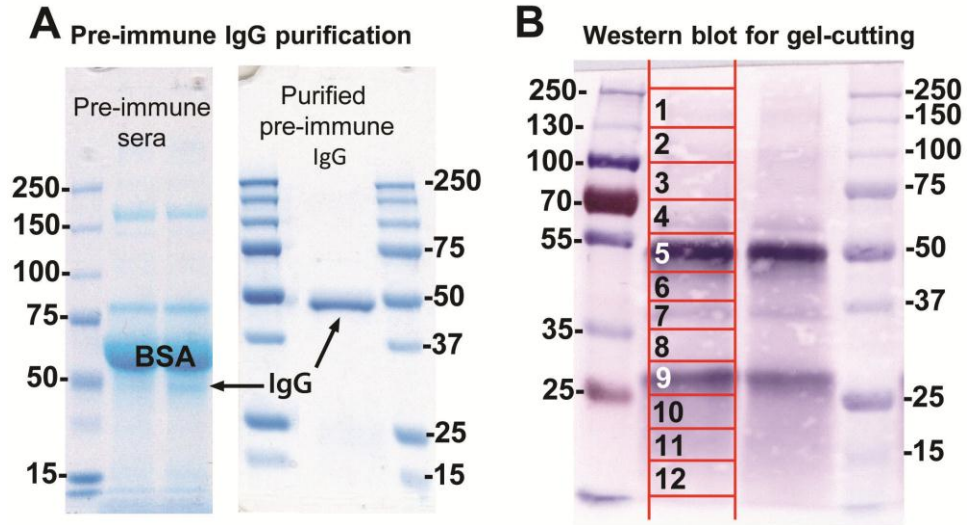


Figure C-3. Pre-immune IgG purification and western blot illustration of gel-cutting for liquid chromatography tandem mass spectrometry.

(A) The left panel shows the pre-immune sera before purification. The right panel shows the pre-immune IgG purified from the pre-immune sera using a HiTrap Protein-A HP 1 ml column. Data are SDS-PAGE gels stained with Coomassie blue.

(B) Two aliquots of 25 μ l immunoabsorbed-GAUT12 fraction were analyzed by western blots, which served as a standard for gel-cutting. The parallel gel run in the same tank with the 500 μ l immunoabsorbed-GAUT12 fractions was cut in the same way as labeled. Twelve gel pieces were obtained and processed as previously described (**Chapter 2**). Protein standard sizes are as labeled.

proteins identified using LC-MS/MS were compiled and processed to yield **Table C-1**. The proteins listed in **Table C-1** were combined proteins identified from all three experiments of the GAUT12-immunoabsorbed fractions, with total peptides number ≥ 4 , total % sequence coverage ≥ 10 . These proteins were also not present in pre-immune IgG fractions. GAUT12 was the most abundant protein in the GAUT12-immunoabsorbed fractions, with 15 specific peptide sequences identified by mass spectrometry that represented ~24% of its entire sequence (**Table C-1**). The results confirmed that the anti-GAUT12 antibody was specific in recognizing GAUT12 but no other GAUT proteins. However, besides GAUT12, all other proteins recovered with high confidence were photosynthesis- and ATP synthesis-related proteins. This result suggests that GAUT12 is unlikely involved in protein complex formation with other glycosyltransferases (or with other wall biosynthesis-related proteins) through covalent linkages. Nevertheless, the possibility remains that GAUT12 may be interacting with wall biosynthesis-related enzymes/proteins through non-covalent interactions, which may be destroyed by the treatment with Triton X-100 during the immunoprecipitation.

Table C-1. Proteomic analyses of GAUT12 immunoabsorbed from detergent-treated wild-type Arabidopsis stem microsomes. Data are analyzed from three and two independent immunoprecipitation experiments using anti-GAUT12 and pre-immune IgG, respectively. The proteins listed are combined proteins identified from all three experiments, with total peptides number ≥ 4 , total % sequence coverage ≥ 10 , and not present in pre-immune IgG fractions.

Gene ID	Sequence Name	Protein Length (a.a.)	MW (kDa)	Total Score	Total Peptides	Total % Seq. Coverage	Total Spectra	Total Protein Probability
AT5G54690	GAUT12, LGT6, IRLX8 (galacturonosyltransferase 12)	535	60.82	749.7	15	24.85981308	100	1
AT1G29910	CAB3, AB180, LHCB1.2 (chlorophyll A/B binding protein 3)	267	28.19	397.12	7	31.46067416	48	0.497997176
AT4G10340	LHCB5 (light harvesting complex of photosystem II 5)	280	30.12	394.45	7	31.07142857	49	1
AT1G78900	VHA-A (vacuolar ATP synthase subunit A)	623	68.75	304.11	6	10.43338684	11	0.914057819
AT3G08580	AAC1 (ADP/ATP carrier 1)	381	41.43	315.81	5	14.69816273	28	0.941097963
AT1G61520	LHCA3 (photosystem I light harvesting complex gene 3)	273	29.15	282.01	5	20.14652015	44	0.773168661
ATCG00480	ATPB, PB (ATP synthase subunit beta)	498	53.88	443.67	8	26.90763052	13	1
ATCG00020	PSBA (photosystem II reaction center protein A)	353	38.89	266.85	5	15.58073654	10	0.99999991

Total score: the sum of the peptide scores (Mascot Ion Scores) for all peptides matching to a protein.

Total peptides: the number of peptides recovered for a protein. Proteins with total peptides number ≤ 4 are not listed.

Total Seq. Coverage: The percentage of recovered peptides counted to total peptide sequence.

Proteins with total seq coverage ≤ 10 are not listed.

Total probability: the estimate of the likelihood that the protein assignment is correct based on the peptide probabilities for the identified peptides mapping to the protein.

Total spectra: the sum of all spectra generating peptide identifications to a given protein, i.e. the number of redundant protein spectra.

APPENDIX D

ENZYMATIC ACTIVITY STUDIES OF GAUT12

To understand how GAUT12 affects lignin and xylan biosynthesis and deposition in *Arabidopsis* secondary cell wall formation, we have attempted to determine its enzymatic activity. GAUT12 is predicted to be a type II transmembrane protein with its presumed C-terminal catalytic domain facing the Golgi lumen. The YFP-tagged GAUT12 is localized to the Golgi apparatus, consistent with its predicted sub-cellular localization (Pena et al., 2007). Stem microsomes have been prepared from *GAUT12* mutants and used to study xylosyltransferases (XylT) activity. Apparently no reduction of XylT activity has been identified in *irx8* microsomes when using UDP-Xyl and xylohexaose as substrates (Brown et al., 2007; Lee et al., 2007a), suggesting that GAUT12 is not synthesizing the xylan backbone. A previous study by Pena *et al.* identified a lack of a xylan reducing-end tetrasaccharide sequence (XRES) β -D-Xylp-(1 $\rightarrow$ 4)- β -D-Xylp-(1 $\rightarrow$ 3)- α -L-Rhap-(1 $\rightarrow$ 2)- α -D-GalpA-(1 $\rightarrow$ 4)-D-Xylp in *irx8* stems, together with a large reduction of xylan, causing the authors to speculate a function of GAUT12 in adding the GalA residue to the terminal xylose in the XRES (Pena et al., 2007). This is *hypothesis 1*.

Whether the reducing-end xylose is free or attached to some moiety during xylan *in vivo* synthesis remains unknown, posing a major difficulty to study this hypothesis. We attempted using 4-methylumbelliferyl- β -D-xylopyranoside (4-MU-Xyl) and 4-nitrophenyl- β -D-xylopyranoside (pNp-Xyl) as substitute reducing-end acceptor substrates, and used wild type

stem microsomes as the enzyme source. However, we recovered no addition of GalA to either of the acceptor substrates (methods see **Chapter 2**).

Due to high levels of GAUT12 protein sequence identity (40%) and similarity (61%) to GAUT1, and the fact that there was a small reduction of GalA in *irx8* stems (Persson et al., 2007), the other hypotheses (*hypothesis 2*) is that GAUT12 is involved in pectin biosynthesis, similar to GAUT1, a homogalacturonan α -1,4-galacturonosyltransferase involved in pectin backbone synthesis (Sterling et al., 2006). A part of this research is presented in Chapter 2, while the excluded experiments, including microsome preparation, assay conditions, detergent concentration optimization, and other acceptors tested, are summarized in this Appendix.

Preparation of microsomes

Microsomal membranes used for enzyme activity assays were prepared as described below. Liquid nitrogen flash-frozen Arabidopsis stems (10 grams) were cut into small pieces with a clean scissors and homogenized on ice in 20 ml of pre-chilled homogenization buffer containing 50 mM Hepes (pH 7.3), 0.4 M sucrose, 0.1 M sodium ascorbate, 0.25 mM MnCl₂, 25 mM KCl, 1% (w/v) polyvinylpyrrolidone (PVPP), and EDTA-free protease inhibitor cocktail (Roche) until the tissues were pureed. The homogenate was filtered through 3 layers of miracloth and the filtrate centrifuged at 4°C for 30 min at 4,000 *g* to remove cell debris and intact cells. The supernatant was ultra-centrifuged at 110,000 *g* for 1 h, yielding the microsome pellet, which was re-suspended on ice in the pre-chilled storage buffer (homogenization buffer without PVPP, 30 μ l buffer/gram fresh weight) with a glass homogenizer. Total protein concentration was measured by the Bradford assay (Bio-Rad Protein Assay 500-0006) using BSA as the standard. Aliquots of the microsomes were used directly or flash frozen in liquid nitrogen and stored at

-80°C for later use in enzyme assays or immunoprecipitation experiments. For suspension cell cultures, the cells were harvested by suction filtration and cells were quickly rinsed once with water. The microsomes were then prepared as described above.

Filter assay and precipitation assay

The HG:GalAT filter assay (Sterling et al., 2005) was designed for fast and easy separation of unincorporated radiolabeled UDP-[¹⁴C]GalA from negatively charged pectic polysaccharides or oligosaccharides of sufficient length to bind to cetylpyridinium chloride (CPC)-treated filter paper squares. The reactions (30 µL) are terminated with 10 µL 400 mM NaOH, quickly vortexed and collected by fast centrifugation, and spotted onto CPC-treated filter squares and washed three to four times each for 15 min with 150 mM NaCl buffer. The filters are air dried, submerged in 4 mL scintillation cocktail fluid and subjected to scintillation counting.

The HG:GalAT precipitation assay (Doong and Mohnen, 1998) is a method to precipitate polymerized sugar products generated in the enzyme reactions. The reactions are terminated with 700 µL chloroform:methanol (3:2), and 100 µg of a sugar carrier, such as 4-*O*-methyl-D-glucurono-D-xylan (Sigma M5144) or OGA (7-23 mer), is added to precipitate the reaction products. The sugar products are pelleted at 12,000 rpm for 5 min and washed in 65% EtOH twice or thrice. Each wash step includes sonication and vortexing to re-suspend the pellet, followed by centrifugation at 12,000 rpm to collect the pellet. The final washed pellet is re-suspended in 100 µL of water and subjected to scintillation counting after the addition of 2 mL scintillation cocktail.

Effect of Triton X-100 on HG:GalAT activity

To test the effect of TX-100 on HG:GalAT activity in Arabidopsis stem microsomes, and to determine the concentration of TX-100 required to solubilize HG:GalAT, 6-week-old wild type Arabidopsis stem microsomes were homogenized in the presence of increasing amounts of TX-100 (0.5%, 1%, 2%, 4%), and the suspensions ultracentrifuged at 100,000 *g* at 4 °C for 1 hour to obtain the supernatant and the pellet fractions. Pellet fractions were re-suspended in storage buffers, and all fractions were assayed using an HG:GalAT filter assay (Sterling et al., 2005). The results were compared to the HG:GalAT activity present in intact microsomes without TX-100 treatment. HG:GalAT activity was most active in the 0.5% and 1% TX-100 treated-pellet fractions, suggesting that partially permeabilized membranes allowed more interactions between the enzyme and its substrates than non-treated microsomes, and that lower HG:GalAT activity was recovered in the 2% and 4% TX-100 pellets (**Figure D-1**). No or very little HG:GalAT activity was recovered in the supernatant fractions under these conditions, and increasing TX-100 concentrations (2% and 4%) caused a dramatic reduction in HG:GalAT activity in both supernatant and pellet fractions. However, microsomes treated with the higher concentrations (2% and 4%) of TX-100 produced better immunoprecipitation results (data not shown).

Both prolonged incubation with higher TX-100 concentration to solubilize the microsomes and residual high concentrations of detergent may affect the HG:GalAT activity in the reaction. Since wild-type stem microsomes were precious samples, the detergent concentration optimization experiment was done using 6-day-old WT Arabidopsis suspension cell culture microsomes. The Arabidopsis suspension culture cells were known to contain high

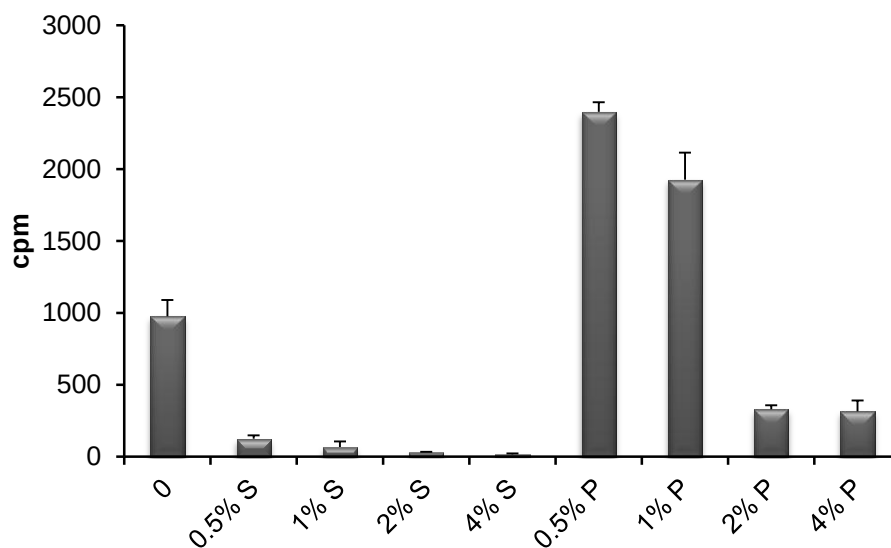


Figure D-1. HG:GalAT activity in Triton X-100 (TX-100)-treated wild type Arabidopsis stem microsomes.

Microsomes from 6-week-old wild type Arabidopsis stems were suspended in 0.5%, 1%, 2% and 4% TX-100 and ultracentrifuged at 100,000 *g* to obtain supernatant (S) and pellet (P) fractions. All fractions were tested for HG:GalAT activity in an HG:GalAT filter assay. HG:GalAT activity was measured in a 30 μ L reaction containing reaction buffer, 80 μ g of OGAs (HG oligosaccharides of DP 7-23), 5.75 μ M UDP- ^{14} C]GalA, and 13 μ L of enzyme (100 μ g protein). Reactions were incubated at 30°C for 3 h, and subsequently processed using the filter assay. The “0” represents HG:GalAT activity in microsomes in the absence of TX-100. Y-value = T_0 -subtracted average cpm incorporated $\pm$ SD (n=2).

levels of HG:GalAT activity, and the microsomes were diluted with storage buffer to a final TX-100 concentration of 0.5% immediately after detergent-homogenization. No centrifugation was used to separate the supernatant and pellet since the aim was to determine the best concentration of detergent for permeabilization and/or solubilization of wild-type Arabidopsis stem microsomes that preserve HG:GalAT activity and support the immunoprecipitation of the target enzyme GAUT12 using the anti-GAUT12 antibody. The same volume of detergent-treated microsomes was used in each reaction, and final protein concentration was determined using the Bradford assay (**Figure D-2**).

It appeared that all fractions contained relatively high HG:GalAT activity, despite the fact that the protein concentration was lower in fractions treated with higher concentrations of TX-100 due to dilution effects (**Figure D-2**). Taking into consideration that higher detergent concentration allows better immunoprecipitation (IP), the conditions used for IP to test for enzymatic activity was to homogenize the microsomes with 4% TX-100 and then immediately dilute with storage buffer to obtain a final concentration of 0.5% TX-100.

The reactions in **Figure D-2** were carried out for 3 hours when presumably the reaction reached saturation and maximum cpm incorporation per reaction was achieved. To investigate the efficiency of the enzyme mixture and to determine the linear time range of the HG:GalAT reaction, a time-course experiment was conducted using 6-day-old wild-type suspension culture cell microsomes treated with 4% TX-100 and diluted to 0.5% TX-100 as the enzyme source. The reaction rate appeared to be linear for 40 min. The reaction started to slow down and form the maximum amount of products at 160 min (**Figure D-3**).

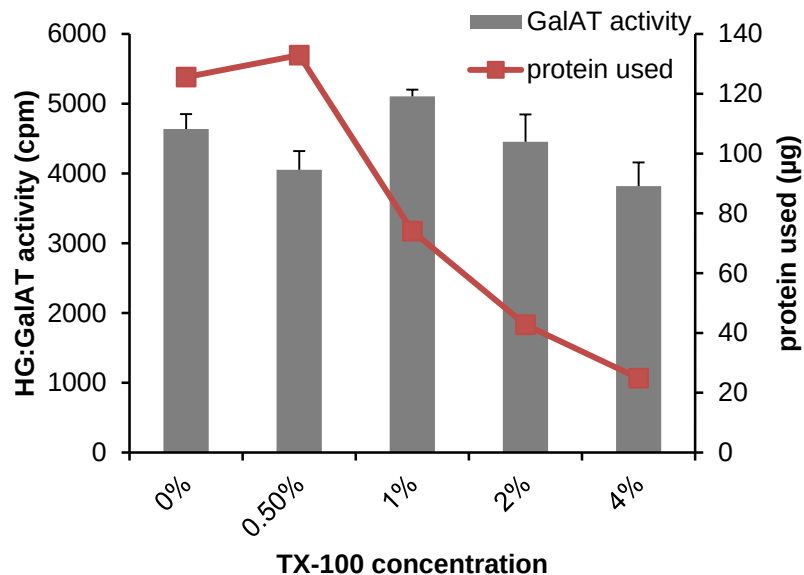


Figure D-2. Effect of Triton X-100 (TX-100) on microsomes prepared from 6-day-old wild-type *Arabidopsis* suspension cell cultures.

Aliquots (50 μ L) of microsomes prepared from 6-day-old WT suspension cell cultures were homogenized with 0.5%, 1%, 2% and 4% TX-100, and diluted to a final concentration of 0.5% TX-100 with the storage buffer. HG:GalAT activity was measured in a 30 μ L reaction containing reaction buffer, 80 μ g of OGAs (HG oligosaccharides of DP 7-23), 5.75 μ M UDP-[^{14} C]GalA, and 13 μ L of enzyme containing the indicated amount of protein (red square symbols). Reactions were incubated at 30°C for 3 h and subsequently processed using the filter assay. The protein concentration was assayed using the Bradford reagent (BIO-RAD) and the total amount of protein used per reaction is displayed using the line chart. Y-value = T_0 -subtracted average cpm $\pm$ SD (n=2).

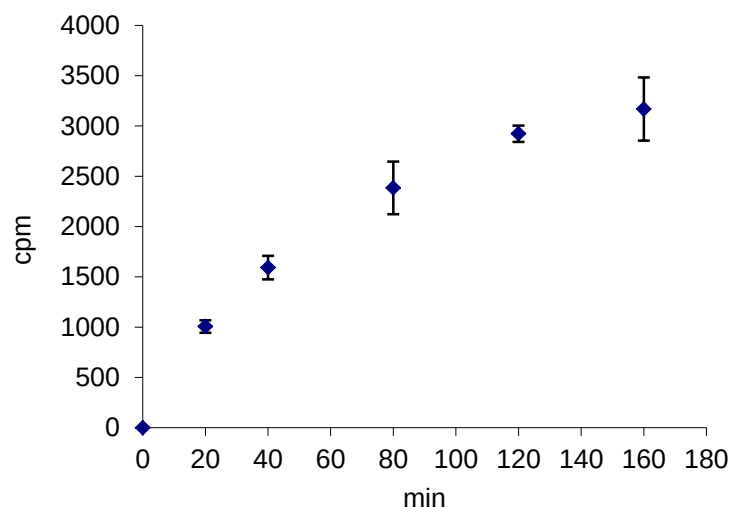


Figure D-3. Time-course of HG:GalAT activity using TX-100-treated microsomes from 6-day-old wild-type suspension cell cultures.

An aliquot (100 μ L) of microsomes from 6-day-old wild-type suspension culture cells was homogenized with 4% TX-100 and diluted to a final TX-100 concentration of 0.5% with the storage buffer. HG:GalAT activity was measured in a 30 μ L reaction containing reaction buffer, 80 μ g of OGAs (HG oligosaccharides of DP 7-23), 5.75 μ M UDP-[^{14} C]GalA, and 13 μ L of enzyme prepared above (25 μ g protein). Reactions were incubated at 30°C for 0, 20, 40, 80, 120, and 160 min, respectively and subsequently processed using the filter assay. Y-value = T_0 -subtracted average cpm $\pm$ SD (n=2).

The *irx8* stem microsomes contain reduced HG:GalAT activity

Multiple groups have attempted to identify GAUT12 enzymatic activity by comparing microsomes isolated from *irx8* and wild-type Arabidopsis stem tissues. Brown et al. (2007) identified a loss of β -(1 $\rightarrow$ 4)-Xylan:XylT activity in *irx9* and *irx14* mutants using UDP-[^{14} C]Xyl and β -(1 $\rightarrow$ 4)-Xyl₆ (xylohexaose) as substrates, while they observed no XylT activity reduction in *irx8* microsomes (Brown et al., 2007). In addition to xylan:XylT activity, Lee et al. (2007) assayed UDP-[^{14} C]GlcA with β -(1 $\rightarrow$ 4)-Xyl₆ as a substrate and found no reduction of xylan glucuronosyltransferase (GlcAT) activity in *irx8* microsomes (Lee et al., 2007a). These findings suggested that GAUT12 do not possess xylan:XylT activity or xylan:GlcAT activity.

To investigate whether GAUT12 is an HG:GalAT as we speculated (*hypothesis 2*), initially I compared total HG:GalAT activity in microsomes isolated from 6-week-old *irx8* and wild type stems using the filter assay (Sterling et al., 2005). The *irx8* stem microsomal membranes had an approximately 40% reduction of UDP-[^{14} C]GalA incorporation onto oligogalacturonide (OGA, DP 7-23) acceptors compared to WT stem microsomes, suggesting a reduced level of HG biosynthesis in the microsomes prepared from *irx8* stems (**Figure D-4B**). The absence of detectable GAUT12 protein in *irx8* microsomes was confirmed by western blot analysis (**Figure D-4A**).

One potential problem of using crude enzyme mixtures such as microsomes is the reduced sensitivity in measuring the enzyme activity of the target enzyme. The activity of GAUT12 may be overshadowed by other glycosyltransferases with similar activities, such as GAUT1. In addition, due to the presence of glycoside hydrolases and enzymes that promote the interconversion of nucleotide sugars, substrates fed to and/or sugar products produced in the reaction tubes can be modified.

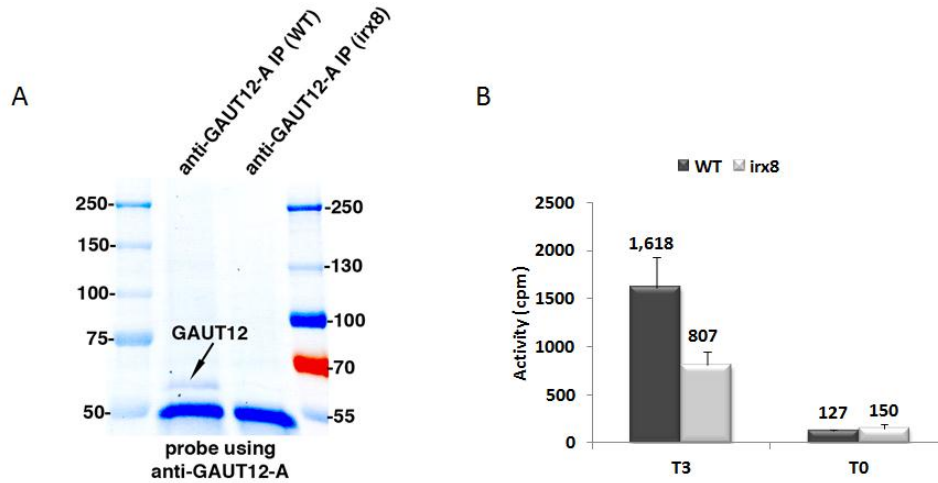


Figure D-4. Microsomes from *irx8* contain reduced HG:GalAT activity and have undetectable GAUT12 protein in western blot analysis.

(A) Anti-GAUT12 antibody-conjugated Dynabeads M-280 Sheep anti-rabbit IgG were incubated with equal amounts of 2% TX-100 permeabilized 6/7-week-old WT and *irx8* stem microsomes (beads to microsome ratio of 1:3, v/v), and the immunoabsorbed products were analyzed by western blots with antibody dilutions of 1:5000 for anti-GAUT12. The presence of GAUT12 is indicated by the black arrow in the WT. GAUT12 was absent in the *irx8* sample.

(B) HG:GalAT filter assay. Microsomes were isolated from stems of 6/7-week-old Arabidopsis plants and equal amounts of WT and *irx8* mutant microsomes (100 μ g total protein) were incubated in 30 μ l reactions containing 15 μ l of microsomes (with 1% Triton X-100), 50 mM Hepes (pH 7.3), 0.2 M sucrose, 0.05% (w/v) BSA, 25 mM KCl, 40 μ g of oligogalacturonides (OGA, DP 7-23), 6.9 μ M UDP-[¹⁴C]GalA (specific activity 180.3 mCi/mmol) (1 Ci = 37 GBq), and 1 mM MnCl₂. The reactions were incubated for 0 min (T₀) or 3 hours (T₃) in a 29°C water bath. The reaction products were processed using the filter assay. Activity = average cpm $\pm$ SD (n=3). The result is representative of two independent experiments using one biological sample set.

Another complication is that the retarded growth of the *irx8* mutant may lead to a reduced amount of HG:GalAT activity, considering that GAUT1, which is expressed in Arabidopsis stems (Atmodjo et al., 2011), may contribute to the majority of HG:GalAT activity in stem tissues. In addition, due to the dwarfed stature of the *irx8* mutant and its sterility, collecting enough stem tissues from this mutant for microsome preparation was difficult. We thus decided to use the partially purified GAUT12 using an anti-GAUT12 polyclonal antibody to confirm whether GAUT12 has the HG:GalAT activity.

Immunoabsorbed-GAUT12 fractions have no HG:GalAT activity using OGAs as acceptor

Three different batches of wild-type Arabidopsis stem (7/8-week-old) microsome preps (S1, S2, S3) were used to obtain the immunoabsorbed-GAUT12 fractions for HG:GalAT assays. For these three batches of stem microsomes (**Figure D-5A**), total HG:GalAT activity was reduced compared to previously used stem microsomes (6-week-old, **Figure D-1**, first bar), since the stems harvested were more mature. The immunoabsorbed-GAUT1:GAUT7 from these samples appeared to have low HG:GalAT activity (**Figure D-5B**), which did not serve as a good positive control for the HG:GalAT activity assay. This was likely due to the developmental stage of stem tissues in which, at 7- to 8-week of growth, primary cell wall production is reduced and the cells are synthesizing mostly secondary cell walls. Log-phase (6-day-old) WT suspension cell culture microsomes contained much more total HG:GalAT activity than 7/8-week-old WT stem microsome samples in similar amounts of microsomes used (**Figure D-5A**). Therefore, GAUT1:GAUT7 immunoabsorbed from 6-day-old WT Arabidopsis suspension cell cultures microsomes, which were prepared in the same manner as the WT stem microsomes, was used as the positive control for the HG:GalAT activity assay. The GAUT12 immunoabsorbed from

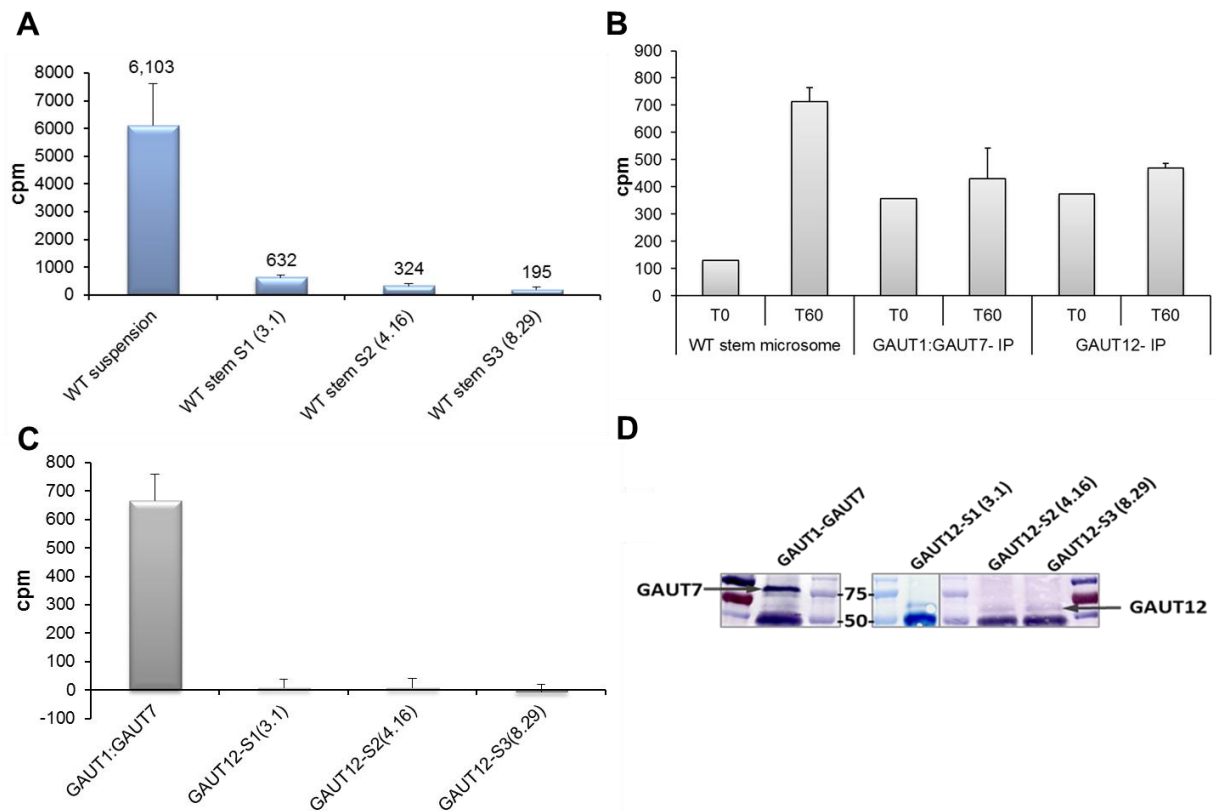


Figure D-5. HG:GalAT activity in anti-GAUT12-immunoabsorbed fractions from WT *Arabidopsis* stem microsomes.

(A) Microsomes from 7/8-week-old WT stems (S1, S2, and S3) were tested for HG:GalAT activity using the filter assay and results compared to microsomes prepared from 6-day-old WT suspension cell cultures. HG:GalAT activity was measured in a 30 μ L reaction containing reaction buffer, 80 μ g of OGA (DP 7-23), 5.75 μ M UDP-[^{14} C]GalA, and 13 μ L of enzyme (91 μ g total protein per reaction). Reactions were incubated at 30°C for 2 h, and subsequently processed using the filter assay.

(B) Immunoprecipitated-GAUT1:GAUT7 and immunoprecipitated-GAUT12 from 7/8-week-old stem microsomes were tested for HG:GalAT activity using the filter assay. Immunoprecipitation (IP) experiment was conducted by incubating 150 μ L of anti-GAUT12- or anti-GAUT7-

conjugated magnetic beads with 250 μ L WT stem microsomes (1750 μ g total protein, treated with 4% TX-100 and diluted to 0.5% final concentration) for 2.5 h at 4°C. The beads were then washed thrice with homogenization buffer on ice and used in assays as IP fractions. HG:GalAT activity was measured in a 30 μ L reaction containing reaction buffer, 80 μ g of OGA (DP 7-23), 5.75 μ M UDP-[¹⁴C]GalA, and 13 μ L of enzyme (equal to 30 μ L of beads per reaction). Reactions were incubated at 30°C for 60 min (T60) and subsequently processed using the filter assay.

(C) Immunoprecipitated-GAUT1:GAUT7 from 6-day-old WT suspension culture cells and immunoprecipitated-GAUT12 from 7/8-week-old stem microsomes (S1, S2, and S3 from A) were tested for HG:GalAT activity using the filter assay. Immunoprecipitation (IP) experiment was conducted by incubating 150 μ L of anti-GAUT12- or anti-GAUT7-conjugated magnetic beads with 250 μ L stem or WT suspension cell culture microsomes (1750 μ g total protein, treated with 4% TX-100 and diluted to 0.5% final concentration) for 2.5 h at 4°C. The beads were then washed thrice with homogenization buffer on ice and used in assays as IP fractions. HG:GalAT activity was conducted in a 30 μ L reaction containing reaction buffer, 80 μ g of OGA (DP 7-23), 5.75 μ M UDP-[¹⁴C]GalA, and 13 μ L of enzyme (equal to 30 μ L of beads per reaction). Reactions were incubated at 30°C for 2 h, and subsequently processed using the filter assay.

(D) GAUT7 protein and GAUT12 protein immunoprecipitated from suspension culture microsomes and WT stem microsomes used in (C) were verified using western blots, respectively. GAUT12 protein (via immunoprecipitation) could not be detected on western blots in WT suspension cell cultures (see Figure D-11C).

7/8-week-old WT stem microsomes contained little HG:GalAT activity. High HG:GalAT activity was present in the GAUT1:GAUT7 immunoabsorbed from 6-day-old WT suspension cell culture microsomes (**Figure D-5C**), used as a positive control for the enzyme assay. Western blot analysis showed the presence of GAUT1:GAUT7 and GAUT12 in the immunoabsorbed fractions used for these assays (**Figure D-5D**).

The fact that little or no HG:GalAT activity was recovered from the immunoabsorbed-GAUT12 fractions, using conditions favorable for GAUT1:GAUT7 HG:GalAT activity, suggests that GAUT12 may not efficiently use OGA (7-23 mer) as acceptors, and thus may not be an HG:GalAT. However, it is possible that the detergent (TX-100) used to treat the microsomes, albeit preserving the GAUT1 activity, inhibits the GAUT12 activity. Alternatively, the reaction catalyzed by GAUT12 may not be processive, and only one GalA residue may be incorporated onto the acceptors which may not have been detected efficiently using the filter assay. Also, the results could not rule out the possibility that GAUT12 may use different pectic or other structures as acceptors.

Do irx8 mutant microsomes contain the proper substrate?

Enzymes that synthesize matrix polysaccharides are thought to reside in the Golgi apparatus and it is reasonable to hypothesize that their substrates may also be present in the Golgi. Mutant microsomes have been viewed as potentially enriched in the acceptor substrate since the enzyme that would use/modify *the* substrate is absent. Heat-inactivated 8-week-old *irx8* stem microsomes, therefore, were tested as exogenous acceptors for the immunoabsorbed-GAUT12 (**Figure D-6**). To ensure that no additional salt was introduced into the reactions, the *irx8* microsomes were first centrifuged and re-suspended in diH₂O to remove the storage buffer.

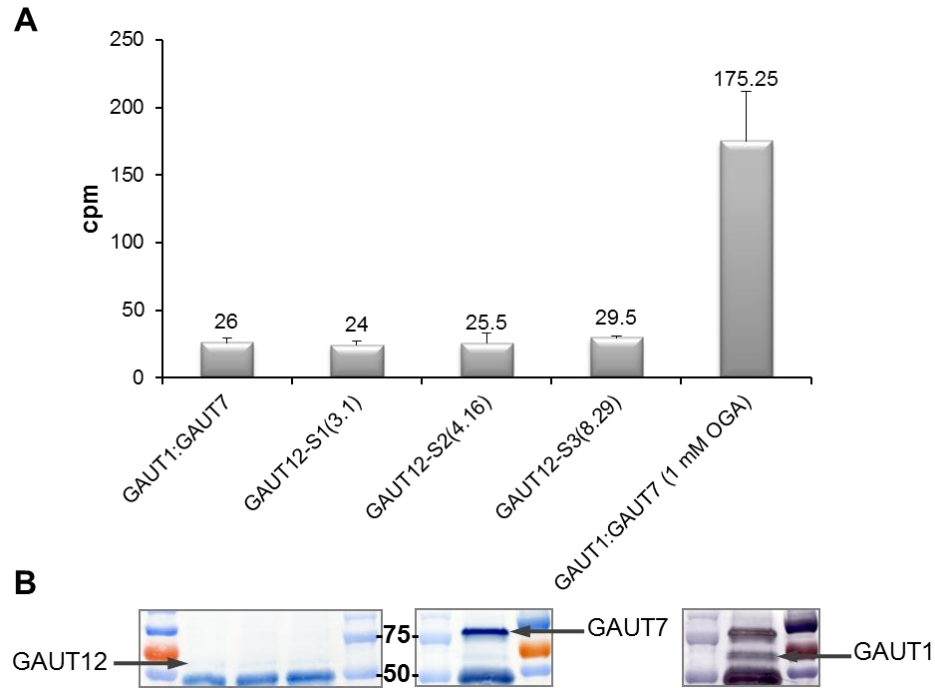


Figure D-6. Heat-inactivated *irx8* stem microsomes as acceptors.

(A) An aliquot (400 μ L) of 8-week-old *irx8* stem microsomes (6 μ g/ μ L) was ultracentrifuged and re-suspended in 210 μ L of water to remove buffer, yielding a final protein concentration of 11.4 μ g/ μ L. The entire 210 μ L of microsomes were boiled at 100°C for 15 min to de-activate enzyme activity and re-homogenized. Aliquots of 8 μ L (equal to 91.4 μ g of total protein) were used in each reaction. IP was done as previously described. GalAT activity was measured in a 30 μ L reaction containing reaction buffer, 8 μ L heat-inactivated microsomes or 80 μ g of OGAs (7-23 mer), 5.75 μ M UDP-[^{14} C]GalA, and 13 μ L of enzyme (30 μ L of beads per reaction). Reactions were incubated at 30°C for 90 min, and subsequently processed using the precipitation assay. Value = T_0 -subtracted cpm $\pm$ SD (n=2).

(B) The presence of GAUT7 and GAUT12 in the IP fractions used in (A) was verified using western blots. GAUT1 was also verified to be present in the GAUT7 IP, demonstrating the successful pull-down of the GAUT1:GAUT7 HG:GalAT core complex.

They were then de-activated at 100 °C for 15 min. An aliquot of 94 µg of heat-inactivated *irx8* microsomes was used in each reaction and immunoabsorbed GAUT12 and GAUT1:GAUT7 were used as the enzyme source. It appeared that GAUT12 immunoabsorbed from all three stem microsome samples contained very low GalAT activity towards heat-inactivated microsomes (**Figure D-6A**). The GAUT1:GAUT7 immunoabsorbed from 6-day-old WT suspension culture cell microsomes also had low activity using heat-inactivated *irx8* stem microsomes as acceptors, while the 1 mM OGA (7-23 mer) appeared to be a better acceptor, suggesting that the heat-inactivated *irx8* microsomes contained little appropriate acceptors for GAUT1:GAUT7. In a separate experiment, heat-inactivated WT stem microsomes were used as an acceptor for immunoabsorbed-GAUT12 and also appeared to be limited to serve as acceptors for GalA incorporation by immunoabsorbed-GAUT12 fractions (**Figure D-7A**).

Although the idea stands that the exact acceptor substrates shall be present in the mutant microsomes since the enzyme is absent to catalyze the reaction and consume the substrate. The results suggested that the amount of such acceptors is either quite low in the microsomes or the products were not recovered using the techniques used. In either case, reaction products could not be detected by precipitation.

Other pectic substrates

It has been shown that GAUT1 has a high substrate specificity for OGAs (7-23 mer) compared to RG-I backbone oligomers [6-24 mer, with either GalA (RG-I-G) or rhamnose (RG-I-R) at the non-reducing end] and RG-II monomer from wine (Atmodjo et al., 2011). Since OGAs appeared not to be an effective acceptor for GAUT12, 1 µM of RG-I-G, RG-I-R, and RG-II monomer from wine were tested as potential acceptors for GAUT12 immunoabsorbed from

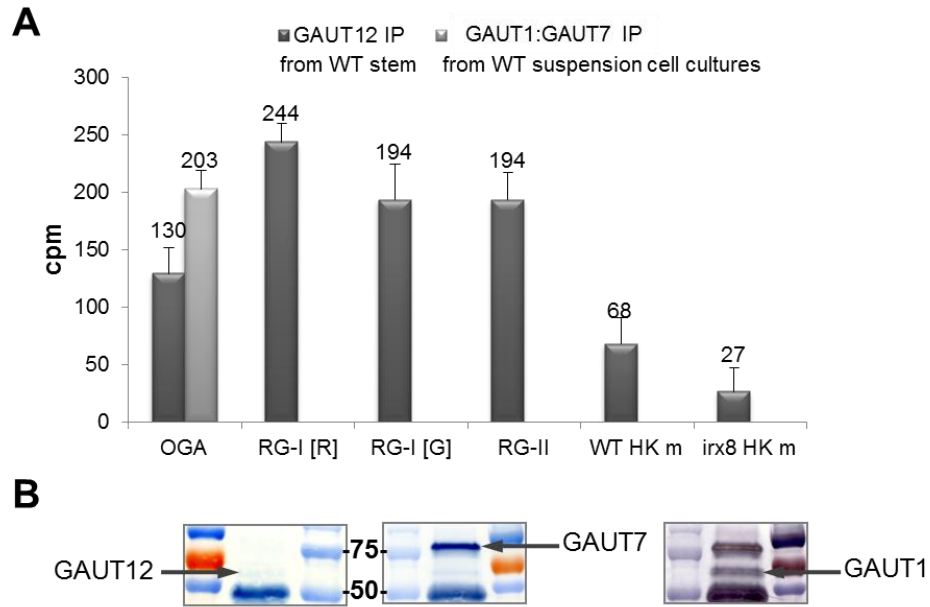


Figure D-7. GAUT12 enzymatic activity using available pectic acceptors and heat-inactivated microsomes.

(A) GalAT activity of immunoabsorbed-GAUT12 (GAUT12 IP) from 7/8-week-old WT stem microsomes using a variety of acceptors including 1 mM OGA (7-23 mer), 1 μ M RG-I-G (with GalA at the non-reducing end), 1 μ M RG-I-R (with Rha at the non-reducing end), 1 μ M RG-II monomer (wine), heat-inactivated WT and *irx8* microsomes (94 μ g of total protein). IP was carried out as described in Figure D-5 legend (C). The GalAT activity was measured in a 30 μ L reaction containing reaction buffer, acceptor substrate, 5.75 μ M UDP-[^{14} C]GalA, and 13 μ L of enzyme (equal to 30 μ L of beads per reaction). GAUT1:GAUT7 immunoprecipitated (IP) from 6-day-old WT suspension culture cells was used as a positive control for the 1 mM OGA acceptor. Reactions were incubated at 30°C for 90 min, and subsequently processed using the precipitation assay. Y-value = T_0 -subtracted cpm $\pm$ SD (n=2).

(B) The presence of GAUT7 and GAUT12 in the IP fractions was verified using western blots. GAUT1 was also verified to be present in the GAUT7 IP demonstrating the successful pull-down of the GAUT1:GAUT7 HG:GalAT core complex.

7/8-week-old WT stem microsomes. In the first experiment compared to OGAs, all three acceptors appeared to be slightly better substrates for GAUT12 (**Figure D-7A**), and the RG-I-R had the highest GalA incorporation, followed by the RG-I-G and the RG-II monomer from wine. All four acceptors provided better GalA incorporation compared to heat-inactivated microsomes from WT and *irx8* stems. The immunoabsorbed-GAUT1:GAUT7 was only used in the reaction containing OGAs in this experiment. In a separate experiment, GAUT12 immunoabsorbed from 7/8-week-old WT stem microsomes catalyzed slightly higher GalA incorporation onto the RG-I-G and the RG-II monomer (from wine) than onto the RG-I-R (**Figure D-8A**). GAUT12 appeared to have slightly higher activity in transferring GalA from UDP-GalA onto these three substrates (RG-I oligomers and RG-II monomer) than the GAUT1:GAUT7 immunoabsorbed from 6-day-old WT suspension culture cell microsomes (**Figure D-8A**). The presence of GAUT12 and GAUT1:GAUT7 in the immunoabsorbed fractions used in the assay was confirmed in western blot (**Figure D-8B**).

It appeared that RG-I oligomers and the RG-II monomer from wine may be potential substrates for GAUT12. However, caution must be taken to interpret these results. The low cpm suggests low activity, and the similar cpm counts towards RG-I and RG-II acceptors suggest the possibility of non-specific activities. At present, we cannot rule out the possibility that GAUT12 transfers single GalA residue onto such acceptors, which could not be identified using the precipitation method. Therefore, future work needs to focus on the characterization of the products formed in these reactions.

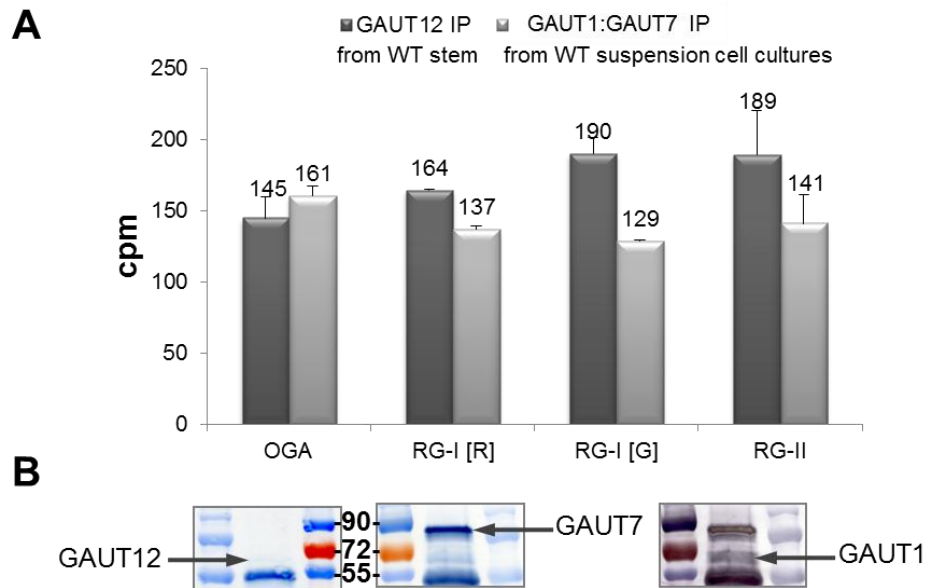


Figure D-8. GAUT12 enzymatic activity using pectic acceptors.

(A) GalAT activity of GAUT2 immunoabsorbed (IP) from microsomes prepared from 7/8-week-old WT stems and GAUT1:GAUT7 immunoabsorbed (IP) from microsomes prepared from 6-day-old suspension culture cells using a variety of acceptors including 1 mM OGA (7-23 mer), 1 μ M RG-I-G, 1 μ M RG-I-R, and 1 μ M RG-II monomer (wine). IP was carried out as described in Figure D-5 legend (C). The GalAT activity was measured in 30 μ L reactions containing reaction buffer, acceptor substrate, 5.75 μ M UDP-[^{14} C]GalA, and 13 μ L of enzyme (equal to 30 μ L of beads per reaction). Reactions were incubated at 30°C for 90 min, and subsequently processed using the precipitation assay. Y-value = T_0 -subtracted cpm $\pm$ SD (n=2).

(B) The presence of GAUT7 and GAUT12 in the IP fractions was verified by western blots. GAUT1 was also verified to be present in the GAUT7 IP demonstrating the successful pull-down of the GAUT1:GAUT7 HG:GalAT core complex.

GAUT12-EGFP fusion protein contains no HG:GalAT activity

Several GAUT12 constructs were stably transformed into WT and *irx8* Arabidopsis suspension cell cultures, including a cauliflower mosaic virus (CaMV) 35S promoter-driven GAUT12-EGFP (*G12*) construct. This construct complements the *irx8* mutant phenotype (**Chapter 2**). Microsomes were prepared from the WT+*G12* (OE) suspension cell cultures on day 6, and western blot analysis was used to confirm the presence of the GAUT12-EGFP fusion protein (~86 KD), which could be immunoprecipitated using anti-GAUT12 antibody (**Figure D-9B**).

The immunoprecipitated (IP) GAUT12-EGFP fractions were tested for HG:GalAT activity using the filter assay. The immunoprecipitated GAUT1:GAUT7 complex from WT suspension cell cultures using the anti-GAUT7 antibody was used as a positive control for the HG:GalAT activity assay. The microsomes before (Pre-IP) and after immunoprecipitation (post-IP) were also tested to verify that the conditions used for the IP did not destroy the HG:GalAT activity. The results (**Figure D-9A**) showed high HG:GalAT activity in the OE microsomes both before and after immunoprecipitation of GAUT12-EGFP from the microsome. However, no HG:GalAT activity was recovered in the GAUT12-EGFP immunoabsorbed by the anti-GAUT12 antibody, as judged by the filter assay. Conversely, GAUT1:GAUT7 immunoabsorbed from WT suspension culture microsomes in parallel had relatively high HG:GalAT activity (**Figure D-9A**). These results indicate that GAUT12 does not have the same HG:GalAT activity as GAUT1.

All three fractions tested in this assay, namely pre-IP, IP, and post-IP, were treated with 4% TX-100 and then quickly diluted with homogenization buffer to a final TX-100 concentration of 0.5%. Both pre-IP and post-IP fractions from both cultures retained relatively high HG:GalAT activity, and so did the GAUT1-7 IP fraction. Combined with previous data

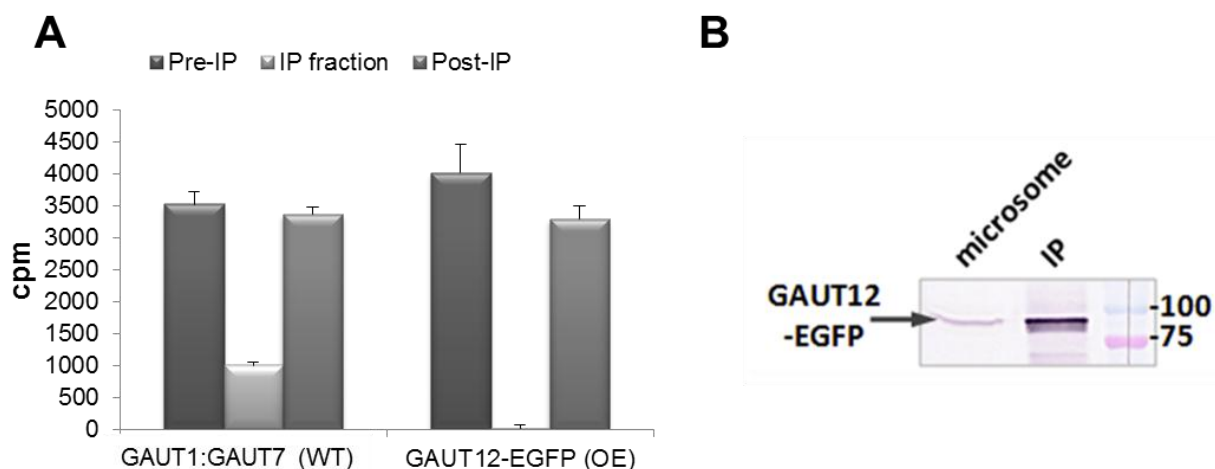


Figure D-9. The GAUT12-EGFP fusion protein has no HG:GalAT activity.

(A) Aliquots (200 μ L) of microsomes (total protein 2000 μ g) from 6-day-old WT and GAUT12-EGFP (OE) suspension culture cells were treated with 4% TX-100 and diluted using storage buffer to a final concentration of 0.5%. Such microsomes (50 μ L) were used as Pre-IP fractions. The rest of GAUT12-EGFP OE and WT microsomes were incubated for 2 h at 4°C with 120 μ L of magnetic beads that were conjugated to either the anti-GAUT12 or the anti-GAUT7 antibodies, respectively. The beads were washed thrice with homogenization buffer on ice and tested for activity as IP fractions. Post bead-incubation microsomes (50 μ L) were used as Post-IP fractions. HG:GalAT activity was measured in a 30 μ L reaction containing reaction buffer, 80 μ g of OGA (DP 7-23), 5.75 μ M UDP-[^{14} C]GalA, and 13 μ L of enzyme (equal to 30 μ L of beads). Reactions were incubated at 30°C for 2 h, and subsequently processed using the filter assay (Sterling et al., 2005). Y-value = T_0 -subtracted average cpm $\pm$ SD (n=3).

(B) The western blot shows the presence of the over-expressed GAUT12-EGFP in 6-day-old OE suspension cell microsomes (40 μ g of total protein) and in the IP fraction.

from enzyme assays using GAUT12 immunoabsorbed from WT stem microsomes, the results indicate that GAUT12 is unlikely to be an HG:GalAT. However, this does not completely rule out the possibility that the permeabilization /solubilization/assay conditions used may not have been optimal for GAUT12 activity.

GalAT activity in xylogenic Arabidopsis suspension cell cultures

GAUT12 is highly expressed in vascular tissues. Thus, the main plant tissue that I used for the isolation of GAUT12 protein was elongating wild type Arabidopsis stems, a process that was time-consuming due to the 7- to 8-week time period required for plant growth. Suspension cell cultures have been widely used for plant cell wall analyses, providing enzymes and sugar substrates for glycosyltransferase studies (Doong et al., 1995; Sterling et al., 2005). Kubo et al. have reported that 1 μ M brassinolide (BL) and 10 mM boric acid can be used to induce wild type Arabidopsis suspension culture cells to transdifferentiate into tracheary elements (TE, i.e. producing secondary cell walls), and that many secondary cell wall biosynthetic genes including *GAUT12* were up-regulated (Kubo et al., 2005). However, no studies have tested whether GAUT12 protein level was induced under the xylogenic conditions. One of our initial goals was to prepare adequate amounts of Arabidopsis tissues for GAUT12 protein preparation using Arabidopsis suspension cell cultures grown on xylogenic medium to induce TE formation. I therefore generated suspension cell cultures from *irx8* and wild type Arabidopsis seeds using methods described by Doelling and Pikaard (Doelling and Pikaard, 1993). The suspension cultures have been maintained by transferring 6-7 ml of packed cells into 100 ml fresh liquid callus inducing media every 10 days (**Chapter 3** and **Appendix F**).

Both wild type and *irx8* cultures were sieved using sterile 500 μ M meshes during the initial period of suspension cell culture generation. Such sieved-through sedimented cells were used to re-inoculate fresh media. However, visible cell clumps formed in the cultures after two subculture periods following such sieving and re-inoculation procedures. However, the suspension cell cultures eventually grew finer over four years of subculture. The formation of tracheary elements in these cells when grown on xylogenic medium were verified by sectioning through 9-day-old cultured cells (**Chapter 3** and **Appendix F**).

Induction of the *GAUT12* transcript level in WT culture cells grown on xylogenic medium was confirmed by qRT-PCR analysis. Prior to the development and characterization of the anti-GAUT12 antibody, microsomes were prepared from WT (WT+BL) and *irx8* (*irx8*+BL) suspension cell cultures grown on xylogenic medium and directly tested for GalAT activity using UDP-[^{14} C]GalA as a substrate. Endogenous acceptors or heat-inactivated *irx8*+BL microsomes were used as acceptor substrates, and the products were processed by the precipitation assay (**Figure D-10A**). The *irx8*+BL microsomes incorporated similar amounts of [^{14} C]GalA onto endogenous acceptors present in the microsomal membranes compared to WT+BL microsomes. In both WT+BL and *irx8*+BL cells (**Figure D-10A**), approximately 50% of the cpm incorporated into precipitated products was hydrolyzed by endopolygalactonase (EPGase, **Figure D-10B**), indicating that at least half the product was unesterified HG. This result suggests that the amount of endogenous HG acceptor is comparable in WT+BL and *irx8*+BL microsomes. The presence of EPGase-resistant product suggests that about half of [^{14} C]GalA-labeled product was either substituted HG that was not accessible to EPGase, or some other GalA-containing products such as RG-I.

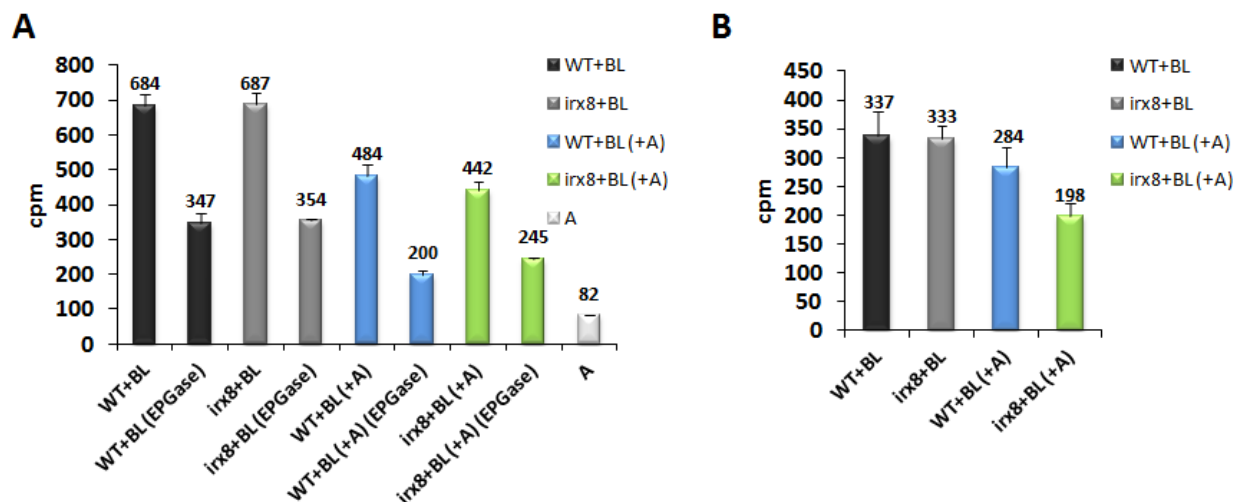


Figure D-10. UDP-[¹⁴C]GalA incorporation using microsomes prepared from 6-day-old WT (WT+BL) and *irx8* (*irx8*+BL) suspension cell cultures grown on xylogenic medium.

(A) The reactions contained 50 mM Hepes (pH 7.8), 0.2 M sucrose, 25 mM KCl, 1.9 mM MnCl₂, 100 µg of microsomes (permeabilized with 0.1% TX-100), 4.8 µM UDP-[¹⁴C]GalA, with (+A) or without the *irx8*+BL heat-inactivated microsomes (labeled as “A”). Reactions were processed using the precipitation assay. EPGase digestion was carried out by re-suspending the pellet in 50 µL ddH₂O and adding 50 µL 100 mM sodium acetate (pH 5.0) containing 1 µL EPGase (7.5 mg/mL, ~1.8 U per reaction). The reactions were incubated at 30°C overnight for 16 h. The undigested (EPGase-resistant) products were recovered using the precipitation method and the cpm was determined by scintillation counting. Y-value = T₀-subtracted average cpm ± SD (n=2).

(B) EPGase-sensitive products generated by WT+BL and *irx8*+BL microsomes with (+A) and without *irx8*+BL heat-inactivated microsomes. Y-value = (Total cpm – EPGase-resistant cpm) ± SD (n=2). EPGase, endopolygalacturonase (EPG).

The addition of the heat-inactivated microsomes appeared to reduce the incorporation of GalA into products, as both the WT+BL and the *irx8*+BL microsomes had 29% and 36% reduced [¹⁴C]GalA incorporation, respectively (**Figure D-10A**). The reduction of [¹⁴C]GalA incorporation into product in the presence of heat-inactivated microsomes may have been a result of additional salts introduced to the system by the addition of such heat-inactivated microsomes. It is noteworthy, however, that in the presence of heat-inactivated acceptor substrates, a smaller percentage of the [¹⁴C]GalA-labeled product produced by the *irx8*+BL microsome compared to the WT+BL microsomes was sensitive to EPGase treatment (45% and 59%, respectively), suggesting that the *irx8*+BL cells may have incorporated more [¹⁴C]GalA onto acceptors other than HG (**Figure D-10B**).

With the generation, purification and characterization of the polyclonal anti-GAUT12 antibody, I then investigated whether the xylogenic suspension culture system produced adequate amounts of GAUT12 protein to explain the difference demonstrated in **Figure D-10B**. GAUT12 was immunoprecipitated from microsomes prepared from WT and *irx8* suspension cell cultures grown on xylogenic (+BL) and control medium on Day 8. GAUT1:GAUT7 complex was immunoprecipitated as a positive control for the HG:GalAT activity assay (**Figure D-11A**). GAUT12-IP fractions did not have HG:GalAT activity, and barely contained detectable amounts of GAUT12 protein as examined by western blot (**Figure D-11C**). The microsomal fraction from xylogenic WT cells (WTX) after incubation with either anti-GAUT7 or anti-GAUT12 was tested for HG:GalAT activity. The residual relatively high HG:GalAT activity in the post-IP fractions showed that the conditions used for the IP conserved HG:GalAT activity and allowed saturation of the magnetic beads with its target protein during incubation (**Figure D-11B**).

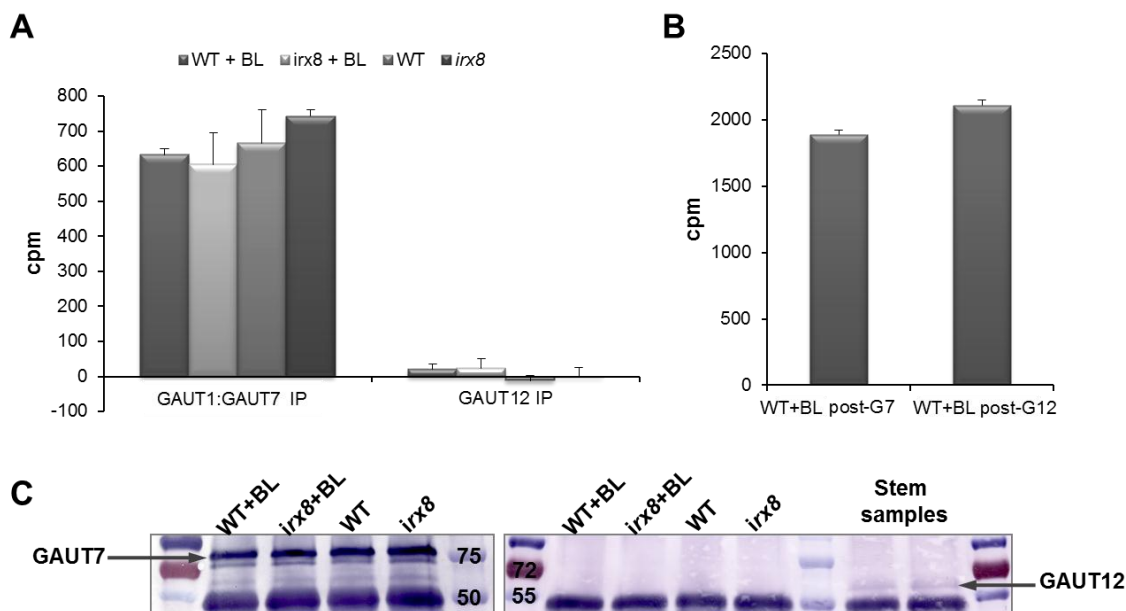


Figure D-11. HG:GalAT activity in wild-type and *irx8* cells grown on xylogenic and control media.

(A) Aliquots (200 μ L) of microsomes (total protein 2000 μ g) from 6-day-old WT and *irx8* suspension culture cells grown on xylogenic (+BL) and control media were treated with 4% TX-100 and diluted to a final concentration of 1% TX-100. Microsomes prepared from WT and *irx8* suspension culture cells grown on xylogenic (WT + BL, *irx8* + BL) and control (WT, *irx8*) media were incubated for 2.5 h at 4°C with 150 μ L of magnetic beads that were previously saturated with anti-GAUT12 or anti-GAUT7 antibodies, respectively. The beads were washed thrice with homogenization buffer on ice and tested as IP fractions. HG:GalAT activity was measured in 30 μ L reactions containing reaction buffer, 80 μ g of OGA (DP 7-23), 5.75 μ M UDP-[^{14} C]GalA, and 13 μ L of enzyme (equal to 30 μ L beads). Reactions were incubated at 30°C for 2 h, and subsequently processed using the filter assay. Y-value = T_0 -subtracted average cpm $\pm$ SD (n=2).

(B) Post-bead-incubation WT+BL microsomes used in (A) were diluted 1:1 (v/v) for a final TX-100 concentration of 0.5% and tested for activity. HG:GalAT activity was measured in a 30 μ L reaction containing reaction buffer, 80 μ g of OGA (DP 7-23), 5.75 μ M UDP-[^{14} C]GalA, and 13 μ L of enzyme (25 μ g protein). Reactions were incubated at 30°C for 2 h and subsequently processed using the filter assay. Y-value = T_0 -subtracted average cpm $\pm$ SD (n=2).

(C) The presence of GAUT7 was verified in western blots from the four microsome preparations used in (A). GAUT12 protein was undetectable in any of the four culture microsome preparations used in (A). GAUT12 IP from stem microsomes was used as a positive control for the immunoprecipitation and western blot analysis.

Xylosyltransferase (XylT) activity

A time-course of UDP-[¹⁴C]xylose incorporation (**Figure D-12**) was conducted. The XylT activity in WT+BL suspension culture microsomes was found to be linear over 30 min. Nevertheless, to allow the synthesis of maximum amounts of products, a reaction time of 60 min was chosen for subsequent reactions.

Radiolabeled [¹⁴C]xylose incorporation was assayed to determine whether there was any change in xylan backbone biosynthesis in microsomes prepared from *irx8*+BL cells compared to WT+BL cells. The first experiment was conducted using endogenous acceptors and heat-inactivated *irx8*+BL microsomes as exogenous acceptor (**Figure D-13A**). Parallel reaction products were treated with β-1,4-xylanase to remove the xylan formed during these reactions. Interestingly, the *irx8*+BL cells had higher [¹⁴C]xylose incorporation upon addition of the heat-inactivated microsomes. Since the amount of xylanase-sensitive products formed in all four reactions was similar (**Figure D-13B**), the results suggest that heat-inactivated microsomes do not provide additional substrates for synthesis of a xylanase-sensitive product.

Another independent experiment was conducted using xylohexaose (Xyl₆) as an exogenous acceptor substrate (**Figure D-14**). WT and *irx8* microsomes were prepared from suspension cultures grown for 6 days on xylogenic and control media. Microsomes prepared from WT+BL cells had increased xylan:XylT activity and greater amounts of xylanase-sensitive products, demonstrating that the induction of TE formation in WT suspension culture cells led to more xylan biosynthesis, as expected. Curiously, in the *irx8* suspension culture cells the greatest amount of XylT activity was present in microsomes prepared from *irx8* cells grown on control medium, while the xylanase-sensitive products produced in *irx8* control cells were similar to, if not less than, those produced by the *irx8*+BL cells. These results suggest that in the absence of

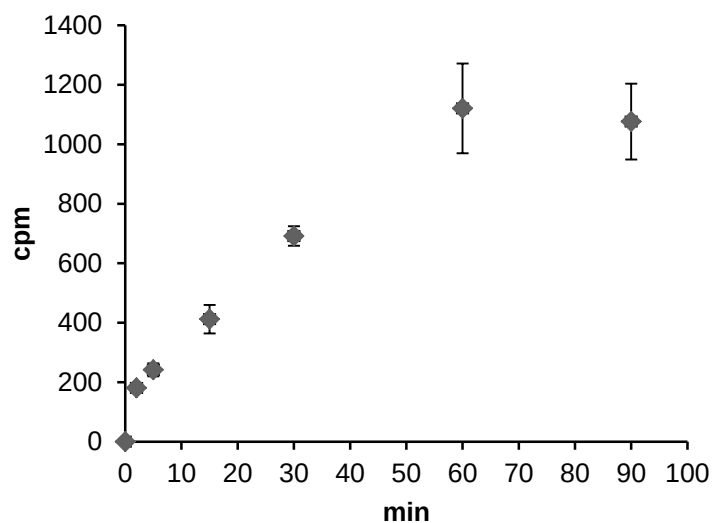


Figure D-12. Time-course of UDP-[¹⁴C]Xyl incorporation using total microsomes from 6-day-old wild-type suspension culture cells grown on xylogenic medium (WT+BL).

The XylT activity assay was carried out in 30 μ L reactions containing 50 mM Hepes pH 7.3, 0.2 M sucrose, 10 mM MgCl₂, 9 μ M UDP-[¹⁴C]Xyl (1.5 kBq), and 100 μ g of microsomes. The reaction was incubated in a 29°C water-bath for 0, 2, 5, 15, 30, 60, and 90 min. The reaction products were processed using the precipitation assay. Y-value = T₀-subtracted average cpm $\pm$ SD (n=3).

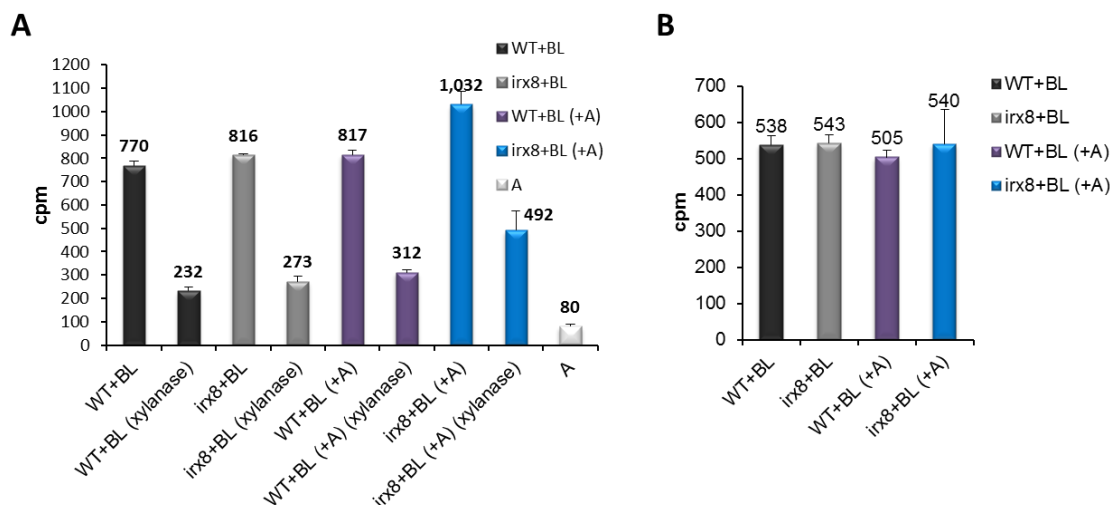


Figure D-13. UDP-[¹⁴C]Xyl incorporation using microsomes prepared from wild-type and *irx8* suspension culture cells grown on xylogenic media (WT+BL and *irx8*+BL).

(A) The reactions contained 50 mM Hepes pH 7.3, 0.2 M sucrose, 10 mM MgCl₂, 9 μM UDP-[¹⁴C]Xyl (1.5 kBq), and 100 μg of microsomes (permeabilized with 0.1% TX-100), and *irx8*+BL heat-inactivated microsomes (+A). The reactions were incubated at 29°C for 1 h for maximum product formation. Reactions were terminated with 700 μL chloroform:methanol (3:2, v/v) and 100 μg OGA (DP 7-23) was added as a carrier to precipitate the product. Reaction products were processed by the precipitation assay. Aliquots of β-xylanase M6 (Megazyme E-XYRU6, lot 51206) in sodium acetate (pH 6.0) was used to treat the reaction product generated per reaction (4.5 U) for 16 h at room temperature. The undigested (xylanase-resistant) products were recovered using the precipitation method and the cpm determined by scintillation counting. Value = average cpm ± SD (n=2).

(B) Xylanase-sensitive products generated by WT+BL and *irx8*+BL microsomes with (+A) and without *irx8*+BL heat-inactivated microsomes. Y-value = (Total cpm – xylanase-resistant cpm) ± SD (n=2).

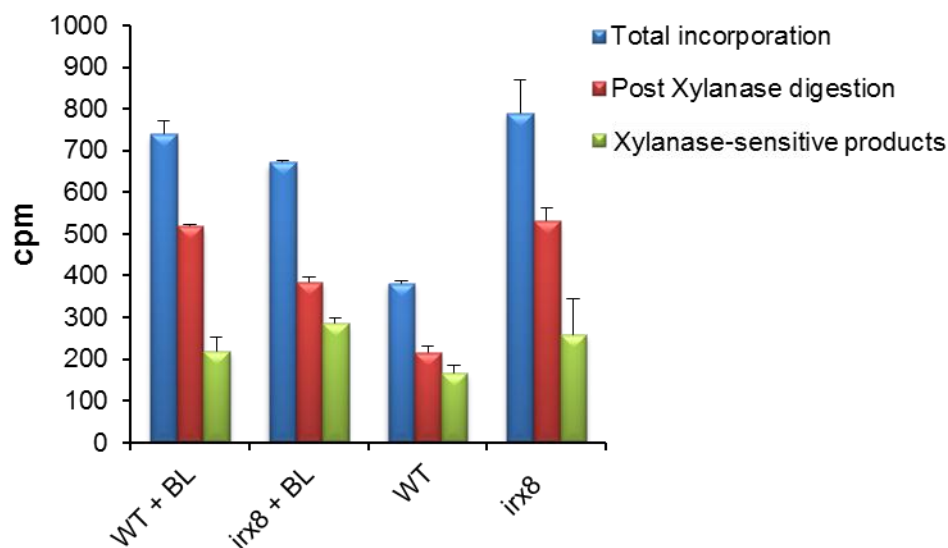


Figure D-14. Xylan:XylT activity in microsomes prepared from 6-day-old WT and *irx8* suspension cells grown on xylogenic (+BL) and control media using xylohexaose as acceptors.

Xylan:XylT activity was measured in 30 μ L reactions containing 50 mM Hepes pH 7.3, 0.2 M sucrose, 10 mM MgCl_2 , 1 mM DTT, 9 μ M UDP-[^{14}C]Xyl, and 1 mM Xyl₆. Y-value = T_0 -subtracted average cpm $\pm$ SD (n=2). The reactions were incubated at 29°C for 1 h for maximum product formation. Reactions were terminated with 700 μ L chloroform:methanol (3:2, v/v) and 100 μ g 4-O-methyl-D-glucurono-D-xylan (Sigma M5144, GX) was added as a carrier to precipitate the products. Reaction products were processed by the precipitation assay (total incorporation). Aliquots of β -xylanase M6 (Megazyme E-XYRU6, lot 51206) in sodium acetate (pH 6.0) was used to treat the pellet generated per reaction (4.5 U) for 16 h at room temperature. The undigested (xylanase-resistant) products were recovered by adding 100 μ g GX as a carrier and precipitated and the cpm was determined by scintillation counting (post xylanase digestion). Y-value (for total and post-xylanase digestion) = T_0 -subtracted average cpm $\pm$ SD (n=2). Y-value (for xylanase-sensitive products) = (Total cpm – xylanase-resistant cpm) $\pm$ SD (n=2).

GAUT12, xylan biosynthesis may be up-regulated in some way (consistent with findings presented in **Chapter 3**). Further studies of these cells will be necessary to elucidate the mechanisms beyond these results.

APPENDIX E

GENERATION OF THE *irx8 wrky12* DOUBLE MUTANT

WRKY12 is a negative regulator of secondary cell wall formation in pith parenchyma cells (**Chapter 1**), and *WRKY12* knock-down mutants have ectopic lignin deposition in pith parenchyma cells of *Arabidopsis* stems (Wang et al., 2010). Since *irx8* has collapsed xylem vessels and reduced fiber thickness due to a lack of secondary wall formation, and since the *wrky12* mutant has increased secondary walls, we investigated whether a double *irx8 wrky12* mutant would result in rescue of the collapsed xylem phenotype of *irx8* and/or reduce the ectopic lignin phenotype of *wrky12*.

I generated an *irx8 wrky12* double mutant by pollinating heterozygous *irx8* pistil with homozygous *wrky12* pollen. Double heterozygous progeny were selected from the F1 population (**Figure E-1A**). The double heterozygous F1 plants were self-fertilized, and F2 plants were grown and selected for *irx8 wrky12* double homozygous mutants by genotyping (**Figure E-1B**). The genotypes of the F2 population fit the Mendelian Law of Independent Assortment (**Figure E-1C**). The double mutant appeared to be slightly taller than *irx8* but did not revert to *wrky12* or wild-type stature (**Figure E-2A**) and genetically contained T-DNA insertions in both *GAUT12* and *WRKY12* genes (**Figure E-2B**). Collapsed xylem remained visible in the double mutant (**Figure E-2F, J**, arrows), suggesting that *GAUT12* is indispensable for normal secondary cell

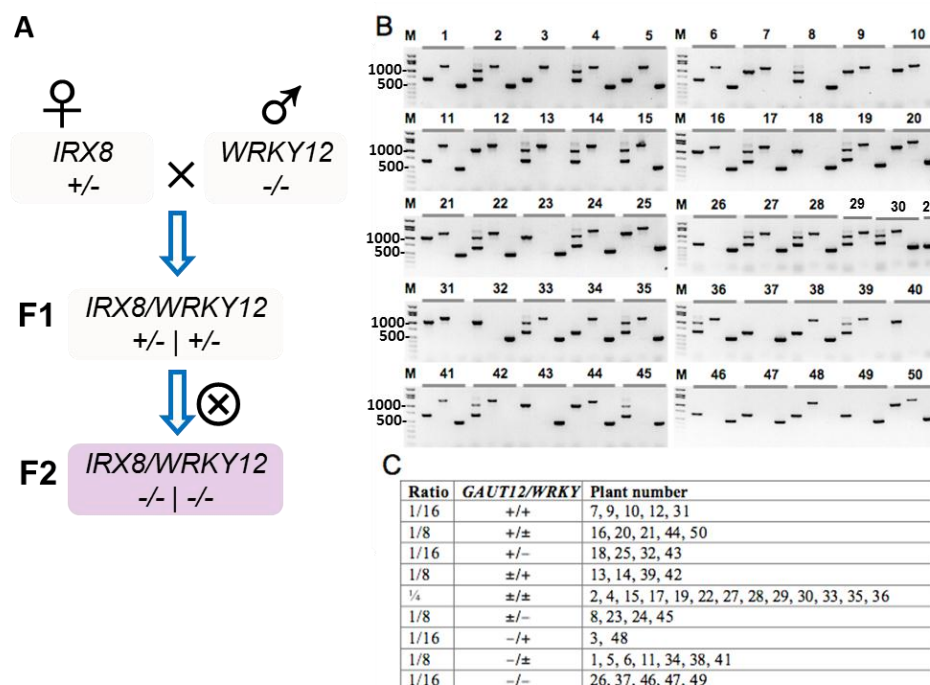


Figure E-1. Generation of the *irx8 wrky12* double mutant.

(A) Schematic diagram of the genetic crosses and progeny selection.

(B) Genotyping results for the selection of the *irx8 wrky12* double mutants. Fifty F2 plants were genotyped and five homozygous double mutants were identified. Plant genomic DNAs were extracted and three PCR reactions were used to analyze each plant. The genotyping of each plant contains 3 PCR reactions (3 lanes on gel). The first lane was the genotyping results for *irx8*. The ~1000 bp band is the *GAUT12* endogenous product (WT), the ~650 bp band is the *irx8* T-DNA insertion product (*irx8*). A double-band corresponds to the heterozygote *irx8*. The second lane contains the *WRKY12* endogenous product (WT), and the third lane contains the *wrky12* T-DNA insertion product. The presence of both bands in lanes 2 and 3 corresponds to the *wrky12* heterozygote. DNA standards (M) of 1000 bp and 500 bp as labeled.

(C) Expected genotypes of the F2 progenies and the genotyping results are summarized for each genotype. “+”, wild type; “-”, homozygous mutant; “±”, heterozygous mutant.

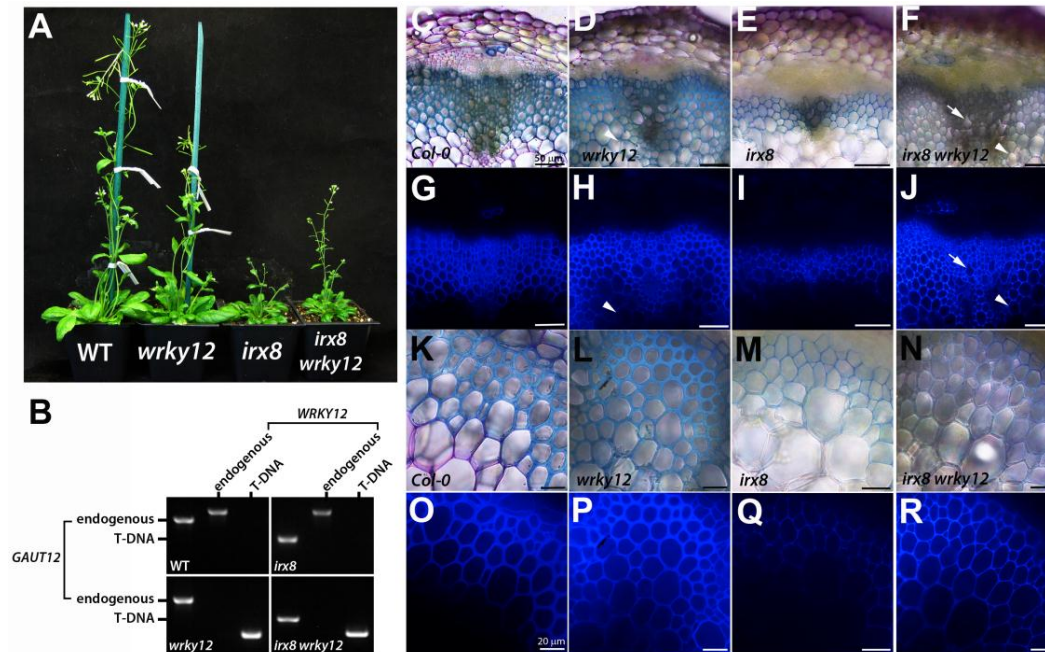


Figure E-2. Double *irx8 wrky12* mutant has reduced ectopic lignin.

(A) Six-week-old wild type *Col-0* (WT), *wrky12*, *irx8*, and *irx8 wrky12* double mutant. The *irx8 wrky12* double mutant plant has a stature close to *irx8* but is slightly taller.

(B) Identification of homozygous *irx8 wrky12* double mutant by PCR. The T-DNA product was obtained by amplification of the T-DNA left border and the gene flanking the T-DNA. Endogenous gene products for *GAUT12* and *WRKY12* were identified by amplification of the gene spanning the T-DNA insertion sites. The corresponding genotypes are labeled in each panel.

The right panel shows toluidine blue O-stained and UV-autofluorescence of lignin in free-hand-cut sections of (C, G) wild type (*Col-0*), (D, H) *wrky12*, (E, I) *irx8*, and (F, J) *irx8 wrky12*. The double mutant has collapsed xylem (arrows) and ectopic lignin (arrowheads) but with reduced cell wall thickness. Interfascicular fiber regions of (K, O) wild type (*Col-0*), (L, P) *wrky12*, (M, Q) *irx8*, and (N, R) *irx8 wrky12* are enlarged. Bar = 50 μ m for vascular bundles and 20 μ m for fibers. Arrows: collapsed xylem vessels; arrowheads: ectopic lignin.

wall formation. Ectopic lignin remained present in the double mutant. However, there was a significant reduction in the lignin content as detected by UV autofluorescence (**Figure E-2R**) and there was also reduced secondary wall thickness based on toluidine blue staining (**Figure E-2N**). Consistent with these results, the *GAUT12* transcript level also increased to 1.5-fold in pith cells of the *wrky12* mutants, indicating a negative regulation of *WRKY12* on *GAUT12* (Huanzhong Wang, R.A.Dixon lab, personal communication).

We also tested the double *irx8 wrky12* mutant for the presence of xylan epitopes using monoclonal antibodies LM10 (recognizing low-substituted xylan) and LM11 (recognizing both low- and high-substituted xylan) (**Figure E-3**). Xylan is reduced in the double mutant, and in particular lacks the epitope recognized by LM10 (**Figure E-3D**), which is consistent with the *irx8* single mutant that lacks LM10 labeling (**Figure E-3C**). The remaining xylan present in the double mutant (**Figure E-3H**) is labeled with LM11, albeit with reduced intensity, similar to the LM11 labeling in the *irx8* single mutant. These results confirmed a role for *GAUT12* in both xylan and lignin formation.

Our data suggest that the product of *GAUT12* is required for lignin deposition, as demonstrated by reduced lignin in *irx8* stems and anther endothecium cell layers (see **Chapter 2**). The transcript level of *GAUT12* increased 1.5-fold in the *wrky12* mutant pith cells compared to wild type (Huanzhong Wang, R.A.Dixon lab, personal communication), indicating that *GAUT12* is negatively controlled by *WRKY12*. The *irx8 wrky12* double mutant retained the collapsed xylem and ectopic lignin phenotypes resembling the single mutants of *irx8* and *wrky12*, respectively. However, the thickness of secondary cell wall was significantly reduced in the double mutant, presumably due to a reduction in both lignin and xylan. The results support the conclusion that *GAUT12* is indispensable for lignin and xylan formation. Interestingly,

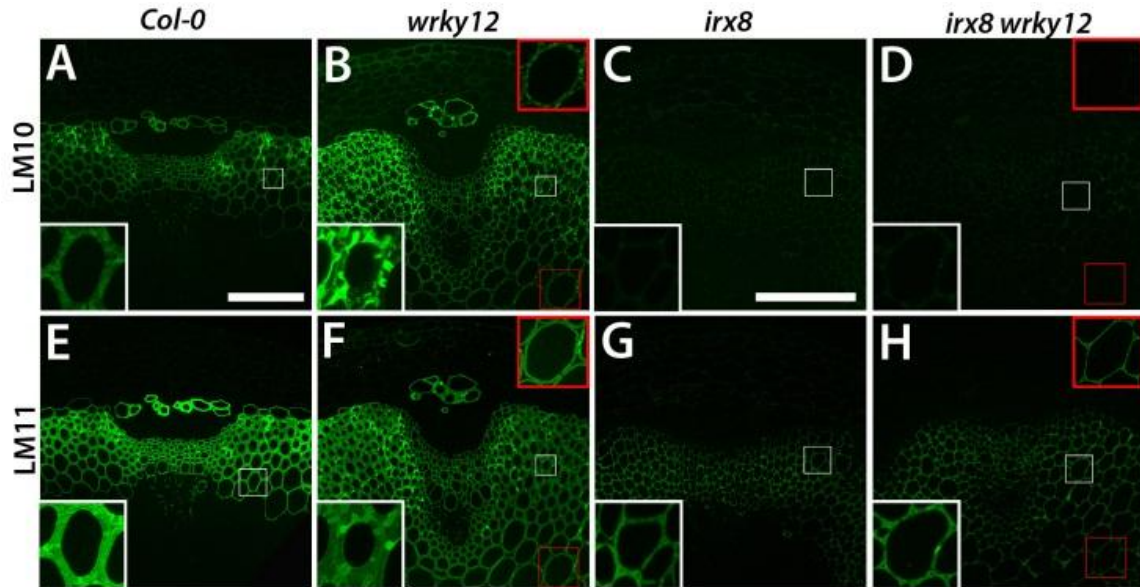


Figure E-3. Immunolabeling of xylan in the *irx8 wrky12* double mutant.

LM10 immunolabeling of (A) WT (*Col-0*), (B) *wrky12*, (C) *irx8*, and (D) *irx8 wrky12*. LM10 is a monoclonal antibody that recognizes 4-*O*-methylated xylan but not arabinoxylan or glucuronoarabinoxylan. LM11 immunolabeling of (E) WT (*Col-0*), (F) *wrky12*, (G) *irx8*, and (H) *irx8 wrky12*. LM11 recognizes both low- and high-substituted xylan. White box insets are enlargements of an interfascicular fiber cell of the corresponding plant, and red box insets are enlargements of an ectopic lignin cell. Bar = 100 μ m for (A, B, E, F) and (C, D, G, H).

GAUT12 transcript was not affected in the over-expression lines of MYB58 and MYB63, which are both lignin-specific transcription factors (Zhou et al., 2009). Thus, the control of secondary wall formation and lignin synthesis can be regulated separately.

APPENDIX F

GENERATION AND CHARACTERIZATION OF ARABIDOPSIS SUSPENSION CELL CULTURES

The major results obtained from the study of the Arabidopsis suspension cell cultures produced from the research described in this thesis research were reported in **Chapter 3**. This appendix describes some details and optimization steps for those studies that were not included in the main body of the thesis.

Arabidopsis suspension culture generation

The Arabidopsis suspension cell cultures were generated by a modification of the methods described by Doelling and Pikaard (1993). The generation scheme is shown in **Figure F-1A**. Seeds of heterozygous *irx8-5* Arabidopsis plants were sterilized with 70% ethanol for 1 min, 10% bleach for 10 min, rinsed with sterilized ddH₂O six to eight times, and plated individually on callus germination media plates containing 0.6% agar, 4.4 g/L MS salt with Gamborg's vitamins (Sigma M0404), 2 mg/L 2,4-D, 0.05 mg/L kinetin, and 20 g/L sucrose (pH 5.7). The sterilized seeds on the plates were grown under a 24-h photoperiod at 23°C. Friable calli from each seed were generated after 8 weeks. The calli were maintained on callus inducing medium (CIM) plates containing 0.6% agar, 3.2 g/L Gamborg's B-5 basal medium with minimal

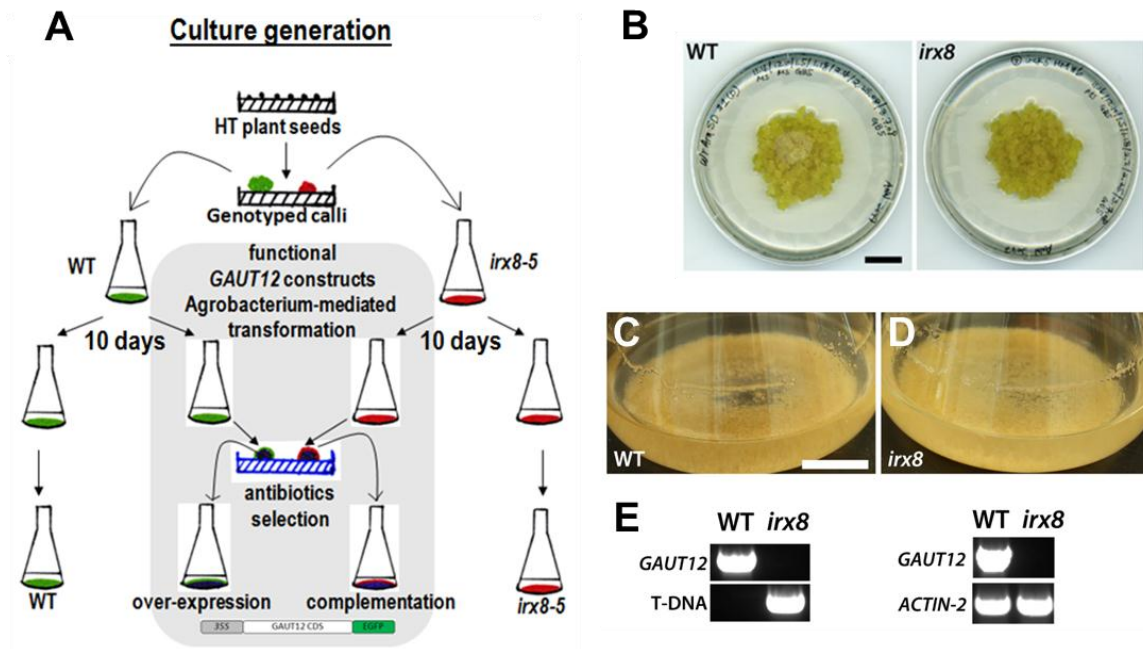


Figure F-1. Generation of Arabidopsis wild-type and *irx8* suspension cell cultures.

(A) Schematic diagram of the generation of the Arabidopsis WT and *irx8* suspension cell cultures, and of *G12*-transformed *G12*-OE and *irx8*+*G12* lines.

(B) WT and *irx8* calli maintained on CIM agar plates.

(C) WT and (D) *irx8* suspension cell cultures.

(E) Genotyping results of WT and *irx8* calli (left panel). *GAUT12* full length transcript was absent in the *irx8* suspension cell culture (right panel).

organics (Sigma G5893 or Phytochemistry), 20 g/L sucrose (Sigma), 2 mg/L 2,4-D, 0.05 mg/L kinetin (Sigma K3253), pH 5.7 (**Figure F-1B**). Genomic DNA was isolated from individual calli germinated from each seed using the PowerPlantTM DNA Isolation Kit (MO BIO 13200-50) following the manufacturer's instruction. PCR was used to genotype each callus (**Figure F-1E** left panel, primer sequences see **Chapter 3**). Homozygous *irx8-5* calli were identified to contain no *GAUT12* gene product (**Figure F-1E** right panel). WT callus that segregated from the *irx8* heterozygote parent and was confirmed to contain no T-DNA insertions within the genome (data not shown) was used as the WT control since it has the most similar genetic background to the *irx8-5* homozygous callus generated from the same seed from the starting heterozygous plants. The *irx8-5* homozygous and WT calli were transferred into 25 ml of liquid CIM in 125 ml flasks and cultured on shakers in the dark for 10-12 days, respectively. Aliquots of six milliliter compact cultured cells were transferred using a serological pipette (with tip removed) into 100 ml of CIM in 500 ml flasks and cultured on gyrotory shakers at 130 rpm in the dark for 10-12 days (**Figure F-C, D**).

Brassinolide (BL)-induced (xylogenic) WT and irx8 suspension cell cultures

Aliquots of 8 ml packed cells were transferred into xylogenic medium and control medium on the sub-culture day, and the newly inoculated cells were designated as "Day 0". A small aliquot of cells (250 μ l) from each cell line was placed into a glass tube and stained with phloroglucinol-HCl on days 0, 2, 4, 6, 8, and 10 (**Figure F-2**). The phloroglucinol-HCl provides a simple and fast way to monitor the BL-induction of lignin by reacting with the coniferaldehyde endgroups in lignin and generating a red color (Lewis and Yamamoto, 1990). There was an increase of red color in the xylogenic WT cells (WT+BL) as time progressed. However, the

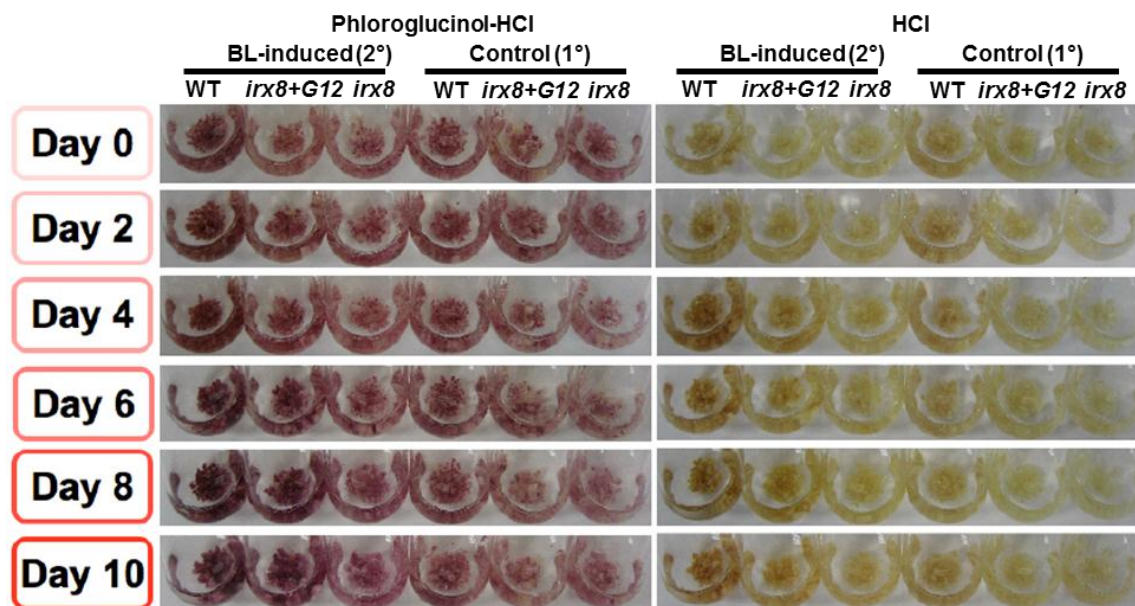


Figure F-2. Time-course of phloroglucinol staining of *Col-0* (WT), *irx8*, and *irx8+G12* suspension cultures during secondary cell wall induction (BL-induced).

All cell lines were induced to produce secondary cell walls (BL-induced, 2°) by growth on xylogenic medium that contain 1μM of brassinolide (BL) and 10 mM H₃BO₃. Control (1°) refers to cultures grown on normal growth (CIM) media (i.e. without BL). Images are 250 μl of cells from each culture at Days 0, 2, 4, 6, 8, and 10 stained for 30 min with acid phloroglucinol in the upper left panel, or only with HCl in the upper right panel. The results suggest a reduction of phloroglucinol-detected monolignol production in *irx8* cultures, and a recovery of red color in the GAUT12-complemented (*irx8+G12*) cell line that closely matches the phenotypes observed in the wild-type cell line. A comparable color increase pattern in the WT and GAUT12-complemented (*irx8+G12*) cell line is observed in the HCl treatment without phloroglucinol (right panel).

irx8+BL cells did not increase in red color as much as the wild type, suggesting a defect in the accumulation of phenolic/lignin compounds upon BL induction of this mutant. The reduction in red color staining by phloroglucinol-HCl was complemented by the *GAUT12-EGFP* (*G12*) expression construct (**Chapter 2**). **Figure F-2** shows one result that is representative of more than 10 culture experiments which compared phloroglucinol-HCl staining of Arabidopsis WT and *irx8* suspension culture cells grown on control versus xylogenic BL-induction media.

To examine whether tracheary elements (TEs) were formed in cells under BL induction, the cells grown on control and BL-induction medium were initially stained with Toluidine blue O and examined by light microscopy (**Figure F-3**). Although some small cell aggregates were found in these cultures (**Figure F-3A, B**), most cells were aggregated into cell clumps in which individual cells in the center of the clump could not be clearly observed (**Figure F-3C-F**). It was noted that WT control cells have oval-round shapes while the WT+BL cells have elongated shapes (**Figure F-3B, D, F**), suggesting that the BL-induced cells were in the process of forming tracheary elements. Occasionally, single cells with a reticulated-secondary wall thickening pattern were observed (**Figure F-4C**), but most of the cells were aggregated into small clumps (**Figure F-4B**). To observe the morphology of cells located in the clump center, both WT and *irx8* cells were collected on day 9 and embedded in LR White (**Figure F-4A**). Cross-sections of the cell blocks were taken and stained with toluidine blue O. **Figure F-4D** shows an example of the *irx8*+BL cells in which the formation of tracheary elements was clearly observed in the center of cell clumps in the *irx8*+BL cultures. The cells of WT, *irx8*, and *G12-OE* grown under both control and BL-induction media conditions were embedded on day 9 and studied for their cell wall changes by immunolabeling of wall glycan epitopes, the results of which were reported in **Chapter 3**.

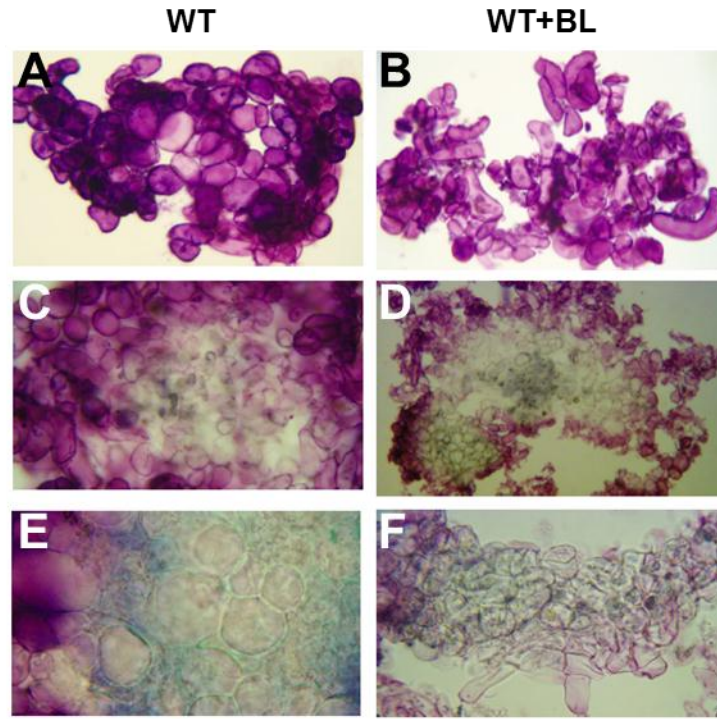


Figure F-3. Toluidine blue O staining of WT cells grown on xylogenes BL-induction medium.

(A) WT control cells have oval to round cell shapes.

(B) WT+BL cells have elongated cell shapes.

(C) and (E) Close-up images of a randomly selected WT control cell clump, where the control cells are less stained or sometimes stained blue/green with relatively round cell shapes.

(D) and (F) Close-up images of a randomly selected WT+BL cell clump, where the control cells are stained green/blue and have elongated shapes. All cells in this figure were examined on day 6.

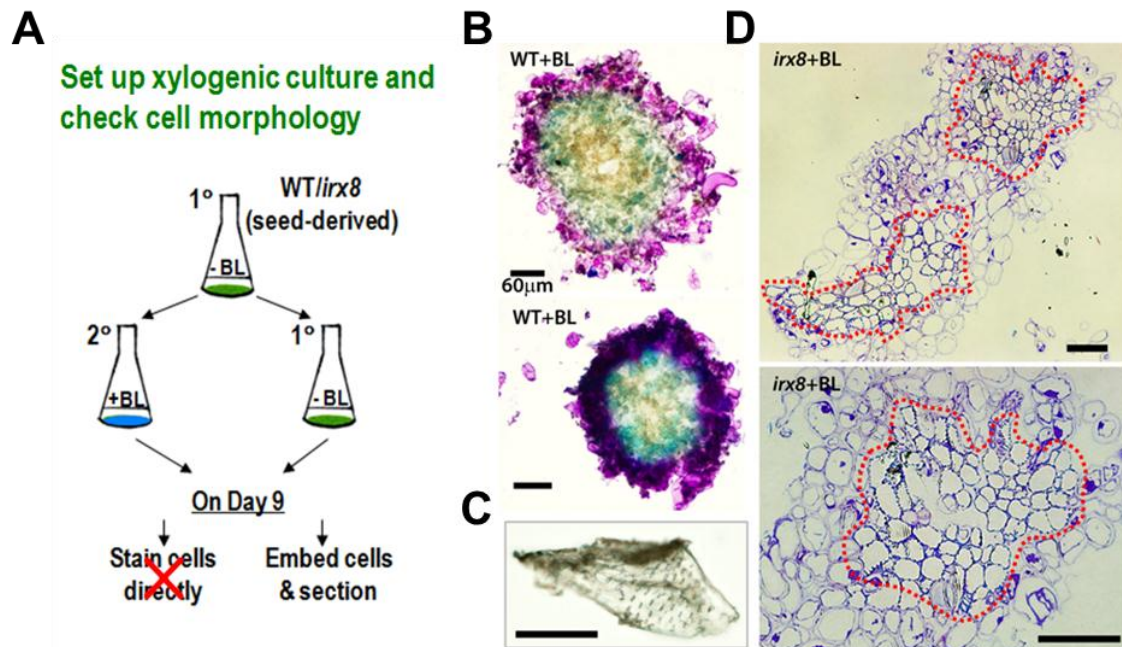


Figure F-4. Analyses of WT and *irx8* cells grown on xylogenic medium.

(A) Schematic diagram showing the BL-induced cultures (+BL) set up in parallel with control cultures (-BL). Cells were taken on Day 9 and analyzed by staining directly or by embedding in LR White for sectioning followed by staining.

(B) Fresh cells were stained with toluidine blue O and visualized under a light microscope. Cells appeared to be aggregated in clumps (common for *Arabidopsis* suspension culture cells), and the inner most cells were stained blue-green, indicating the presence of lignin.

(C) Occasionally single cells with secondary wall reticulated-patterns were observed.

(D) LR White-embedded *irx8* cells grown on xylogenic medium (*irx8*+BL) were sectioned, and tracheary elements were found in the center of these cell clumps in clusters, as outlined with red dotted lines. Bar = 60 µm.

Analyses of wall biosynthesis-related gene expression

A reduction in labeling of xylan with the monoclonal antibodies LM10 and LM11 was observed in TEs formed in the *irx8*+BL cultures (**Chapter 3**). We then asked whether xylan biosynthesis-related gene expression was changed in *irx8* cells grown on xylogenic medium (i.e. during TE formation). Three cell lines were included in this study, namely WT, *irx8*, and *G12*-OE. The cells were grown on both xylogenic and control media in parallel, and aliquots of 1 ml-packed cells were collected on days 0, 3, 6, and 9 for RNA isolation (**Figure F-5A**). Selected gene expression related to cell wall biosynthesis was studied using quantitative PCR, including 10 genes involved in lignin biosynthesis, 13 genes involved in xylan biosynthesis, *CESA1* and *CESA4* involved in primary and secondary wall cellulose biosynthesis, 4 *GAUT* genes involved in both primary and secondary wall pectin biosynthesis, and two transcription factor genes involved in secondary cell wall formation (**Figure F-5B**). The results of this study were reported in **Chapter 3**.

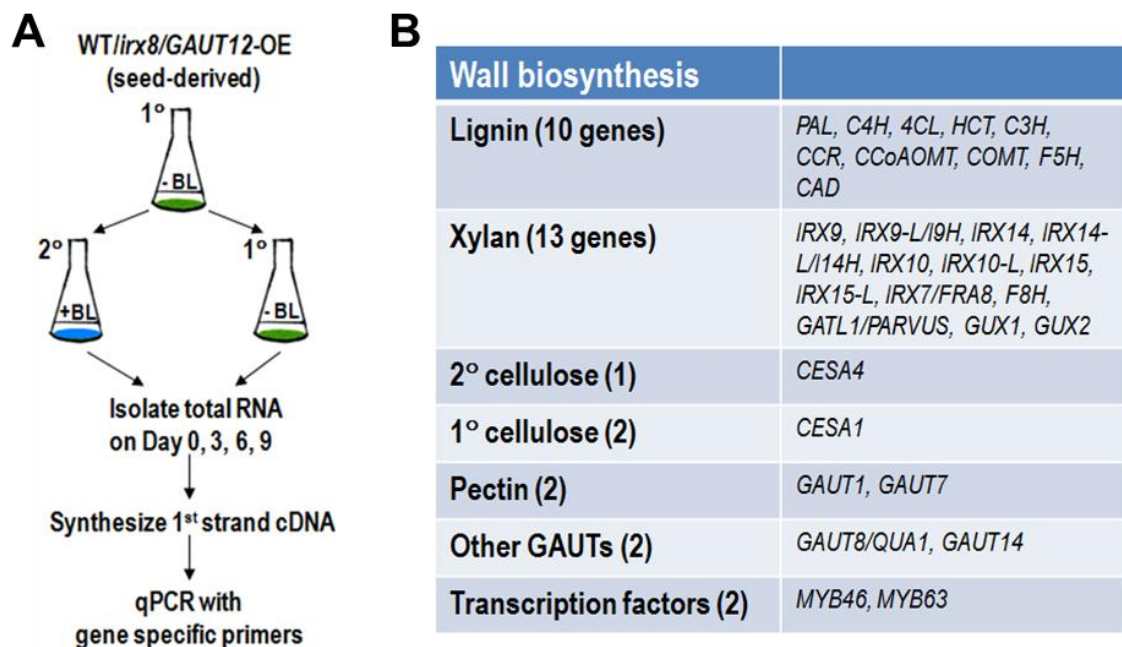


Figure F-5. Analyses of wall biosynthesis-related gene expression in WT, *irx8*, and *G12*-OE cells grown on control and xylogenic media.

(A) Schematic diagram of the experiment outline.

(B) List of selected genes analyzed in this study. The results of the transcriptome analyses were described in Chapter 3.