

EXPRESSION AND CHARACTERIZATION OF GALACTURONOSYLTRANSFERASE-6
(GAUT6) OF THE GALACTURONOSYLTRANSFERASE-1 (GAUT1)-RELATED GENE
FAMILY OF *ARABIDOPSIS THALIANA*

by

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(Under the Direction of Debra Mohnen)

ABSTRACT

Pectin is an important component of the plant primary wall that contributes to wall structural integrity, cell adhesion and modulation of wall expansibility. The function of pectic polysaccharides also extends to the elicitation of plant defense response-related signal transduction pathways. The pectic polysaccharides are defined by the presence of α -1,4-linked galacturonic acid residues in their backbone structures. The current knowledge of pectin structure, function and biosynthesis is being elucidated in the post-genomic era, however, the complexity of pectic polysaccharide structure, the difficulty of isolating membrane bound glycosyltransferases and the tolerance of plants to changes in wall structure, continue to hinder rapid progress towards the elucidation of the pectin biosynthetic process and machinery. Indeed, many hurdles were overcome by the identification of the *Arabidopsis thaliana* GALACTURONOSYLTRANSFERASE-1 (GAUT1), a protein that catalyzes the transfer of GalA from UDP-GalA onto oligogalacturonides, an activity consistent with pectin homogalacturonan galacturonosyltransferase activity. The identification of GAUT1 has led to the identification of 14 additional proteins that make up the Arabidopsis GAUT1-related gene

family. Here it has been shown that most members of the *Arabidopsis* GAUT1-related gene family have conserved orthologs in *Populus trichocarpa* (black cotton wood; a woody dicot) and *Oryza sativa* (rice; a grassy monocot). In a survey of GAUT gene mutant cell walls, it was found that at least five other GAUT genes, GAUT 6, 8, 9, 10, and 11, are putative GalATs that function in pectin biosynthesis, but that at least three members, GAUT 12, 13 and 14, are putative GalATs involved in xylan synthesis. GAUT6 was selected for further analysis based on the *gaut6* mutant wall compositional phenotype and by the relatedness of GAUT6 to GAUT1. A fractionation of *gaut6* walls, compared to WT, was carried out and showed that *gaut6* walls appeared to be altered in the synthesis of a pectic component indicative of homogalacturonan or rhamnogalacturonan-I. Additionally, GAUT6 expressed in *Nicotiana benthamiana* and *Escherichia coli* produced protein that had detectable HG:GalAT activity, proving that GAUT6 is able to transfer [¹⁴C]GalA from UDP-[¹⁴C]GalA onto oligogalacturonides, consistent with a function as an HG:GalAT and with a role in pectin biosynthesis.

INDEX WORDS: pectin, biosynthesis, glycosyltransferase, galacturonosyltransferase, Golgi, *Arabidopsis thaliana*, CAZy GT family 8, T-DNA insertion mutant, *Nicotiana benthamiana*, green fluorescent protein, GAUT.

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DEDICATION

I dedicate this work to my husband, Zachary Farr Caffall, for the unconditional support and love that he has given me, always.

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.....	v
FORWARD.....	1
CHAPTER	
1 LITERATURE REVIEW: THE STRUCTURE, FUNCTION AND BIOSYNTHESIS OF PLANT CELL WALL PECTIC POLYSACCHARIDES.....	4
Introduction.....	5
Pectin Structure.....	7
A Model of the Primary Cell Wall.....	21
Structural Characterization of Pectic Polysaccharides.....	39
Function of Pectic Polysaccharides.....	53
Biosynthesis of Pectic Polysaccharides.....	62
Conclusions and Relevance.....	97
2 COMPARATIVE PHYLOGENETIC ANALYSIS AND CELL WALL COMPOSITION OF <i>ARABIDOPSIS THALIANA</i> T-DNA MUTANTS IMPLICATE GAUT GENES IN CELL WALL AND SEED TESTA PECTIN AND XYLAN BIOSYNTHESIS	98
Abstract.....	99
Introduction.....	101
Results.....	105
Discussion.....	124
Conclusion.....	132
Materials and Methods.....	133
3 STRUCTURAL ANALYSIS OF <i>ARABIDOPSIS</i> GAUT6 T-DNA MUTANT CELL WALL AND MUTANT SEED EXTRACTABLE POLYSACCHARIDES.....	138
Abstract.....	139
Introduction.....	141

Results.....	145
Discussion.....	166
Conclusion.....	172
Materials and Methods.....	173
 4 THE CLONING AND EXPRESSION OF ATGAUT6 IN <i>N. BENTHAMIANA</i> AND <i>E. COLI</i>	 178
Abstract.....	179
Introduction.....	180
Results.....	184
Discussion.....	208
Conclusion.....	210
Materials and Methods.....	211
 REFERENCES.....	 218
 APPENDIX A.....	 246
 APPENDIX B.....	 253
 APPENDIX C.....	 259

FORWARD

Plant cell walls are essential organs in plant biology that are made up of carbohydrates, protein and phenolic compounds. Diverse functions that contribute to proper growth and development of plants are attributed to the plant cell wall. The carbohydrate component makes up more than 90% of the primary wall, and is accordingly extremely important in wall function and plant biology. There is a wide variety of carbohydrate structures that make up the wall, and which belong to one of three groups based on structural characteristics: cellulose, hemicelluloses and pectins.

The pectins are enriched in the plant primary cell wall and middle lamellae, and are a class of molecules whose defining character is inclusion of galacturonic acid residues within their structure. A detailed synopsis of the existing literature encompassing pectin structure, function and biosynthesis is presented in Chapter 1. In brief, the pectic polysaccharides are the galacturonans (homogalacturonan and the substituted galacturonans) and rhamnogalacturonan-I. The galacturonans have a linear backbone that is made up of α -1,4-linked galacturonic acids that may or may not have further substitution. The identification of glycosyltransferases involved in pectin synthesis is important to the many uses of pectin in industry and human health, and is essential to the study of pectin function in plant growth and development.

Previous studies carried out by Dr. Jason Sterling identified the *Arabidopsis thaliana* GALACTURONOSYLTRANSFERASE-1 (GAUT1) that was shown to have homogalacturonan galacturonosyltransferase (GalAT) catalytic activity. A BLAST search of the *Arabidopsis thaliana* genome resulted in the discovery of 14 additional GAUT1 isoforms that have greater

than 37% amino acid identity and greater than 56% amino acid similarity to GAUT1. Additionally a GALACTURONOSYLTRANS-FERASE-LIKE (GATL) family was also identified that has lower amino acid similarity to GAUT1 and that clusters independently from the GAUTs in phylogenetic analyses. Based on the catalytic activity of GAUT1 and evidence to suggest that GAUT8, GAUT12 and GATL1 are involved in cell wall biosynthesis, it is hypothesized that additional unstudied GAUTs function in the synthesis of pectic polysaccharides and have GalAT activity. The studies described in this dissertation are aimed at the identification of additional pectin biosynthetic glycosyltransferases in the *Arabidopsis thaliana* GAUT1-related family of proven and putative cell wall biosynthetic GalATs.

T-DNA insertion seed lines were obtained with tentative insertion sites in 14 out of the 15 GAUT genes for application to a screen for involvement of the mutated genes in pectin biosynthesis (Chapter 2). Homozygous mutants were obtained in all of the GAUT genes except for GAUT1 and GAUT4, yielding 26 mutant lines corresponding to 13 of the 15 GAUT genes. Mutant plants were screened for altered wall structure by assaying mutants compared to similarly isolated wild-type walls and for altered wall polysaccharide structure by glycosyl residue composition analysis. The results of the study designated GAUT 6, 9, 10 and 11 as putative GalAT candidate genes involved in pectin biosynthesis, based on the reduced galacturonic acid wall composition of the corresponding mutant plants compared to wild-type. In addition, GAUT13 and GAUT14 are hypothesized to function in wall biosynthesis, and the elevated wall galacturonic acid and reduced wall xylose of these mutants suggests a possible role in xylan or RG-I synthesis.

The next section of this dissertation describes the detailed structural characterization of mutant GAUT6 walls, a putative pectin biosynthesis mutant compared to wild-type. The

comparative analysis of *gaut6* walls compared to wild-type walls has a two-fold purpose: To further narrow the wall polysaccharide structures affected by the mutations and to clarify the function of a GAUT gene that clusters to a previously uncharacterized clade of the GAUT1-related gene family (Chapter 3). The *gaut6* and WT leaf wall and seed wall polysaccharides were selectively solubilized to yield pectic, hemicellulosic or cellulosic fractions that were analyzed by glycosyl residue composition and linkage analyses. The detailed analysis of *gaut6* mutants has demonstrated that GAUT6 affect plant growth and has a distinct role in wall polysaccharide biosynthesis. More specifically, the results of detailed leaf wall and seed wall polysaccharide analysis revealed that GAUT6 is involved in the synthesis of (1) a subfraction of HG that is likely to be an RG-I:HG linker region or (2) RG-I.

In order to clarify the catalytic function of GAUT6, the native and tagged GAUT6 protein was transiently expressed in the leaves of *Nicotiana benthamiana* by *Agrobacterium tumefaciens*-mediated transformation. The HG:GalAT activity was assessed by two different assay systems. Increased HG:GalAT activity was observed in leaf tissues transformed with GAUT6 over untransformed or empty-vector transformed *Nicotiana*, suggesting that GAUT6 has GalAT activity or has a direct affect on GalAT levels in the plant. Two soluble truncated versions of GAUT6 were heterologously expressed in *E. coli* and also shown to have detectable GalAT activity. These results demonstrate that GAUT6 is an HG:GalAT involved in wall biosynthesis.

CHAPTER 1

LITERATURE REVIEW: THE STRUCTURE, FUNCTION AND BIOSYNTHESIS OF PLANT CELL WALL PECTIC POLYSACCHARIDES.

INTRODUCTION

The cell wall of plants is a distinguishing character of plant cell biology and is essential to plant survival. Plants are relatively stationary organisms that are limited in their mobility and thus are limited in their ability to avoid undesirable environmental conditions and to escape from attack. As a consequence of limited mobility, plants have become very plastic in their ability to withstand a variety of harsh environmental conditions and to affront attack by pathogen and herbivore. The plant cell wall is an extremely complex super-structure that surrounds and protects the cell. The walls of the plant contribute in many ways to the ability of plants to thrive under non-ideal conditions. The polysaccharides, proteins and aromatic compounds that make up the cell wall enable the plant cell, and thus plants, to flourish in almost any environmental niche on Earth.

The plant cell wall structure is not static, but is modified as the plant develops and as the environmental conditions vary. The plant cell lays down the middle lamella and the primary wall during initial growth and expansion of the cell. In many cells, the wall is thickened and further strengthened by the addition of a secondary wall (Figure 1.1). The primary wall is characterized by less relative cellulose and greater pectin compared to secondary walls. The primary wall contributes to cell wall structural integrity, cell adhesion and signal transduction. The major fraction of primary wall non-cellulosic polysaccharides in the Type-I walls of dicot and non-graminaceous species are the pectic polysaccharides. The pectic polysaccharides play a major role in plant development and in the reproductive success of the plant. It is the focus of this literature review to bring together the available knowledge of the fine structure, analysis, function and biosynthesis of the pectic polysaccharides of the plant cell wall, with respect to plant growth and development.

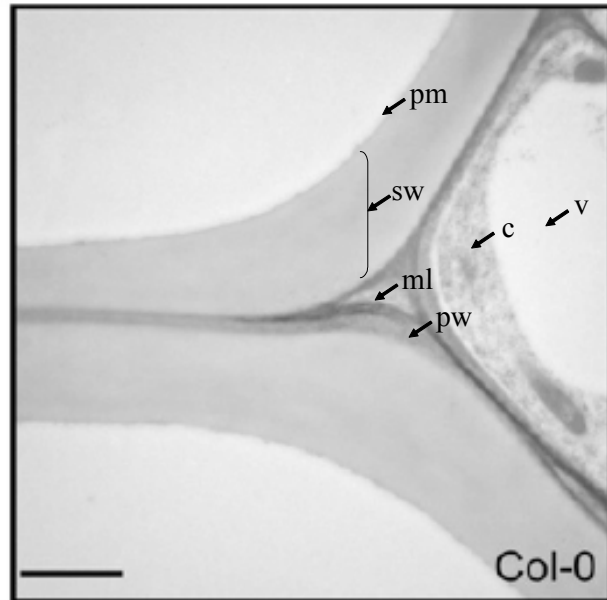


Figure 1.1. The cell wall of *Arabidopsis thaliana*.

Transmission electron micrograph of WT *Arabidopsis thaliana* Columbia-O transverse root section showing the clearly delineated middle lamella (ml), primary wall (pw) and secondary wall (sw). Additional labeled features of the cell are the plasma membrane (pm), cytosol (c) and vacuole (v). Bar = 2 μ M. Adapted from Persson et al. (2007).

PECTIN STRUCTURE

The pectic polysaccharides comprise a class of GalA containing polysaccharides that are abundant in the plant cell wall; comprising as much as 30% of dicot, gymnosperm and non-Poales monocot walls (Ridley et al., 2001; O'Neill and York, 2003). The walls synthesized by the order Poales (formerly the Gramineae) and related orders contain considerably less pectin; approximately 10% by weight (Carpita, 1996). It has been estimated that ~90% of the uronic acids in the wall derive from the GalpA residues of pectic polysaccharides (Selvendran and O'Neill, 1987). The structural classes of pectic polysaccharides are homogalacturonan (HG), xylogalacturonan (XGA), apiogalacturonan (AGA), rhamnogalacturonan II (RG-II) and rhamnogalacturonan I (RG-I), (Ridley et al., 2001). The fine structures of the pectic polysaccharides govern the biological roles of these molecules within the cell wall. Understanding how the structure of pectin is modified during growth, in response to environmental stimuli is essential to understanding the role of these biological molecules in plant biology.

Homogalacturonan

HG is a linear polymer of α -1,4-linked-D-galacturonic acid (Figure 1.2) that can account for greater than 60% of pectin in the plant cell wall (Ridley et al., 2001). HG is abundant in potato (*Solanum tuberosum*) primary walls and is particularly dense in the middle lamellae of this species (Bush et al., 2001). HG comprises at least 23% of *Arabidopsis thaliana* leaf walls (Zabackis et al., 1995) and ~10% of sycamore suspension culture cell walls (*Acer pseudoplatanus*) (Talmadge et al., 1973). The walls of tomato have up to 35% uronic acid (Seymour et al., 1990), while mango walls are ~52% uronic acid (Muda et al., 1995).

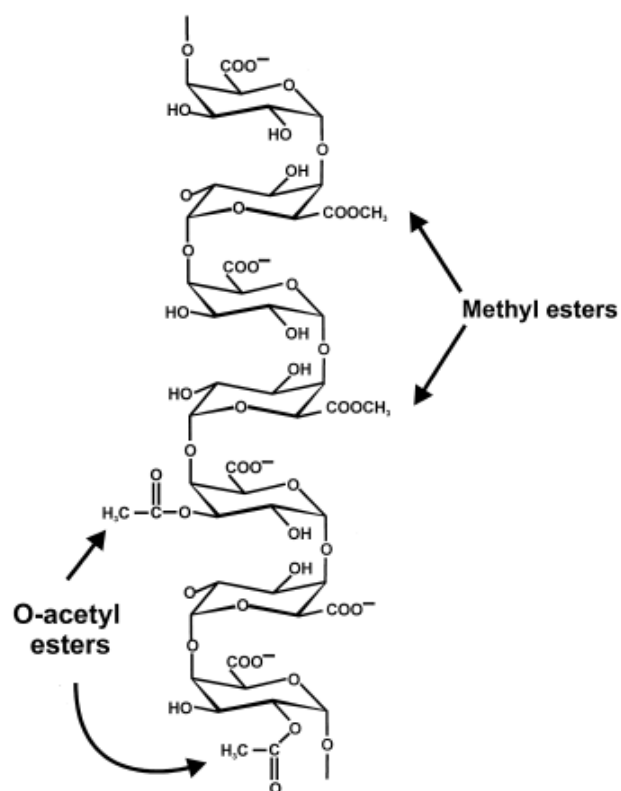


Figure 1.2. Homogalacturonan structure and modification. The structure of the pectic polysaccharide homogalacturonan (HG) as a linear polymer of α -(1,4)-linked galacturonic acid (GalA) residues. The sites of methylesterification at the C-6 and *O*-acetylation at the O-2 or O-3 of the carbohydrate ring are shown (Ridley et al., 2001).

HG can be methyl-esterified at the C-6 carboxyl or acetylated at the O-2 or O-3 of GalA residues (Figure 1.2) (Ridley et al., 2001). The pattern and degree of methylesterification and acetylation may vary. Suspension-cultured cotton HG was shown to be ~50% methylated with non-random distribution (Mort et al., 1993). Methylesterification is hypothesized to be tightly regulated by the plant in a developmental and tissue-specific manner in *Arabidopsis* (Willats et al., 2001a).

The unmethylated C-6 of GalpA residues of HG are negatively charged and may coordinate Ca^{+2} cations forming a stable gel with other pectic molecules if >10 consecutive unmethyl-esterified GalA residues are coordinated (Liners et al., 1989). *In vitro*, citrus peel pectin was used to demonstrate that a pectin gel can be formed by addition of monovalent salts to pectin de-methylesterified by orange peel PME. It was postulated that the gel formation was mediated by salt cations in solution, hydrogen bonding and hydrophobic interactions (Yoo et al., 2003). The hypothesized *in vivo* structure of the HG-calcium complex is referred to as the egg-box model (Figure 1.3) (Liners et al., 1989). NMR spectroscopy of a calcium pectate gel prepared from orange peel pectin established that the HG backbone takes a two-fold helical structure (2_1).

HG is covalently crosslinked to RG-I, RG-II and xyloglucan.

The backbone of HG is covalently linked to RG-I and RG-II and is also hypothesized to be covalently crosslinked to xyloglucan (XG) hemicellulose polysaccharides *in muro* (Popper and Fry, 2007). It has long been observed that pectic polymers are released by endopolygalacturonases (EPG) that hydrolyze the glycosidic bonds of the HG backbone to produce monomeric, dimeric or oligomeric fragments (Benen et al., 1999). The egg-box model

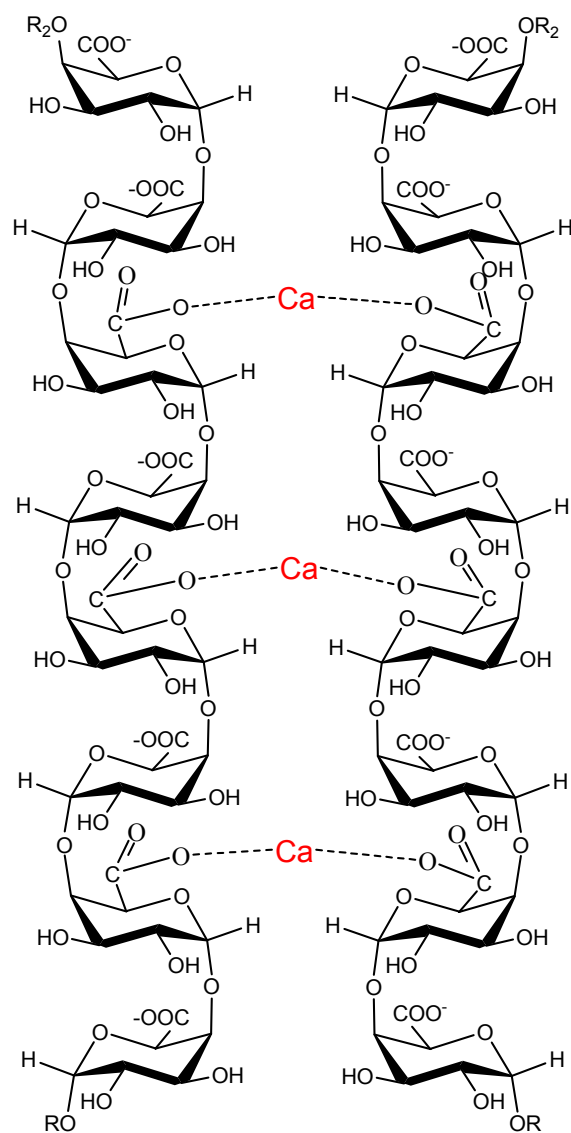


Figure 1.3. The egg-box model of calcium crosslinking in HG polysaccharides.

describes the close packing of HG polysaccharides that occurs upon Ca^{+2} -induced gelling in the walls of plants, which accounts for ~70% of the pectic gel in the cell walls of plants, however, a small amount of the 3_1 helical structure occurs naturally (Jarvis and Apperley, 1995). HG, RG-I and RG-II polysaccharides fail to resolve independently by size exclusion chromatography prior to fragmentation by EPGase digestion (Keegstra et al., 1973; York et al., 1996). In soybean soluble polysaccharides, α -(1,4)-linked (GalpA)₄₋₁₀ oligosaccharides were found flanked by RG-I fragments providing evidence that HG and RG-I are directly and covalently connected through backbone residues (Nakamura, 2002). It has also been suggested that HG polysaccharides are covalently attached to xyloglucan hemicellulosic wall polysaccharides. Fragments of XG were readily solubilized from walls by treatment with EPG, but not before (Talmadge et al., 1973). An XG diagnostic fragment, isoprimerverose, was released from the acidic or pectic fractions of driselase digested and alkali-extracted walls of Arabidopsis, rose, sycamore, tomato, spinach, maize and barley (Popper and Fry, 2005) suggesting a crosslink.

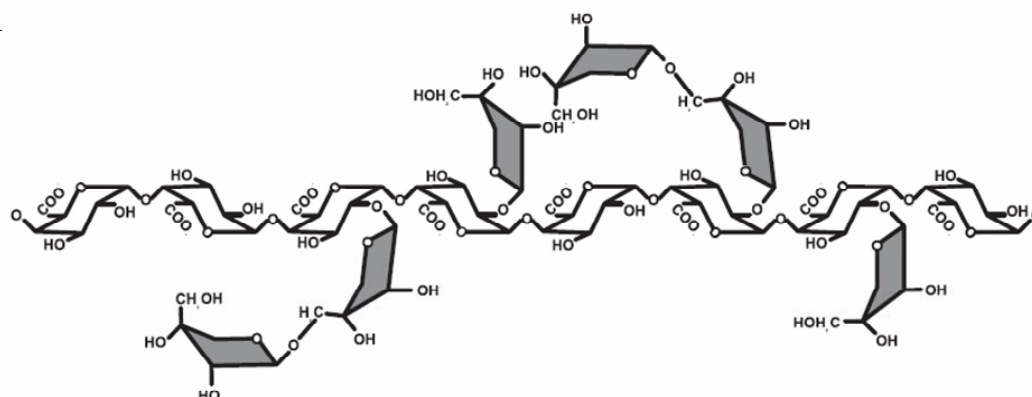
The length of HG polysaccharides may vary in a developmental or functional manner. The *in vitro* synthesis of HG onto endogenous acceptors has yielded very large polymers with a degree of polymerization (DP) of up to 150 residues (Doong and Mohnen, 1998), suggesting that HG regions of up to 150 residues may be present *in muro*. The endogenous acceptors in this study were not characterized, thus the results reported may represent longer chains than might be found *in muro*. In agreement with this finding of long HG chains, however, were long chains of HG in pectin derived from apple, commercial beet pectin and commercial citrus pectin that were 21,000, 19,000 and 24000 da in size, which translates to approximately 72 to 100 GalA residues in length (Thibault et al., 1993). Comparably, HG domains isolated from dried citrus peel were between 17,000 and 20,600 da in size (Yapo et al., 2007) suggesting that long chain HGs are

found in the walls of these species. Reliable sources of EPG of high purity have made digestion of walls with EPG the method of choice to solubilize cell walls for analysis. However, this method prevents further characterization of HG domain chain length. The HG intra-RG-I linkers identified in soybean were found to be 4 to 10 residues in length (Nakamura, 2002). These reinforced the theory that HG and RG-I are covalently crosslinked by backbone residues. The DP of the fragments were, however, much shorter than HG previously characterized in citrus and beet walls. Because the fragments were isolated from soybean cotyledons, it is unknown if the structure extends to other species or other tissues. Further characterization of the structure of HG polysaccharide domains and linker regions is essential to understand the function of HG, and thus, our understanding of the role of HG in plant growth and development.

Substituted Galacturonans, Apiogalacturonan and Xylogalacturonan

The D-apiose substituted apiogalacturonan (AGA) is a pectic polysaccharide found in the walls of aquatic plants such as the duckweeds (*Lemnaceae*) (Hart and Kindel, 1970) and the marine seagrasses (*Zosteraceae*) (Ovodov et al., 1971). The apiose residues are found 2-linked, 3-linked and 2- and 3-linked to single GalA residues of the galacturonan backbone (Figure 1.4A). The characterization of AGA obtained by the mild extraction of *Lemna* walls showed that the substitution of the galacturonan backbone can also occur as apibiose (Apif-1,3'-Apif-1-), a disaccharide of apiose (ONeill and York, 2003). The level of apiosylation of HG, assessed by the GalA to Api ratio, may range from 4 to 1 in *Zosteraceae*, to 4 to 5 in *Lemnaceae* (Hart and Kindel, 1970; Ovodov et al., 1971). The content of AGA in plant walls fluctuates widely from 0.2% to 20% of non-cellulosic polysaccharides in the dormant buds, or turions, and the green fronds of giant duckweed (Longland et al., 1989). The abundance of AGA suggests that these

A



B

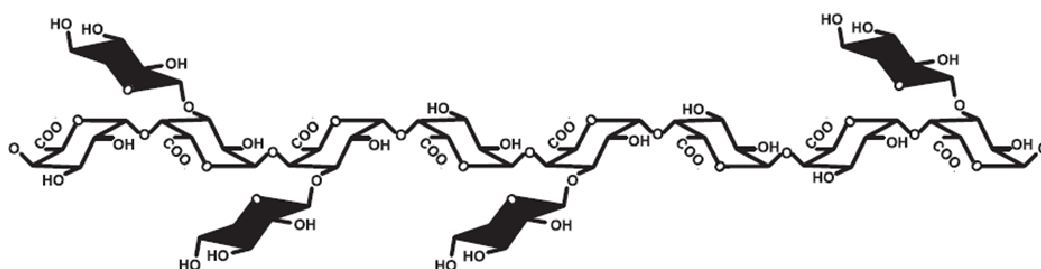


Figure 1.4. The substituted galacturonans xylogalacturonan and apiogalacturonan.

Apiogalacturonan (A) is characterized by apiose and 3-linked apibiose at the C-2 of galacturonan backbone. Xylogalacturonan (B) is characterized by xylose and 2-linked xylobiose (not shown) at the C-3 of galacturonan backbone. Adapted from Rose et al., 2003.

structures are specifically important in the structural framework of the walls of these water-born plants.

Xylogalacturonan (XGA) is HG substituted by xylose residues at the C-3 of GalA backbone residues (Schols et al., 1990; Nakamura et al., 2002; O'Neill and York, 2003) (Figure 1.4B). XGA fractions in pectic extracts of a marine seagrass (*Zosteraceae*) were characterized and shown to have HG substituted with a xylose disaccharide (Xylp-(1,2)-Xylp-(1,3)-GalpA) (Ovodov et al., 1971), while XGA extracted from pea hulls (*Pisum sativum*) (Le Goff et al., 2001) was substituted mostly by single xylose residues and only occasionally by an additional 2-linked xylose to form the disaccharide. Results similar to those found in pea have been found in apple (Schols et al., 1995) and *Arabidopsis thaliana* (Zandleven et al., 2007), albeit with variation in the extent of xylosylation of the HG chains. XGA isolated from soybean soluble polysaccharides (*Glycine max*) (Nakamura et al., 2002) produced a fragment of α -(1,4)-linked GalA residues substituted at the C-3 with chains of approximately one to seven β -(1,4)-linked xylose residues that might also be branched at the C-2. Because the fragment isolated from soy has not been observed previously in plant cell walls, it is likely to make up a relatively minor component of the wall or be a structure specific to soy walls. The most abundant XGA polysaccharide structure, which has been observed in multiple species, is the galacturonan backbone substituted at the O-3 by Xyl and by Xyl branched at the O-2 by another Xyl residue.

Substituted Galacturonan, Rhamnogalacturonan II

Rhamnogalacturonan II (RG-II) is a substituted galacturonan that is a ubiquitous, component of plant walls making up ~4% of suspension-cultured sycamore walls

(Darvill et al., 1987) and ~8% of *Arabidopsis* leaf walls (Zablackis et al., 1995). An RG-II molecule is recognized as a stretch of HG backbone approximately seven to nine residues long with four well defined side chains, designated sidechain A through D (Figure 1.5). The structure of RG-II is highly complex with 12 different types of glycosyl residues, including the rare sugar species 2-*O*-methyl xylose, 2-*O*-methyl fucose (Darvill et al., 1987), aceric acid (Spellman et al., 1983), 2-keto-3-deoxy-D-lyxo heptulosaric acid (Dha) (Stevenson et al., 1988) and 2-keto-3-deoxy-D-manno octulosonic acid (Kdo) (York et al., 1985a). Despite its complexity, the conservation of RG-II structure across higher and lower land plants (reviewed in (O'Neill et al., 2004), suggests that RG-II must play an important role in wall function.

RG-II molecules are known to self associate, forming RG-II dimers via a boron diester bond that was first definitively demonstrated by NMR of *in vitro* RG-II-borate complexes derived from sugar beet (*Beta vulgaris* s.) (Ishii and Matsunaga, 1996). Early studies showed the boron was an essential microelement in plant growth (Whittington, 1959). The pectic component of walls harbors greater than 60% of total boron content in squash leaves (*Curcubita Pepo* L.) and suspension-cultured tobacco cell walls (*Nicotiana tabacum*) (Hu and Brown, 1994). Studies of radish root (*Raphanus sativus*) showed that a single pectic polysaccharide was associated with boron (Kobayashi et al., 1996; Matoh and Kobayashi, 1998). Ultimately, the borate ion was found to bind the apiofuranosyl residues of RG-II sidechain A, but not that of sidechain B (Ishii and Matsunaga, 1996) (Figure 1.6). The 3-dimensional conformation of RG-II sidechain A was found to be mostly stationary in solution, while sidechain B was dynamic. The stationary behavior of sidechain A may provide a binding surface for borate and also contribute to the mechanical stability of the RG-II dimer (Mazeau and Perez, 1998; Rodriguez-Carvajal et al., 2003).

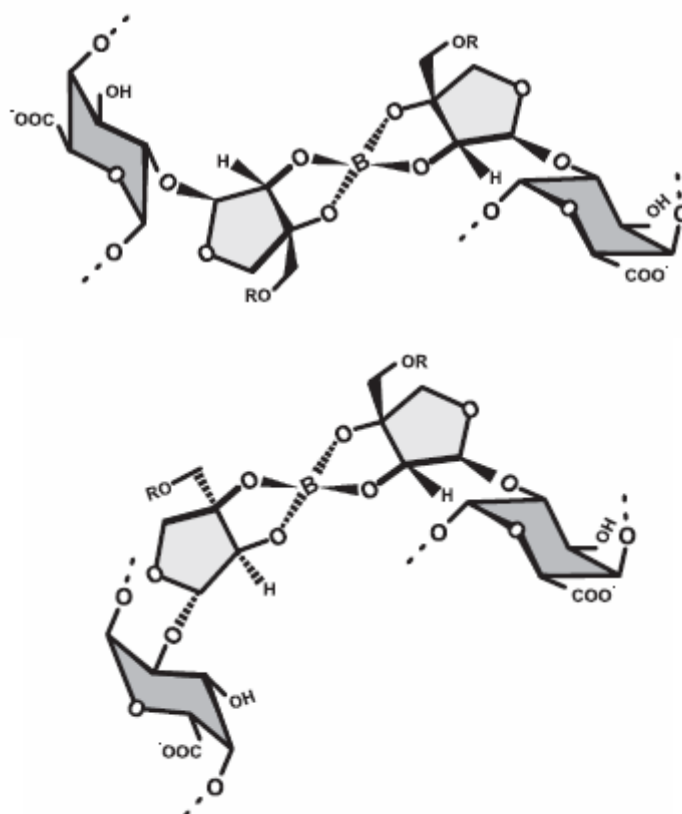


Figure 1.6. The apiosyl residues of rhamnogalacturonan-II sidechain A coordinate boron atoms in the wall.

The structure of the two possible isomers of the reversible RG-II-boron diester found in the walls of plants is shown. Adapted with permission from O'Neill and York (2003).

Rhamnogalacturonan I

The backbone of Rhamnogalacturonan I (RG-I) has repeating units of $[\rightarrow 2)\text{-}\alpha\text{-L-Rhap-(1-4)\text{-}\alpha\text{-D-GalpA-(1}\rightarrow]_n$ as characterized from suspension-cultured sycamore walls (*Acer pseudoplatanus*) (Lau et al., 1985) and soybean soluble polysaccharides (Nakamura, 2002) (Figure 1.7). Large relative amounts of RG-I are found in the mucilage extruded from the seeds of myxospermous species (Western et al., 2000) and in the primary wall and middle lamella of potato (*Solanum tuberosum*) (Bush et al., 2001; Oomen et al., 2002; Skjot et al., 2002). Suspension-cultured sycamore walls have ~7% RG-I (McNeil et al., 1980), while potato tuber walls (*Solanum tuberosum*) have ~36% dry weight RG-I polysaccharides (Obro et al., 2004). The extended conformation of the RG-I backbone is predicted to take that of a three-fold helix (3_1) (Engelsen et al., 1996). The RG-I isolated from seed mucilage is unbranched (Western et al., 2004), while RG-I isolated from walls is branched at approximately half of the rhamnose residues at the C-4 position by arabinan, galactan or arabinogalactan side chains (Lau et al., 1985). The abundance of RG-I sidechains is developmentally and differentially regulated (Bush et al., 2001; Willats et al., 2001a; Bochicchio et al., 2006).

The $\alpha\text{-(1,5)}$ -linked-L-arabinofuranose and $\beta\text{-(1,4)}$ -linked-D-galactopyranose chains are 4-linked to approximately half of the rhamnose residues of the RG-I backbone (Carpita and Gibeaut, 1993). The RG-I arabinan and galactan side chains from the walls of apple (Schols et al., 1994), sugar beet (Sakamoto and Sakai, 1995), soy (Huisman et al., 2001; Nakamura, 2002), persimmon (Duan et al., 2003) and potato (Bush et al., 2001; Skjot et al., 2002; Obro et al., 2004) showed a great deal of heterogeneity in structure from source to source. The RG-I arabinan found in sugar beet and soy was $\alpha\text{-(1,5)}$ -linked with terminal 3-linked arabinose residues and occasionally with terminal galactose residues (Sakamoto and Sakai, 1995; Huisman et al., 2001).

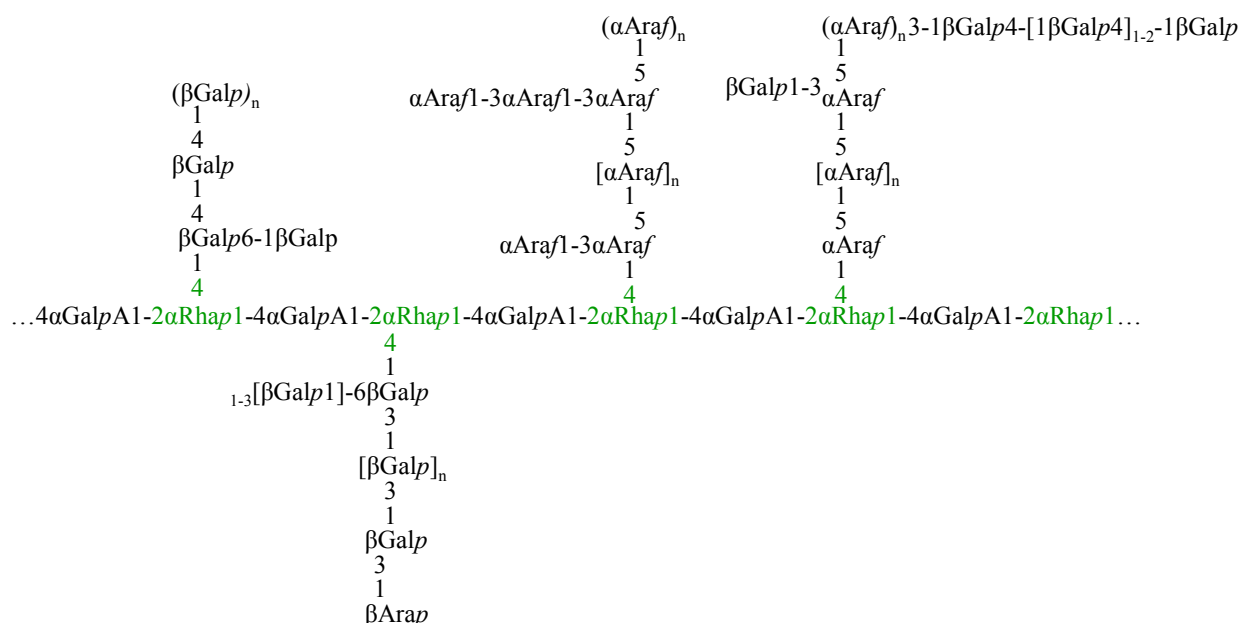


Figure 1.7. The structure of RG-I backbone and representative sidechains.

The structure of RG-I with sidechains of α -(1,5)-arabinan, β -(1,4)-galactan and Type-I and Type-II arabinogalactan. Arabinan chains may have long chains 3-linked branches of mono- or dimeric arabinan. In some instances mono-, di- or oligomeric branches of β -(1,4)-linked Gal occur at the 3-linked branch points of arabinan. Type-II arabinogalactan may have branches of 6-linked or 3,6-linked galactose residues. Adapted from O'Neill et al., 2003.

However, galactan oligosaccharides have been found linked to RG-I arabinan, for example four or more β -(1,4)-linked galactose residues were observed in potato (Obro et al., 2004) and up to five in soy (Nakamura, 2002).

Three types of galactan polysaccharides have been isolated in association with RG-I polysaccharides: galactan, and Type-I and Type-II arabinogalactan (AG). RG-I galactan from soybean reached 43 to 47 β -(1,4)-D-galactose residues in length (Nakamura, 2002). Type-I AG is the most abundant RG-I-associated AG. Type-I AG is characterized by single interspersed α -(1,5)-linked arabinofuranose residues in a β -(1,4)-linked galactan chain that has branches of one or more arabinofuranose residues or single terminal arabinopyranose residues (Huisman et al., 2001). The Type-II AG found in the wall is largely attributed to the post translational modification of arabinogalactan proteins (AGPs), but some Type-II AG in wall preparations is associated with pectic polysaccharides. Type-II AG is characterized by a backbone of β -(1,3)-D-galactan with branch points of 6-linked β -D-galactose of one, two or three residues in length, and are sometimes capped by single β -Arap residues (Luonteri et al., 2003).

The RG-I backbone may also be modified by single GlcpA and 4-O-methyl-GlcpA residues that have been identified in association with RG-I galactan (An et al., 1994). Wall fragments isolated from acid-hydrolyzed suspension-cultured sycamore RG-I showed single GlcpA residues (1,6)-linked and (1,4)-linked to galactose residues, suggesting that these residues decorate RG-I galactan chains. Thus far, GlcpA residues have not been found linked directly to the RG-I backbone (An et al., 1994) and not linked to RG-I arabinan (Lerouge et al., 1993).

The polysaccharides known as pectin (HG, AGA, XGA, RG-I and RG-II), are a collection of complex molecules produced solely by plant species. The complexity of these

molecules and their conservation to a greater or lesser degree throughout the plant kingdom, infers specific and important biological function in the plant cell wall.

A STRUCTURAL MODEL OF THE PRIMARY CELL WALL

Current models of the primary plant cell wall are based on the hydrogen, covalent and ionic bonding between two or more structural components of the wall. To determine how the many described components of the wall come together as a complete functional wall *in vivo* is an objective of cell wall science: i.e. how the matrix polysaccharides of the primary wall function within the frame-work of the cellulose-xyloglucan structural network. The structure and role of cellulose and hemicellulose in primary walls and the integration of known cellulose, hemicellulose and pectin structure into a practical 3-dimensional model of the primary wall are discussed.

Cellulose in primary walls

Cellulose is the foremost load bearing network of the primary and secondary wall. The % dry weight of cellulose in a dicot such as *Arabidopsis* ranges from 15% of leaves (Zabackis et al., 1995) to 33% of stems (Zhong et al., 2005). The walls of monocot grass species have approximately 6 to 10% cellulose in leaves and 20 to 40% in stems (Gibeaut et al., 2005; Smole et al., 2005). Cellulose is a polymer of β -(1,4)-D-glucopyranose residues that associate with other cellulose chains by hydrogen bonding and Van der Waals forces (Nishiyama et al., 2002).

The cellulose chains of plant walls are synthesized at the plasma membrane by cellulose synthase complexes comprised of multiple cellulose synthase (CesA) subunits that form a rosette structure. The rosette refers to the appearance of the CesA complex when visualized by scanning

electron microscopy. The rosettes synthesize growing cellulose chains that form a single strand of 6 to 10 cellulose chains (Ha et al., 1998; Delmer, 1999), which are referred to as 2 nm fibers. Six of the 2 nm fibers then may associate to form microfibrils of approximately 36 glucan chains (Herth, 1983). The microfibrils average 30 nm in width, a size that may be visualized by spectroscopic methods.

The cellulose chains of the primary wall were found to be of low molecular weight compared to cellulose chains of the secondary wall (Brett, 2000; Brown, 2004). Cellulose chains may align in a parallel (Type I) or antiparallel (Type II) fashion (Sugiyama et al., 1991). Only the Type I conformation is known to naturally occur in plants; however concentrated alkaline treatments may cause Type II cellulose to form during harsh extraction procedures. The cellulose chains may form the Type I α or Type I β conformation depending on the extent of staggering of the chains in relation to each other. Type-I α and Type-I β are recognized by the triclinic or monoclinic unit cell, respectively, of the crystalline cellulose (Sugiyama et al., 1991). The inter-conversion of Type I α and Type I β allomers may be induced by mild alkali (Sugiyama et al., 1991) or by the bending of the cellulose chains (Jarvis, 2000), not unlike the reorienting that cellulose microfibrils undergo to run parallel with the surface of the plasma membrane after synthesis. It is also thought that the interaction of cellulose microfibrils with hemicelluloses may effect the ratio of Type I α to Type I β cellulose (Jarvis, 2000). For example, the developing tracheid of the Japanese hinoki cypress (*Chamaecyparis obtusa*) formed greater amounts of the metastable I α in the primary wall, with greater amounts of the stable I β in the secondary wall (Kataoka and Kondo, 1998). Primary wall cellulose microfibrils are highly crystalline and oriented parallel to the direction of elongation, contrary to that found in secondary walls (Kataoka and Kondo, 1998). The differences in the size, conformation, crystalline form,

crystallinity and orientation of primary wall cellulose microfibrils are attributed to the stresses that cellulose microfibrils undergo during rapid cell expansion in the pectinaceous primary wall environment (Kataoka and Kondo, 1998; Brett, 2000).

Hemicellulose

The hemicelluloses are often described as those wall polymers that (1) are solubilized from the wall by alkaline solvents and (2) are β -(1,4)-linked pyranosyl residues that have the O-4 in the equatorial position (O'Neill and York, 2003). These are characteristics that result in a cellulose-like conformation and cause a tendency to hydrogen-bond to cellulose chains. Xylans, mannans and xyloglucan fit this technical definition, but arabinogalactan may also be considered a hemicellulose. The hemicelluloses are more abundant in secondary walls than in the primary walls of both dicots and monocot species. Monocot species, however, have significantly more hemicellulose and less pectin than dicots, and also have mixed linkage glucans that make up a significant proportion of monocot hemicellulose polysaccharides (Aspinall et al., 1967).

Xylan polysaccharides are comprised of linear chains of β -(1,4)-D-xylopyranosyl residues and may be found as arabinoxylan (AX), glucuroarabinoxylan (GAX), glucuronoxylan (GX) or the unsubstituted homoxylan (Figure 1.8). Xylans also are decorated by acetyl groups at the O-2 or O-3 position (Chesson et al., 1983). The arabinose residues of AX are primarily terminal residues linked to the 2-position of the xylose backbone in dicots and non-graminaceous species (Darvill et al., 1980; Carpita, 1996). Alternate forms of AX have been identified in rye wholemeal having arabinose O-2 and O-3 doubly substituted xyloses, substituted arabinoses, terminal xylose and terminal galactose substitutions (Vinkx et al., 1995). These structures have not, thus far, been confirmed in the primary walls of dicots, suggesting that they are specialized

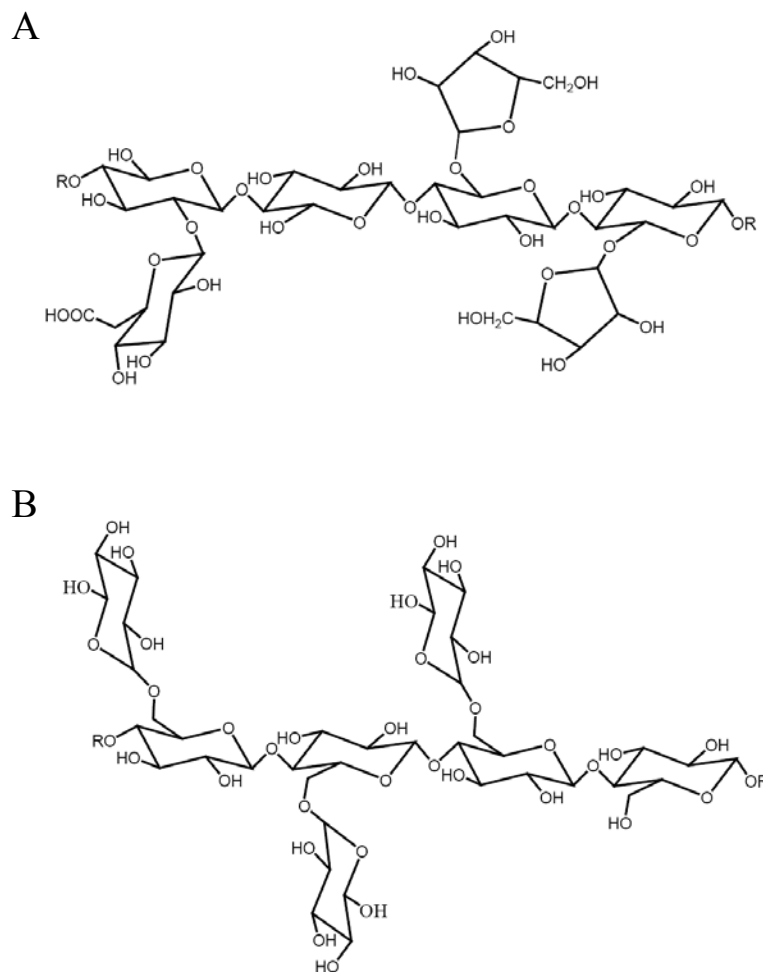


Figure 1.8. The basic structure of xylan and xyloglucan.

Glucuronoarabinoxylan (A) is β -(1,4)-D-xylan substituted by glucuronic acid and arabinose at the C-2 and C-3. Xyloglucan (B) is β -(1,4)-D-glucan, like cellulose, but is substituted at the C-6 by xylose that may be further modified, in a regular pattern of 3-substituted glucose residues followed by a single free glucose. Adapted from ONeill (2003).

features of cereal walls. AX and GAX are the most abundant xylans in the primary walls of dicots making up ~5% of primary walls of sycamore suspension cultured cells, while GAX constitutes ~25% of monocot species (Darvill et al., 1980). Xylans of higher plants may also be substituted by 4-*O*-methylglucuronosyl residues (Ebringerova and Heinze, 1999). Incorporation of 4-*O*-methylglucuronic acid to form GX occurs in dicot secondary walls, but is not generally found in the walls monocots or the primary walls of dicots (Ebringerova and Heinze, 1999).

The mannans include the galactomannans (GM) and the galactoglucomannans (GGM) that are structurally important components of the cell wall as well as an important source of storage polysaccharide. Mannans have a similar 3-dimensional structure to cellulose. Mannan found in some sea weeds of the *Codiaceae* and the *Dasycladaceae* families are known to take the mannan I form, analogous to cellulose I, based on crystallographic methods (Frei and Preston, 1968). In these few unusual species, the mannans form a fibril-like structure 100 Å wide that takes the place of cellulosic fibrils and is the primary structural component in the walls of these sea alga species (Frei and Preston, 1968). The specific functions of mannans in the plant cell wall are unclear, but they appear to play a role in the growth of pollen tubes (Goubet et al., 2003) and roots (Zhu et al., 2003). In xylogenic cultures of zinnia (*Zinnia elegans*) supplemented with GGM oligosaccharides, GGM induced significantly higher rates of differentiation of cells to metaxylem tracheary elements (Benova-Kakosova et al., 2006). It has been shown that the mannan synthase genes in *Arabidopsis* (*Arabidopsis thaliana*), poplar (*Populus trichocarpa*) and moss (*Physcomitrella patens*) CslA gene families are widely and differentially expressed (Liepman et al., 2007). While mannan synthase gene expression correlated well with secondary wall synthesis, which is expected for a hemicellulose, mannan synthase genes are also expressed

during primary wall synthesis, potentially indicating functional importance of mannans in the structure of the primary wall (Liepman et al., 2007).

Xyloglucan (XG) is the most abundant hemicellulose in dicot primary walls making up 21% of angiosperm (*Acer pseudoplatanus*) (Bauer et al., 1973) and 10% of gymnosperm suspension-cultured cell walls (*Pseudotsuga menziesii*) (Thomas et al., 1987). The walls of the graminaceous monocots, or grasses, are more than 50% hemicellulosic polysaccharides but only 2% to 5% of this is xyloglucan (Carpita, 1989; Popper and Fry, 2005). Like cellulose, XG has a core backbone structure of β -(1,4)-D-glucopyranose residues; however XG is heavily decorated with side chains of α -D-xylose residues linked to the C-6 of backbone glucose residues (Figure 1.8). In addition, the structural modification of XG by *O*-acetylation of backbone Glc residues and sidechain Gal and Ara residues has been observed (Jia et al., 2005). XG may be hydrolyzed by a XG-specific endo-glucanase to yield characteristic oligosaccharides and facilitating structural characterization (Pauly et al., 1999b). The XG structure most frequently found in dicotyledonous flowering plants is that of a repeating heptamer of four Glc residues that have substitutions of α -D-xylopyranosyl residues at three consecutive Glc backbone residues followed by a single unsubstituted Glc backbone residue. The heptamer was first isolated from suspension-cultured sycamore (*Acer psuedoplatanus*) and is considered diagnostic for the presence of XG polysaccharides in dicot species (Bauer et al., 1973; Wilder and Albersheim, 1973). Repeating heptamer blocks have not been found in graminaceous monocots but instead release isoprimeverose, a disaccharide (Xylp- α -(1,6)-Glc_p), from xyloglucan-specific endoglucanase digestion of grass walls. The smaller fragments of XG and the reduced xylosylation of the xyloglucan backbone (30% to 40%) in the grasses compared to dicots (60% to 75%) (Fry, 1989) suggests that the grasses do not synthesize the heptamer blocks. The XG of

graminaceous monocots, instead, consist of 1 or 2 adjacent α -(1,6)-linked xylosyl residues with ~3 intervening unsubstituted β -(1,4)-linked glucosyl backbone residues (Kato et al., 1981).

An assortment of xyloglucan sidechains have been characterized to date and are assigned a single-letter shorthand classification to facilitate the description and discussion of xyloglucan structures (Fry et al., 1993) (Table 1.1). The unsubstituted Glc residues are abbreviated G, while the terminal Xyl branch points are abbreviated X. The core heptamer structure, abbreviated XXXG, may vary in the number and arrangement of Xyl branch points in a species specific manner (Fry et al., 1993). Solanaceous species, such as tomato and tobacco, were found to have arrangements of XXGG (York et al., 1996) and rarely XGGG or GXGG. In addition, the existence of XXG and GGGG blocks in XG polysaccharides have been postulated in the literature (Hayashi et al., 1987). It seems likely that the XXG found in these samples is an artifact of an XXGG structure (York et al., 1996; Vincken et al., 1997). The core structure XXGGG was isolated from species of the *Lamiaceae* family (Hoffman et al., 2005). The *O*-acetylation of the third and fourth Glc residues is believed to have facilitated the isolation of the complete fragment without further hydrolysis of reducing end Glcp residues by endo-glucanase digestion. The five Glc core is atypical of that found in dicot species and it is hypothesized that the XG structures found in this family represent an evolutionary divergence from species having the XXXG core (Bremer et al., 2004). The X substitutions that decorate the β -(1,4)-glucan backbone of XG in dicots are frequently further elongated at the Xyl C-2 by a terminal β -D-Galp (abbreviated L), a disaccharide of α -L-Fucp-(1,2)- β -D-Galp (abbreviated F) or by an increasingly wide variety of less common structural variants (see Table 1.1). The fucosylation of XG is differentially distributed in the walls of dicot plants in a species-specific and tissue-specific manner (Puhlmann et al., 1994; Freshour et al., 2003). For example, F chains do not occur in the

Table 1.1. The structure and classification of xyloglucan sidechains.

Xyloglucan sidechains have variable structure depending on the source of the walls from which the xyloglucan is isolated. ^(a) The species from which the xyloglucan was isolated. ^(b) A single letter abbreviation is used to designate specific XG structures (Fry et al., 1993). ^(c) The XG backbone Glcp residue.

Source ^a	Structure of xyloglucan sidechain	Designation ^b	Reference
<i>Acer Pseudoplatanus</i>	Glcp ^c	G	(Bauer et al., 1973)
<i>Acer Pseudoplatanus</i>	T- α -D-Xylp -1, 6-Glcp ^c	X	(Bauer et al., 1973)
<i>Acer Pseudoplatanus</i>	T- β -D-Galp -1, 2- α -D-Xylp -1, 6-Glcp ^c	L	(Bauer et al., 1973)
<i>Acer Pseudoplatanus</i>	T- α -D-Fucp - β -D-Galp -1, 2- α -D-Xylp -1, 6-Glcp ^c	F	(Bauer et al., 1973)
<i>Lycopersicon esculentum</i>	T- α -L-Araf- α -D-Xylp -1, 6-Glcp ^c	S	(York et al., 1995)
<i>Lycopersicon esculentum</i>	T- β -D-Araf- α -D-Araf- α -1, 2-D-Xylp -1, 6-Glcp ^c	T	(York et al., 1995)
<i>Argania spinosa</i>	T- β -D-Xylp -1, 2- α -D-Xylp -1, 6-Glcp ^c	U	(Ray et al., 2004)
<i>Simmondsia chinensis</i>	T- α -L-Galp -1, 2- β -D-Galp -1, 2- α -D-Xylp -1, 6-Glcp ^c	J	(Hauntus et al., 1997)
<i>Acer Pseudoplatanus</i>	T- α -L-Araf-1, 2- & T- α -D-Xylp -1, 6-Glcp ^c	A	(York et al., 1995b)
<i>Acer Pseudoplatanus</i>	T- β -D-Xylp -1, 2- & T- α -D-Xylp -1, 6-Glcp ^c	B	(York et al., 1995b)
<i>Acer Pseudoplatanus</i>	T- α -Araf-1, 3- β -D-Xylp -1, 2-(T- α -D-Xylp -1, 6-)-Glcp ^c	C	(York et al., 1995b)

Convovulaceae, *Solanaceae* or *Oleaceae* families, however, it is frequently found in the walls of other dicots. Unique XG sidechains found in tomato, tobacco and olive fruit were α -L-Araf-(1,2)- α -D-(1,2)-Xylp (abbreviated S) and β -Araf-(1,3)- α -L-Araf-(1,2)- α -D-Xylp (abbreviated T) and it is believed that the resulting XXSG and XLSG may develop in species that have a XXGG primary core structure (York et al., 1996) (Vierhuis et al., 2001) found in these species. Interestingly, further xylosylation of the XG glucan backbone in bilberry was shown by the β -D-Xylp-(1,2)- α -D-Xylp substituent (abbreviated at U) (Helz et al., 2007). Only one other related species, the argan tree (*Argania spinosa*) of the order Ericales, has been identified that has the U chain (Ray et al., 2004).

Despite the structural variability found among different species, the function of XGs in plant growth and development are hypothesized to be conserved among all species of flowering plants. XG is thought to primarily function in a structural capacity in the cellulose-xyloglucan network of the plant cell wall. XG, however, also has a role in supplying energy stores in the seeds of plants (Carpita and Gibeaut, 1993; Levy et al., 1997) and as a signal molecule (York et al., 1985a; Fry, 1989; McDougall and Fry, 1990).

The cellulose-xyloglucan network in primary walls

The contribution of the cellulose-xyloglucan network to the structural integrity of the plant cell wall has been studied for many years and the subtleties of the interaction of XG, in all of its forms, with cellulose in the primary wall continues to unravel. The strong binding of XG to cellulose has been observed (Aspinall et al., 1969). The strength of the interaction derives from the strong non-covalent and additive interaction of hydrogen bonds between XG molecules and cellulose microfibrils (Keegstra et al., 1973; Valent and Albersheim, 1974).

The release of XG from cellulose by concentrated alkali indicates that the binding of XG to cellulose is pH dependent and that it is an extremely strong non-covalent interaction, however the potential contribution of other base-sensitive linkages cannot be discounted (Hayashi et al., 1987). In cultures of *Acetobacter xylinum*, purified XG coated and disrupted the native cellulose of these organisms. This data suggests that the XG of plant walls is likely to interact with cellulose microfibrils as they are synthesized into the primary wall matrix, causing microfibrils of smaller diameter than those found in secondary walls (Hayashi et al., 1987). The binding of XG to cellulose is also known to weaken cellulose networks (Whitney et al., 2006), but increase the expansibility of such networks (Chanliaud et al., 2002); mechanical properties suited to the expansion and stresses characteristic of conditions during primary wall synthesis.

The XG is bound to cellulose microfibrils in three distinct domains; (1) XG that is endoglucanase accessible, (2) XG that is solubilized by concentrated alkali and (3) XG that is neither enzyme accessible nor alkali soluble (Pauly et al., 1999a). The enzyme accessible domain constitutes the XG that bridges cellulose microfibrils or is unbound by cellulose, and thus is enzyme accessible. The strong alkali soluble XG domain is that which is bound to the surface of cellulose microfibrils. The remaining unsolubilized, enzyme inaccessible domain is XG that is partially buried by cellulose microfibrils. Molecules of XG have been microscopically visualized to coat and tether the cellulose microfibrils (McCann et al., 1990; Whitney et al., 1995) and by virtue of the repeating unit structure of XG polysaccharides, bring order to the cellulose network (Levy et al., 1991). It has also been observed that XG from different sources (i.e. with distinct populations of the different side chains) bind differently to the cellulose microfibrils (Whitney et al., 2006). The L and F sidechains are abundant XG constituents in dicotyledons. XG lacking the L-chain were shown to self-associate, competing

with cellulose for binding, while fucosylated XGs (F-chain) promoted higher crosslinking with cellulose microfibrils than galactosylated XGs. The data demonstrate that the sidechains of XG modulate the binding of XG to cellulose and thus are important in regulating the mechanical properties of the cellulose-XG network. In addition, the *Arabidopsis* xyloglucan xylosyltransferase (XXT) double mutant (*xxt1xxt2*) is devoid of XG and was found to have swelling of root hairs and reduced wall stiffness; root swelling was also observed in the XG XXT5 mutant (*xxt5*). These results suggest that XG plays a role in root hair development and wall integrity.

The primary cell wall pectic network

The covalent crosslinking of the pectic polysaccharides HG, RG-I and RG-II has been demonstrated repeatedly in the literature by the EPGase-dependent release of pectic polysaccharides from the wall (O'Neill et al., 1996). The available data suggest that the RG-I and RG-II backbones are continuous with the HG backbone, not that of RG-I sidechains, as suggested by Vincken and colleagues (2003). If the backbones of the pectins are continuous, the pectic network may be thought of as a macromolecular structure having specific domains of HG, RG-I and RG-II, however the arrangement of these domains *in vivo* is not known (Figure 1.9). The linkage of HG, RG-I and RG-II through backbone glycosidic linkages is just one way in which the pectins are crosslinked. The pectic network is based on multiple levels of crosslinking that includes but is not limited to, backbone glycosidic linkages, calcium crosslinking, borate ester crosslinking and covalent linkages to phenolic compounds.

The HG domains of pectin may self-associate depending on the degree of methylesterification and thus the affinity of HG for calcium ions. RG-I has a unique backbone

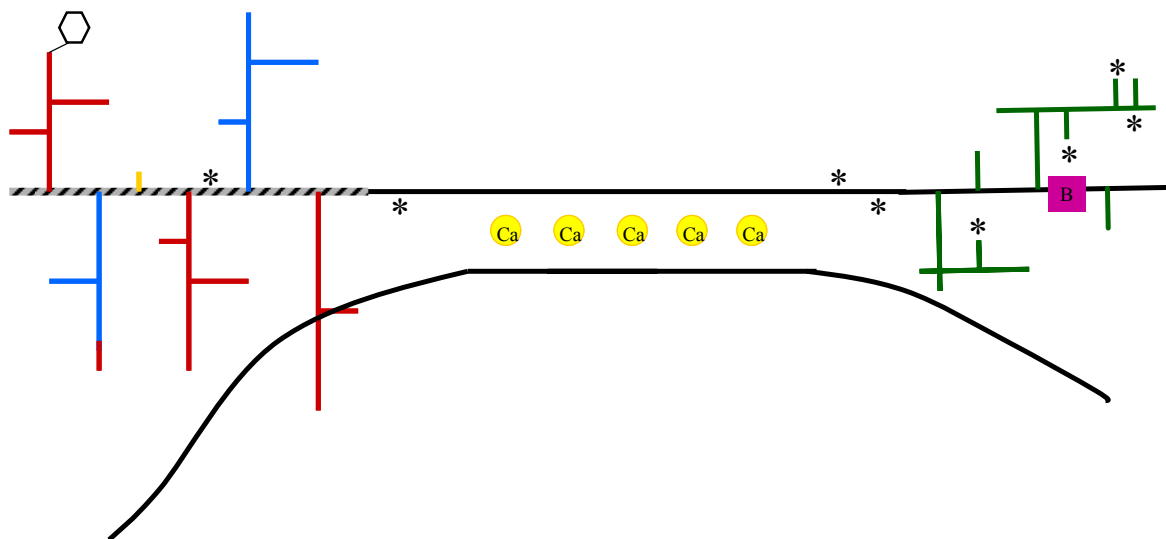


Figure 1.9. The pectic network of HG, RG-I and RG-II. The figure depicts one possible configuration of the pectin-pectin network.

Experimental evidence suggests that the pectic polysaccharides are covalently crosslinked through the backbones of homogalacturonan (HG), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II). The polysaccharide structures are HG (solid black bars), RG-I backbone (slashed black bars), arabinan (red bars), galactan (blue bars), glucuronic acid (orange bars) and RG-II (green bars). Sites of methylation are shown as asterisks (*). Phenolic compounds are shown as a black hexagon.

of alternating 2-linked Rha_p and 4-linked Gal_pA residues. Some rhamnose residues are branched by arabinan, galactan and/or AG sidechains (McNeil et al., 1980) that may be crosslinked to other wall components such as xylans, xyloglucans, proteins and lignins. RG-II domains form crosslinks to other RG-II molecules via a borate diester linkage, to form a dimer that contributes to wall strength and has an effect on the pore size and flexibility of the pectic network (Ishii and Matsunaga, 1996; Fleischer et al., 1999). Greater than 95% of RG-II molecules participate in dimer complexes of RG-II (ONeill et al., 2004). The linkages that pectic polysaccharides make to other pectins, as well as to other wall molecules, combine to assemble the pectic network of the plant cell wall. The complexity of the pectic network structure and the modulation of the pectic crosslinks contribute strength, flexibility and functionality to the pectic network, and thus, to the primary cell wall.

Pectic polysaccharides may be crosslinked to hemicelluloses, phenolics and proteins

There is evidence in the literature to suggest that pectic polysaccharides may also be crosslinked to hemicelluloses, phenolic compounds and to wall proteins. The crosslinking of pectic polysaccharides to other wall components provides added structural and functional complexity to the wall.

The structure of an oligosaccharide isolated from the mild acid hydrolysates of soy sauce acidic polysaccharides demonstrated what could be a linkage between HG and xylan polysaccharides (Nakamura et al., 2002). In the acidic fraction, (α -(1,4)-Gal_pA)₃₋₄ residues were branched at the C-3 by (β -(1,4)-Xyl_p)₄₋₇. Similar fragments have previously been isolated from soybean cotyledon meal (Aspinall et al., 1967) and soy sauce acidic polysaccharide (Kikuchi and Sugimoto, 1976). Because the xylan-HG oligosaccharide fragment has only been identified in

soya products, and also may be a modified XG, but presents the possibility that xylan-HG crosslinks exist in the walls of some species.

Xyloglucan from the walls of sycamore suspension cells was found to co-chromatograph with a neutral sugar-rich acidic polysaccharides (Bauer et al., 1973). The sycamore xyloglucan/acidic polysaccharide fractions failed to be resolved into separate fractions by endopolygalacturonase treatment alone, but were released only when urea, base or endoglucanase was applied. Similar phenomena have been observed in kidney bean, rose, tomato, spinach, maize, barley and Arabidopsis wall fractions (Wilder and Albersheim, 1973; Popper and Fry, 2005). Further investigation of this crosslink has suggested that xyloglucan may be linked to the neutral sidechains of RG-I (Keegstra et al., 1973). The anionic component of the *rosa* that make up ~30% of total XG (Thompson and Fry, 2000), was not separable by HPLC, electrophoresis, 8 M urea, NaOH or protease treatment. The anionic component and XG were ultimately found to be separable by cellulase, arabinase, galactanase and endopolygalacturonase treatment, indicating that the anionic component is likely to be RG-I (Thompson and Fry, 2000). Walls from Arabidopsis cell cultures pulse-labeled with Arabinose were used to further investigate the stage at which XG becomes linked to the anionic pectic polysaccharide (Popper and Fry, 2007). Labeling of XGs occurred within 4 minutes, which is too short a time for vesicles to reach the apoplastic space indicating that the XG-pectin complex must form during the synthesis of the polysaccharides in the endomembrane system and not after incorporation into the wall. This work also showed that because the majority of anionic XGs were incorporated into the wall, the XG-pectin complex aids in retention of XG molecules preventing their loss to the medium (Popper and Fry, 2007). Based on these analyses, the mechanism of the XG-pectin

complex formation is likely to occur in a conserved manner among angiosperms (Popper and Fry, 2005).

Pectin molecules are crosslinked by phenolic compounds that make up >2% of the wall (ONeill and York, 2003). The most abundant phenolic species found in the walls of *Arabidopsis* are *p*coumaryl and feruloyl acids. Complexes of feruloylated-xyloglucan and a *para*-coumaroylated-arabinoxylan have been isolated from spinach leaf walls (Ishii, 1991). Feruloylated α -(1,5)-linked arabinan and β -(1,4)-linked galactan (Ishii and Tobita, 1993) were also isolated from spinach walls. The pulp of spruce and pine wood yielded lignin-carbohydrate β -(1,4)-D-galactan complexes. Interestingly, a small relative amount of arabinose was also found in conjunction with the lignin-carbohydrate complexes, not associated with arabinoxylan based on carbohydrate linkage analysis (Laine et al., 2004). As such, the data implicate crosslinking via ferulic and/or *p*coumaric esters to arabinogalactan, α -(1,5)-linked arabinan and β -(1,4)-linked galactan in these complexes, which is consistent with the structure of RG-I sidechains.

The structural proteins of the wall make up 2 to 10% of wall dry weight (Keegstra et al., 1973; ONeill and York, 2003) and are comprised of a variety of wall associated proteins. The wall residue of sycamore remaining after endopolygalacturonase, endoglucanase and alkali extraction produced a residue from which further pectic polysaccharides were only released by protease treatment. The release of pectins by protease treatment is likely due to a linkage with the structural protein of the cell wall (Keegstra et al., 1973). The arabinogalactan proteins (AGPs), proline rich proteins (PRPs), glycine rich proteins (GRPs) and wall associated kinases (WAKs) are wall associated proteins and are hypothesized to aid in the wall structural reinforcement and regulatory pathways. The arabinogalactan proteins (AGPs), RG-I and GAX of maize and proso millet ammonium oxalate extractable polysaccharides were not separable by

gradient anion exchange chromatography suggesting an interaction between these components (Carpita, 1989). AGPs are highly glycosylated, similar to animal proteoglycan glycoproteins, and are localized to the cell surface by a glycosphosphatidylinositol-lipid anchor at the plasma membrane (Showalter, 2001a). AGPs may be hydroxyproline rich (classical AGP), but some AGPs have domains enriched with asparagine, cysteine or histidine amino acids (non-classical AGP) (Showalter, 2001a). AGPs are typically glycosylated by arabinogalactan sidechains that are 3-linked-D-galactan branched at the C-6 by terminal galactose or arabinose residues (Type I arabinogalactan). *AtAGP30*, a non-classical AGP, functions in signal mediation of the phytohormone ABA (van Hengel and Roberts, 2003), which implicates AGPs in plant hormone signaling, although this has yet to be determined for each specific AGP. The PRPs are wall associated proteins that are secreted into the wall matrix where they ultimately become crosslinked, conferring strength to the wall (Battaglia et al., 2007). PRPs have repeating proline-rich motifs that are specific to one of three subclasses of PRPs. The expression and incorporation of PRPs into the wall can be induced by oxidative bursts that occur during responses to stress (Battaglia et al., 2007), suggesting that these proteins play a role in the defense responses of the plant. The expression of GRPs is also induced by stress (Keller et al., 1988). GRPs are hypothesized to interact with components of signaling pathways, and thus, may be regulators of wall structure. For example, *AtGRP-9* was shown, by yeast two-hybrid, to interact with a cinnamoyl alcohol dehydrogenase, *AtCAD-5*, that is involved in lignin biosynthesis (Chen et al., 2007). *AtGRP-3* was shown to bind to a wall associated kinase (WAK-1) and a kinase-associated protein phosphatase (KAPP) (Park et al., 2001). WAK-1 is a member of a family of membrane bound serine/threonine receptor kinases in which the kinase domain faces the interior of the cell and an epidermal growth factor (EGF)-like domain faces the cell

wall. WAK-1 reversibly binds to pectin-calcium complexes in a calcium dependent manner (Decreux and Messiaen, 2005). WAKs have been implicated in cell elongation, morphogenesis (Lally et al., 2001; Wagner and Kohorn, 2001) and defense against pathogens (He and Kohorn, 1998).

The ultrastructural model of the plant cell wall

Ultrastructural models of the plant cell wall have been formulated based on known cell wall structures in an attempt to integrate available knowledge into a functional structural wall model. The model presented in recent reviews of wall structure argues for two independent networks within the primary cell wall; the pectin-pectin and xyloglucan-cellulose network (O'Neill and York, 2003). In this model, the polysaccharides of the pectic-network, proteins and phenolic compounds are organized independently around the framework of the cellulose-xyloglucan network. This model utilizes the well-established models of the pectin-pectin network and XG-cellulose network. There is well established evidence to show that a covalent pectin-pectin network exists through the linear backbones of the pectic polysaccharides and that the XG polysaccharides have a strong affinity for cellulose and that XG functions in part to coat and tether cellulose microfibrils to form the XG-cellulose networks. Realistic wall models must integrate the pectic network, the cellulose xyloglucan network and the available knowledge of other wall structural components that have been characterized. A better wall model would demonstrate the highly crosslinked wall wherein pectin-pectin, pectin-XG, pectin-phenolics, pectin-protein and XG-cellulose networks provide a cohesive wall network (Keegstra et al., 1973). The available wall structural data demonstrate that pectins and hemicelluloses of the wall

are likely to attain a level of heterogeneous crosslinking not addressed by the current wall models.

STRUCTURAL CHARACTERIZATION OF PECTIC POLYSACCHARIDES

The current understanding of wall structure is dependent on the ability of researchers to resolve the fine structure of the polysaccharides that can be extracted from the plant cell wall. The polysaccharides of plant walls vary in abundance and composition according to the species, the tissue and the cells from which the walls are harvested. The source, from which walls are extracted, as well as the method of wall extraction and analysis, can influence the results obtained, and thus, the interpretation of wall carbohydrate structural elements. The structural characterization of oligosaccharide fragments from larger cell wall carbohydrate networks involves the initial extraction of the walls, or a subfraction of the wall, followed by glycosyl residue composition, glycosyl residue linkage, and structural sequence determination of the glycosyl residues, as well as identifying any structural modifications, such as *O*-acetylation or methylation. A brief overview of available wall sources and the most commonly used methods of extraction, fractionation and analysis are presented in order to give perspective to the structural models of the cell wall that have been proposed based on these most commonly used methods of wall polysaccharide structural analysis.

Wall isolation

The nature of the pectic polysaccharides recovered from wall samples is dependent on the species of plant, the specific tissue and the manner in which the walls were harvested. Plant cell walls are commonly isolated from ground plant tissues that are extracted to separate the cellular

contents from the wall residue (Talmadge et al., 1973). The properties of extracted wall polysaccharides are directly related to the starting plant material from which the walls were extracted, because the pectic components of plant cell walls are regulated both spatially and temporally in plant organs and in individual cell types. According to histochemical analyses using RG-I sidechain specific probes, the pectic polysaccharides, as well as their degree of esterification, are differentially expressed in different cell types of carrot and *Arabidopsis* root (Knox et al., 1990; Willats et al., 1999). By selectively harvesting specific tissue types, large amounts of homogeneous wall material may be obtained for wall analysis. For example, apple fruit cortex (Knee et al., 1975), potato tubers (Ishi, 1997) and the hypocotyls of young seedlings (Sterling et al., 2001) yield large amounts of relatively homogeneous walls. Laser capture micro-dissection (LCM) is a technique introduced in the 1990's that is useful in specifically isolating a specific tissue or cell type (Emmert-Buck et al., 1996). However, LCM is inappropriate where large quantities of material are required. LCM has great potential in application to the cell wall field to isolate the specialized walls of a specific cell types for analysis where techniques are available to identify structural elements in small quantities of wall material (Kerk et al., 2003).

Plant suspension-cultured cells are fast-growing, relatively undifferentiated cells that are also amenable to genetic manipulations and that yield abundant homogeneous wall material rich in pectic polysaccharides. Transformation by particle-bombardment/biolistics (Iida et al., 1991), mutagenesis by *Agrobacterium tumefaciens* (An, 1985) and induction of defense responses (Davis and Hahlbrock, 1987), xylogenesis and programmed cell death (Fukuda et al., 1998) have been developed in plant cell culture systems. The usefulness of cell-suspension cultures is evident in their long standing use for wall analyses in a variety of species including, but not

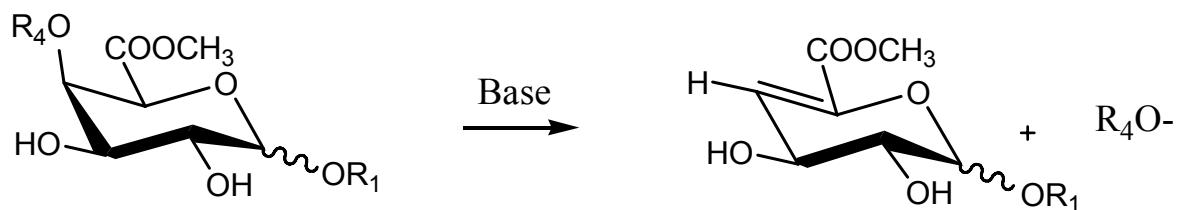
limited to sycamore maple (Keegstra et al., 1973), Douglas fir (Thomas et al., 1987), tobacco (Olson et al., 1969), tomato (Jia et al., 2005) and Arabidopsis (Leboeuf et al., 2005). A pectin-enriched fraction may be extracted from the walls of suspension cultured cells, or alternatively from the medium of suspension-cultures as secreted extracellular polysaccharides (SEPs). SEPs from sycamore cell-suspension cultures represented primarily pectins, with some xyloglucan and xylan components (Talmadge et al., 1973).

Multiple methods are available for the extraction of whole cell walls from plant tissues or suspension cultured cells. Phosphate buffers can be used to wash such cell walls; however a portion of the pectic polysaccharides may be solubilized and lost to the buffer. Phosphate buffer solubilized up to 34% of walls from Arabidopsis leaves (Zabackis et al., 1995) and therefore, is not recommended for extractions targeting the pectic polysaccharides. Pectic polysaccharides are not readily solubilized by organic solvents and thus, the walls extracted as alcohol insoluble residue (AIR) have minimal loss of wall polysaccharides. AIR is obtained when ground tissues are extracted with large quantities of 80% to 100% cold or boiling ethanol, over 30 minutes to 24 hours, to denature and remove cell contents (Capodicasa et al., 2004). Frequently, the walls are further extracted with chloroform:methanol (1:1) to remove the lipid component (Brown and DuPont, 1989). Finally, the walls are washed with 100% acetone to remove residual moisture and to aid in drying the hygroscopic cell walls. Walls extracted as AIR from Arabidopsis leaf, silique, stem and inflorescence constituted approximately 4 to 20% of the dry weight per fresh weight plant material, depending on the tissue from which the walls were derived (Caffall Ph.D. dissertation, Appendix B).

Extraction of pectic polysaccharides

The methods used to solubilize and enrich pectic polysaccharides have an affect on the specific properties of the pectic fractions recovered. The matrix polysaccharides may be selectively solubilized or extracted from AIR with water, buffers or chelating agents to yield one or more fractions of pectic polysaccharides. Further extractions may selectively solubilize hemicelluloses, leaving a cellulose-enriched pellet.

Pectin-enriched fractions may be extracted directly from plant tissue or from AIR walls with hot or boiling water to yield RG-I-enriched polysaccharides. Water extracted walls constituted 7.3% wall dry weight in *Diospyros kaki* leaf walls, and the extracted polysaccharides were substituted with arabinan, galactan and to a lesser extent, Xyl residues (Duan et al., 2003; Duan et al., 2004). Extractions carried out with hot water or hot neutral solutions, however, may cause the beta-elimination of uronic acid residues that are a major component of pectic polysaccharides (Figure 1.10) (Albersheim et al., 1960; Westereng et al., 2007). Beta-elimination of uronic acids causes fragmentation of the pyranose ring of the sugar followed by further degradative fragmentations causing the loss of these residues. Calcium chelators effectively release the pectic polymers held in the wall by Ca^{2+} ionic crosslinking (Western et al., 2001; Leboeuf et al., 2005). Sodium carbonate (50 mM), potassium or ammonium oxalate (0.2 to 1%) solutions, sometimes in combination with ethylene glycol-*bis*-N, N'-tetra-acetic acid (EGTA), are used in this way to extract pectin-enriched fractions. The negative charges of these chemicals in solution compete for the calcium cations. Pectin-enriched fractions from *Arabidopsis* extracted with 1% potassium oxalate contained up to 66 mol% GalA residues (Leboeuf et al., 2005). Chemical extraction of wall components with basic buffers induces the saponification of acetyl-esters, methyl-esters and ferulic esters that are important characteristics



Galacturonic Acid

4, 5 unsaturated Galacturonic Acid

Figure 1.10. The beta-elimination of GalpA residues.

Beta-elimination of uronic acid residues can occur in basic or hot neutral solutions causing the loss of the substituent on the beta carbon, carbon C-4, of the sugar ring. The 4,5-unsaturated structure is unstable and the sugar ring is then susceptible to subsequent degradations, and thus the loss of these residues.

of cell wall polysaccharides (Davies et al., 2002). Conversely, acidic extractions of pectins, cause the degradation of *Arafuranosyl* residues present in pectin side-chains (Westereng et al., 2007). Extraction procedures must be chosen with care and consideration of the effect that the extraction procedure will have on the polysaccharides extracted and on the stability of the pectic polysaccharides in the solvent (Westereng et al., 2007).

Polygalacturonase enzymatic release of pectic polysaccharides

Pectic polysaccharides may be released from cell walls by use of wall degrading enzymes such as polygalacturonases (PGs), that specifically hydrolyze the α -(1,4)-linked glycosidic bonds of the GalA residues of the HG backbone. The PGs used are frequently derived from phytopathogenic fungi. Different types of PGs exist that hydrolyze interior residues (endo-PG) or terminal residues (exo-PG) (Benen et al., 1999). The products of endo-PG digest are monomeric GalA, dimeric GalpA, oligogalacturonides, RG-I and RG-II polysaccharides (York et al., 1985b). The endo-PGases derived from *A. niger* have sensitivity to the degree of methylation of GalA residues of HG, preferring non-methylesterified substrates (Benen et al., 1999). Thus, the degree and distribution of methylesterification of HG affects the outcome of wall digests with PGs. On a dry weight basis, less polysaccharide is released by enzyme hydrolysis than by chemical means. However, the relatively pure pectin obtained by enzyme hydrolysis can immediately be subjected to chromatography to further isolate RG-I and RG-II (York et al., 1985b). The HG content, but not its character, may be approximated from the yield of hydrolyzed GalA residues in PG-released extracts. Hydrolysis of HG by PG digestion is considered a mild treatment because it minimizes both the degradation of non-HG polysaccharides and the de-acetylation and

de-methylation of sugar residues of the wall polysaccharides, but it does degrade unmodified HG polysaccharides.

Glycosyl residue composition

The glycosyl residue composition of whole walls can be determined by high performance anion exchange chromatography (HPAEC) (Gardner et al., 2002; Obro et al., 2004) or two different methods of glycosyl residue derivatization and gas chromatography mass spectrometry (GC-MS): alditol acetates (AA) (Albersheim, 1967) that quantifies neutral sugars (Figure 1.11), or trimethylsilyl derivatives (TMS) (Sweeley et al., 1963; Doco et al., 2001) that allows quantification of neutral sugars, uronic acids and amino sugars (Figure 1.12). Glycosyl residue composition by TMS derivatization is able to semi-quantitatively identify and separate the uronic acids that are a major component of pectins, and therefore is the method of choice for pectin analysis.

The pectic polysaccharides RG-II and RG-I have characteristic glycosyl residue profiles (Table 1.2); for example, Kdo, 2-*O*-methyl-xylose and 2-*O*-methyl-fucose are diagnostic for RG-II in wall samples. RG-II polysaccharide compositions have two to five mol% fucose (more than purified RG-I) and seven to 12 mol% galactose (less than RG-I). Conversely, purified samples of RG-I are devoid of Kdo, 2-*O*-methyl-fucose and 2-*O*-methyl-xylose (Aboughe-Angone et al.). The arabinose content of RG-I polysaccharides is variable, but generally high (12 to 60 mol %) as is galactose content (17 to 71 mol %). The backbone of RG-I has alternating residues of GalpA and Rhap, which will yield a Rhap/GalA ratio of ~1.0, although ratios of 0.5 to 1.0 have been observed (Schols et al., 1990). The composition of HG is largely GalA with minor amounts of other residues. The composition of HG isolated from citrus had small amounts of Xyl (0.7

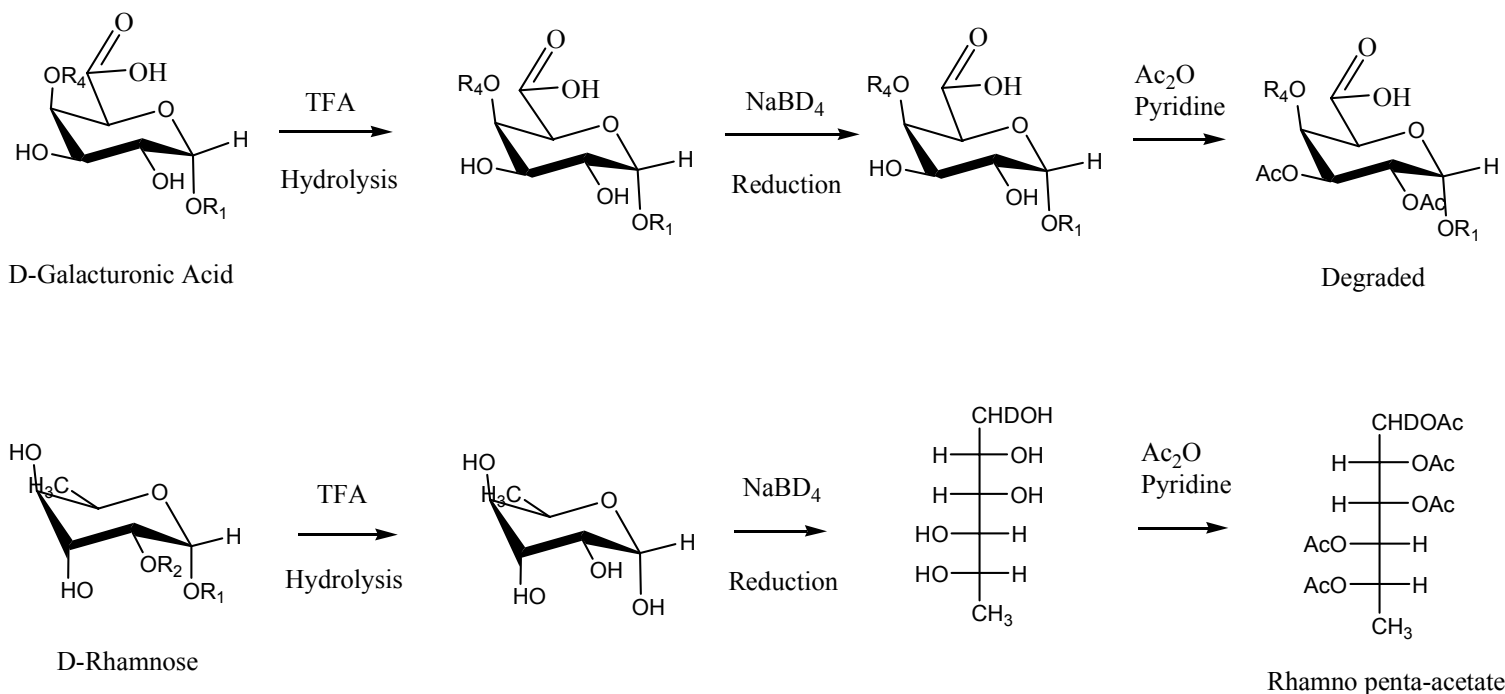


Figure 1.11. The principle of alditol acetates derivatization of polysaccharides for glycosyl residue composition analysis.

The production of the alditol acetate (AA) derivatives is a method for the analysis of neutral sugars but does not efficiently hydrolyze stable polymers of uronic acids to monomers and does not allow subsequent reduction of uronic acids to an open chain monomer. Therefore, uronic acids have limited detection in the AA procedure because they are not well hydrolyzed, which prevents full derivatization. In addition the acetylated uronic acid is unstable leading to degradation of the derivative and thus low levels of detection with the AA procedure.

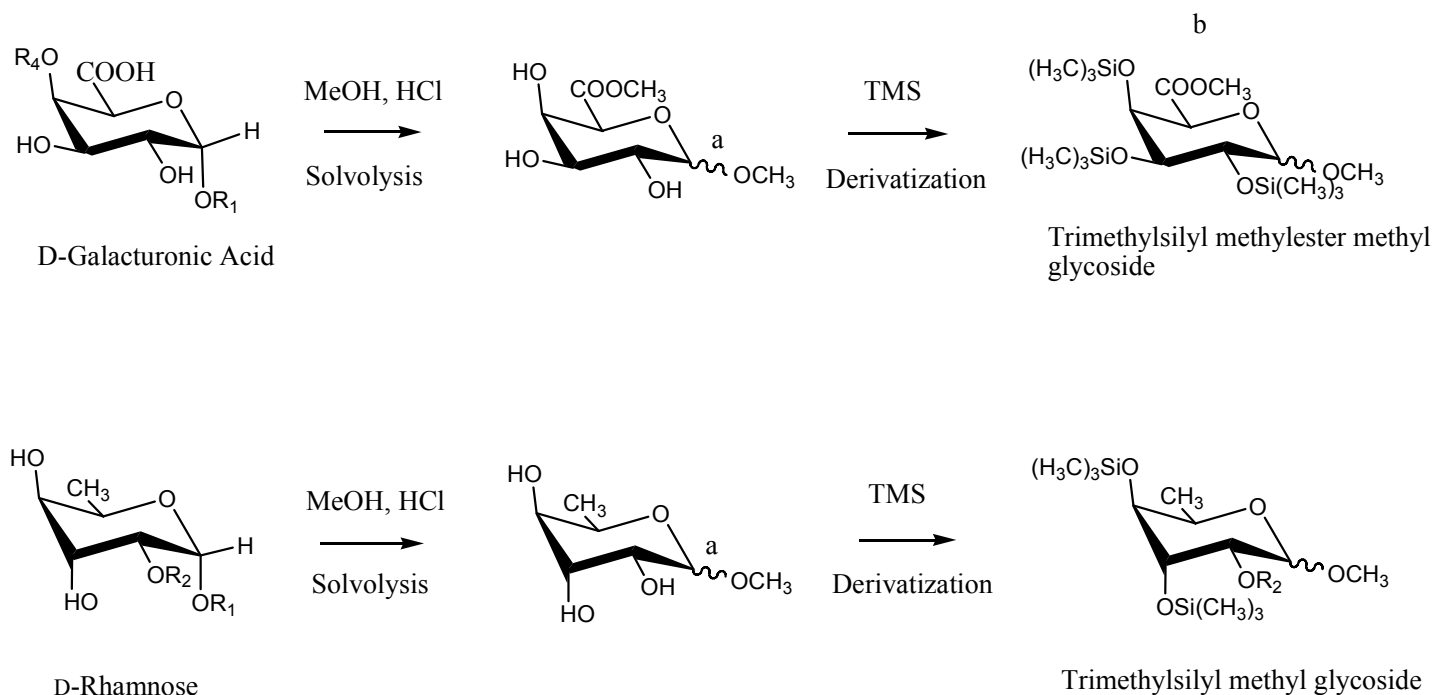


Figure 1.12. The principle of trimethylsilyl derivatization of polysaccharides for glycosyl residue composition.

Uronic acids and neutral sugars are solvolysed by methanolic-HCl for 18 hours at 80°C to form monomeric sugars that undergo trimethylsilyl (TMS) derivatization and are subsequently separated and identified by gas chromatography mass spectrometry (GC-MS). (a) As sugars undergo solvolysis in acidic methanol the sugar ring may reform as a pyranose (shown here) or furanose ring (not shown) and as either alpha or beta conformers, resulting in at least four possible species that represent a single residue type. (b) The C-6 carboxyl group of uronic acids does not undergo silylation.

Table 1.2. The glycosyl residue composition of purified RG-I and RG-II polysaccharides.

The glycosyl residue compositions of purified RG-I and RG-II isolated from plant walls of various species and the corresponding reference. Glycosyl residues are arabinose; Ara, rhamnose; Rha, fucose; Fuc, 4-*O*-methyl fucose; MeFuc, xylose; Xyl, 4-*O*-methyl xylose; MeXyl, galacturonic acid; GalA, galactose; Gal, glucuronic acid; GlcA, glucose; Glc and 2-keto-3-deoxy-D-mannooctulosonic acid; Kdo. ^(a) The species from which the pectic polysaccharide originated. ^(b) The specific tissue source from which the pectic polysaccharide originated.

Polymer	Species ^a	Source ^b	Ara	Rha	Fuc	MeFuc	Xyl	MeXyl	GalA	Gal	GlcA	Glc	Kdo	Reference
RG-II	<i>Acer psuedoplatanus</i>	suspension-culture	17	21	4	4	ND	5	20	12	3	2	3	(York et al., 1985)
	<i>Arabidopsis thaliana</i>	leaf	17	10	3	1	3	2	44	9	2	2	3	(Zabackis et al., 1995)
	<i>Arabidopsis thaliana</i>	rosette	13	11	3	4	1	3	37	7	5	ND	ND	(Reuhs et al., 2004)
	<i>Arabidopsis thaliana</i>	root	18	9	3	2	3	3	31	18	3	ND	1	(Nguema-Ona et al., 2006)
	<i>Argania spinosa</i>	fruit pulp	34	20	5	10	6	7	11	17	8	7	ND	(Aboughe-Angone et al., 2007)
	<i>citrus</i>	peel	12	17	4	1	3	2	43	11	2	1	0	(Yapo et al., 2007)
	<i>Curcubia moschata</i>	leaf	9	7	2	3	ND	4	43	10	5	ND	4	(Ishii et al., 2001)
RG-I	<i>Acer psuedoplatanus</i>	suspension-culture	35	10	2	ND	1	ND	18	31	ND	3	ND	(York et al., 1985)
	<i>Arabidopsis thaliana</i>	leaf	14	27	1	ND	5	ND	34	17	1	1	ND	(Zabackis et al., 1995)
	<i>Solanum tuberosum</i>	root	18	5	ND	ND	ND	ND	6	72	ND	ND	ND	(Obro et al., 2004)
	<i>Argania spinosa</i>	fruit pulp	60	12	ND	1	1	1	3	21	3	1	ND	(Aboughe-Angone et al., 2007)
	<i>citrus</i>	peel	36	19	ND	ND	1	ND	20	24	ND	ND	ND	(Yapo et al., 2007)

mol%) and Glc (0.3 mol%) (Yapo et al., 2007). The glycosyl residue composition of a wall sample is an important initial characterization of the polysaccharide content of the sample.

Linkage analysis

The generation of partially methylated alditol acetates (PMAA) for linkage analysis of wall polysaccharides yields information about the specific sites of covalent bonds on the sugar ring of residues present in a given wall sample, which includes the branch points and ring configuration (pyranose or furanose) (Figure 1.13) (Hakamori, 1964). In linkage preparations, bonded and unbonded sites of the carbohydrate ring are differentially derivatized such that when analyzed by GC-MS, specific patterns of fragmentation can sensitively reveal the specific linkages of residues in the sample. The results are semi-quantitative due to the differential stability of certain residues undergoing the extensive derivatization procedure, which may cause breakdown of some carbohydrate species (Hisamatsu et al., 1992). This is particularly true for Apif and GalpA residues present in pectin samples. Apiose is sensitive to degradation and is often lost during wall extractions and linkage analyses. In addition, GalpA residues are not detected by PMAA analysis unless prior reduction of the C-6 carboxyl group to galactose is carried out. Linkage analysis is often a necessary step toward the determination of wall carbohydrate structures. Linkage data interpretation is time consuming, but can be facilitated by resources offered by the Complex Carbohydrate Research Center web sites (<http://www.ccrc.uga.edu/specdb/ms/pmaa/pframe.html>), which lists the derivatization structure and the corresponding fragmentation patterns of many sugars present in wall samples.

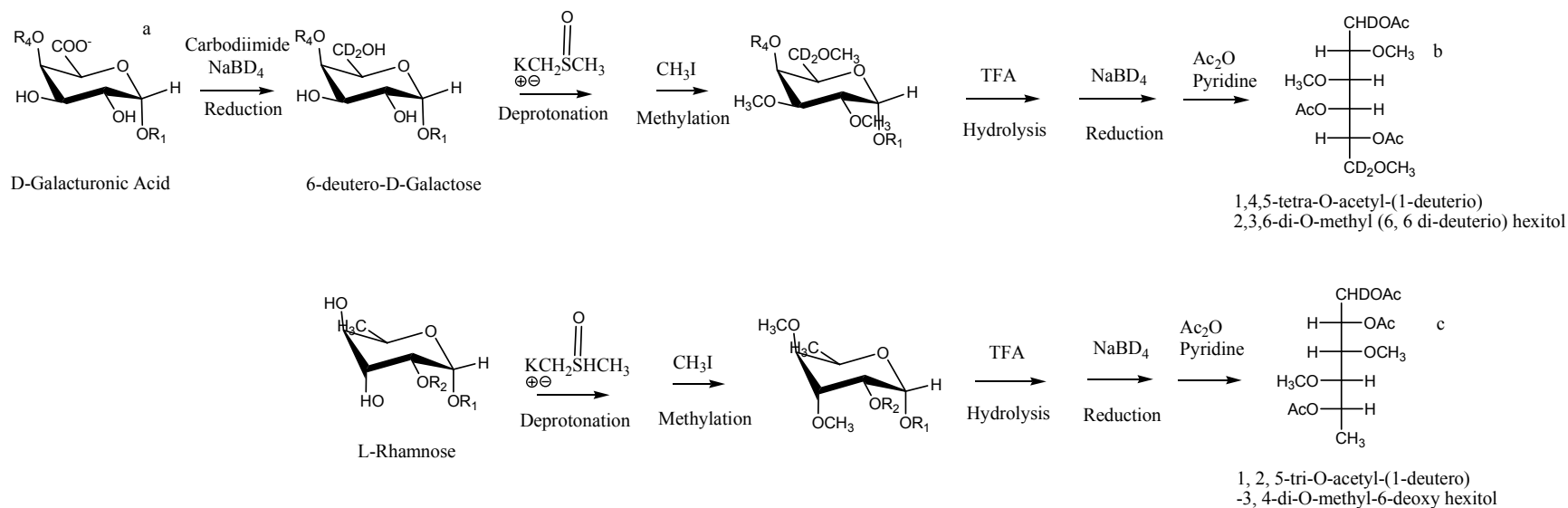


Figure 1.13. The principle of partially methylated alditol acetates (PMAA) analysis.

Partially methylated alditol acetates (PMAA) can be used to determine the branch points, ring structure and modification of residues in a sample of polysaccharides. Uronic acids are reduced to neutral sugars (a) prior to methylation to label, and to allow for efficient hydrolysis and thus detection as 6,6-di-deutero derivatives (b) of these residues in wall samples. The 6-deoxyhexose residues of rhamnose are not acetylated at the C-6 (c) to yield a CH_3 and not the CH_2OCH_3 that would result for a neutral hexose.

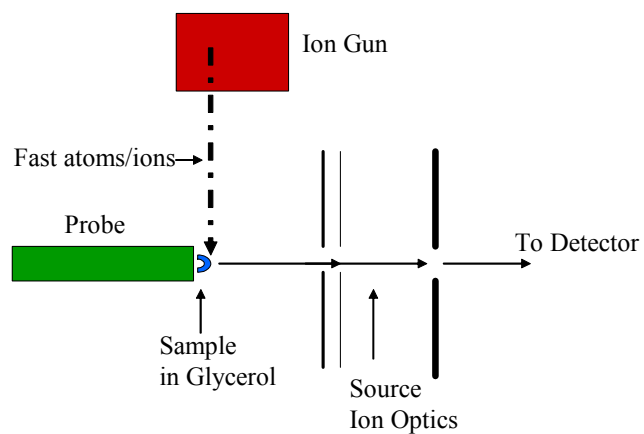
Mass spectrometry

Mass spectrometry (MS) has become increasingly useful as new methods become available for the specific analysis of carbohydrate structure. In principle, a compound or substance may be ionized and accelerated towards an anode or cathode, depending on the mode of ionization, at the end of which lies a detector (Figure 1.14). Three different types of MS are particularly useful in wall polysaccharide analysis and structure determination: fast atom bombardment mass spectrometry (FAB-MS), matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) and electrospray ionization mass spectrometry (ESI-MS).

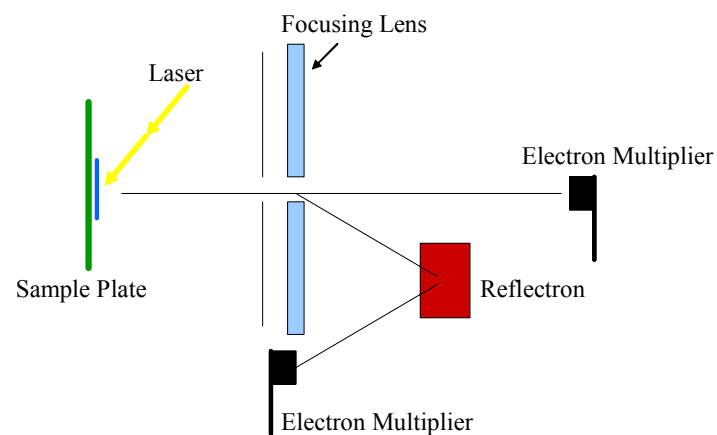
The sequence of oligosaccharides can be determined with FAB-MS. FAB-MS yields fragmentation ions from which the structure and sequence of the oligosaccharide may be deduced (David et al., 1986). Ions are directed at the sample suspended in glycerol, which causes ionization and fragmentation of the sample. In FAB-MS experiments, an oligosaccharide will be fragmented, one sugar at a time. Fragmentation of the sugar ring, however, does not generally occur with this method. The different molecular weights of sample species will determine the sequence of an oligosaccharide.

The usefulness of MALDI-TOF-MS lies primarily in molecular weight determination with-out fragmentation. Because the detector may be run in both positive and negative mode, neutral and acidic polysaccharides may be detected. However, the detection of acidic sugars by MALDI-TOF-MS is typically less well resolved than neutral sugars (ONeill and York, 2003). MALDI-TOF-MS may be modified to yield carbohydrate sequence information by fragmentation: post-source decay (PSD), which occurs as a consequence of fragmentation at ionization; in-source decay (ISD) caused by

Fast atom bombardment ionisation



Field Ionisation of Matrix Assisted Laser Desorption Ionisation



Electrospray Ionisation Mass Spectrometry

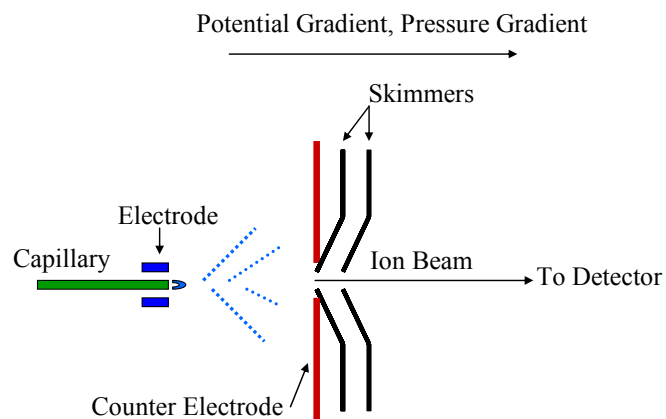


Figure 1.14. Mass spectrometry methods used in carbohydrate research (Adapted from Harvey 1999).

matrix induced fragmentation; and collision-induced fragmentation (CID) where a collision cell is introduced for fragmentation (Harvey, 1999).

ESI-MS is becoming an extremely useful technique in carbohydrate and cell science because this method utilizes a continuous flow of sample that is vaporized and subsequently ionized before entering the mass detector. As a result of the continuous flow of charged ions into the detector, ESI-MS has more sensitive detection of polar or charged molecules than MALDI-TOF-MS, an aspect particularly important for analysis of acidic pectic polysaccharides. In addition, ESI-MS allows detection well below 500 Da where matrix molecules normally interfere in MALDI and FAB methods of MS at this size scale (Figure 1.14) (Griffiths et al., 2001).

Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) spectroscopy is a technique that can determine the chemical identity of carbohydrates and other biomolecules in solution (Jia et al., 2003). NMR identifies the resonance frequency or chemical shift of magnetically active nuclei, such as protons (^1H) or carbons (^{13}C), which is unique based on the neighboring nuclei that are bound to it. Information extracted from NMR spectra may offer the distances between nuclei (nuclear overhauser effect/dipolar coupling), the molecular geometry (residual dipolar coupling) and the geometry between two coupled nuclei (scalar coupling) (Prestegard and Kishore, 2001). NMR is a method that also can identify sites of O-acetylation (Jia et al., 2005) and methylation (Glushka et al., 2003). Carbohydrates are often not well resolved due to the dynamic or floppy nature of carbohydrates in solution. Regardless, the powerful magnetic fields of ultra-high field NMR (> 600 MHz) have vastly improved resolution of carbohydrates making NMR an important tool for

structural characterization of plant wall polysaccharides (Blundell et al., 2006). Oligosaccharides extracted from plant walls frequently include repeating units, as is the case with the backbones of HG, RG-I and RG-II (Blundell et al., 2006), which generate overlapping chemical shifts. This problem is being addressed by the cell wall community by establishment of enzymatic or chemical wall fragmentation methods to yield non-repeating structural units for analysis (Deng et al., 2006). NMR techniques are rapidly becoming essential to wall structure elucidation. Databases have become available for interpretation of the complex spectra of all carbohydrate species (SugaBase; <http://www.boc.chem.uu.nl/sugabase/Sugabase.html>) and xyloglucan specifically (XG NMR: www.cerc.uga.edu/specdb/specdbframe.html). More widespread NMR facilities and means of data interpretation have allowed NMR to become more accessible to a greater number of researchers.

FUNCTION OF PECTIC POLYSACCHARIDES

The plant cell wall plays a functional role in many aspects of plant growth and development such as contributing to structural integrity, cell adhesion and mediation of defense responses to protect the plant cell against wounding and pathogen attack. The specific role of pectic polysaccharides in these processes is quickly being elucidated. The primary wall is formed during cell expansion. The primary wall of dicot plants is composed of the cellulose microfibrils that are extensively crosslinked by hemicelluloses, such as xyloglucan, and embedded in a matrix of pectic polysaccharides. Often this arrangement is described as analogous to reinforced cement, where the cellulose-xyloglucan network is represented by rebar and the matrix polysaccharides are represented as cement that fills around the rebar. This model is easy to comprehend but is overly simplistic in that a static and unresponsive nature is inferred.

The wall is responsive to growth, differentiation and environmental stimuli. HG, RG-I and RG-II are structurally diverse polysaccharides that contribute to many aspects of primary wall function with regard to cell strength, cell adhesion, stomatal function and defense response mediation.

HG-calcium complexes contribute to wall strength

HG is a polymer of α -1,4-galacturonopyranosyl residues (Ridley et al., 2001). The extent and pattern of methyl-esterification directly affects the affinity of HG for calcium cations involved in the gelation of HG chains (Yoo et al., 2003). Calcium crosslinking contributes to wall strength by bringing blocks of unmethylesterified HG chains into a tightly packed conformation that is dependent on three characteristics; the intramolecular conformation of HG, the charge separation between two GalA molecules in an HG chain, and the efficiency with which HG chains pack together (Grant et al., 1973). The hypothesized *in vivo* configuration of HG chains bound to calcium ions is referred to as the ‘egg-box model’, which describes the close packing of 2 or more HG chains brought about by calcium crosslinking (Liners et al., 1989). The egg-box model proposes that the HG chains take a two-fold helical structure (2₁). The conformation of the two-fold helical structure of the egg-box model was confirmed by NMR of *in vitro* and *in vivo* pectate gels (Jarvis and Apperley, 1995).

A decline in wall expansibility and an increase in wall stiffening has been correlated with a decrease in arabinan and galactan RG-I sidechains and an increase in HG-calcium complexes. In bean pods (*Phaseolus vulgaris* L.), RG-I neutral sugar sidechains and HG steadily increased during exponential growth or cell expansion. At maturation, arabinan and galactan were degraded, while the HG continued to accumulate forming HG-calcium complexes (Stolle-Smits

et al., 1999). The loss of RG-I side chains coincided with de-methylation of the pectic component, facilitating HG-calcium complexation. In addition to tracking the wall polymers at five stages in pea pod development, the enzyme activities of α -arabinase, β -galactanase, pectinmethylesterase (PME), polygalacturonase (PG) and peroxide (POD) activities were assayed. Dramatic changes in enzyme activity were observed in fully mature (24 to 55 days after flowering) and senescing (>55 days after flowering) pea pods. The activities of α -arabinase and β -galactanase and PME gradually increased up to the fully mature stage; thereafter β -galactanase and PME dramatically increased in senescing pods. The data suggests that the loss of RG-I sidechains in combination with the de-methylation of HG, but not the degradation of HG, contributes to the locking of wall components (Stolle-Smits et al., 1999). In soybean, glycerinated hollow cylinders (GHCs) isolated from hypocotyls were used as a tool to study the effect of Ca^{2+} on wall tension and wall expansibility (Ezaki et al., 2005). Addition of a calcium chelator to the system dramatically increased wall expansibility, a response that ceased with addition of calcium. The calcium-induced wall stiffening may play a role in decreased wall expansibility and strength (Ezaki et al., 2005).

Transgenic plant lines expressing polygalacturonase (PG) enzymes have been produced in apple (*Malus domestica*) (Atkinson et al., 2002), tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* (Capodicasa et al., 2004), in order to study the changes in wall structure and the developmental abnormalities caused by *in vivo* pectin degradation. The HG extracted from PG-expressing apple tree leaf walls had reduced molecular weight and overall amount. In addition, wall weakening contributed to epidermal tearing in stomatal guard cells in apple leaves (Atkinson et al., 2002). Tobacco plants expressing the *Aspergillus niger* endo polygalacturonase-II (*AnPG-II*) had a dwarfed phenotype and a general weakening of walls that

were unable to maintain cell shape and size against the force of turgor pressure (Capodicasa et al., 2004). The transgenic expression of EPG in apple, tobacco and Arabidopsis clarify that HG, and likely, HG-calcium complexes, play a role in wall strengthening.

RG-II-borate complexes contribute to wall strength

Boron is hypothesized to function specifically in membrane proteins, plant reproduction, nitrogen fixation and in plant cell wall strengthening (Blevins, 1998). The first definitive proof of a boron requirement in plants was in 1923 (Warington, 1923). Boron is an essential micronutrient in plants. Approximately 80% of cell wall boron is associated with pectic polysaccharides (Kobayashi et al., 1996; Matoh and Kobayashi, 1998). A large number of reviews have documented the progression boron research in plant biology through the years and a selected number are provided: (Skok, 1941), (Lewis, 1980), (Pilbeam and Kirkby, 1983), (Loomis and Durst, 1992), (Matoh and Kobayashi, 1998) and (O'Neill et al., 2004). In regards to the wall pectic polysaccharides, boron atoms are involved in the reversible dimerization of two RG-II molecules. RG-II dimerization plays a critical role in the expansive strength of the plant cell wall and has a specialized role in meristematic and reproductive systems of the plant.

Boron deficiency in plants causes slowed root growth, degeneration of new growth, degeneration of meristematic regions and abnormal morphology of reproductive organs (Blevins, 1998). The Arabidopsis *bor1-1* (high boron requiring) mutant is perpetually boron deficient, even in boron concentrations that are satisfactory for normal growth of WT plants. The *bor1-1* plants are dwarfed with stems that fail to elongate and have a loss of apical dominance (Noguchi et al., 1997). The Arabidopsis *mur1-1* mutant (Bonin et al., 1997) is morphologically similar to the *bor1-1* mutant, but is deficient in the production of L-fucose, an essential component of RG-

II structure caused by a lesion in GDP-mannose-4,6-dehydratase, the enzyme that synthesizes the substrate for addition of fucose into wall polysaccharides; GDP-L-fucose (ONEILL et al., 2001). Normal plants have ~95% of the RG-II molecules in the dimer form, whereas *mur1-1* has only ~50% of RG-II molecules in the dimer form (ONEILL et al., 2001). The L-fucose in sidechain A and B of *mur1-1* RG-II is replaced by L-galactose, but this interesting *in vivo* substitution only partially rescues RG-II dimerization in the mutant (ONEILL et al., 2001; Reuhs et al., 2004).

The *nolac-H18* mutant (*non-organogenic callus with loosely attached cells*) has a T-DNA insertion in the *NpGUT1* (*Nicotiana plumbaginifolia* glucuronosyltransferase 1) gene in tobacco (Iwai et al., 2002). The *NpGUT1* mutant is deficient in a putative glucuronosyltransferase that has homology to animal glucuronosyltransferases, and fails to incorporate a GlcpA residue, and the corresponding Gal branch (α -L-Galp-(1,2)- β -D-GlcpA-(1, $\rightarrow$), into RG-II sidechain A (Iwai et al., 2002). RG-II sidechain A specifically interacts with boron to form a tetrahedral borate diol ester via the apiosyl residue. The coordination of boron by RG-II is dependent on the specific conformation of RG-II sidechain A, which is compromised in the *mur1-1* and *nolac* mutants (Ishii and Matsunaga, 1996; Ishii and Ono, 1999). The consequence of RG-II-boron complex disruption is a lack of wall expansibility that results in plants that have dwarfed stature, compromised cell adhesion and defects in reproductive tissue function (Iwai et al., 2002; Iwai et al., 2006). The disruption of meristematic regions in *nolac* shoots was similar to that found in boron deficient pumpkin plants (*Curcubita moschata*) (Ishii et al., 2001; Iwai et al., 2002). The loss of RG-II dimerization and cell adhesion is hypothesized to be a factor in *nolac* and boron-deficient pumpkin meristematic regions. The pistils of GUT1A tobacco plants (*Nicotiana tabaccum*), that had GUT1 gene silencing, remained immature and did not develop pectin-rich transmitting tissue, which normally allows the passage of pollen tubes

(Iwai et al., 2006). The pollen tube tips of GUT1 silenced plants lacked boron, suggesting that RG-II borate dimers were not forming due to incorrectly synthesized RG-II structure (Iwai et al., 2006). In summary, RG-II dimerization via boron crosslinking has a major impact on the growth and development of plants.

HG-calcium complexes and RG-I sidechains contribute to cell-adhesion

Cellular adhesion in plant tissues is mediated by the extracellular matrix or pectic polysaccharides of the plant cell wall. Cell adhesion is reduced in mutants that have insufficient HG-calcium complexes, branched RG-I polysaccharides and RG-II dimerization. Tomato fruits undergo a defined program of cellular changes during ripening (Gray et al., 1994; Wilkinson et al., 1995). The colorless non-ripening mutant (*Cnr*), isolated from plantings of commercial tomato (*Lycopersicon esculentum* Mill. cv Liberto), grow similar to wild type cv Liberto fruits up to the mature-green stage, but do not ripen. When Liberto fruits are ripe-red, *Cnr* tomatoes are yellow with white flesh. The mealy texture of *cnr* tomato flesh (Thompson et al., 1999) and the large intercellular spaces in *Cnr* pericarp compared to WT (Orfila et al., 2001), was an indication of altered cell adhesion. Compared to wild type, a greater proportion of cells were released by placing blocks of tissue in water, however a smaller proportion of *Cnr* cells were released by the chelating agent CDTA (trans-1,2-cyclohexylenedinitrilo-tetraacetic acid) (Thompson et al., 1999). Decreased calcium crosslinking of pectins in *Cnr* tomatoes was suggested by increased solubility in water and an overall decrease in chelator soluble pectins, which was confirmed by EELs spectroscopy (Orfila et al., 2001). The glycosyl residue composition of *Cnr* mature-green walls showed decreases in Rha, Xyl and uronic acids with increases in galactose compared to WT. The PAM antibody, which recognizes a region of

unesterified homogalacturonan, showed dramatically reduced binding to *Cnr* middle lamellae compared to WT (Orfila et al., 2001). Interestingly, the binding of LM6 that binds to α -(1,5)-arabinan, was lacking in the walls of *Cnr*, but binds to cytosolic vesicles suggesting that arabinan is not being incorporated into the walls of these plants. Ultimately, the changes in cell walls of the *Cnr* tomato mutant correlate with reduced calcium complexed HG and a lack of wall arabinan incorporation, which implicates these pectic polysaccharides in cell adhesion.

HG-calcium complexes and RG-I arabinan sidechains affect stomatal function

Epidermal strips of the species *Commelina communis* were used to study the changes in stomatal opening in response to wall manipulation by purified wall degrading enzymes (Jones et al., 2003). Guard cells were used as a model for cell wall architecture because turgor pressure drives a daily cycle of expansion (opening) and contraction (closing) in stomata that necessitate great wall strength as well as expansibility. The tremendous turgor pressure that builds up during stomatal opening (up to 5 MPa) causes a volume expansion of each guard cell of up to 70% as the stomatal pore is opened (Franks et al., 2001). Guard cells were induced to open in epidermal peels by fusicoccin and induced to close by ABA such that normal opening of the stomata can be measured and tested after treatment with an assortment of wall degrading enzymes (Jones et al., 2003). Surprisingly, only pectinolytic enzymes and a feruloyl esterase had an appreciable affect on pore opening. Degradation of cellulose and hemicellulose by cellulase and xylanase enzymes had no affect on fusicoccin induced stomatal opening. Endoarabinase, that specifically hydrolyzes α -(1,5)-arabinan, completely blocked pore opening, while feruloyl esterase FaeA inhibited but did not stop guard cell pore opening. Open stomata treated with arabinase and induced to close with either ABA or mannitol failed to close, suggesting that the

walls were ‘locked’ into place. Interestingly, a combination of endopolygalacturonase (EPG) and pectinmethylesterase (PME) produced much more widely open pores than either treatment alone. The addition of EPG/PME treatment after arabinase-induced wall locking then allowed the stomata to “unlock” and close. The locking of guard cell walls is also reversed by treatment with strong calcium chelators. Based on these experiments, it is hypothesized that *in vivo*, the feruloylated RG-I arabinan form ester linkages either to other feruloylated RG-I arabinans, or to other wall molecules, providing a mechanism for spatial buffering of HG polymers, and thus, by not allowing the HG chains to come into close proximity the HG is inhibited from locking into place by calcium crosslinking (Figure 1.15) (Jones et al., 2003).

Pectic polysaccharides mediate defense; a barrier and signaling mechanism

The wall provides a physical barrier that pathogens must break down in order to gain entry into the cells of the plant to establish an infection. Both bacteria and fungal phytopathogenic organisms produce wall hydrolytic enzymes as essential virulence factors that allow entry into plant cells. The wall hydrolytic enzymes produce wall fragments that become signaling molecules to the plant of an impending infection. An early response by the plant can stave off, or end, an infection by a phytopathogenic organism. The action of oligosaccharides as signaling molecules in plant defense is well documented and a number of reviews cover these phenomena: (Aldington and Fry, 1993), (Cote et al., 1998), (Ridley et al., 2001), (Shibuya and Minami, 2001), and (Vorwerk et al., 2004). Chitin, chitosan, β -glucan and oligogalacturonides are oligosaccharide elicitors of defense responses (Shibuya and Minami, 2001). Oligogalacturonides or OGAs, derived from pectic polysaccharides, have specialized function.

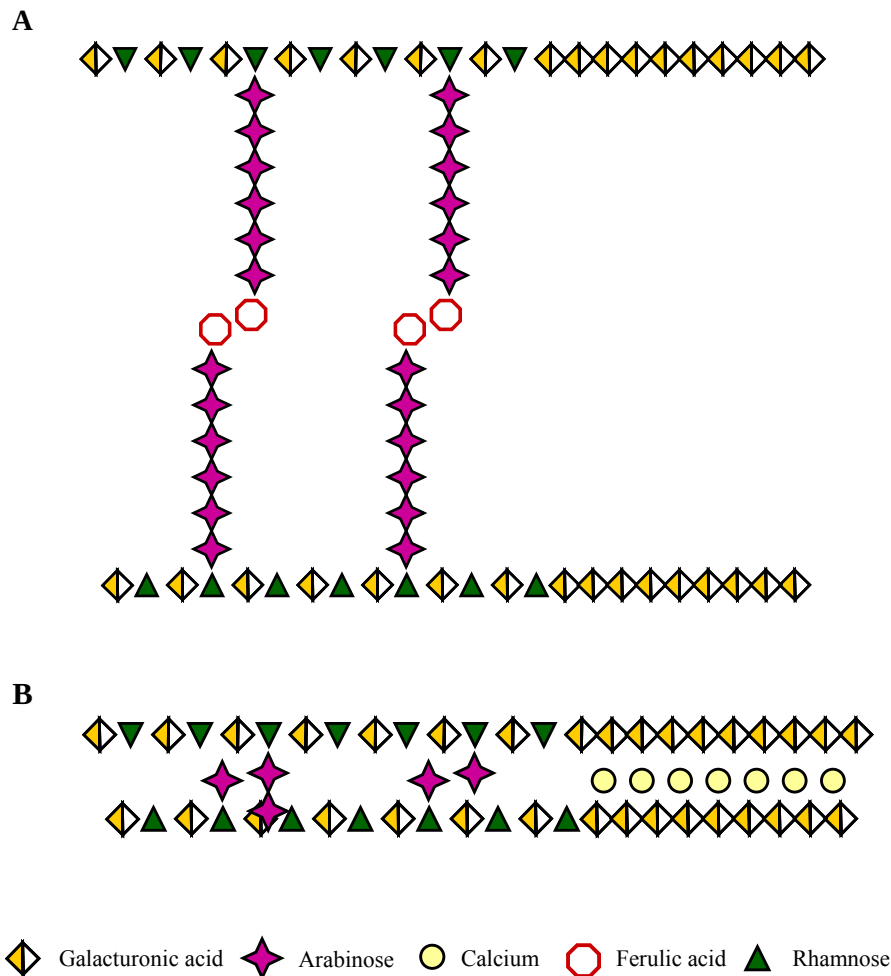


Figure 1.15. The model of arabinan and HG-calcium complexes based on the behavior of stomatal pore openings in response to wall degrading enzymes.

The α -(1,5)-arabinan of RG-I may be esterified to ferulic acids that are thought to dimerize providing a linkage between RG-I molecules (A). According to Jones et al. (2003), the arabinan provides a mechanism for the inhibition of HG calcium complexes (yellow/white diamonds (GalA/HG); light yellow circles (calcium)), which function to “lock” the wall (B). Removal of the ferulic acid crosslinks by a α -(1,5)-arabinan-specific endoarabinase or ferulic acid esterase promotes the formation of HG-calcium complexes leading to the locking of walls. Adapted from Jones et al. (2003).

OGAs extracted by water or cleaved by *Erwinia carotovora* pectin lyase have been shown to have functions beyond those of structural components in the elicitation of phytoalexins (antibiotic) and reactive oxygen species (ROS) (Hahn et al., 1981; Davis et al., 1986). Treatment of sodium polypectate or cowpea (*Vigna unguiculata*) walls with EPG also produced OGAs that were elicitors, also termed oligosaccharins (Davis et al., 1986; Chervone et al., 1987). The elicitor activity did not depend on the cleavage enzyme or if the resulting OGA was terminally 4,5-unsaturated. Elicitor activity did depend on a galacturonic acid structure and DP of 9 to 16 (Cervone et al., 1989; Mathieu et al., 1991). Phytoalexin-elicitor active OGAs cause changes in gene expression in addition to the induction of genes in a pathway for coumarin phytoalexin biosynthesis, demonstrating that OGA elicitor activity works to induce defense response pathways (Davis and Hahlbrock, 1987; Ferrari et al., 2007a). PG-expressing tobacco plants were also constitutively upregulated in defense responses as a result of wall architectural changes brought about by the fragmentation of HG by EPG enzymes (Ferrari et al., 2007b).

BIOSYNTHESIS OF PECTIC WALL POLYMERS

The polysaccharides that are important to plant cell wall structural integrity and defense against plant pathogens are synthesized by glycosyltransferases (GTs). Pectin biosynthetic GTs require specific nucleotide-sugar substrates and acceptors for activity. Pectin wall biosynthesis occurs in the lumen of the Golgi where the substrates and acceptors must also be available. The current model of pectin biosynthesis illustrates Golgi luminal GT active sites and nucleotide-sugar substrates, which are thought to be imported into the Golgi lumen by membrane spanning protein transporters (Figure 1.16). Aspects of pectin biosynthesis have been reviewed in the literature: (Scheible and Pauly, 2004), (Ridley et al., 2001), (Goubet and Mohnen, 1999),

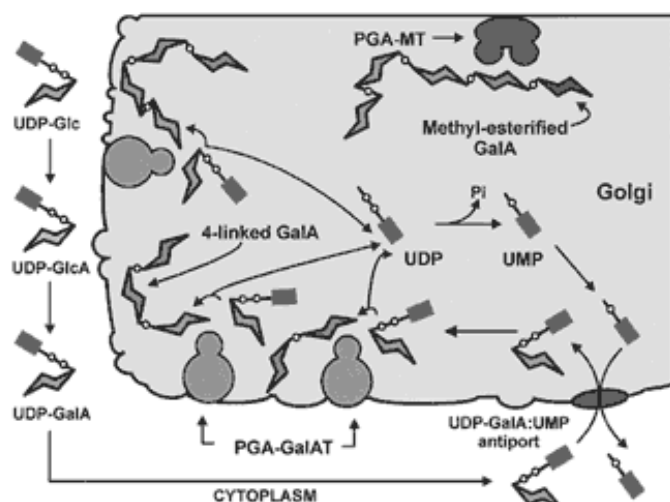


Figure 1.16. The current model of pectin biosynthesis in the Golgi apparatus.

Pectin biosynthesis in the Golgi occurs by utilization of the appropriate nucleotide-sugar substrates that are likely to be largely synthesized in the cytoplasm and imported to the lumen of the Golgi by membrane bound transporters (UDP-GalA:UMP antiport). Although it cannot be ruled out that some of the nucleotide interconverting enzymes (NIEs), like UDP-GlcA4-epimerase, may have a catalytic site in the Golgi. Here HG synthesis is specifically depicted by membrane bound homogalacturonan galacturonosyltransferase (HG-GalAT or PGA-GalAT) and pectin methyltransferase (PMT or PGA-MT) in the Golgi lumen. Adapted from O'Neill CCRC web-site.

(Mohnen, 2008) and (Mohnen et al., 2008). Here the pertinent historical and progressive research in the synthesis of nucleotide sugars, glycosyltransferases, pectinmethyltransferases and *O*-acetyltransferases that contribute to the construction of plant cell wall pectic polysaccharides is summarized.

Subcellular localization of pectin biosynthetic glycosyltransferases

The pectic polysaccharides are synthesized in the Golgi apparatus of the plant cell, sorted to vesicular compartments, and secreted to the plasma membrane, to the apoplastic space (Zhang and Staehelin, 1992). The Golgi apparatus is a complex organelle that is made up of stacks of flattened vesicles that contain enzymes specific to the sorting and processing of cargo in the specific vesicles of the Golgi. A Golgi stack has four defined regions; the cis, medial, trans Golgi and the trans-Golgi network (Dunphy and Rothman, 1985) (Figure 1.17). Cargo intended for secretion is transported through the endoplasmic reticulum (ER) to the cis face of the Golgi body. Wall polysaccharides are continually synthesized and moved through Golgi stacks from the cis face to the trans face of the Golgi where they are sorted and packaged into the vesicles of the trans Golgi network (TGN) for transport to the plasma membrane, the cell plate of dividing cells or the vacuole (Denecke et al., 1990).

In plants, transport from the ER to the Golgi may occur via clathrin coated COPII vesicles or by direct connections that physically link ER and Golgi membranes. Movement through the Golgi is thought to occur via two complementary mechanisms as described by the vesicle shuttle model and the cisternae maturation model (Neumann et al., 2003). The vesicle shuttle model proposes that each cisterna is stable and functionally specific, while the cargo is transported stack to stack by vesicular shuttles. The cisternae maturation model

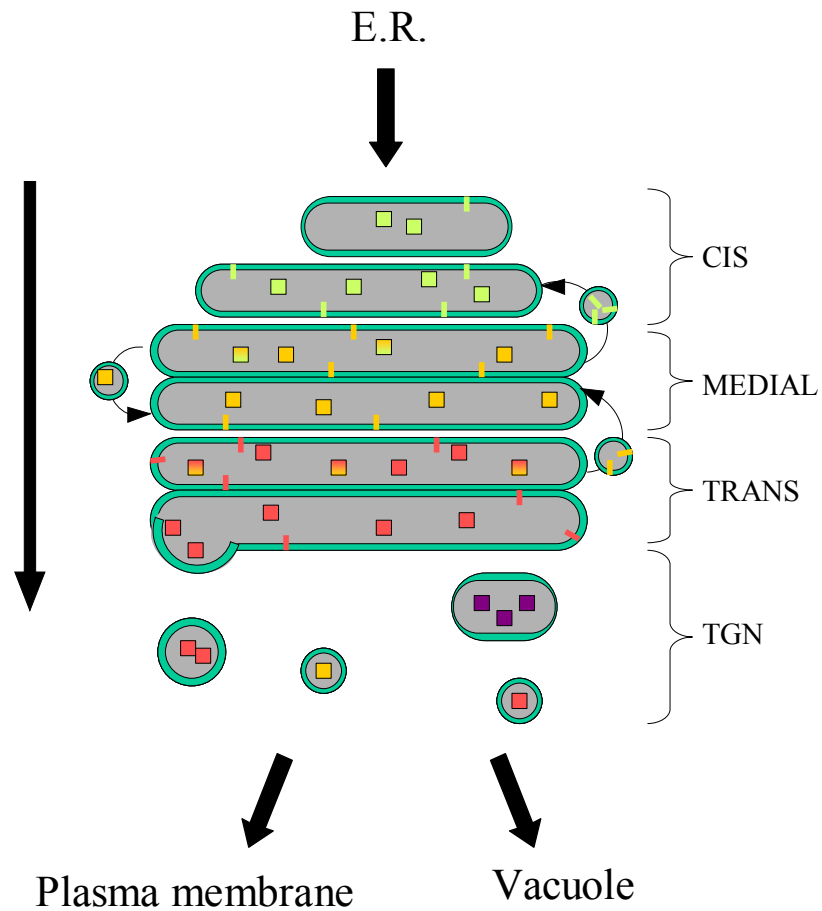


Figure 1.17. The plant Golgi apparatus.

The Golgi apparatus is composed of flattened vesicles within which glycoproteins and wall polysaccharides (cargo-colored squares) are synthesized by membrane bound glycosyltransferases (colored sticks). The direction of cargo flow is depicted by the large black arrows. The Golgi cargo is received from the endoplasmic reticulum (E.R.) at the cis-face of the Golgi and traverses to the medial, trans and the trans-Golgi network (TGN) for further processing and export.

proposes that the resident processing enzymes may be regulated by retrograde transport of enzymes in COPI vesicles while cargo is moved in bulk within the cisternae; from cis to trans Golgi (Opat et al., 2001). Transport in the secretory system is known to involve a host of proteins regulating and orchestrating the initiation, formation and direction of secretory vesicle movement associated with the Golgi. Vesicle trafficking in the Golgi is a complex process for which there are many valuable reviews (Sanderfoot and Raikhel, 1999; Nebenfuhr and Staehelin, 2001; Nebenfuhr, 2002; Neumann et al., 2003; Hawes and Satiat-Jeunemaitre, 2005; Guo et al., 2007).

The synthesis of polysaccharides in the Golgi and their movement to the plasma membrane has been tracked by histochemical analysis of plant cells. Antibody probes that recognize epitopes of carrot extensin-1, sycamore maple deesterified RG-I/HG and purified sycamore maple XG had differential binding to specific Golgi stacks. RG-I epitopes appeared in the cis and medial Golgi and the XG epitopes were present in the medial, trans and TGN, while the extensin epitopes were observed throughout the Golgi (Moore et al., 1991; Zhang and Staehelin, 1992). The spatially distinct localization of these Golgi products demonstrated that glycoproteins and wall polysaccharides are both simultaneously synthesized in the Golgi, but appear to have different sorting and export programs (Moore et al., 1991). The differential localization of RG-I, XG and extension epitopes also suggest the separation of glycoprotein, as well as acidic and neutral polysaccharide biosynthetic machinery, within the Golgi apparatus.

The localization of pectic polysaccharide epitopes within the cisternae of the Golgi sets the stage for biochemical localization of the activities necessary for pectin biosynthesis. Multiple activities are necessary for the synthesis of HG, RG-I and RG-II (Table 1.3). Many glycosyltransferases may be required for the complete synthesis of pectic polysaccharides

Table 1.3. Predicted glycosyltransferases required for pectin biosynthesis^{a,b}.

(^a) Adapted from Mohnen (2002), Mohnen et al. (2008) and Sterling Ph.D. Dissertation (2004). (^b) The glycosyltransferases listed are based on the current understanding of pectic polysaccharide structure as determined by the corresponding structure references. (^c) The activity of the homogalacturonan (HG) GalAT listed may in fact be carried out by several GalAT's for synthesis of apiogalacturonan (AGA), xylogalacturonan (XGA) and rhamnogalacturonan II (RG-II).

Glycosyltransferase	Parent polymer	Enzyme Acceptor	Reference for structure
<i>HG glycosyltransferases</i>			
α -1, 4-GalAT ^c	HG/RG-II	D-Galp A- α -(1 $\rightarrow$ 4)-D-Galp A- α -(1 $\rightarrow$	(O'Neill et al., 1990)
<i>RG-I glycosyltransferases</i>			
α -(1,2)-GalAT	RG-I	L-Rhap - α -(1 $\rightarrow$ 4)-D-Galp A- α -(1 $\rightarrow$	(McNeil et al., 1980; Lau et al., 1985; O'Neill et al., 1990)
α -(1,4)-GalAT	RG-I/HG	D-Galp A- α -(1 $\rightarrow$ 2)-L-Rhap - α -(1 $\rightarrow$	(Nakamura et al., 2002a)
α -(1,4)-GalAT	RG-I/HG	D-Xylp- ?	(Pena et al 2007; Nakamura et al, 2002)
α -(1,4)-RhaT	RG-I	D-Galp A- α -(1 $\rightarrow$ 2)-L-Rhap - α -(1 $\rightarrow$	(McNeil et al., 1980; Lau et al., 1985; O'Neill et al., 1990)
α -(1,4)-RhaT	HG/RG-I	D-Galp A- α -(1 $\rightarrow$ 4)-D-Galp A- α -(1 $\rightarrow$	(Nakamura et al., 2002b)
β -(1,4)-GalT	RG-I	L-Rhap - α -(1 $\rightarrow$ 4)-D-Galp A- α -(1 $\rightarrow$	(Lau et al., 1987; O'Neill et al., 1990; Schols et al., 1995)
β -(1,4)-GalT	RG-I	D-Galp - β -(1 $\rightarrow$ 4)-L-Rhap - α -(1 $\rightarrow$	(Lau et al., 1987; O'Neill et al., 1990)
β -(1,4)-GalT	RG-I	D-Galp - β -(1 $\rightarrow$ 4)-D-Galp - β -(1 $\rightarrow$	(Aspinall et al., 1967; Aspinall, 1980; Lau et al., 1987; O'Neill et al., 1990; Nakamura 2001)

β -(1,6)-GalT	RG-I	D-Galp - β -(1 $\rightarrow$ 4)-D-Galp - β -(1 $\rightarrow$	(Lau et al., 1987; O'Neill et al., 1990; Nakamura et al., 2001)
β -(1,3)-GalT	RG-I/AGP	D-Galp - β -(1 $\rightarrow$ 3)-D-Galp - β -(1 $\rightarrow$	(O'Neill et al., 1990; Carpita and Gibeaut, 1993)
β -(1,6)-GalT	RG-I/AGP	D-Galp - β -(1 $\rightarrow$ 3)-D-Galp - β -(1 $\rightarrow$	(O'Neill et al., 1990; Carpita and Gibeaut, 1993)
β -(1,6)-GalT	RG-I/AGP	D-Galp - β -(1 $\rightarrow$ 6)-D-Galp - β -(1 $\rightarrow$ 3)-D-Galp - β -(1 $\rightarrow$	(O'Neill et al., 1990; Carpita and Gibeaut, 1993)
β -(1,5)-GalT	RG-I	L-Araf -? β -(1 $\rightarrow$ 4)-D-Galp - β -(1 $\rightarrow$	(Huisman et al., 2001)
α -(1,4)-ArafT	RG-I	L-Rhap - α -(1 $\rightarrow$ 4)-D-Galp A- α -(1 $\rightarrow$	(Lau et al., 1987)
α -(1,5)-ArafT	RG-I	L-Araf - α -(1 $\rightarrow$ 5)-L-Araf - α -(1 $\rightarrow$	(Lerouge et al., 1993)
α -(1,5)-ArafT	RG-I	D-Galp - β -(1 $\rightarrow$ 4)-D-Galp - β -(1 $\rightarrow$	(Huisman et al., 2001)
α -(1,3)-ArafT	RG-I	D-Galp - β -(1 $\rightarrow$ 4)-L-Rhap - α -(1 $\rightarrow$	(Lau et al., 1987; O'Neill et al., 1990)
α -(1,2)-ArafT	RG-I	L-Araf - α -(1 $\rightarrow$ 3)-D-Galp - β -(1 $\rightarrow$	(Lau et al., 1987; O'Neill et al., 1990)
α -(1,5)-ArafT	RG-I	L-Araf - α -(1 $\rightarrow$ 2)-L-Araf - α -(1 $\rightarrow$	(Lau et al., 1987; O'Neill et al., 1990)
α -(1,3)-ArafT	RG-I	L-Araf - α -(1 $\rightarrow$ 5)-L-Araf - α -(1 $\rightarrow$	(Carpita and Gibeaut, 1993)
α -(1,2)-ArafT	RG-I	L-Araf - α -(1 $\rightarrow$ 5)-L-Araf - α -(1 $\rightarrow$	(Carpita and Gibeaut, 1993)
α -(1,3)-ArafT	RG-I	L-Araf - α -(1 $\rightarrow$ 3)-L-Araf - α -(1 $\rightarrow$	(Carpita and Gibeaut, 1993)
α -(1,5)-ArafT	RG-I	L-Araf - α -(1 $\rightarrow$ 3)-L-Araf - α -(1 $\rightarrow$	(Carpita and Gibeaut, 1993)
α -(1,3)-ArafT	RG-I	D-Galp - β -(1 $\rightarrow$ 4)-D-Galp - β -(1 $\rightarrow$	(Aspinall et al., 1967; Aspinall et al., 1980; Carpita et al., 2001)
α -(1,5)-ArafT	RG-I	L-Araf -? β -(1 $\rightarrow$ 3)-D-Galp - β -(1 $\rightarrow$	(Aspinall et al., 1967; Aspinall, 1980)
α -(1,6)-ArafT	RG-I	D-Galp - β -(1 $\rightarrow$ 4)-D-Galp - β -(1 $\rightarrow$	(Nakamura et al., 2001)
α -(1,4)-ArafT	RG-I	D-Galp - β -(1 $\rightarrow$ 4)-D-Galp - β -(1 $\rightarrow$	(Huisman et al., 2001)
α -(1,4)-Arap T	RG-I	D-Galp - β -(1 $\rightarrow$ 6)-D-Galp - β -(1 $\rightarrow$	(Nakamura et al., 2001)
β -(1,3)-Arap T	RG-I	L-Araf - α -(1 $\rightarrow$ 5)-L-Araf - α -(1 $\rightarrow$	(Ishii et al., 2005)
α -(1,3)-ArafT	RG-I/AGP	D-Galp - β -(1 $\rightarrow$ 6)-D-Galp - β -(1 $\rightarrow$	(Carpita and Gibeaut, 1993)
α -(1,6)-ArafT	RG-I/AGP	D-Galp - β -(1 $\rightarrow$ 6)-D-Galp - β -(1 $\rightarrow$	(Carpita and Gibeaut, 1993)
α -(1,2)-FucT	RG-I	D-Galp - β -(1 $\rightarrow$ 4)-D-Galp - β -(1 $\rightarrow$	(Lau et al., 1987; O'Neill et al., 1990)
β -(1,4)-GlcAT	RG-I	Gal-?	(An et al., 1994)
β -(1,6)-GlcAT	RG-I	Gal-?	(An et al., 1994)

β -(1,4)-XylT	RG-I	L-Rhap- α -(1,4)-D-GalpA- α -($\rightarrow$	(Nakamura et al., 2002)
β -(1,4)-XylT	RG-I	D-Xylp- β -(1,4)-L-Rhap- α -(1,4)-D-GalpA- α -($\rightarrow$	(Nakamura et al., 2002)
<i>XGA glycosyltransferases</i>			
β -(1,3)-XylT	XGA	D-Galp A- α -(1 $\rightarrow$ 4)-D-Galp A- α -(1 $\rightarrow$	(Schols et al., 1995; Kikuchi et al., 1996; Yu and Mort, 1996; Nakamura et al., 2002)
β -(1,4)-XylT	XGA	D-Xylp - β -(1 $\rightarrow$ 3)-D-Galp A- α -(1 $\rightarrow$	(Nakamura et al., 2002)
β -(1,2)-XylT	XGA	D-Xylp - β -(1 $\rightarrow$ 3)-D-Galp A- α -(1 $\rightarrow$	(Ovodov et al., 1971)
β -(1,4)-XylT	XGA	D-Xylp - β -(1 $\rightarrow$ 4)-D-Xylp - β -(1 $\rightarrow$	(Nakamura et al., 2002)
<i>AGA glycosyltransferases</i>			
β -(1,2)-ApiT	AGA	D-Galp A- α -(1 $\rightarrow$ 4)-D-Galp A- α -(1 $\rightarrow$	(Cheng and Kindel, 1997; Golovchenko et al., 2002)
β -(1,3)-ApiT	AGA	D-Galp A- α -(1 $\rightarrow$ 4)-D-Galp A- α -(1 $\rightarrow$	(Cheng and Kindel, 1997; Golovchenko et al., 2002)
β -(1,3)-ApiT	AGA	D-Apif - β -(1 $\rightarrow$ 2)-D-Galp A- α -(1 $\rightarrow$	(Cheng and Kindel, 1997; Golovchenko et al., 2002)
β -(1,3)-ApiT	AGA	D-Apif - β -(1 $\rightarrow$ 3)-D-Galp A- α -(1 $\rightarrow$	(Cheng and Kindel, 1997; Golovchenko et al., 2002)
<i>RG-II glycosyltransferases</i>			
α -(1,2)-GalAT	RG-II-A	L-Rhap - β -(1 $\rightarrow$ 3')-D-Apif -(1 $\rightarrow$	(Thomas et al., 1989; Puvanesarajah et al., 1991; Reuhs et al., 2004)
β -(1,3)-GalAT	RG-II-A	L-Rhap - β -(1 $\rightarrow$ 3')-D-Apif -(1 $\rightarrow$	(Thomas et al., 1989; Puvanesarajah et al., 1991; Reuhs et al., 2004)
β -(1,3')-RhaT	RG-II-A/B	D-Apif - β -(1 $\rightarrow$ 2)-D-Galp A- α -(1 $\rightarrow$	(Thomas et al., 1989; Puvanesarajah et al., 1991; Reuhs et al., 2004)
α -(1,3)-RhaT	RG-II-B	L-Arap - α -(1 $\rightarrow$ 4)-D-Galp - β -(1 $\rightarrow$	(Thomas et al., 1989; Pellerin et al., 1996; Glushka et al., 2003; Reuhs et al., 2004)

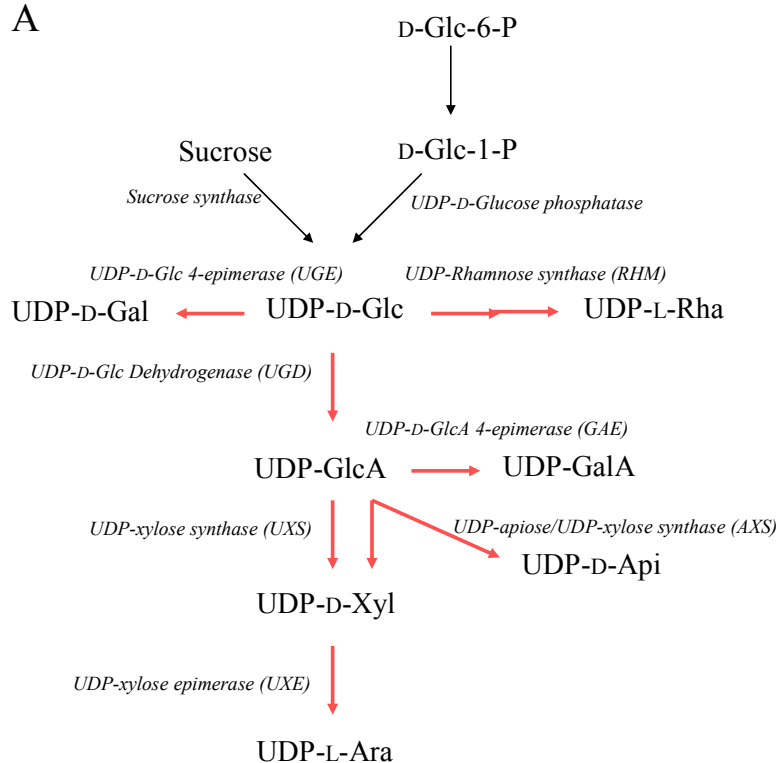
α -(1,2)-RhaT	RG-II-B	L-Arap α -(1 $\rightarrow$ 4)-D-Galp β -(1 $\rightarrow$	(Pellerin et al., 1996; Reuhs et al., 2004)
α -(1,5)-RhaT	RG-II-C	D-Kdop α -(2 $\rightarrow$ 3)-D-Galp A- α -(1 $\rightarrow$	(York et al., 1985; Reuhs et al., 2004)
α -(1,2)-L-GalT	RG-II-B	D-Glcp A- β -(1 $\rightarrow$ 4)-L-Fucp α -(1 $\rightarrow$	(York et al., 1985; Thomas et al., 1989; Reuhs et al., 2004)
β -(1,2)-GalT	RG-II-B	L-Acef A- α -(1 $\rightarrow$ 3)-L-Rhap β -(1 $\rightarrow$	(Thomas et al., 1989; Puvanesarajah et al., 1991; Reuhs et al., 2004)
α -(1,4)-Arap T	RG-II-B	D-Galp β -(1 $\rightarrow$ 2)-L-Acef A- α -(1 $\rightarrow$	(Thomas et al., 1989; Puvanesarajah et al., 1991; Reuhs et al., 2004)
β -(1,2)-AraT	RG-II-B	L-Rhap α -(1 $\rightarrow$ 2)-L-Arap α -(1 $\rightarrow$	(Pellerin et al., 1996; Reuhs et al., 2004)
β -(1,5)-AraT	RG-II-D	D-Dhap β -(2 $\rightarrow$ 3)-D-Galp A- α -(1 $\rightarrow$	(Stevenson et al., 1988; Puvanesarajah et al., 1991; Reuhs et al., 2004)
α -(1,4)-L-FucT	RG-II-A	L-Rhap β -(1 $\rightarrow$ 3')-D-Apif β -(1 $\rightarrow$	(Thomas et al., 1989; Reuhs et al., 2004)
α -(1,2)-L-FucT	RG-II-B	D-Galp β -(1 $\rightarrow$ 2)-L-Acef A- α -(1 $\rightarrow$	(Thomas et al., 1989; Puvanesarajah et al., 1991; Reuhs et al., 2004)
β -(1,2)-ApiT	RG-II-A/B	D-Galp A- α -(1 $\rightarrow$ 4)-D-Galp A- α -(1 $\rightarrow$	(Thomas et al., 1989; Puvanesarajah et al., 1991; Reuhs et al., 2004)
α -(1,3)-XylT	RG-II-A	L-Fucp α -(1 $\rightarrow$ 4)-L-Rhap β -(1 $\rightarrow$	(Thomas et al., 1989; Reuhs et al., 2004)
β -(1,4)-GlcAT	RG-II-A	L-Fucp α -(1 $\rightarrow$ 4)-L-Rhap β -(1 $\rightarrow$	(Thomas et al., 1989; Iwai et al., 2002; Reuhs et al., 2004)
α -(2,3)-KdoT	RG-II-C	D-Galp A- α -(1 $\rightarrow$ 4)-D-Galp A- α -(1 $\rightarrow$	(York et al., 1985; Reuhs et al., 2004)
β -(2,3)-DhaT	RG-II-D	D-Galp A- α -(1 $\rightarrow$ 4)-D-Galp A- α -(1 $\rightarrow$	(Stevenson et al., 1988; Puvanesarajah et al., 1991; Reuhs et al., 2004)
α -(1,3)-AceAT	RG-II-B	L-Rhap β -(1 $\rightarrow$ 3')-D-Apif β -(1 $\rightarrow$	(Thomas et al., 1989; Puvanesarajah et al., 1991; Reuhs et al., 2004)

(Mohnen, 1999; Mohnen et al., 2008). The subcellular localization of pectin biosynthetic HG α 1,4-GalAT (Sterling et al., 2001), RG-I β 1,4-GalT (Geshi et al., 2004) and α 1,5-AraT (Nunan and Scheller, 2003) has been investigated. HG:GalAT activity was detected exclusively in Golgi enriched fractions based on the correlation with latent UDPase activity, a Golgi resident activity (Sterling et al., 2001). Pea homogenates (*Pisum sativum*) were subjected to discontinuous sucrose density gradient, which separates membrane fractions derived from ER, Golgi and the plasma membrane according to relative density. Proteinase K treatment of GalAT activity-positive Golgi fractions, with and without dissolution of membranes by Triton X-100 detergent, demonstrated that GalAT activity is preserved in the presence of Proteinase K without Triton X-100 but is lost after addition of detergent: a situation that can exist only if the active site of the HG:GalAT is located in the lumen of the Golgi, thus being protected from Proteinase K degradation (Sterling et al., 2001). Pectin biosynthetic β 1,4-GalT activity in potato stems (*Solanum tuberosum*) (Geshi et al., 2004) and arabinosyltransferase (AraT) activity in mung bean (*Vigna radiate*) (Nunan and Scheller, 2003) were also localized specifically to the Golgi, suggesting that the pectin biosynthetic machinery is likely located within the Golgi apparatus.

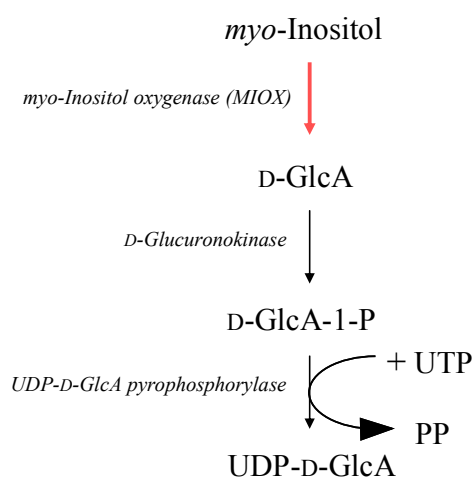
Nucleotide sugar interconverting enzymes in pectin biosynthesis

Complex biosynthetic pathways lead to the synthesis of the specific nucleotide-sugars required for plant pectin biosynthesis (Figure 1.18). Progress has been rapid in the elucidation of the *Arabidopsis thaliana* nucleotide sugar interconverting pathway due to the completion of the *Arabidopsis* genome sequence (Nature 408, 796-815, 2000) (Table 1.4). The nucleotide-sugars may be formed from sugar-phosphates as substrates in a reaction with nucleotide triphosphates to yield nucleotide-sugar diphosphates. Such nucleotide sugars are then converted into a diverse

A



B



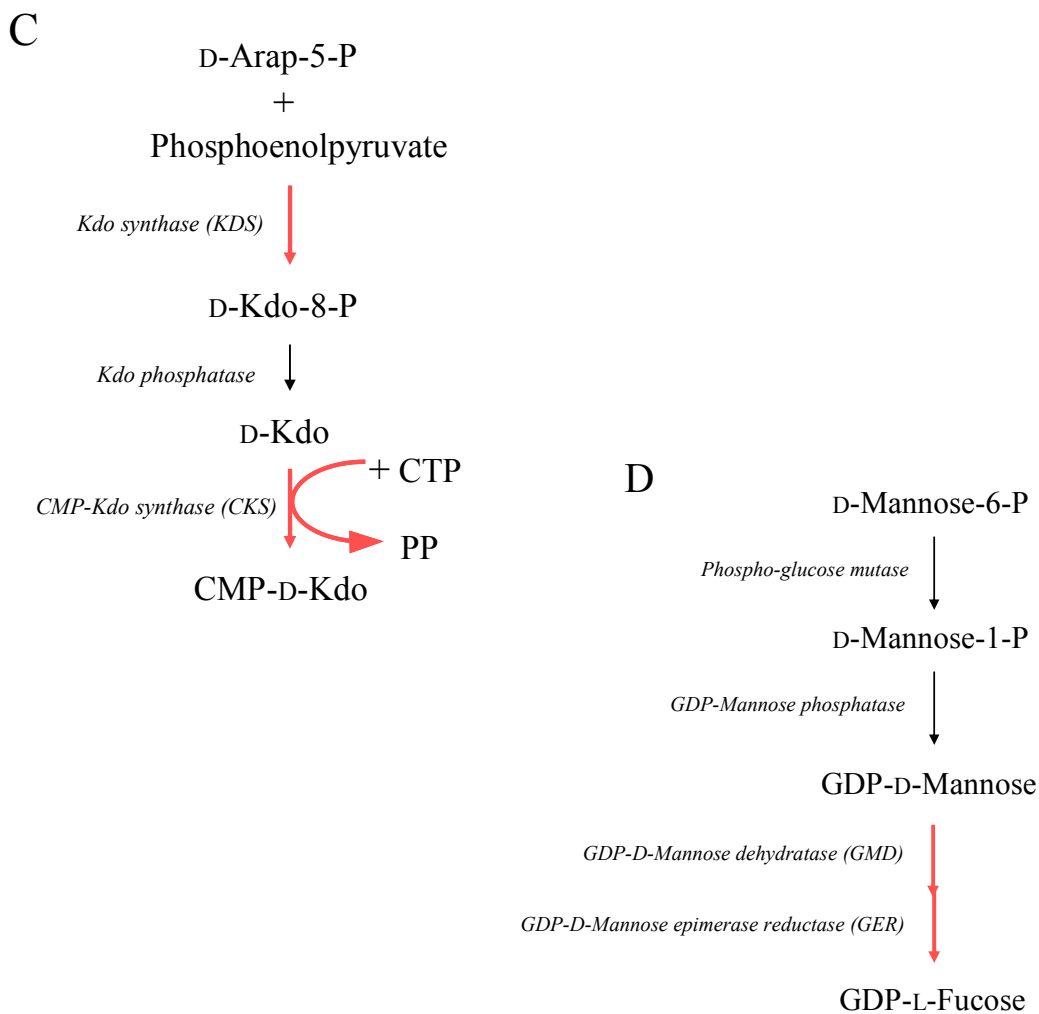


Figure 1.18. The plant nucleotide-sugar interconverting pathway.

(A) Eight different species of nucleotide sugars are created from UDP-D-Glc. (B) UDP-D-GlcA may also be formed from myo-inositol in an alternate pathway. (C) The proposed mechanism of CMP-D-Kdo synthesis in plants. (D) GDP-L-Fucose is synthesized from GDP-D-mannose by the concerted actions of two enzymes.

Table 1.4. Predicted and proven *Arabidopsis thaliana* nucleotide-sugar interconverting enzymes required for pectin biosynthesis^a.

(^a) Adapted from Seifert et al., 2004. (^b) The Enzyme Commission number (E.C.x.x.x.x) of each enzyme activity is listed based on the chemical reaction carried out. (^c) The locus of the *Arabidopsis* gene is given as the AGI code that refers to the position of the gene on *Arabidopsis* chromosomes. (^d) The RHM has catalytic activities equivalent to the bacterial E.C. 5.1.3.13 and E.C. 1.1.1.133 in addition to E.C. 4.2.1.76. (^e) E.C. numbers were not assigned for these activities. (^f) CMP-Kdo synthase enzymes have not been identified in *Arabidopsis*, however the protein and corresponding gene has been identified in maize (*ZmCKS*) and a putative protein accession number has been identified in *Arabidopsis* (AC007202).

Nucleotide sugar interconverting enzyme	Enzymatic reaction ^b	Isoform	Locus ^c	Reference
UDP-glucose epimerase (UGE)	E.C.5.1.3.2	UGE1	At1g12780	(Dormann et al., 1998; Rosti et al., 2007)
		UGE2	At4g23920	(Rosti et al., 2007)
		UGE3	At1g63180	(Rosti et al., 2007)
		UGE4/RHD1	At1g64440	(Seifert et al., 2002; Andeme-Onzighi et al., 2002; Seifert et al., 2004; Rosti et al., 2007)
		UGE5	At4g10960	(Rosti et al., 2007)
UDP-xylose synthase (UXE)	E.C.5.1.3.5	UXE1/MUR4	At1g30620	(Burget et al., 1999; Burget et al., 2003)
		UXE2	At4g20460	
		UXE3	At2g34850	
		UXE4	At5g44480	

UDP-glucuronic acid epimerase (GAE)	E.C.5.1.3.6	GAE1/UGlcAe3	At4g30440	(Gu et al., 2004)
		GAE2/UGlcAe5	At1g02000	(Gu et al., 2004)
		GAE3/UGlcAe4	At4g00110	(Gu et al., 2004)
		GAE4/UGlcAe1	At2g54310	
		GAE5/UGlcAe6	At4g12250	
		GAE6/UGlcAe2	At3g23820	
UDP-glucose dehydrogenase (UGD)	E.C.1.1.1.22	UGD1	At5g39320	(Seitz et al., 2000; Klinghammer et al., 2007)
		UGD2	At3g29360	(Klinghammer et al., 2007)
		UGD3	At5g15490	(Klinghammer et al., 2007)
		UGD4	At1g26590	(Klinghammer et al., 2007)
UDP-xylose synthase (UXS)	E.C.4.1.1.35	UXS1/AUD3	At3g53520	(Harper et al., 2002)
		UXS2/AUD1	At3g62830	(Harper et al., 2002)
		UXS3/SUD2	At5g59290	(Harper et al., 2002)
		UXS4/AUD2	At2g47650	
		UXS5/SUD1	At3g46440	
		UXS6/SUD3	At2g28760	
UDP-rhamnose synthase (RHM)	E.C.4.2.1.76 ^d	RHM1/URS1	At1g78570	
		RHM2/MUM4	At1g53500	(Usadel et al., 2004; Western et al., 2004; Oka et al., 2007)
		RHM3	At3g14790	
		UER1	At1g63000	
UDP-apiose/UDP-xylose synthase (AXS)	^e	AXS1	At2g27860	(Molhoj et al., 2003)
		AXS2	At1g08200	
GDP-mannose dehydrogenase (GMD)	E.C.4.2.1.47	GMD1	At5g66280	(Bonin et al., 2003; Freshour et al., 2003)
		GMD2/MUR1	At3g51160	(O'Neill et al., 2001; van Hengel et al., 2002; Bonin et al., 2003; Freshour et al., 2003; Nakayama et al., 2003)
GDP-mannose epimerase/reductase (GER)	^e	GER1	At1g73250	(Bonin et al., 2000; Nakayama et al., 2003)
		GER2	At1g17890	
GDP-mannose epimerase (GME)	E.C.5.1.3.18	GME1	At5g28840	(Woluka et al., 2001; Woluka et al., 2003; Major et al., 2005)

Kdo synthase (KDS)	E.C.2.5.1.55	KDSA1/KDOPS	At1g79500	(Matsuura et al., 2003; Wu et al., 2004)
		KDSA2	At1g16340	(Matsuura et al., 2003)
CMP-Kdo synthase (CKS)	E.C.2.7.7.38			^f
<i>myo</i> -inositol oxygenase (MIOX)	E.C.1.13.99.1	MIOX4	At4g26260	(Lorence et al., 2004)
			At5g56640	(Lorence et al., 2004)
			At2g19800	(Lorence et al., 2004)
			At1g14520	(Lorence et al., 2004)

array of sugar donor molecules by the nucleotide-sugar interconverting enzymes (NIEs). For this discussion the advances taking place in Arabidopsis NIEs will be covered as a means to condense the material currently existing in the literature in reference to plant and bacterial NIEs.

Nucleotide-sugars are substrates for the enzyme-catalyzed transfer of a sugar group to an acceptor molecule in polysaccharide biosynthesis. If the appropriate nucleotide sugar is not synthesized in the plant, synthesis is hindered. Nucleotide-sugars are supplied to wall biosynthetic glycosyltransferases by NIEs that regulate wall polysaccharide biosynthesis and are themselves regulated by elements of the NIE pathway (Dalessandro and Northcote, 1977; Orellana and Mohnen, 1999). Evidence of NIE regulation of nucleotide-sugar availability to wall biosynthetic enzymes is observed in the biological shift from primary wall to secondary wall synthesis: the abundance of nucleotide sugars and their precursors are coordinately shifted to reflect an up-regulation in hemicellulose and cellulose nucleotide-sugar substrates and a down-regulation in pectic polysaccharide nucleotide-sugar substrates (Dalessandro and Northcote, 1977).

Several Arabidopsis mutants have been isolated that demonstrate the important role of NIEs in pectin biosynthesis. The nucleotide-sugar interconverting pathway mutant, *murI*, showed disrupted RG-II synthesis, stemming from a lesion in a GDP-D-mannose-4,6-dehydrogenase gene (GMD1). GMD1 is a key component in the synthesis of GDP-fucose that is necessary for the correct synthesis of cell wall RG-II and of fucosylated xyloglucans. The RG-II of *murI* does not contain any fucose and as a result does not form stable RG-II borate dimers in the wall, leading to morphological phenotypes indicative of borate deficiency (O'Neill et al., 2001). UDP-D-4-epimerase (UGE) catalyzes the epimerization of UDP-D-Glc to UDP-D-Gal, which has an effect on the specific incorporation of Gal onto XG sidechains, Type-II

arabinogalactan (β -1,6-galactan) and to a lesser extent RG-I (Seifert et al., 2002). The *Arabidopsis* root hair deficient mutants (*rhdl-1-1*, *reb1-1/rhdl-2*, *reb1-2/rhdl-3* and *rhdl-4*) are deficient in UGE4, lack root hairs and show weakened swollen walls of root trichoblast cells (Scheifelbein and Somerville, 1990; Baskin et al., 1992; Ding and Zhou, 1997; Andeme-Onzighi et al., 2002). Monoclonal antibodies specific to fucosylated XG (CCRC M1) and fucosylated AGPs (CCRC M7) showed an absence of CCRC M1 label in *rhdl* root sections and a clear reduction in CCRC M7 label (Seifert et al., 2002). In addition, binding of the AGP-specific monoclonal antibodies, JIM14 and LM2, were reduced in trichoblast cells specifically, indicating the specific and dramatic alterations brought about by a mutation in the NIE UGE4 (Andeme-Onzighi et al., 2002).

Evidence for NIE involvement in, and the regulation of, wall biosynthesis is provided by biochemical and kinetic data on the activities of specific NIEs that are themselves regulated by components of the nucleotide sugar interconversion pathway (Orellana and Mohnen, 1999; Seifert et al., 2004). UDP-D-Xyl is synthesized by the decarboxylation of UDP-D-GlcA by UDP-D-Xyl synthase (UXS), and UDP-D-Xyl is in turn utilized for the synthesis of UDP-D-Ara (Seifert et al., 2004). Representatives of the UXS gene family were expressed in *E. coli* (Harper and Bar-Peled, 2002). The product of UXS (UDP-Xyl) down-regulates upstream components of the nucleotide-sugar interconverting pathway by negative feedback: UDP-Glc dehydrogenase and UDP-Glc pyrophosphorelase are strongly inhibited by UDP-Xyl (Klinghammer and Tenhaken, 2007). The activity of UXS is itself down-regulated by UDP-Xyl, UMP, UDP and UTP (Harper and Bar-Peled, 2002). The activity of an additional NIE is also regulated by other products of the nucleotide-sugar interconverting pathway. The UDP-Api/UDP-Xyl synthase (AXS) synthesizes UDP-Api or UDP-Xyl from UDP-GlcA, depending on reaction conditions.

AXS activity is inhibited by UDP-GalA by as much as 69% (Molhoj et al., 2003). It is proposed that UDP-GalA is a regulator of AXS *in vivo* (Molhoj et al., 2003). Rhamnose biosynthesis (RHM) is responsible for the synthesis of UDP-L-Rha from UDP-D-Glc by three distinct activities; 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 (Oka et al., 2007). The activity of RHM is inhibited by UDP-Rha, UDP-Xyl and UMP and thus regulates the levels of UDP-Glc and UDP-Rha in the cell (Oka et al., 2007). Regulation of NIEs in plant cells by negative feedback inhibition is a mechanism by which the biosynthesis of pectins and other wall polysaccharides are, in-part, controlled (Seifert, 2004).

HG glycosyltransferases

The HG backbone is a polymer of α 1,4-linked GalpA residues and is proposed to require several α 1,4-GalATs (HG:GalATs) to synthesize the entire complement of HG required throughout plant development. HG:GalATs specifically catalyze the transfer of D-GalpA from UDP-D-GalpA to a growing stretch of HG (E.C. 2.4.1.43) via a lumenally facing HG:GalAT catalytic domain (Sterling et al., 2001). GAUT1, the only known HG:GalAT has been shown to be Golgi localized, and the other GAUT1-related gene family members are also predicted to be Golgi localized Type-II membrane proteins (Sterling et al., 2006). These predictions are consistent with crystallographic structures of the related lipopolysaccharide α 1,4-galactosyltransferase LgtC from *Neisseria meningitidis* (Gastinel et al., 2001; Persson et al., 2001; Lairson et al., 2004).

HG:GalAT activity has been documented in several species with variable degrees of kinetic analysis and product characterization (Table 1.5). The synthesis of plant HG:GalAT was

Table 1.5. Pectin biosynthetic galacturonosyltransferase (GalAT) activity.

(^a) The activity was detected from membrane particulate preparations (particulate), detergent-permeabilized membranes (permeabilized) or detergent-solubilized membranes (solubilized). (^b) The acceptor may be endogenous (that existing in the preparation) or exogenous (previously extracted pectins of variable purity). N.D: Not determined.

Plant Source	Fraction ^a	pH optimum	Apparent Km for UDP-Galp A (μM)	V _{max} (pmol min ⁻¹ mg ⁻¹)	Acceptor ^b	Reference
<i>Vigna radiata</i>	particulate	6.3-7.0	1.7	4700	endogenous	(Villemez et al., 1966)
<i>Vigna radiata</i>	particulate	ND	ND	ND	endogenous	(Lin et al., 1966)
<i>Brassica rapa</i>	particulate	ND	ND	ND	endogenous	(Lin et al., 1966)
<i>Lycopersicon esculentum</i>	particulate	ND	ND	ND	endogenous	(Lin et al., 1966)
			770			(Bolwell et al., 1985)
<i>Nicotiana tabacum</i>	particulate	7.8	8.9	150	exogenous/endogenous	(Doong et al., 1995;
<i>Nicotiana tabacum</i>	solubilized	6.3-7.8	37	290	exogenous	(Doong and Mohnen 1998;
						Scheller et al., 1999)
<i>Vigna angularis</i>	permeabilized	6.8-7.8	140	1650	exogenous	(Takeuchi and Tsumuraya
<i>Pisum sativum</i>	permeabilized	ND	ND	ND	exogenous/endogenous	(Sterling et al., 2001)
<i>Petunia axillaris</i>	solubilized	7.0	170	640	exogenous	(Akita et al., 2002)
<i>Cucurbita moschata</i>	particulate	6.8-7.3	1700	15,000	exogenous	(Ishii et al., 2002)
<i>Arabidopsis thaliana</i>	solubilized	ND	ND	ND	exogenous	(Sterling et al., 2006)
<i>Pisum sativum</i>	particulate	ND	ND	ND	exogenous	(Abdel-Massih et al., 2007)

activity first critically evaluated in pea (Villemez et al., 1966). Unmethylated UDP-D-GalA was the preferred nucleotide-sugar substrate for elongation of endogenous acceptors in particulate membrane fractions of mung bean (*Vigna radiata*) (Villemez et al., 1966). HG:GalAT activity has also been characterized in tomato (*Lycopersicon esculentum*), turnip (*Brassica rapa*) (Lin et al., 1966; Bolwell and Northcote, 1983b), tobacco (*Nicotiana tabacum*) (Doong et al., 1995), azuki bean (*Vigna angularis*) (Takeuchi and Tsumuraya, 2001), pea (*Pisum sativum*) (Sterling et al., 2001), petunia (*Petunia axillaris*) (Akita et al., 2002), pumpkin (*Cucurbita moschata*) (Ishii, 2002) and Arabidopsis (*Arabidopsis thaliana*) (Sterling et al., 2006; Abdel-Massih et al., 2007).

The solubilization of HG:GalAT activity from tobacco microsomal membrane particulate preparations was achieved in HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) pH 6.8, 40 mM CHAPS detergent (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) and 2 mM EDTA (ethylenedinitrilotetraacetic acid) (Doong and Mohnen, 1998). The solubilized enzyme catalyzed the transfer of GalA from UDP-D-GalpA to the non-reducing end of OGA acceptors of DP 10 or greater (Scheller et al., 1999). The products formed were of an α -(1,4)-configuration as demonstrated by the endo- or exopolygalacturonase degradation of the reaction products to yield GalpA (Doong and Mohnen, 1998). The tobacco solubilized enzyme, under the conditions used, added only single GalpA residues in a non-processive manner (Doong and Mohnen, 1998). However, the pumpkin detergent-permeabilized GalAT activity catalyzed the addition of 5 GalpA residues (Ishii, 2002), while petunia solubilized GalAT activity added up to 27 GalpA residues (Akita et al., 2002), with the number of consecutive GalAs added being dependent on the concentration of UDP-GalA used. HG oligos are subject to the consecutive addition of multiple GalA residues, however, the bulk of the *in vitro* HG:GalAT data strongly suggest that HG:GalATs are not processive.

The first gene encoding a HG:GalAT was isolated from Arabidopsis suspension cultures (Sterling et al., 2006). LC-MS-MS of trypsin-digested partially purified solubilized Arabidopsis GalAT active fractions identified two proteins in a NCBI protein-protein BLAST (<http://www.ncbi.nlm.nih.gov/>) corresponding to JS36/GAUT1 (locus; At1g61130) and JS33/GAUT7 (locus; At2g38650). Anti-JS36 polyclonal antibodies were able to immunoadsorb GalAT activity from the partially purified Arabidopsis solubilized GalAT enzyme preparations, providing evidence that the JS36/GAUT1 gene encodes a pectin biosynthetic HG:GalAT (Sterling et al., 2006).

The open reading frames of JS36/GAUT1 (JS36 Δ 1-43) and JS33/GAUT7 (JS33 Δ 1-41) were amplified from Arabidopsis total RNA and truncated to remove the transmembrane domain for expression in human embryonic kidney (HEK293) cells. A *typanosoma cruzi* mannosidase signal sequence and C-terminal HA epitope tag was engineered onto JS36 and JS 33 allowing the recombinant protein to be secreted directly to the culture medium. The truncated JS36/GAUT1 and JS33/GAUT7 proteins were recovered from the HEK cell medium by immunoprecipitation with anti-HA antibodies from the cell medium. JS36/GAUT1, but not JS33/GAUT7, immunoprecipitates yielded incorporation of [¹⁴C]GalpA into discreetly-sized OGA acceptors when incubated with UDP-[¹⁴C]GalpA. JS36/GAUT1, however, catalyzed elongation of OGA acceptors in an α -(1,4)-configuration; an activity consistent with a role of JS36/GAUT1 as an HG:GalAT in pectin biosynthesis.

The DNA sequences encoded by JS36/GAUT1 and JS33/GAUT7 correspond to glycosyltransferases in a distinct subfamily of the GT8 family of the CAZy database (<http://www.cazy.org/>) (Coutinho et al., 2003), which includes 15 GAUT genes and 10 GATL genes. The GAUT1-related family is defined by a C-terminal amino acid motif common to both

the GAUT and GATL proteins, but that is not a characteristic of other GT8 member proteins: [H-[FWY]-[DNS]-G-x (2)-K-P-W-x (2)-[ILH]-[ADGS]] (Sterling et al., 2006). The GAUT and GATL proteins are each defined by additional amino acid motifs unique to each group (Figure 1.19). It has been proposed that the GAUT1-related gene family may encode GalATs required to synthesize the many specific Gal α A linkages of the wall complex polysaccharides (see Table 1.3). Thus, further study of this gene family may lead to the discovery of many unique GalAT activities. In addition, many new structures may be identified in the process of defining the expression patterns and the specific linkages catalyzed by the genes of the GAUT1-related gene family.

Pectin methyltransferase

The modification of pectic polysaccharides by addition of methyl groups at the C-6 or acetyl groups at the O-2 or O-3 of GalA residues is regulated in a developmental manner (Willats et al., 2001b). Pectin methyltransferases (PMTs) act specifically on pectic polysaccharides during synthesis in the Golgi apparatus. Pectins are secreted in a highly methylated form (Li et al., 1997; Lennon and Lord, 2000). After the deposition of pectins at the apoplast, pectic methylesterases (PMEs) selectively remove the methyl groups. The extent and pattern of methylation of pectic polysaccharides affects the functional characteristics of the pectic polysaccharides (Powell et al., 1982), for example, by altering the affinity of pectins for calcium ions and altering the accessibility of pectins to wall hydrolases (Benen et al., 1999). The pectic polysaccharides are methylated by PMTs that catalyze the transfer of a methyl group from the

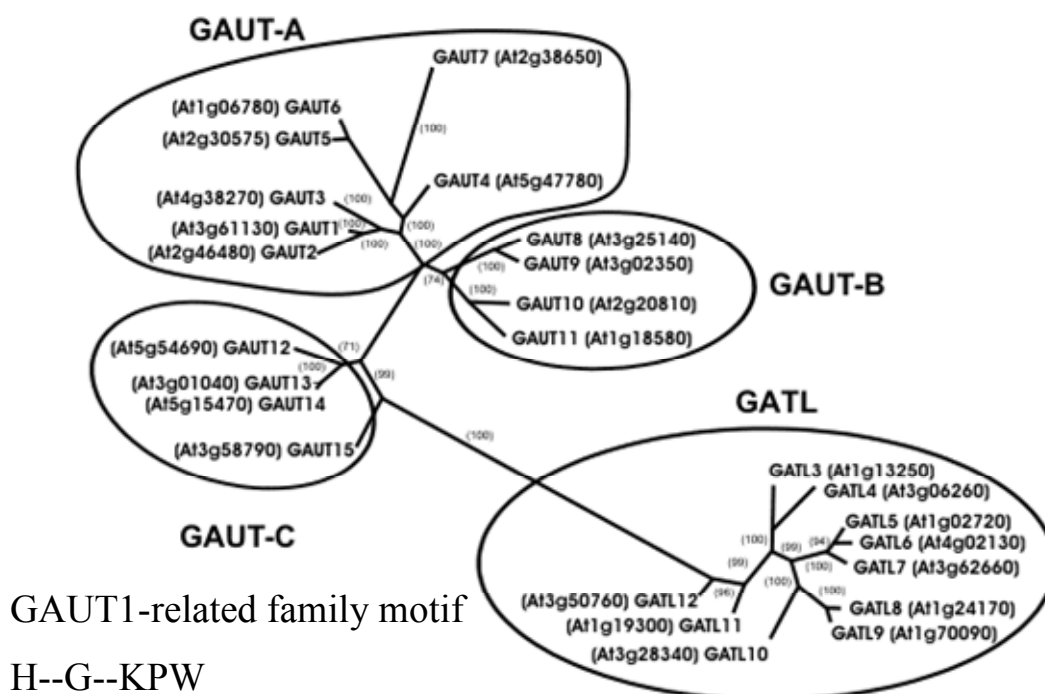


Figure 1.19. The *Arabidopsis thaliana* GAUT1-related gene family as defined by Sterling et al., (2006).

The *Arabidopsis thaliana* galacturonosyltransferase-1 (GAUT)-related gene family has 15 GAUT genes and 10 GAUT-like (GATL) genes. Proteins in the GAUT1-related gene family have shared amino acid motifs not conserved among other GT8 proteins. The GAUT and GATL proteins also have unique defining amino acid motifs.

donor S-adenosyl-methionine (SAM) to the target pectic polysaccharide (Villemez et al., 1966; Kauss et al., 1967; Bruyant-Vannier et al., 1996). The import of SAM into the Golgi lumen is necessary for the methylation of HG (Ibar and Orellana, 2007) where PMT activities have been localized (Goubet and Mohnen, 1999; Dunkley et al., 2006; Mouille et al., 2007). The catalytic activity of PMTs has been detected and partially characterized in the extracts of multiple plant species (Table 1.6): mung bean (*Vigna radiata*) (Kauss and Hassid, 1967), flax (*Linum usitatissimum*) (Vannier et al., 1992), tobacco (*Nicotiana tabacum*) (Goubet et al., 1998) and soy bean (*Glycine max*) (Ishikawa et al., 2000) (Table 1.6).

Multiple PMT enzymes are required for pectin synthesis as evidenced by the discovery of PMT isoforms in flax (*Linum usitatissimum* L.) Golgi membranes separated in sucrose density gradients. The PMT isoforms were identified as PMT activities that had distinct pH optima and also had a differential preference for substrates with high or low levels of pectin methylation (Bourlard et al., 1997). Indeed, an assortment of PMTs may be required to form the methylesters of HG in a regulated fashion.

The biosynthesis of pectin is dependant on the action of PMTs. It was shown in flax (Bourlard et al., 1997) and mung bean (Kauss and Swanson, 1969) that addition of exogenous pectin acceptors increased rates of PMT activity. Excessive SAM substrate, however, does not have this affect on PMT activity. Flax microsomal membranes yielded three partially purified polypeptides of distinct sizes with PMT activity: PMT5 (40 kDa), PMT7 (110 kDa) and PMT18 (18 kDa). The PMT18 protein appeared to harbor PMT activity and was found in partially purified preparations of both PMT5 and PMT7, suggesting that PMT18 may be the catalytic subunit of PMT5 and PMT7. Confirmation that these proteins are PMTs has not yet been presented.

Table 1.6. Pectin biosynthetic pectin methyltransferase (PMT) activity.

(^a) Particulate (particulate), detergent-permeabilized (permeabilized) or detergent-solubilized (solubilized) membranes were used. (^b) The HG acceptor, with variable levels of further characterization except where noted (high MeOxyl or low MeOxyl-HG). (^c) The PMT activity was further purified from a solubilized membranes preparation to yield PMT5, PMT7 and PMT18. N.D.: Not determined.

Plant Source	Fraction ^a	pH optimum	Apparent Km for SAM (μ M)	$V_{\max}$ (pmol min ⁻¹ mg ⁻¹)	Acceptor ^b	Reference
<i>Vigna radiata</i>	particulate	6.8	59	2.7	HG	(Kauss & Hassid, 1967; Kauss et al., 1967; Kauss & Swanson, 1969)
<i>Linus usitatissimum</i>	particulate	6.8	10-30	ND	HG	(Vannier et al., 1992)
<i>Linus usitatissimum</i>	solubilized				HG	(Bruyant-Vannier et al., 1996)
<i>Linus usitatissimum</i>	particulate	5.5	20	ND	low MeOxy-HG	(Bourlard et al., 1997; Bourlard et al., 1997b; Bourlard et al., 2001)
<i>Linus usitatissimum</i>	particulate	7.0	20	ND	high MeOxy-HG	(Bourlard et al., 1997; Bourlard et al., 1997b; Bourlard et al., 2001)
<i>Nicotiana tabacum</i>	particulate	7.8	38	49	HG	(Goubet et al., 1998, Goubet and Mohnen, 1999)
<i>Nicotiana tabacum</i>	solubilized	7.8	18	7.3	HG	(Goubet et al., 1999)
<i>Glycine max</i>	permeabilized	6.8	230	600-1300	HG	(Ishikawa et al., 2000)
<i>Linus usitatissimum</i>	solubilized ^c	ND	ND	ND	HG	(Bourlard et al., 2001)

Mutagenesis of the Arabidopsis genome has led to the identification of a putative PMT mutant at the At1g78240 gene locus that is predicted to encode a ~43 kDa protein with sequence similarity to known methyltransferases. The mutant is referred to as the *quasimodo-2/tumorous shoot development-2* (*qua2/tsd2*) mutant (Krupkova et al., 2007; Mouille et al., 2007). Based on the conservation of the MT domain of QUA2/TSD2, the Arabidopsis genome contains 29 putative PMT homologs, while rice has at least 14 conserved homologs. The catalytic activity of QUA2/TSD2 has not been definitively determined, either *in vivo* or *in vitro*. Evidence for QUA2/TSD2 PMT function in pectin biosynthesis is its pleiotropic phenotype similar in several relevant aspects to *qual1*, a putative pectin HG:GalAT (Bouton et al., 2002). *Qua2/tsd2* has reduced cell adhesion, particularly at the meristems, and is specifically reduced in HG, but not RG-I pectic polysaccharides (Krupkova et al., 2007; Mouille et al., 2007). Interestingly, the QUA2 transcript was found to be co-expressed with *QUA1/GAUT8*, suggesting a functional linkage between QUA1 and QUA2. The transcripts of additional QUA2 isoforms were co-expressed with GAUT9 and GAUT1 (Mouille et al., 2007). The cooperativity of PMT activity with exogenously added HG acceptors in mung bean (Kauss et al., 1967) and the co-expression of GAUT and PMT transcripts, may reflect a physical dependence of HG:GalATs on PMTs.

Pectin acetyltransferase

Pectin *O*-acetyltransferase (PAT) activity catalyzes the transfer of an acetyl group from acetylCo-A to a pectic polysaccharide acceptor. Acetyl groups decorate the GalA residues of pectic polysaccharides at the O-2 or O-3 positions (Ishi, 1997). Acetyl groups may decorate the GalpA residues of HG and RG-I, however, acetylation was not detected on the RG-II backbone (Ishi, 1997). *O*-acetylation was detected, however, on the 4-*O*-methyl-fucose residue and aceric

acid residue of RG-II sidechain B (Whitcombe et al., 1995). The functional consequences of acetylation are not clear but may play a role in preventing pectin breakdown by microbial hydrolases. PAT activity has been detected in microsomal membrane preparations of potato (*Solanum tuberosum*) (Pauly and Scheller, 2000). The PAT activity at 30°C was found to have a pH optimum of 7.0, an apparent K_m of 35 μM for acetyl-CoA and a V_{max} of 0.9 pkat mg^{-1} protein (Pauly and Scheller, 2000).

RG-I backbone glycosyltransferases

The biosynthesis of RG-I requires multiple glycosyltransferase activities to synthesize a backbone of $[\rightarrow 2)\text{-}\alpha\text{-L-Rhap-(1,4)-}\alpha\text{-D-GalpA-(1}\rightarrow]$ disaccharide repeats that are branched at the C-4 of approximately half of the rhamnose residues by 5-linked and 3,5-linked arabinan, 4-linked and 4,6-linked galactan, as well as Type-I and Type-II AG (Ridley et al., 2001). Potentially 34 specific activities may be required for the synthesis of the RG-I backbone and complex arabinan and galactan sidechains (see Table 1.3) (Mohnen, 2002).

The $\alpha 1,4$ -rhamnosyltransferase ($\alpha 1,4$ -RhaT) and α -1,2-galacturonosyltransferase ($\alpha 1,2$ -GalAT) responsible for synthesis of the RG-I backbone have not been identified. Bacterial genes are useful as potential models with which to query plant genomes and may be of use in identification of the RG-I:RhaT. Recently, an $\alpha 1,3$ -RhaT (WapR) and an $\alpha 1,6$ -RhaT (MigA) were identified in *Pseudomonas aruginosa* that are responsible for the transfer of rhamnosyl residues to the core glycan structure of lipopolysaccharide (LPS) (Poon et al., 2008). Bacterial nucleotide interconverting genes have successfully been used to identify the nucleotide interconverting enzymes UGE1 in *Arabidopsis thaliana* from *Streptococcus pneumoniae* Cap1J bacterial UGE (Munoz et al., 1999; Gu and Bar-Peled, 2004). The RHM2 in *Arabidopsis*

thaliana was identified from bacterial protein family hidden markov models (HMM) of NDP-rhamnose synthesis (PFAM; PF01370) (Usadel et al., 2004). In addition, hydrophobic cluster analysis in conjunction with the identified conserved glycosyltransferase domains of *Acetobacter xylinum* cellulose synthase (CesA) were used to identify CesA homologs in plants (Saxena et al., 1994; Delmer, 1999). Hydrophobic cluster analysis aids in the identification of homologs across species that may not retain great conservation of the primary amino acid sequence but retain function based on the secondary protein structure predicted by hydrophobic amino acid clustering (Callebaut et al., 1997). These methods provide hope for identification of additional pectin GTs in the future.

RG-I galactosyltransferases

The galactan side chains of RG-I are composed primarily of β -1,4-D-galactan with some branches of β -1,6- and β -1,3-Gal. The galactosyltransferases (GalTs) catalyze transfer of D-Galp residues from UDP-D-Galp to an acceptor molecule. Pectin GalTs are hypothesized to catalyze the initiation of galactan side chains directly onto the backbone Rhap residues of RG-I, elongate galactan chains, initiate branch points onto galactan chains and elongate galactan side chains. Synthesis of these structures may require up to 10 or more different pectin specific GalTs (see Table 1.3) (Mohnen, 2002).

The characterization of GalT activities necessary for RG-I galactan biosynthesis has been carried out in flax (*linus usitatissimum*) (Goubet and Morvan, 1993), mung bean (*Vigna radiate*) (Brickell and Reid, 1996), potato (*Solanum tuberosum*) (Geshi et al., 2000), radish (*Raphanus sativus*) (Kato et al., 2003) and soy bean (*Glycine max*) (Konishi et al., 2004) (Table 1.7). The β 1,4-GalT activity of potato (Geshi et al., 2004), radish (Kato et al., 2003) and pea homogenates

Table 1.7. Pectin biosynthetic galactosyltransferase (GalT) activity.

^(a) Particulate (particulate), detergent-permeabilized (permeabilized) or detergent-solubilized (solubilized) membranes were used. ^(b)

The acceptor molecules used in the references listed were both endogenous and exogenous have been characterized to a greater or lesser degree; additional information is noted as available. N.D.: Not detected.

Plant Source	Fraction ^a	pH optimum	Apparent Km for UDP-Galp (μM)	V _{max} (pmol min ⁻¹ mg ⁻¹)	Linkage	Acceptor ^b	Reference
<i>Linum usitatissimum</i>		8.0	38	75	β-1,4-	endogenous	(Goubet and Morvan, 1993; Goubet and Morvan, 1994)
<i>Linum usitatissimum</i>		5.0	38	75	β-1,3 or β-1,6-	endogenous	(Goubet and Morvan, 1993; Goubet and Morvan, 1994)
<i>Vigna radiata</i>		6.5	ND	ND	β-1,4-	endogenous	(Brickell and Reid, 1996)
<i>Solanum tuberosum</i>	particulate	6.0	ND	ND	β-1,4-	endogenous RG-I/exogenous galactan	(Geshi et al., 2000; Geshi et al., 2004)
<i>Linum usitatissimum</i>	solubilized	8.0	460	180	β-1,4-	exogenous RG-I/galactan	(Peugnet et al., 2001)
<i>Solanum tuberosum</i>	solubilized	7.5	ND	ND	β-1,4-	exogenous RG-I/galactan	(Geshi et al., 2002)
<i>Solanum tuberosum</i>	solubilized	5.6	ND	ND	?	exogenous RG-I/galactan	(Geshi et al., 2002)
<i>Pisum sativum</i>	particulate	7.0-8.0	ND	ND	β-1,4-	endogenous RG-I/galactan/XG	(Abdel-Massih et al., 2003)
<i>Raphanus sativus</i>	particulate	5.9-6.3	410	1000	β-1,6-	exogenous RG-I/galactan	(Kato et al., 2003)
<i>Vigna radiata</i>	permeablized	6.5	32	200	β-1,4-	exogenous β-1,4-galactan	(Ishii et al., 2004)
<i>Glycine max</i>	permeablized	6.5	1200	2,000-3,000	β-1,4-	exogenous β-1,4-galactan	(Konishi et al., 2004)
<i>Glycine max</i>	solubilized	6.5	ND	17,000	β-1,4-	exogenous β-1,4-galactan	(Konishi et al., 2004; Konishi et al., 2007)

(Boydoun et al., 2001) was localized to the Golgi apparatus by sucrose density gradient centrifugation. Fractionation of Golgi membranes into high-, medium- and low-density microsomes showed that the galactan β 1,4-GalT activity had a differential distribution in density gradients from glycoprotein GalT activity (Boydoun et al., 2001). The products of the above pectin GalT activity were not characterized, and thus, may represent both an elongating and branching galactan GalT activity.

Pectin RG-I:GalT activity that specifically elongates existing Gal branches on the RG-I backbone was recovered from microsomal membrane preparations of mung bean. The GalTs catalyzed the transfer of a Galp onto β -1,4-galactan acceptor molecules (Brickell and Reid, 1996; Ishii et al., 2004). Cleavage of the reaction products specifically by endo- β -1,4-galactanase verified the linkage catalyzed. Similar experiments were carried out in potato (Geshi et al., 2000) and soybean (Konishi et al., 2004). The GalT activity in pea microsomal membranes elongated a β -1,4-galactan acceptor that was associated with a high molecular weight PG- and RG-A lyase-digestible pectin (Abdel-Massih et al., 2003). The partially purified pectin biosynthetic GalT activity isolated from soybean was able to transfer more than 25 galactosyl units onto an oligogalactan of DP 7, which is approaching the length of native pectin galactan branches isolated from soybean RG-I (Konishi et al., 2007).

The GalT activity that elongates a growing galactan chain is discerned from GalT activity that initiates a Gal linkage onto the rhamnosyl C-4 of the RG-I backbone or onto the C-4 of an existing Gal sidechain of the RG-I backbone. GalT activity that utilizes an $[\beta$ -D-Galp-(1 $\rightarrow$ 4)- α -L-Rhap-(1 $\rightarrow$] acceptor was identified in potato microsomal membranes (Geshi et al., 2002). This GalT activity was suggestive of a chain initiation GalT that adds a single Gal onto the firstly added Gal on the RG-I backbone. Such an activity would catalyze the second initiating step in

RG-I galactan synthesis. This activity catalyzed the addition of Gal residues from UDP- $[^{14}\text{C}]$ galactose onto small RG-I backbone fragments with a low degree of galactosylation, but not onto RG-I with a high degree of galactosylation, RG-I without galactosylation, galactan or galactooligomers. In addition, the activity was separable from a different β 1,4-GalT activity that did incorporate $[^{14}\text{C}]$ Gal onto highly galactosylated RG-I (Geshi et al., 2002). Similarly, a GalT activity isolated from flax elongated existing single Gal residues of RG-I; a potential GalT involved in galactan chain initiation (Peugnet et al., 2001).

The β -1,3-galactan and β -1,6-galactan structures are primarily associated with wall AGPs, however, some have been isolated in association with RG-I sidechains. A GalT activity isolated from 6-day old radish root microsomal membrane preparations showed elongation of β -1,3- and β -1,6-galactooligomers (Kato et al., 2003). The elongation of de-arabinosylated AGs from AGP polysaccharides were also elongated by the GalT in these fractions (Kato et al., 2003).

The Arabidopsis *MUR3* encodes a glycosyltransferase in GT47 that is predicted to be an XG-GalT. GT47 has 9 subfamilies (A, B, C1, C2, C3, D1, D2, E and F) that show varying similarity to animal exostosins. *MUR3* is a putative GalT based on wall glycosyl residue composition phenotype that has a distinct reduction in wall Gal content. Further characterization of the *mur3* mutant plants had a wild-type-like phenotype but wall analyses showed that the xyloglucan of *mur3* mutants plants had reduced galactosylation. The data suggest that *MUR3* encodes a galactosyltransferase that is specific for transfer of a galactose to the third xylose substituted Glc in a xyloglucan XXXG unit (Madson et al., 2003).

RG-I arabinosyltransferases

The synthesis of α -1,5-linked arabinan by α 1,5-ArafT involves the transfer of an Araf residues from UDP-L-Araf to acceptor molecules in an α -(1,5) configuration. As many as 18 AraTs may be required for the synthesis of the complex branched arabinans of pectic polysaccharides (Mohnen, 2002). AraT activity has been characterized in mung bean (*Vigna radiata*) (Odzuk and Kauss 1967) and French bean (*Phaseolus vulgaris*) (Bolwell and Northcote, 1983a) (Table 1.8). Pectin biosynthetic AraT activity is derived from membrane fractions and requires Mn^{+2} ions.

The synthesis of arabinan by an AraT was first observed in mung bean permeabilized microsomal membranes (Odzuck and Kauss, 1972). The product of addition of UDP- $[^{14}C]$ arabinose to mung bean permeabilized membranes was an arabinan, as characterized by paper chromatography of the sugars released by acid hydrolysis (Odzuck and Kauss, 1972). Similar experiments carried out in french bean (*Phaseolus vulgaris*) produced products that were pectinase-degraded (Bolwell and Northcote, 1983a). Further characterization of the reaction products was not carried out, and thus, the structure of the acceptor was not identified. In 1992 a partially purified 70 kDa putative AraT was recovered from active fractions using a monoclonal antibody found to inhibit AraT activity in microsomal membranes (Rodgers and Bolwell, 1992). Despite the repeated solubilization of AraT activity and the partial purification of a putative AraT protein, the AraT was not identified and the enzyme products of the putative AraT were not rigorously characterized. Recently, however, an authentic α 1,5-ArafT activity was observed by the incubation of UDP-L-Araf with mung bean hypocotyl Golgi membranes and exogenous α -1,5-L-arbinooligosaccharides. The products were determined to be α -1,5-linked by α -L-arabinofuranosidase cleavage and NMR structure determination (Konishi et al., 2006).

Table 1.8. Pectin biosynthetic arabinosyltransferases (AraT) activity.

^(a) Particulate (particulate), detergent-permeabilized (permeabilized) or detergent-solubilized (solubilized) membranes were used. ^(b)

The acceptors utilized in the referenced studies were endogenous or exogenous with variable degrees of characterization; added information is listed where available. N.D.: Not detected

Plant Source	Fraction ^a	pH optimum	Apparent Km for UDP-Ara (μM)	V_{max} ($\text{pmol min}^{-1} \text{mg}^{-1}$)	Linkage	Acceptor ^b	Reference
<i>Vigna radiata</i>	permeablized	6.0-6.5	ND	ND	? Ara	endogenous	(Odzuk and Kauss, 1972)
<i>Phaseolus vulgaris</i>			178			endogenous	(Bolwell and Northcote, 1981; Bolwell, 1983)
<i>Phaseolus vulgaris</i>	solubilized ^c			199,000	?-Ara	endogenous	(Rogers and Bolwell, 1984)
<i>Vigna radiata</i>	solubilized	6.5	33,000	ND	?-Ara _p	exogenous α -1,5-arabinan	(Nunan et al., 2003)
<i>Vigna radiata</i>	solubilized	6.5-7.0	550	1700	β -1,3-Ara _f	exogenous α -1,5-arabinan	(Ishii et al., 2005)
<i>Vigna radiata</i>	solubilized	6.0-6.5	330	200	β -1,4-Ara _p	exogenous β -1,4-galactan	(Ishii et al., 2005b)
<i>Vigna radiata</i>	solubilized	6.5-7.0	234	ND	α -1,5-Ara _f	exogenous α -1,5-arabinan	(Konishi et al., 2006)

The complex branched arabinans of pectic RG-I polysaccharides also require the activities of a β 1,3-ArapT that was identified in mung bean membrane preparations (Nunan and Scheller, 2003; Ishii et al., 2005a; Ishii et al., 2005b). The mung bean Golgi-enriched membranes incorporated L-[^{14} C]Arap onto endogenous 1,5-linked arabinofuranooligos, but very inefficiently. The reaction products were not released with an α 1,5-arabinofuranosidase and only approximately 25% of the radio-labeled products were released by an arabinofuranosidase that cleaved 1,2-, 1,3- or 1,5- linkages (Nunan and Scheller, 2003). Similar activity assays were carried out with mung bean Golgi membranes incubated with UDP- β -L-Arap and 2-aminobenzamide (2-AB) labeled 1,5-linked arabinofuranoheptasaccharides. The reaction products were found to be β -1,3-Arap residues linked to the terminal Ara β residues of the 2-AB labeled acceptors (Ishii et al., 2005a). The linkage and ring conformation of the terminal β -linked residue was determined by linkage analysis of partially methylated alditol acetates and NMR spectroscopy (Ishii et al., 2005a).

A β 1,4-ArapT activity was identified that catalyzes the transfer of a terminal β -Arap from UDP- β -L-Arap onto the terminal non-reducing residue of a β -1,4-galactooligosaccharide labeled at the reducing end with 2-AB (Ishii et al., 2005b). The conformation of the reaction products was determined by ESI-MS-MS, linkage analysis and NMR. The linkage described naturally occurs in RG-I galactan sidechains (Huisman et al., 2001). The Ara1Gal7-2-AB molecule was not further utilized as an acceptor for either AraT or GalT activity, suggesting that the terminal Ara residue may act as a cap or terminator of RG-I sidechain synthesis (Ishii et al., 2005b).

The *Arabidopsis* T-DNA insertion mutant *arad1*, had reduced cell wall arabinan, with little change in the composition of other glycosyl residues and no apparent physical phenotype and is hypothesized to be a putative cell wall AraT (Harholt et al., 2005). The mutant wall

compositional phenotype was complemented by the ARAD1 transgene under the 35S promoter. ARAD1 (locus At2g35100) is a GT47 glycosyltransferase that has 7 additional Arabidopsis homologs and 4 rice homologs (Harholt et al., 2005).

RG-II glycosyltransferases

The backbone of RG-II is α -1,4-D-GalpA-linked HG. GAUT1 has recently been discovered, which catalyzes the addition of GalA residues onto HG oligomers (Sterling et al., 2006), and could, conceivably, synthesize the backbone of RG-II. There are 15 GAUT genes in the GAUT1-related gene family, and it is also possible that one of these may function to specifically synthesize the backbone of RG-II. Further work in this area will be needed to explore this possibility, because the acceptor specificities for the GAUTs have not been determined.

A putative glucuronosyltransferase (GlcAT) was identified in a screen for intercellular adhesion defects in T-DNA transformants of *Nicotiana plumbaginifolia nolac-H18/irx10*. The gene identified that causes the *nolac* phenotype had nucleotide sequence similarity to the human, *C. elegans* and *D. melanogaster* heparin sulfate GlcAT exostosin 2 (EXT2) (Iwai et al., 2002). Complementation of the mutant with *NpGUT1* under the 35S promoter returned WT-like cellular adhesion to the cells. Because the mutant was shown to have reduced efficiency of RG-II borate dimer formation and reduced GlcA in purified RG-II preparations, the linkage disrupted was hypothesized to be the RG-II sidechain A GlcA residue that is linked to the C-4 of the Fuc residue. The absence of this residue would also eliminate a Galp residue linked to the C-2 of the GlcpA residue. Unfortunately, the activity of the protein encoded by GUT1/IRX10 has not been

determined. Currently, other GTs that act to synthesize RG-II sidechain structures are not known, leaving large informational gaps in the biosynthesis of RG-II.

CONCLUSIONS AND RELEVANCE

The research of pectin structure, techniques for pectin analysis, pectin function in plant growth and pectin biosynthesis is rapidly advancing in the genomic age. Genes may be identified by sequence similarity to those in databases and targeted for cloning or mutagenesis. Clearly, today the tools are available to tackle the existing gaps in the knowledge of pectin structure, function and biosynthesis.

Areas of pectin research that are lagging include the identification of GTs responsible for the biosynthesis of pectin. Progress has been made in recent years, however GT activities for less than a handful of linkages have been characterized and fewer genes have been identified. Generally speaking, the substrates and acceptors frequently must be generated by each researcher in order to carry out the analyses. Undoubtedly, the lack of discoveries in pectin biosynthesis are in-part due to the difficulty with which the activities may be assayed.

The goals of the projects described in this dissertation are designed to push the knowledge of pectin biosynthesis forward by aiding in the identification of additional pectin biosynthetic HG:GalATs and by characterizing the function of GalATs in plant growth and development.

CHAPTER 2

**COMPARATIVE PHYLOGENETIC ANALYSIS AND CELL WALL COMPOSITION
OF *ARABIDOPSIS THALIANA* T-DNA MUTANTS IMPLICATE GAUT GENES IN
CELL WALL AND SEED TESTA PECTIN AND XYLAN BIOSYNTHESIS²**

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ABSTRACT

The 25 member *Arabidopsis thaliana* galacturonosyltransferase-1 related gene family encodes 15 galacturonosyltransferase (GAUT) proteins and 10 galacturonosyltransferase-like (GATL) proteins with 56-84% and 42-53%, respectively, amino acid sequence similarity to GAUT1; an α 1,4-D-galacturonosyltransferase (GalAT) that transfers galacturonic acid from uridine 5'-diphosphogalacturonic acid onto homogalacturonan (Sterling et al., 2006). Previous phylogenetic analyses of *At*GAUTs indicated three GAUT clades: A through C. A comparative phylogenetic analysis of the *At*GAUT family with that of poplar and rice supports the subclassification of the GAUTs into seven clades. *Arabidopsis* GAUT clade A is subdivided into clades A, E, F and G; *At*GAUT clade B into clades B and D; while *At*GAUT clade C remains clade C. The corresponding GAUTs in each clade are: A (1 to 3); E (4), F (7) and G (5 and 6); B (8 and 9), D (10 and 11); and C (12 to 15). The *Arabidopsis* GAUTs have a distribution comparable to the poplar orthologs with the exception of GAUT2. Rice, however, has major distinctions in the number of GAUT 2, 7, 8 and 12 apparent orthologs compared with either *Arabidopsis* or poplar. An analysis of the plant cell wall glycosyl residue compositions of 26 homozygous *Arabidopsis* mutants carrying T-DNA insertions in 13 of the 15 GAUT genes reveals that the glycosyl residue composition of mutant cell walls of *gauts* 6, 8, 9, 10, 11, 12, 13 and 14 are significantly and reproducibly different from that of wild type *Arabidopsis* walls. The observed changes in galacturonic acid, xylose, rhamnose, galactose and arabinose composition of mutant walls implicate pectin and xylan polysaccharides as being affected by the loss of GAUT function. The wall glycosyl residue compositional phenotypes observed among the GAUT mutants suggests that at least six different biosynthetic linkages in pectins and/or xylans are affected by the lesions in these GAUT genes. Evidence is also presented to support a role of

GAUT11 in seed mucilage expansion and in seed wall and mucilage composition. These data support a role of GAUTs in pectin and xylan synthesis.

INTRODUCTION

The plant cell wall is composed of many structural elements that interact with specificity and elasticity to accommodate cell growth, cell shape and cell differentiation. The cell wall protects the plant from external environmental and compressive forces, wounding, pathogen attack and internal turgor pressure (Ridley et al., 2001). Carbohydrates, proteins and phenolics make up the bulk of the structural framework of the plant cell wall. A detailed understanding of the structure, synthesis and interaction of these components is important to understand wall function.

The load-bearing structural network of the wall is formed largely by high molecular weight cellulose microfibrils in both the primary and secondary walls. Cellulose microfibrils are composed of β -(1,4)-D-glucan chains that are synthesized at the plasma membrane by macromolecular polymeric complexes. The cellulose chains associate by inter- and intra-chain hydrogen bonding to form large microfibrils that also hydrogen bond with hemicelluloses, such as xyloglucan and xylan, to make up the cellulose-hemicellulose network (Herth, 1983; Nishiyama et al., 2002). Hemicelluloses also play a role in the organization of cellulose microfibrils, their crystallinity, as well as the ratio of cellulose I α to I β (McCann et al., 1990; Jarvis, 2000). Proteins such as wall glycosyl hydrolyases, extensins, expansins, arabinogalactan proteins, hydroxyproline-rich glycoproteins, proline-rich proteins, wall associated kinases and others contribute to wall structural integrity and the regulation of wall structural components (reviewed in (Cosgrove, 2000; Showalter, 2001b)). The pectic polysaccharides form the wall matrix by interacting in known and unknown ways with each other, and with hemicelluloses, wall proteins and phenolic compounds to yield the complex and functional walls of higher plants; reviewed in (Ridley et al., 2001).

The hallmark of pectic polysaccharides is the presence of 4-linked α -D-galacturonic acid (GalA) residues. There are three major classes of pectic polysaccharides: homogalacturonan (HG), rhamnogalacturonan-I (RG-I) and the substituted galacturonans rhamnogalacturonan-II (RG-II), xylogalacturonan and apiogalacturonan. HG is a polymer of α -(1,4)-D-GalA that is largely unbranched by sugar groups but may be modified by occasional acetyl groups at the O2 and O3 and methylesters at C6 of many GalA residues (Mort et al., 1993). The tricellular junctions, middle lamellae and primary wall are especially rich in HG, which may constitute up to 69% of primary wall pectin (Ridley et al., 2001). The galacturonan of some aquatic plants species may be substituted by terminal xylose residues, or by a disaccharide (Xylp-(1,2)-Xylp $\rightarrow$) at O3 of GalA residues in xylogalacturonan (Ovodov et al., 1971; Le Goff et al., 2001), or by 2-linked and 3-linked apiose residues in apiogalacturonan (Hart and Kindel, 1970). RG-II is a highly conserved substituted galacturonan that represents approximately 10% of pectin. An RG-II structural unit, which is embedded within a larger HG region, consists of four highly complex side chains (A-D) attached to an HG backbone of 7 to 9 GalA residues. RG-II sidechain A is involved in the dimerization of two RG-II molecules, via a borate diester, which confers strength to the wall; reviewed in (ONEILL et al., 2004). RG-I has a repeating disaccharide backbone of $[\rightarrow\alpha\text{-D-GalpA-1,2-}\alpha\text{-L-Rhap-1,4}\rightarrow]_n$. The GalA residues in the RG-I backbone may be O-acetylated as in HG. The rhamnose residues of the RG-I backbone may be branched with α -(1,5)-linked arabinan, β -(1,4)-linked galactan, and a variety of arabinogalactan structures (Carpita and Gibeaut, 1993). The regulation of RG-I sidechain structure and abundance is developmentally determined, suggesting that RG-I sidechains have important and diverse structure-function relationships in primary walls (Willats et al., 1999). The pectic polysaccharides are covalently linked as evidenced by the release of RG-I, RG-II and HG

oligosaccharides upon digestion of the wall with endopolygalacturonase, which cleaves the glycosidic linkages of the HG backbone (Keegstra et al., 1973). Additional evidence exists for the possible cross-linking of pectic polysaccharides to xylan (DuPont and Selvendran, 1987), xyloglucan (Popper and Fry, 2007) and phenolics (Ishii and Tobita, 1993), suggesting a high level of structural complexity in the plant cell wall matrix.

Despite the increasing detail with which primary wall structure is understood, few pectin biosynthetic genes have been identified. Thus far, only a single gene (*GAUT1*) has been definitively shown to have HG-GalAT activity. The *AtGAUT1* gene encodes a Golgi localized type-II membrane protein that catalyzes the elongation of HG oligosaccharides (oligogalacturonides) in an α 1,4-configuration, consistent with HG-GalAT function. A BLAST search (<http://www.ncbi.nlm.nih.gov/>) of the Arabidopsis genome identified 24 additional members of the GAUT1-related gene family. The Arabidopsis GAUT1-related gene family is made up of 15 so-called galacturonosyltransferase (*GAUT*) genes and 10 galacturonosyltransferase-like (*GATL*) genes (Sterling et al., 2006). Mutants of several GAUT1-related family genes have been studied and their phenotypes suggest that some GAUTs, in addition to GAUT1, likely have GalAT activity and are involved in the synthesis of cell wall polysaccharides.

The Arabidopsis IRX8 and PARVUS1 proteins are encoded by the *GAUT12* and *GATL1* genes, respectively, and have 61% and 49% amino acid similarity to GAUT1. The cell walls of *irx8-1* and *irx8-5* have reduced β -(1,4)-linked xylose and α -(1,4)-linked GalA residues (Persson et al., 2007) and had less of the reducing end pentasaccharide: [$\rightarrow$ 4)- β -D-Xylp-(1,4)- β -D-Xylp-(1,3)- α -L-Rhap-(1,2)- α -D-GalpA-(1,4)-D-Xylp] purified from a subfraction of xylan (Pena et al., 2007). It has been suggested that the α 1,4-linked GalA in this xylan reducing-end

pentasaccharide may be inserted by IRX8/GAUT12 (Pena et al., 2007). Alternatively, it has been hypothesized that IRX8/GAUT12 may synthesize a subfraction of HG to which xylan is attached (Persson et al., 2007). Recent studies of *parvus-3/gatl1* have demonstrated that *parvus* and *irx8* have a similar reduction in Xyl and β 1,4-XylT activity, and therefore, may be involved in a similar xylan biosynthetic pathway (Brown et al., 2007; Lee et al., 2007). The data thus far provide descriptive evidence for possible functions of IRX8/GAUT12 and PARVUS/GATL1, however, no biochemical evidence of enzymatic activity has been obtained to confirm these hypotheses.

QUA1/GAUT8 has 77% amino acid similarity to GAUT1. *Qual-1* is a severely dwarfed mutant that has reduced cell adhesion in expanding leaves and callus tissue and has cell walls with 25% reduced GalA levels (Bouton et al., 2002; Leboeuf et al., 2005). Membrane preparations from *qual-1* stem tissue are reduced in both α 1,4-GalAT and β 1,4-XylT activities (Orfila et al., 2005), providing ambiguous results and preventing the definitive identification of QUA1/GAUT8 function. QUA1/GAUT8 has higher amino acid similarity to GAUT1 than either GAUT12 or GATL1, and QUA1/GAUT8 is hypothesized to be an α 1,4-GalAT involved in HG biosynthesis (Orfila et al., 2005). However, the associated reductions in xylan and XylT activity of the *qual-1* mutant remain to be clarified.

In order to probe the function of the family of GAUT genes in wall biosynthesis, Arabidopsis T-DNA mutants corresponding to 13 GAUT genes were isolated and analyzed for changes in wall structure. The cell wall glycosyl residue compositions of the 26 GAUT mutants described here demonstrate aberrant wall compositional phenotypes among the mutants of GAUTs 6, 8, 9, 10, 11, 12, 13 and 14. The changes in the mol% of galacturonic acid, rhamnose, xylose, galactose and arabinose in the walls of mutants of these eight GAUT genes, compared to

wild type, have distinct patterns, suggesting that these GAUT genes have at least six unique functions in pectin and/or xylan biosynthesis. Our results also confirm wall phenotypes of IRX8/GAUT12 mutants and lethality phenotypes associated with IRX8/GAUT12 and QUA1/GAUT8 mutants. Finally, GAUT11 is shown to be involved in the production of Arabidopsis seed cell wall and mucilage.

RESULTS

The GAUT family of Arabidopsis, poplar and rice

The 25 member Arabidopsis GAUT1-related gene family encodes 15 GAUT proteins and 10 GATL proteins with 56-84% and 42-53% amino acid sequence similarity, respectively, to GAUT1 (Sterling et al., 2006). Previous phylogenetic analyses of the Arabidopsis *GAUT1*-related gene family identified three *GAUT* clades and one *GATL* clade (Sterling et al., 2006). The *GATL* clade, which consists of genes that cluster tightly and somewhat independently of the *GAUT* genes were not included in the study reported here. It was previously determined that some Arabidopsis *GAUT* genes, which have 56 to 84% amino acid similarity to GAUT1, had conserved orthologs among other species of both vascular and non-vascular plants (Sterling et al., 2006). The genome of rice (*Oryza sativa*) and poplar (*Populus trichocarpa*) have now been sequenced and a phylogenetic analysis of Arabidopsis, poplar and rice *GAUT* genes has been carried out to assess the degree of conservation among specific *GAUT* genes in herbaceous dicot (Arabidopsis), woody vascular dicot (poplar), and monocot (rice) species (Figure 2.1). Arabidopsis has 15 *GAUT* genes, while poplar and rice have 21 and 22 genes, respectively. There are one to two poplar orthologs for each Arabidopsis *GAUT*, likely due to a recent genome duplication event in *Populus* (Tuskan et al., 2006). These results indicate that poplar has a

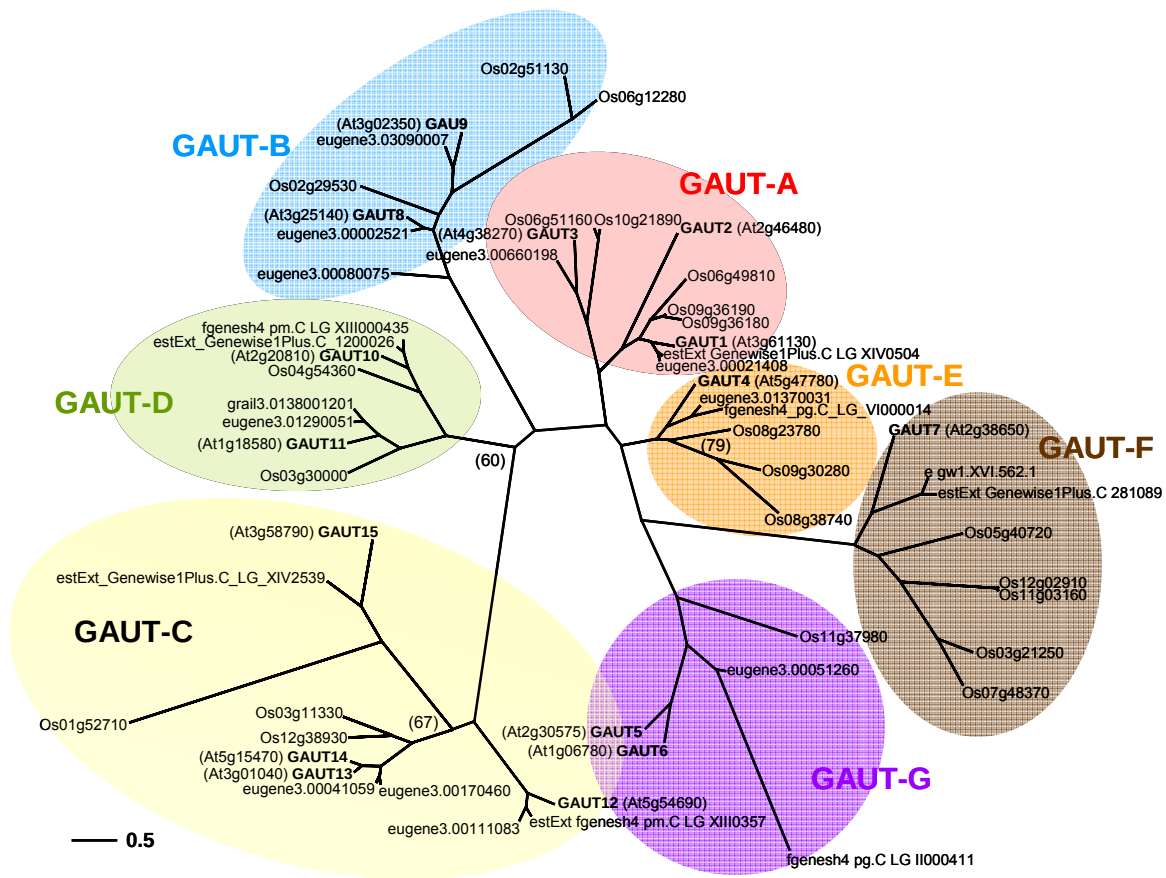


Figure 2.1. The GAUT gene family of Arabidopsis, poplar and rice.

Phylogenetic analysis of the GAUT Family in *Arabidopsis thaliana*, *Oryza sativa*, and *Populus trichocarpa*. Alignment of the complete protein sequences of the GAUT Family was carried out with ClustalX (Thompson et al., 1997) using suggested parameters (Hall, 2004) for protein alignments. Bayesian analysis employing MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) was used to infer phylogenetic relationships between the members of the family and group the protein sequences into related clades. The analysis was carried out for 500000 generations, using a mixture of amino acid transition parameter models. The phylogram presented here is the majority rule tree. Only those percentage branch credibility values less than 90 are shown (in parentheses).

similar distribution of GAUTs as *Arabidopsis*, with the exception of GAUT2, for which there is no poplar ortholog. Rice, conversely, has major distinctions from both *Arabidopsis* and poplar in its distribution of *GAUT* gene orthologs. Rice has no GAUT2 or GAUT12 ortholog, and potentially no GAUT8 ortholog. In addition, rice has multiple apparent isoforms of GAUTs 1, 4, 7 and 9, suggesting an expansion of the role of these GAUTs in rice.

The prior phylogenetic analysis of the *Arabidopsis* GAUTs indicated three GAUT clades: clades A through C. Upon including the rice and *Populus* genes in the comparative phylogenetic analysis, the three GAUT clades originally proposed resolved into seven clades. In order to preserve previous clade identity between the original three *Arabidopsis* clades (Sterling et al., 2006) and the more finely resolved seven clades presented here, the following clade identities are assigned. *Arabidopsis* GAUT clade A is subdivided into clades A, E, F and G; GAUT clade B is subdivided into clades B and D; and GAUT clade C remains clade C. The corresponding GAUTs in each clade are: A (1 to 3); E (4), F (7) and G (5 and 6); B (8 and 9), D (10 and 11) and C (12 to 15).

GAUT gene transcript expression in Arabidopsis tissues

The transcript expression of *AtGAUTs* available from the Whole Genome Array, Massively Parallel Signature Sequence and Genevestigator bioinformatic databases have been investigated (Table 2.1). Qualitative and semi-quantitative RT-PCR was also carried out to investigate the expression of *Arabidopsis* GAUT genes in the tissues that were to be harvested for wall glycosyl residue composition analyses and to assess the validity of the available bioinformatics data. Total RNA from wild type (WT) inflorescence, silique, stem and leaf tissues of eight week old plants was used for RT-PCR. Qualitative RT-PCR using *GAUT* gene-

Table 2.1. Bioinformatic analysis of Arabidopsis GAUT gene transcript expression.

(^a) GAUT gene designation (Sterling et al., 2006). (^b) Expression of GAUT gene transcript was detected (+) or not (-) according to the Whole Genome Analysis (WGA) of Arabidopsis (Yamada et al., 2003). (^c)Relative expression of the designated GAUT gene transcript for different tissues, available through the Massively Parallel Signature Sequences (MPSS) (Meyers et al., 2004) web site (<http://mpss.udel.edu/at/>). The MPSS information is listed as the code for each sample parameter: INF (Inflorescence - mixed stage, immature buds, classic MPSS), LEF (Leaves - 21 day, untreated, classic MPSS), LES (Leaves - 21 day, untreated, signature MPSS????), ROF (Root - 21 day, untreated, classic MPSS), SIF (Silique- 24 to 48 hr post-fertilization, classic MPSS), SIS (Silique - 24 to 48 hr post-fertilization, signature MPSS), CAF (Callus - actively growing, classic MPSS), CAS (Callus - actively growing, signature MPSS). (^d) GENEVESTIGATOR Expression Potential is the average of the top 1% signal value of a probe for the designated GAUT gene across all tissue expression arrays (Zimmermann et al., 2004).

Locus	Gene ^a	WGA ^b	INF ^c	LEF	LES	ROF	SIF	SIS	CAF	CAS	Expression Potential ^d
At3g61130	GAUT1	+	114	48	46	42	22	25	18	0	14093
At2g46480	GAUT2	-	0	0	0	0	0	0	0	0	1493
At4g38270	GAUT3	+	0	11	12	2	13	58	31	50	6851
At5g47780	GAUT4	+	87	161	0	142	154	0	152	0	18061
At2g30575	GAUT5	-	11	19	1	14	7	18	5	20	0
At1g06780	GAUT6	+	0	4	0	0	0	0	0	0	11224
At2g38650	GAUT7	+	68	69	111	62	40	218	53	236	7126
At3g25140	GAUT8	+	405	125	72	230	285	664	117	329	27875
At3g02350	GAUT9	+	74	78	28	450	249	106	93	69	15384
At2g20810	GAUT10	+	39	29	50	42	13	0	42	0	7087
At1g18580	GAUT11	+	19	15	22	29	38	17	26	12	6915
At5g54690	GAUT12	+	44	5	2	19	37	3	0	0	12028
At3g01040	GAUT13	+	24	11	8	58	4	1	22	10	9670
At5g15470	GAUT14	+	5	14	15	25	4	46	3	9	5386
At3g58790	GAUT15	+	0	0	0	0	16	0	4	12	6717

specific primers detected *GAUT* transcripts in all WT tissues tested, except for *GAUT2*, which may be expressed at a very low level or at different stages of development that has not yet been tested. Semi-quantitative RT-PCR of WT total RNA was carried out to more finely assess the relative transcript levels of *GAUT* genes in WT tissues (Figure 2.2).

The GAUTs are expressed in Arabidopsis inflorescence, leaf, stem, silique and root. The qualitative RT-PCR results only partially agree with the published transcript expression data (see Table 2.1). In several instances we detected GAUT transcript in tissues where it had not been previously reported. The data available from the Whole Genome Analysis (Yamada et al., 2003) did not detect *GAUT5*, while the Massively Parallel Signature Sequence data did not indicate detection of *GAUTs 7, 10, 11* and *12* in leaf, *GAUTs 1, 3* and *7* in stem, and *GAUTs 1, 3, 4, 8, 9, 10, 13* and *15* in silique (Meyers et al., 2004). Overall, the data supplied by Whole Genome Analysis and Massively Parallel Signature Sequences underestimated GAUT gene transcript expression. The relative transcript expression of the *GAUT* genes, however, generally agrees with that reported by Genevestigator (Zimmermann et al., 2004). However, Genevestigator does not list a probe for *GAUT5*, and therefore has no expression data for this gene. The MPSS database, however, reports low to moderate expression of *GAUT5*, which agrees with the results reported here.

RT-PCR also indicated that relative transcript expression was highest in *GAUTs 1, 4, 8, 9* and *12*, moderate in *GAUTs 3, 5, 6, 10, 14, 15* and low in *GAUTs 2, 7, 11* and *13*. It should be noted that RT-PCR of *GAUT7* repeatedly produced two bands, one of the expected size and a minor band of a smaller size. Since the RT-PCR data indicated that the GAUT genes were expressed at some level in all tissues tested, inflorescence, silique, leaf and stems were used for the molecular genetic studies of the GAUT mutants.

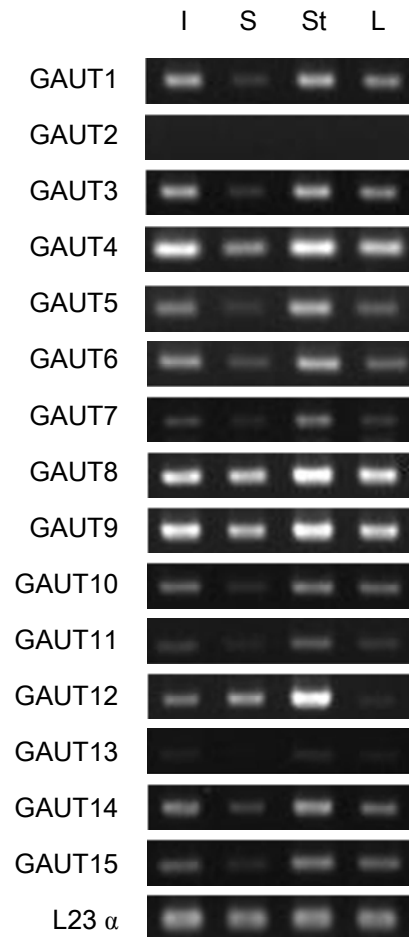


Figure 2.2. Transcript levels of GAUT genes in WT Arabidopsis tissues.

Semi-quantitative RT-PCR of total RNA isolated from inflorescence (I), silique (S), stem (St) and leaf (L) was used to assess transcript level in Arabidopsis tissues. Gene specific primers were used to amplify 800 bp fragments from the 5' end of each GAUT open reading frame. Similar results were obtained in three independent experiments. Control: RT-PCR using primers to L23α small ribosomal protein.

Isolation of homozygous mutants of 13 of the 15 GAUT genes

Twenty-six *Arabidopsis* homozygous T-DNA insertion seed lines in 13 distinct GAUT genes were isolated from mutagenized seed obtained from the SALK Institute (<http://signal.salk.edu/cgi-ben/tdnaexpress>) through the Arabidopsis Biological Resource Center (Alonso et al., 2003). Mutant seed lines were preferentially selected with the T-DNA insertion site in the exon, 5' UTR or intron of target GAUT genes, if such lines were available. SALK insertion seed lines in *GAUT1* were not available and neither homozygous nor heterozygous mutants were recovered from the SALK insertion seed lines for *GAUT4*. RT-PCR of total RNA isolated from homozygous GAUT mutant lines identified 10 knock-out mutants and 10 knock-down mutants (Table 2.2).

Growth phenotypes of GAUT mutants

The 26 *gaut* mutants were visually inspected for obvious growth phenotypes, such as dwarfing and/or organ malformation. *Arabidopsis* WT and GAUT mutants were grown for eight weeks under a light/dark cycle [14 h (19°C)/10 h (15°C)] and continuous 60% relative humidity. The average light intensity during the day cycle was 150 $\mu\text{Ei m}^{-2}$. Major abnormalities were not observed in plant growth or morphology of the majority of *gaut* mutants that were isolated for this study, except for *gaut8* and *gaut12*. The presence of subtle growth phenotypes cannot be discounted and may require more sensitive methods than those applied here. It is possible that functional redundancy among the GAUT proteins may contribute to the lack of readily detectable phenotypes in many of the *gaut* mutants. Estimates put forth by Østergaard and Yanofsky (2004) predict that mutations in approximately 10% of genes may result in growth phenotypes, which is in line with the predicted outcome (Østergaard and Yanofsky, 2004).

Table 2.2. Arabidopsis T-DNA insertion seed lines analyzed.

The Sterling et al. (2006) ^(a) and the new ^(b) clade designation for GAUT genes. ^(c) The amino acid sequence identity and similarity (I/S) of each GAUT gene to GAUT1 (Sterling et al., 2006). ^(d) The tentative location of the T-DNA insertion site: exon (E), 5' untranslated region (5'), intron (I), promoter (P) or 3' untranslated region (3'). ^(e) Transcript levels of GAUT T-DNA insertion mutant lines: Knock-Out, KO; Knock-Down, KD; WT-like, W. ^(f) SALK T-DNA insertion lines were unavailable in the GAUT1 gene. ^(g) Transcript for GAUT2 was not detectable in WT. ^(h) T-DNA insertions were not detected in plants from GAUT4 SALK_034472 and SALK_001026 seed. ⁽ⁱ⁾ GAUT8 T-DNA insertion seed lines SALK_039214, 041919 and 102380 yielded heterozygous, but no homozygous, mutant plants.

Locus	Gene	Clade ^a	Clade ^b	I / S ^c	SALK	Name	L ^d	KO/KD/W ^e
At3g61130	GAUT1	A	A	100/100				^f
At2g46480	GAUT2	A	A	65/78	122209	gaut 2-1	P	^g
At4g38270	GAUT3	A	A	68/84	001920	gaut 3-1	I	KO
					113167	gaut 3-2	5'	KD
At5g47780	GAUT4	A	E	66/83	034472	gaut 4-1	5'	^h
					001026	gaut 4-2	5'	^h
At2g30575	GAUT5	A	G	45/67	050186	gaut 5-1	E	KO
					058223	gaut 5-2	P	KD
At1g06780	GAUT6	A	G	46/64	007987	gaut 6-1	E	KO
					056646	gaut 6-2	E	KO
					073484	gaut 6-3	5'	KD
At2g38650	GAUT7	A	F	36/59	015189	gaut 7-1	E	KD
					046348	gaut 7-2	P	KD
At3g25140	GAUT8	B	B	58/77	030075	gaut 8-1	3'	KD
					039214	gaut 8-2	E	ⁱ
					041919	gaut 8-3	I	ⁱ
					102380	gaut 8-4	I	ⁱ
At3g02350	GAUT9	B	B	57/76	135312	gaut 9.1	E	W
					115588	gaut 9.2	E	W
					040287	gaut 9.3	E	KD
At2g20810	GAUT10	B	D	50/72	029319	gaut 10.1	E	KO
					082273	gaut 10.2	E	KD
At1g18580	GAUT11	B	D	51/71	104761	gaut 11.1	5'	KD
					148781	gaut 11.2	3'	KD
At5g54690	GAUT12	C	C	40/61	044387	gaut 12.1	I	KO
					014026	gaut 12.2	E	KO
At3g01040	GAUT13	C	C	43/62	122602	gaut13.1	E	W
At5g15470	GAUT14	C	C	43/62	000091	gaut 14.1	E	KO
					029525	gaut 14.2	3'	KO
At3g58790	GAUT15	C	C	37/56	113194	gaut 15.1	I	W
					117272	gaut 15.2	P	W
					070957	gaut 15.3	I	KO

detectable mutant phenotypes due to gene redundancy among large gene families in higher organisms. Thus far, 2 out of 13 *GAUT* genes (~15%) have yielded mutants with severe phenotypes. Insertion mutants of *qual-1* that are allelic to *gaut8* were previously analyzed and had severe dwarfing, sterility and bumpy epidermal surfaces as a result of reduced cell adhesion (Bouton et al., 2002). *Gaut8-2*, *gaut8-3* and *gaut8-4* produced only heterozygous and WT progeny, suggesting an embryo-lethal phenotype. The *gaut8-1* homozygous plants did not show the expected *qual-1* phenotype and were experimentally determined to have detectable GAUT8 transcript by RT-PCR, which may account for the WT-like phenotype of these plants.

The *gaut12-1* (*irx8-1*) and *gaut12-2* (*irx8-5*) mutant plants were severely dwarfed and sterile, which necessitated recovery of homozygous plants from the progeny of heterozygous parental plants, as previously reported (Persson et al., 2007). The phenotype of *gaut12-1* and *gaut12-2* was recognized in plants at least 4-weeks-old. Such plants were small and with darkened leaves compared to WT. Surprisingly, the *gaut12-5* promoter mutant (SALK_038620) did not produce homozygous progeny, even from the progeny of heterozygous plants. In addition, *gaut12-5* heterozygous mutants were dwarfed compared to WT, and more severely dwarfed compared to the *gaut12-1* or *gaut12-2* heterozygotes.

RT-PCR of RNA from homozygous *gaut12-1* and *gaut12-2* plants did not yield PCR products using 5'- and 3'-end coding region-specific primers, showing that the full-length *GAUT12* transcript was not produced. However, PCR products were amplified using selected internal *GAUT12*-specific primers (data not shown). The location of the T-DNA insertion in the promoter region of *GAUT12*, for which no homozygous plants were obtained, is likely to completely eliminate transcription of *GAUT12* and account for the apparent reduced viability of *gaut12-5* compared to *gaut12-1* and *gaut12-2* homozygotes. Because of the lethal phenotype,

only heterozygous *gaut12-5* was obtained and therefore was not included in our analyses of *gaut* homozygous mutants.

Strategy to identify glycosyl residue composition differences between GAUT mutant and WT walls

Gas chromatography-mass spectrometry (GC-MS) has been successfully used to detect the changes in glycosyl residue composition in cell walls arising from mutations in cell wall related genes (Reiter et al., 1997). Analysis of wall glycosyl residue composition by GC-MS of trimethylsilyl (TMS) derivatives allows for detection of acidic and neutral sugars in a single analysis (Doco et al., 2001), in contrast to composition analysis by formation of alditol acetate derivatives that detect neutral but not acidic sugars (Reiter et al., 1997). Since uronic acids make up the largest proportion of glycosyl residues in the non-cellulosic wall polysaccharides of WT *Arabidopsis* tissues (Figure 2.3), the TMS method was used here to analyze *gaut* mutant walls. A statistical assessment of the TMS method showed that at least 4 independent TMS analyses per wall sample are necessary to detect a 15% difference between the glycosyl residue compositions of different wall samples with 90% or greater statistical confidence. The mutant glycosyl residue composition results were normalized to the composition of WT plants grown in the same experiment, in order to minimize the variability observed in the glycosyl residue compositions of plants grown in different experiments and to enable the comparison of data from experiment to experiment. Thus, for example, rhamnosyl compositions would be normalized according to the following formula:

$$\text{Normalized Rha} = [(\text{mutant mol \% Rha} / \text{WT mol \% Rha}) \times 100]$$

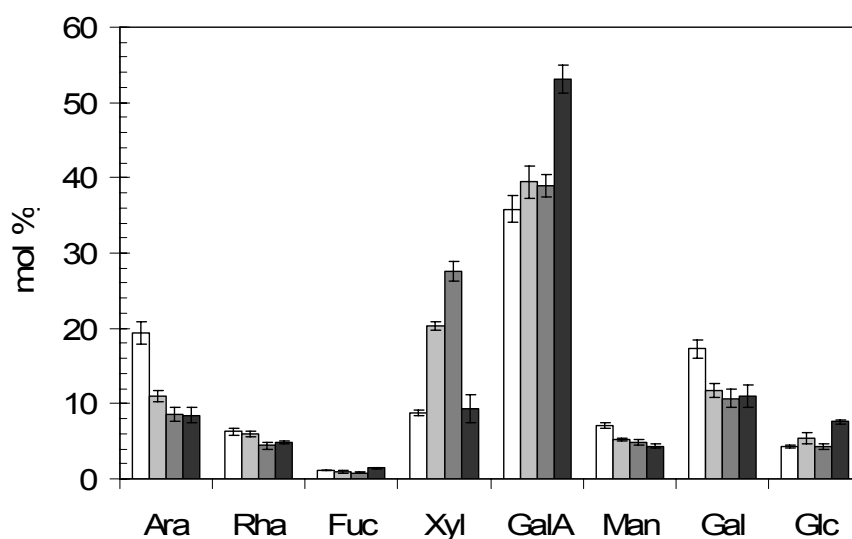


Figure 2.3. Glycosyl residue composition of Arabidopsis WT cell walls.

The glycosyl residue composition of walls determined by GC-MS of TMS derivatives was quantified from inflorescence (white bars), silique (light gray bars), stem (dark gray bars) and leaf (black bars) tissues. Glycosyl residues are abbreviated as arabinose (Ara), rhamnose (Rha), fucose (Fuc), xylose (Xyl), galacturonic acid (GalA), mannose (Man), galactose (Gal) and glucose (Glc).

Normalization of mutant glycosyl residue composition to WT controls allowed for mutant wall composition phenotypes to be compared between experiments. The tissues chosen for the cell wall analyses of each specific GAUT mutant were based on transcript expression of the corresponding GAUTs according to the Whole Genome Array (Yamada et al., 2003) and Massively Parallel Signature Sequences (Meyers et al., 2004) databases (see Table 2.1). To identify GAUT mutant wall glycosyl residue compositions that were statistically different from that of WT walls, the normalized compositions were evaluated by ANOVA procedures ($t_{\alpha(2)} = 0.1$). As an extra measure of stringency, a 15 percentage point or greater departure from the normalized WT mean, in addition to a statistically different outcome by ANOVA, was required to conclude that mutant walls were different from WT.

Wall glycosyl residue composition is altered in multiple GAUT gene mutants

TMS glycosyl residue composition analyses of walls from two or more tissues of WT and the 26 GAUT mutant lines representing 13 GAUT genes, revealed that distinct GAUT mutants have unique wall composition changes that include *increases* and *decreases* in GalA, as well as significant changes in other glycosyl residues (Table 2.3). The wall glycosyl residue compositions that were statistically different in the GAUT mutants are shown in bold in Table 2.3. The data indicate that mutants of eight GAUT genes had statistically different mol% levels of GalA, Xyl, Rha, Gal or Ara in at least two mutant alleles of the same gene or in at least two tissues of the same mutant allele compared to WT. The analysis of the data revealed six patterns of glycosyl residue composition changes in the walls of GAUT mutants (Table 2.4). These reproducible mutant phenotypes were identified by comparing the natural log transformed data

Table 2.3. Percent cell wall glycosyl residue composition^a of Arabidopsis GAUT mutants compared to WT.^a

(^a) Data are four independent TMS GC-MS reactions from four independent wall extractions. Residues are abbreviated according to Figure 2.3. SALK T-DNA seed lines were unavailable for GAUT1 and were unable to be isolated from SALK seed received for GAUT4. (^b) The walls used for glycosyl residue analysis were harvested from inflorescence (I), silique (S), leaf (L) and stem (T). (^c) Bold values indicate mutant glycosyl residue compositions that were statistically and $\pm 15\%$ different from the WT mean.

Mutant	Tissue ^b	(mutant mol%/WT mol%*100)							
		Ara	Rha	Fuc	Xyl	GalUA	Man	Gal	Glc
<i>gaut2-1</i>	S	160^c	116	108	114	84	103	103	77
	L	152	173	98	124	91	89	112	123
<i>gaut3-1</i>	I	74	64	74	111	104	108	125	125
	S	90	62	72	82	110	104	126	118
<i>gaut3-2</i>	I	99	102	181	118	99	89	97	131
	S	132	112	109	118	87	98	86	120
<i>gaut5-1</i>	I	117	112	110	105	85	131	102	73
	S	109	132	117	130	94	148	112	61
<i>gaut5-2</i>	I	98	99	97	97	106	103	93	97
	S	102	118	43	95	105	141	78	71
<i>gaut6-1</i>	I	193	222	154	161	80	162	65	128
	S	123	173	127	133	85	141	74	153
	L	168	204	156	158	75	167	89	107
<i>gaut6-2</i>	I	69	126	95	122	114	133	99	170
	S	87	137	108	126	112	150	73	125
	L	103	142	115	129	87	131	79	153
<i>gaut6-3</i>	I	113	111	102	100	88	111	98	92
	S	161	114	135	118	78	104	103	86
	L	139	142	106	109	92	102	102	112
<i>gaut7-1</i>	I	91	113	104	110	102	89	93	126
	L	114	130	117	90	96	107	93	114
<i>gaut7-2</i>	I	100	96	87	98	114	89	96	105
	L	112	102	100	110	113	102	108	51
<i>gaut8-1</i>	I	65	67	72	81	111	106	119	116
	S	59	55	35	137	102	102	95	111

<i>gaut9-1</i>	I	130	167	156	154	99	159	82	139
	S	89	113	118	122	92	154	99	136
	T	101	131	153	148	80	146	72	127
<i>gaut9-2</i>	I	77	79	70	100	106	100	119	99
	S	82	72	207	103	104	282	99	85
	T	100	90	96	105	81	58	129	106
<i>gaut9-3</i>	I	139	130	151	137	108	102	91	114
	S	147	137	178	128	82	100	98	112
	T	100	100	100	100	100	100	100	100
<i>gaut10-1</i>	I	103	98	93	107	89	120	112	86
	S	103	103	110	116	83	113	92	108
<i>gaut10-2</i>	I	152	154	128	115	87	94	92	75
	S	131	104	85	103	83	85	110	78
<i>gaut11-1</i>	I	110	96	99	137	85	100	105	146
	S	151	135	125	109	81	86	84	117
	L	222	207	128	133	86	90	131	124
<i>gaut11-2</i>	I	59	50	56	86	108	99	135	125
	S	110	73	76	108	91	112	114	95
	L	75	83	52	88	115	95	121	83
<i>gaut12-1</i>	I	148	120	97	101	82	89	102	142
	S	147	115	112	33	114	100	127	121
	T	179	124	130	55	103	66	168	132
<i>gaut12-2</i>	I	163	137	105	130	82	80	91	115
	S	65	67	176	25	129	102	126	169
	T	198	154	126	58	117	60	148	109
<i>gaut13-1</i>	I	62	58	63	68	125	111	120	123
	S	24	26	117	89	137	99	110	159
<i>gaut14-1</i>	I	42	41	47	88	132	109	135	113
	S	70	50	54	98	117	110	124	97
	L	40	62	41	73	133	81	156	78
<i>gaut14-2</i>	I	74	84	63	105	121	90	117	74
	S	136	86	204	104	86	121	111	64
	L	102	102	61	88	98	67	143	98
<i>gaut15-1</i>	I	87	83	89	76	104	105	119	166
	S	171	107	117	118	85	86	96	84
<i>gaut15-2</i>	I	111	161	67	134	99	213	72	81
	S	98	147	90	190	82	156	60	112
<i>gaut15-3</i>	I	77	78	71	109	111	109	117	98
	S	130	84	95	112	93	103	109	84

Table 2.4. Phenotypic grouping of GAUT mutants.

(^a) Due to the lethality of GAUT8 homozygous mutants, the *qual-1* leaf compositions were used for the phenotypic grouping of GAUT8 (Bouton et al., 2002). (b) Mutants showing the qual phenotype were not obtained and therefore, the data obtained by Bouton et al. (2002) was used for phenotypic analyses. (c) Similar results were also obtained for *irx8* mutants by Brown et al. (2005), Pena et al. (2007) and Persson et al. (2007).

Wall Phenotype	GalA	Xyl	Rha	Gal	Ara	GAUT
1	down	up	up	down	no change	6
2	down	down	no change	no change	no change	8 ^b
3	down	up	variable	variable	no change	9
4	down	no change	no change	no change	no change	10
4	down	no change	variable	variable	no change	11
5	up	down	no change	up	no change	12 ^c
6	up	down	down	up	down	13
6	up	down	down	up	down	14

for all mutants that had statistically different mol% GalA, Xyl, Rha, Gal and Ara levels compared to WT. Type-1 mutant wall phenotype patterns were observed in the silique tissues of *gaut6-1* and *gaut6-3*, which were consistently reduced in GalA, increased in Xyl, Rha and Fuc and had no reproducible change in Gal or Ara wall composition. Type-2 mutant wall compositions were observed in leaves of *qual-1* that were previously analyzed (Bouton et al., 2002). Viable GAUT8 homozygous knock-out mutants were not able to be isolated and therefore the wall composition of *qual-1* is used to establish a phenotype grouping for GAUT8 mutants. The wall composition data generated (Bouton et al., 2002) were decreased in GalA and Xyl, but were not changed in Rha or other sugars. Type-3 mutant wall compositions were observed in *gaut9-1*, which were reduced in wall GalA and increased in Xyl and Fuc. Type-4 mutant wall compositions were observed in *gaut10-1*, *gaut10-2* and *gaut11-1*, which were consistently reduced in silique GalA only. Type-5 mutant wall compositions were observed in *gaut12-1* and *gaut12-2* mutant stems, which were severely reduced in Xyl, coincident with elevated Ara, Rha and Gal. *Gaut12-1* and *gaut12-2* are analogous to *irx8-1* and *irx8-5* and consequently show similar stem glycosyl residue composition as previously reported (Brown et al., 2005; Pena et al., 2007; Persson et al., 2007). Type-6 mutant wall compositions were observed in *gaut13-1*, *gaut14-1* and *gaut14-2*, which had increased GalA and Gal and reduced Xyl, Rha, Ara and Fuc.

There were a few significant changes in the mutant walls of GAUTs 2, 3, 5, 7 and 15, but those that did occur, were not generally consistent between two or more mutants or in more than one tissue of a single mutant. There were also some changes in Fuc, Man and Glc in walls of several GAUT mutants. For example,; increased Fuc was observed in *gaut6-1*, *gaut6-2*, *gaut6-3*, *gaut9-1*, *gaut9-2* and *gaut9-3*; decreased Fuc in *gaut8-1*, *gaut11-2*, *gaut14-1* and *gaut14-2*;

increased Man in *gaut5-1* and *gaut5-2*; increased Glc in *gaut3-1*, *gaut3-2* and *gaut6-2*; and decreased Glc in mutants of *gaut5-1*, *gaut5-2* and *gaut10-2*.

Survey of seed mucilage reveals GAUT11 involved in mucilage extrusion

The seeds of myxospermous species, such as *Arabidopsis*, extrude mucilage from the seed coat epidermal cells when hydrated to protect against desiccation and aid in seed dispersal. The mucilage of WT and GAUT mutant seeds was investigated by ruthenium red staining as a facile method to determine if specific GAUT genes are involved in mucilage polysaccharide extrusion or synthesis. The mucilage extruded from *Arabidopsis* seeds is enriched in the pectic polysaccharide RG-I, which is efficiently coordinated by ruthenium red stain that coordinates the negative charges of GalA residues of mucilage pectin. This method has been successfully employed to identify mucilage or testa polysaccharide biosynthesis mutants (Western et al., 2001). The quantity of seed mucilage was evaluated by observing the staining intensity of mucilage and measuring the mucilage thickness under a dissecting scope after application of aqueous 0.05% ruthenium red to the seeds of WT and the 26 *gaut* mutant lines. A single mutant (*gaut11-2*) was identified that displayed a reproducible reduced mucilage thickness phenotype compared to WT seed mucilage thickness. Ruthenium red staining of WT and *gaut11-2* seeds (Figure 2.4A through 2.4C) revealed that ~68% of *gaut11-2* seeds were largely lacking mucilage, while the remaining *gaut11-2* seeds (~32%) were reduced in the thickness of the mucilage layer to approximately half that of WT. Samples of WT and *gaut11-2* seed were tested three separate times with similar results obtained in seed derived from different parental plants (Table 2.5).

The water-extracted mucilage (WEM) of *gaut11-2* and WT seed was analyzed by uronic acid assay, phenol sulfuric acid assay, glycosyl residue composition and linkage analysis.

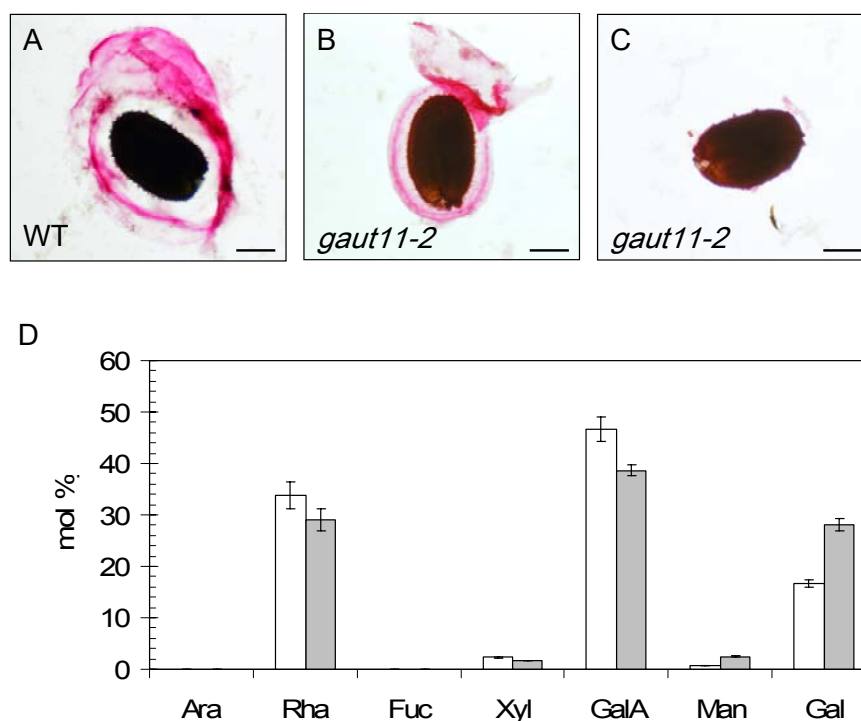


Figure 2.4. Ruthenium red staining of WT and *gaut11-2* seeds.

Ruthenium red (0.05 %) was applied directly to Arabidopsis seeds without shaking. WT seeds (A) clearly show a thick mucilage layer and a dark-staining mucilage envelope that sloughs off of the seed. The GAUT11-2 seeds (B and C) extrude less mucilage than similarly treated WT seeds (B) or appear to lack mucilage extrusion almost entirely (C). The *gaut11-2* seed mucilage in panel B also shows different staining properties than the WT mucilage in panel A. Inset bar = 100 μ m. The composition (D) of WT (white bars) and GAUT11-2 (gray bars) hot water extracted mucilage was determined by TMS (mol%).

Table 2.5. WT and *gaut11.2* mucilage expansion and uronic acid content. ^(a) The data are the average (%) seeds with expanded mucilage after staining with aqueous ruthenium red. ^(b) The data are the uronic acid content of hot water extracted mucilage per 200 seeds of WT and *gaut11-2* as assayed by the *m*-hydroxybiphenyl reagent assay.

Experiment	Mucilage (% seeds) ^a		UA (ug UA/200 seeds) ^b	
	WT	<i>gaut11-2</i>	WT	<i>gaut11-2</i>
Experiment # 1	92	16	59	46
Experiment # 2	100	41	58	46
Experiment # 3	87	39	56	45
Experiment # 4			61	48
Experiment # 5			58	53
Average	93.0±7	31.8±14	58.8±2	47.8±3
	P = 2.3 ⁻³		P = 2.2 ⁻⁴	

The WEM of WT had 59 µg uronic acid per 200 extracted seeds while similar *gaut11-2* mucilage had 48 µg uronic acid per 200 extracted seeds (see Table 2.4). The total carbohydrate extracted was similar for WT and *gaut11-2* WEM. This suggests that even though very little mucilage was observed by ruthenium red staining, a similar amount of carbohydrate was able to be extracted over several hours, but that the uronic acid content of that mucilage was reduced by 19%. The *gaut11-2* WEM was subjected to glycosyl residue composition analysis (see Figure 2.4) and was found to have statistically significant reductions in GalA and Xyl content and increases in Man and Gal content, as determined by ANOVA ($t_{\alpha(2)} = 0.05$). The glycosyl residue composition of residual seed material that represents the remaining mucilage, some testa wall and possibly some storage polysaccharide, was also reduced in GalA (69%) and Gal (68%) and increased in Ara (110%), Man (128%) and Glc (138%) compared to WT.

DISCUSSION

There are seven GAUT gene clades in Arabidopsis, poplar and rice

The relatedness of GAUT genes has been re-evaluated based on the analysis of phylogenetic relationships of Arabidopsis, poplar and rice GAUT genes. This comparative phylogenetic analysis distinguished seven clades, A through G (Figure 2.1) instead of three as previously proposed by Sterling et al. (2006). The previous Arabidopsis clade GAUT clade A that included *AtGAUT1*-GAUT7 has been subdivided into four clades; GAUT clade A (*AtGAUT1* through 3), GAUT clade C (*AtGAUT4*), GAUT clade F (*AtGAUT7*), and clade G (*AtGAUT5* and *AtGAUT6*). The former Arabidopsis clade B has been subdivided into GAUT clade B (*AtGAUT8* and *AtGAUT9*) and GAUT clade D (*AtGAUT10* and *AtGAUT11*). The former

Arabidopsis GAUT clade C, has been retained as clade C and contains *AtGAUT12* through *AtGAUT15*.

GAUT2 does not appear to have a direct ortholog in either rice or poplar. It is hypothesized that GAUT2 is not a complete copy of a GAUT1 duplication event. GAUT2 also does not have detectable transcript in the tissues tested and GAUT2 T-DNA insertion mutants did not have reproducible phenotypes. These data, combined with the phylogenetic analysis of GAUT2, support the hypothesis that GAUT2 may be a pseudogene. It cannot be discounted, however, that GAUT2 may have very low abundant transcript and a unique function in Arabidopsis alone, however this seems unlikely based on the current data.

The Arabidopsis, poplar and rice genomes have one, two and five copies of GAUT7, respectively. Thus, rice has more than twice the number of GAUT7 genes than poplar or Arabidopsis. There is considerable evidence that the *AtGAUT7* protein resides in a complex with *AtGAUT1*, a complex that has α 1,4-GalAT activity (M. A. Atmodjo 2008, unpublished data). GalAT activity was detected in immunoprecipitates from HEK cells transiently transfected with GAUT1, but not in HEK cells transiently transfected with GAUT7 (Sterling et al., 2006). Based on these data, GAUT7 may be expressed in an inactive state with limited activity itself or may be a chaperone or some other ancillary protein necessary for GAUT1-associated GalAT activity. Whatever the role of GAUT7, its function appears to be dramatically expanded in rice. Because the role of GAUT7 in wall polysaccharide biosynthesis is currently unknown, the underlying biological reason for five copies of GAUT7 in rice remains to be determined.

GAUT9 and GAUT8 appear to be descended from a common ancestor. Poplar and rice each have putative orthologs of GAUT9: eugene3.03090007 (poplar), *Os06g12280* (rice) and

Os02g51130 (rice). Poplar also has at least one putative ortholog of GAUT8 (Eugene3.00002521), while there is not an obvious ortholog in rice. A rice gene that may be a GAUT8 candidate by parallel evolution lies at an earlier position on the branch, indicating that it may also possibly be an additional ortholog of GAUT9. It is also possible that the rice genome may have lost, or did not receive a copy of, GAUT8. The confirmation of these speculations will require the functional analysis of *OsGAUT8*. The implications of such an outcome however, are intriguing in that a homolog of GAUT12 has thus far also not been identified in monocots. The functions of GAUT8 and GAUT12 have been linked in that they both appear to have distinct roles in HG and xylan synthesis. It is interesting to hypothesize functions that might be unique to dicot secondary walls and vasculature, and that rely on the coordinated actions of both GAUT8 and GAUT12.

The putative functions that have been hypothesized for GAUT12 include an α 1,4-GalAT that adds GalA into a primer or cap for xylan synthesis or as a novel linkage in xylan or pectic polysaccharides (Brown et al., 2005; Pena et al., 2007; Persson et al., 2007). GAUT12 has been shown to be essential for normal growth and more specifically in the synthesis of secondary wall glucuronoxylan and/or wall HG synthesis. Rice does not have an apparent homolog of GAUT12, and appear to produce secondary wall xylan and glucuronoarabinoxylan, but not 4-O-methylglucuroxylan (Ebringerova and Heinze, 1999). Thus, GAUT12 may have a specialized function in glucuronoxylan synthesis of dicot plants. GAUT12 transcript has been shown to be localized closely with glucuronoxylan-rich vascular tissues suggesting that GAUT12 has a specialized role in secondary wall glucuronoxylan of dicot walls (Persson et al., 2007). GAUT12 has an expression profile distinct from that of other GAUT genes according to semi-quantitative RT-PCR; it is much more highly expressed in stem than in other tissues compared to

other GAUT transcripts. The unique transcript expression, role in secondary wall methylglucuroxylan synthesis and exclusivity among the dicot species suggests that GAUT12 has undergone a differentiation that has rendered it essential in dicots, but nonessential in monocots.

GAUT gene transcripts are expressed ubiquitously in Arabidopsis tissues

The transcript expression of GAUT8 and GAUT12 has been associated with vascular tissues in Arabidopsis stem (Orfila et al., 2005; Persson et al., 2007). The GAUT12 results described here agree with previous analyses of GAUT12/IRX8 gene expression by RT-PCR analysis (Persson et al., 2007) and GAUT8 RT-PCR data agree with reports of QUA1 expression (by Northern blot) in ‘Flowers II’ and ‘Rosette Leaves II’ RNA, but do not agree with the low transcript expression reported in ‘Stems II’ by Bouton and colleagues (2002). We report high relative expression of GAUT8 in stems. In situ PCR of QUA1/GAUT8 in WT stems, however, did show prominent expression in that tissue, which is more closely aligned with our results (Orfila et al., 2005). The detectable expression of all of the GAUT genes in all of the tissues tested correlates with a function in wall biosynthesis, as this is a process required by all plant cells. GUS reporter gene studies have shown that QUA2, a putative pectinmethyltransferase involved in pectin biosynthesis, also has ubiquitous transcript expression (Mouille et al., 2007).

The Wall Compositions of Multiple GAUT Mutants are Altered Compared to WT

Mutants of *GAUTs* 6, 9, 10 and 11 had statistically significant reductions in GalA content in more than one mutant sampling. Other GAUT mutants, *gaut13* and *gaut14* had statistically significant increased wall GalA content. In addition, specific other glycosyl residues

were reproducibly altered in the walls of these *GAUT* mutants. Analysis of the walls of *GAUT* mutants using the TMS method allowed the GalA content of the walls to be quantified (Doco et al., 2001). An accurate quantification of wall GalA content is important in attempting to identify mutants of putative pectin biosynthesis genes, because GalA is a major component of the pectic polysaccharides (Ridley et al., 2001). The wall compositional phenotypes of the *GAUT* mutants are discussed below, taking into account the degree of relatedness of each *GAUT* gene to *GAUT1* and *GAUT8/QUA1* or *GAUT12/IRX8*.

The wall glycosyl residue composition phenotype of *GAUT6* provides compelling evidence that *GAUT6* is a putative pectin biosynthetic GalAT. *GAUT6* has 64% amino acid similarity to *GAUT1* and *gaut6* has reduced wall GalA that coincides with higher levels of Xyl and Rha wall compositions. It is possible that the increased Xyl and Rha content signify the compensatory reinforcement of the wall by xylans and an apparent enrichment of RG-I in proportion to reduced HG polymers. Further work is necessary to test this hypothesis; however preliminary results are in agreement with this hypothesis (Caffall, Ph.D. dissertation Chapter 3, 2008).

The *qual-1* mutant has a T-DNA insertion in the *GAUT8/QUA1* gene that cluster with *GAUT9* in clade B and *GAUT10* and *GAUT11* in clade D. These *GAUTs* have been placed in separate clades, however mutants in all the *GAUT* genes show marked reductions in wall GalA content. *Qual-1* mutant plants have walls with both reduced GalA and Xyl, and microsomal membrane protein preparations from *qual-1* stems had reduced GalAT and Xylan synthase activity compared to WT (Orfila et al., 2005; Brown et al., 2007). The cumulative experimental evidence argues in favor of *QUA1* as a putative pectin biosynthetic GalAT as the walls of *qual-1* are significantly reduced in homogalacturonan and also have a strong defect in cell adhesion

(Bouton et al., 2002; Leboeuf et al., 2005). Deficiencies in cell adhesion have been associated with changes in pectin synthesis (Iwai et al., 2002) and pectin localization (Shevell et al., 2000). In addition, the transcript expression of a pair of Golgi-localized putative pectinmethyltransferases are strongly correlated with GAUT8 expression, as well as with the expression of GAUT9 and GAUT1 (Mouille et al., 2007). The *gaut9*, *gaut10* and *gaut11* mutant plants did not have any obvious physical growth or cell adhesion defects, but the wall compositional phenotypes of the corresponding *gaut* mutants, and the high amino acid similarity with QUA1/GAUT8 suggest that these GAUTs are putative pectin biosynthetic GalATs. The mutant alleles of GAUT9, GAUT10 and GAUT11 have reduced wall GalA content but were not decreased in Xyl, which has been observed in some mutants thought to be involved in xylan synthesis (Brown et al., 2007; Lee et al., 2007; Pena et al., 2007; Persson et al., 2007). Based on the evidence reported here, a role for the genes in GAUT clades A as well as clades G, B and D in pectin biosynthesis is proposed.

Contrary to QUA1, GAUT12 is believed to function in glucuronoxylan synthesis essential for secondary wall function. The *gaut12-1* and *gaut12-2* mutant plants have reduced Xyl composition with increases in the GalA composition in stem and silique walls, consistent with previous reports and consistent with the proposed function of GAUT12 in the synthesis of a oligosaccharide essential for xylan synthesis. GAUT12 and other putative xylan biosynthetic genes, IRX7, IRX8, IRX9, IRX14 and PARVUS-3, have similar wall compositional phenotypes (Pena et al., 2007; Persson et al., 2007). GAUT12 may play a specialized role, among the GAUT genes, in secondary wall synthesis and vascularization in dicot species (Brown et al., 2007). Xylans are abundant in stem and silique tissues, where the Xyl compositional phenotype is observed, however reductions in Xyl are not observed in inflorescence where GAUT12 is also

expressed. In inflorescences, *gaut12* mutants show a reduction in GalA to 82% that of WT. The changes brought about by the lesion in GAUT12 additionally impact the pectin component of the wall, which is evident in the inflorescence tissue composition. The underlying causes for the reduced GalA content in inflorescence may be of significance to understand how pectin and xylan synthesis are regulated.

The walls of *gaut13* and *gaut14* have increased GalA and Gal content and reduced Xyl and Rha content compared to WT. It seems unlikely that a mutant showing an increased wall GalA phenotype is involved in the synthesis of HG. However, reduced Rha, primarily a component of RG-I, may lead to walls enriched in HG, driving up GalA content. A Gal containing wall component in *gaut13* and *gaut14* (and also *gaut12*) is increased in the walls of *gaut13* and *gaut14*. Pectic galactans have been associated with wall strengthening (McCartney et al., 2000) and are also increased in *irx8* walls (Persson et al., 2007). A galactan in *gaut13* and *gaut14* may be up-regulated in response to wall weakening in a similar manner. GAUT13 and GAUT14 are very closely related to GAUT12, which would also suggest that the Xyl containing polysaccharide that is reduced in these mutants is also a xylan and that GAUT13 and 14 share overlapping function with GAUT12. Based on the strong transcript expression of GAUT12, most notably in the stem tissues of 8-week-old Arabidopsis plants, it is conceivable that *gaut13* or *gaut14*, that have WT-like growth phenotypes, may be partially rescued by existing GAUT12 expression, if function is shared between GAUT12, GAUT13 and GAUT14, thus resulting in mild or undetectable growth phenotypes.

GAUT11 effects mucilage extrusion

The *gaut11-2* mutant has reduced mucilage expansion and reduced GalA content of extracted mucilage, suggesting a role in the synthesis of mucilage polysaccharides. The *gaut11-2* seed mucilage and testa has reduced GalA composition. The *gaut11-2* has reduced GalA in silique walls, while *gaut11-1* has reduced GalA in inflorescence, silique and leaf walls. The *gaut11-1* seeds, however did not appear to have inhibited mucilage expansion. The predicted insertion site location of the T-DNA insertion present in *gaut11-2* is in the 3'UTR, a location that may alter the targeting or regulation of GAUT11 expression rather than knocking out function (Lai, 2002) and account for the difference in phenotype between *gaut11-1* and *gaut11-2*.

The composition and linkage analysis of *gaut11-2* mucilage suggests a minor reduction in RG-I-like extractable polysaccharides. The visible phenotype of *gaut11-1* is similar in character to the *mucilage modified (mum)* mutants (Western et al., 2001; Western et al., 2004). Three types of MUM mutants have been described: mutants of pectin modification (*mum2* and *mum1*), mutants affecting cytoplasmic rearrangement (*transparent testa glabra-1*; *tig1*, *glabra-2*; *gl2*) and mutants of mucilage biosynthesis (*mum3*, *mum5* and *mum4*) (Western et al., 2001). Preliminary data suggests a role for GAUT11 in wall modification or biosynthesis based on the reduction in GalA in the extractable mucilage and based on the observation that the majority of the polysaccharides may be extracted over time, but are inefficiently released from the seed epidermal cells. It is known that unbranched RG-I or reductions in intact RG-I may lead to increased Ca^{2+} crosslinking of HG in the wall (Jones et al., 2003). This may induce wall locking and thus inhibit expansion and release of mucilage by hydration. Additionally, accumulation of less RG-I in the epidermal cells of the seed coat may prevent extrusion of the mucilage by

reducing the internal pressure that is required to break through the epidermal cell wall necessary to release mucilage (Western et al., 2000).

Lethality of GAUT mutants: something lost, something gained

GAUT1 is an HG-GalAT. GAUT1 was the most abundant glycosyltransferase isolated from Arabidopsis suspension culture microsomal membrane fractions (Sterling et al., 2006). GAUT1 and GAUT4 are expressed highly in the tissues of 8-week old plants according to semi-quantitative RT-PCR and to the GENEVESTIGATOR and MPSS databases (Figure 2.2 and Table 2.1) (Meyers et al., 2004; Zimmermann et al., 2004). Proteins that share high amino acid similarity often have a similar function and it is likely that GAUT4 (83 % amino acid similarity to GAUT1) also has a function in synthesizing HG in the walls of Arabidopsis similar to that of GAUT1. The lack of recoverable mutants for GAUT1 and GAUT4 may speak to the importance of these genes in plant growth and development. Indeed, a GAUT1 GABI-KAT mutant yielded only heterozygous and WT progeny; homozygotes were not obtained to date (M. A. Atmodjo and I. Petrascu 2008, unpublished data). In addition, a degree of lethality has been demonstrated in GAUT8 and GAUT12 mutants, both in this paper and elsewhere (Bouton et al., 2002; Persson et al., 2007). *Qual-1*, *irx8-1* and *irx8-5* mutants are severely dwarfed and semi-sterile (Brown et al., 2005; Orfila et al., 2005). Lethality among plant mutants is problematic experimentally, but may signify that the gene of interest is both essential and non-redundant with other genes and that the protein is of great importance to cell wall biosynthesis and plant growth and development. More vigorous attempts to isolate and characterize mutants of GAUT1 and GAUT4 will undoubtedly aid in the clarification of the role of these genes in wall biosynthesis.

CONCLUSION

The data presented establish the foundation for multiple hypotheses regarding GAUT gene function. The rigorous testing of these hypotheses is expected to lead to the identification of additional genes involved in specific wall biosynthetic pathways. The wall compositional phenotypes support the proposition that (1) GAUT genes function in wall biosynthesis, (2) GAUT 6, 9, 10 and 11, that have the highest amino acid similarity to GAUT1, have putative function in plant pectin biosynthesis and (3) GAUT13 and 14 are likely to have putative function in xylan biosynthesis like that of GAUT12 or pectin RG-I biosynthesis. The mutant wall composition phenotypes presented here are not sufficient to prove GAUT function but serve to develop hypotheses regarding GAUT gene function in the GAUT1-related gene family of *Arabidopsis*. The data demonstrate that mutants corresponding to more than half of the GAUT genes have significantly altered wall polysaccharides and strongly support a role for the entire family in pectin and pectin/xylan synthesis.

MATERIALS AND METHODS

Sequence alignment of GAUT family genes and phylogenetic analysis

GAUT protein sequences were identified by BLAST searches of the *Arabidopsis thaliana* (<http://www.arabidopsis.org/index.jsp>), *Oryza sativa* (<http://www.tigr.org/tdb/e2k1/osa1/>), and *Populus trichocarpa* (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) genomes, using *AtGAUT1* as the search probe. The GAUT protein sequences were aligned using ClustalX (Thompson et al., 1997) and suggested protein alignment parameters (Hall, 2004). Phylogenetic Bayesian analysis was carried out employing MrBayes (Huelsenbeck and Ronquist, 2001;

Ronquist and Huelsenbeck, 2003). Full length protein sequences were used in the analysis for all proteins except Os09g36180, whose C-terminal 404 amino acid extension was excluded.

Plant materials and growth conditions

Arabidopsis thaliana var. Columbia S6000 T-DNA insertion mutant seeds were obtained from the Arabidopsis Biological Resource Center (<http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/abrchome.htm>). The seeds were sown on pre-moistened soil and grown to maturity with a 14/10 photoperiod at $150 \mu\text{Ei m}^{-2}\text{s}^{-1}$ and temperatures of 19°C/14°C at 60% constant relative humidity. The plants were fertilized (Peters 20/20/20 with micronutrients) once a week or as needed. Seeds were sown in ‘growth sets’ consisting of approximately 20 plants from WT and T-DNA mutant lines. Walls were harvested from multiple 8-week-old plants and pooled together for wall glycosyl residue composition.

DNA extraction and mutant genotyping

Fresh, flash-frozen leaf tissue (100-200 mg) was ground with mortar and pestle and suspended in 0.5 ml extraction buffer (100 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 250 mM NaCl, $100 \mu\text{g ml}^{-1}$ proteinase K and 1% n-lauroylsarcosine) and extracted with an equal volume phenol:chloroform:isoamyl alcohol (49:50:1, v v⁻¹). RNA was degraded by addition of 2 μl of DNase-free RNase-A (10 mg ml^{-1}) for 20 minutes at 37°C. The DNA was precipitated twice with 70% ethanol and suspended in a final volume of 50 μl . Primers used for mutant genotyping were designed by ISECT tools (www.tdnexpress/isect). The genotype of mutant plants was determined based on the ability of the LB primers to anneal and produce T-DNA

specific PCR products when combined with the appropriate GAUT gene-specific primer. Gene specific primer pairs were similarly used to determine the presence of intact GAUT genes.

Isolation of cell walls

Cell wall samples were harvested from selected tissues of multiple 8 week old plants from WT and mutant lines ($n = 4$). The plant tissues for cell wall extraction were weighed (100-200 mg), flash frozen in liquid N_2 and ground to a fine powder. The tissues were consecutively extracted with 1 ml of 80% ethanol, 100% ethanol, chloroform:methanol (1:1, v v⁻¹) and 100% acetone. Centrifugation in a table top centrifuge at 6,000 x g for 10 minutes was used to pellet the sample in between all extractions. The remaining pellet was immediately treated with α -amylase (Sigma, porcine Type-I) in 100 mM ammonium formate pH 6.0. The remaining pellet was washed three times with sterile water and twice with acetone, and dried in a rotary vacuum dryer overnight at 40°C and weighed.

Mucilage extraction

Mucilage was extracted from 200 Arabidopsis seeds with sterile water at 60°C, with vortexing. Each hour over a 6 hour period the seeds were centrifuged and the supernatant transferred to a sterile tube. Pooled supernatants were lyophilized and resuspended in 600 μ l of sterile water. Phenol-sulfuric (Dubois et al., 1956) and *m*-hydroxybiphenyl (Blumenkrantz and Asboe-Hansen, 1973) assays were carried out with 100 μ l each. Duplicate 200 μ l aliquots were used for glycosyl residue composition analyses. To analyze the remaining seed coat material after extraction, the water extracted seeds were aliquoted in water to glass tubes and 20 μ g of

inositol was added. The seeds were lyophilized to dryness and used for glycosyl residue composition analyses.

TMS GC-MS glycosyl residue composition

The cell walls were aliquoted (1-3 mg) to individual tubes in acetone and allowed to air dry. Twenty μg of inositol was added to each tube and the samples were lyophilized. The dry samples were hydrolyzed for 18 h at 80°C in 1 M methanolic-HCl. The samples were cooled and evaporated under a stream of dry air and further dried two additional times with anhydrous methanol. The walls were derivatized with 200 μl of TriSil Reagent (Pierce-Endogen, Rockford IL USA) and heated to 80°C for 20 minutes. The cooled samples were evaporated under a stream of dry air, resuspended in 3 ml of hexane and filtered through packed glass wool. The dried samples were resuspended in 150 μl of hexane and 1 μl of sample was injected into the gas chromatograph.

Statistical analyses

The variance ratio test ($\alpha=0.05$) was used to compare the variances of standards and samples. ANOVA analyses, standard deviation, variance, t and the mean of sample were calculated using SAS 9.1.3 software (© 2002-2004 produced by the SAS Institute Inc. Cary N.C. USA). Significant differences between WT and mutant compositions were determined with $t_{\alpha(2)} = 0.1$ (90% confidence), but was set to 0.05 (95% confidence) for all other analyses. The appropriate sample size was predicted using equation 7.7, pp105 of Biostatistical Analysis, Fourth edition (Zar, 1999).

RNA extraction and RT-PCR

The total RNA was extracted from 0.5 g of stem, inflorescence, silique and leaf tissue from 8 week old plants. The tissues were homogenized in 10 ml of Homogenization Buffer (2% SDS in 50 mM Tris-HCl pH 7.8 and 40% water saturated phenol) and shaken for 15 minutes at 25°C. The tissue samples were centrifuged for 10 minutes at 8000 x g and 4°C and the supernatant removed to a clean tube. The sample was extracted two times with phenol:chloroform:isoamyl alcohol (25:24:01, v v⁻¹) and the aqueous phases were pooled. The RNA was precipitated overnight with 0.1 volume 3 M sodium acetate and 2.5 volumes of cold ethanol. The samples were DNase treated with RQ1 RNase-Free DNase (Promega, Madison WI USA) according to the manufacturer's instructions.

RT-PCR products were generated using primer sequences unique to each of the 15 GAUT genes. Each GAUT gene primer set was designed to span at least one intron such that unique PCR products were produced from RNA for each GAUT gene. Control RT reactions were carried out along side GAUT-specific reactions utilizing primers designed to the small ribosomal protein L23 alpha (Volkov et al., 2003). Qualitative RT-PCR experiments were carried out using 5 µg of total RNA in the RT reaction and 2 µl of the RT reaction was amplified through 30 cycles of PCR. Semi-quantitative RT-PCR used 2 µg of total RNA in the RT reactions and 1.5 µl of this was amplified through 26 cycles of PCR. Mutant transcript levels were assessed as follows: knock-outs (KO) were defined as mutants with RT-PCR reactions that yielded no detectable PCR product using gene specific primers. Knock-down (KD) mutants were those that yielded a PCR product with significantly decreased intensity compared to the WT.

CHAPTER 3

STRUCTURAL ANALYSIS OF THE *ARABIDOPSIS GAUT6* T-DNA MUTANT CELL WALLS AND MUTANT SEED EXTRACTABLE POLYSACCHARIDES³

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ABSTRACT

Pectic polysaccharides make up approximately one third of the dry weight of primary wall polysaccharides and are intricately involved in the function of the plant cell wall and the growth and development of the plant as a whole. Thus, the identification of glycosyltransferases directly involved in the synthesis of pectic polysaccharides is of a high priority. Uronic acids are a major component of pectic polysaccharides and many galacturonosyltransferases are required for pectin synthesis. Thus far, GAUT1, a homogalacturonan galacturonosyltransferase, is the only pectin biosynthetic glycosyltransferase gene of known biochemical activity. However, additional members of the GAUT1-related gene family of *Arabidopsis thaliana* are hypothesized to be pectin biosynthetic GalATs based on wall compositional phenotypes. GAUT6 has 46% amino acid similarity and 64% amino acid identity to GAUT1 and belongs to clade G of the GAUT1-related gene family. Furthermore, initial characterization of the cell wall phenotypes of mutants of 13 of the 15 *Arabidopsis* GAUT genes showed that GAUT6 belonged to a different wall phenotype group than other GAUT mutants. The total walls of *gaut6* mutant plants had reduced GalA. The detailed analysis of *gaut6* plants compared to WT carried out here has demonstrated that GAUT6 plays a role in plant growth and has a distinct role in wall polysaccharide biosynthesis. More specifically, the results of detailed leaf wall and seed polysaccharide analysis revealed that *gaut6* walls have ~30% less pectin in an HG-enriched fraction that is accessible to PME/EPG digestion and ~23% more pectin in the sodium carbonate/EGTA soluble fraction. The remaining pectin in the HG-enriched fraction of *gaut6* plants was reduced in Rha. The *gaut6* sodium carbonate/EGTA extractable wall fraction was increased in GalA and reduced in Rha compared to WT. The hemicellulose-enriched (4 N KOH soluble wall fraction) and the cellulose-enriched (4 N KOH insoluble wall fraction) were

reduced. Taken together these results suggest that GAUT6 encodes a putative pectin HG biosynthetic glycosyltransferase. We hypothesized that GAUT6 is likely to play a role in the synthesis of (1) stretches of HG linking RG-I domains or (2) a novel subfraction of HG.

INTRODUCTION

The cell wall of plants serves to protect and mechanically support the plant cell throughout growth and development and accordingly the wall is an important aspect of plant biology. The cell wall of plants is largely composed of carbohydrate, protein and phenolic compounds. The carbohydrate component of the wall is made up of cellulose, hemicelluloses and matrix polysaccharides. The matrix polysaccharides, or pectins, are abundant in the primary wall and have diverse structural characteristics that contribute greatly to wall function.

The primary wall of dicot plants is enriched in pectic polysaccharides that are essential for plant growth and development, cell adhesion and signaling. There are three major classes of pectin: homogalacturonan (HG), rhamnogalacturonan-I (RG-I), and the substituted galacturonan rhamnogalacturonan-II (RG-II). HG is a homopolymer of α -1,4-linked galacturonic acid (GalA) residues that may be modified by O-acetylation at the O-2 or O-3 and methylesterification at the C-6. RG-I has a backbone of alternating α -1,4-linked GalA residues and α -1,2-rhamnose residues. The rhamnose residues of RG-I are frequently branched at the C-4 by stretches of α -1,5-L-arabinofuranose, β -1,4-D-galactopyranose and arabinogalactan molecules. The occurrence of arabinan, galactan and arabinogalactan branches appears to be regulated (Willats et al., 2000) and these structures are frequently themselves branched. For example RG-I arabinan may be linked at the C-3 position of arabinose residues by terminal arabinofuranose, terminal galactose (Sakamoto and Sakai, 1995) (Huisman et al., 2001) or up to five β -1,4-linked galactose residues (Obro et al., 2004) (Nakamura et al., 2001). RG-I galactan may occur as a minimally branched β -1,4-galactan or as Type-I arabinogalactan or the less abundant Type-II arabinogalactan. Type-I arabinogalactan may have a single interspersed α -(1,5)-linked arabinofuranose residue in a galactan chain, that also has branches of one or more arabinofuranose residues or terminal

arabinopyranose residues (Huisman et al., 2001). The Type-II arabinogalactan is largely attributed to arabinogalactan proteins (AGPs), however some Type-II AG has been identified in association with pectic polysaccharides. Type-II AG is characterized by a backbone of β -(1,3)-D-galactan with branch points of 6-linked β -D-galactose of one, two or three residues in length. Type-II arabinogalactan may sometimes be capped by single β -Arap residues (Luonteri et al., 2003). RG-II molecule consists of a stretch of HG of approximately seven to nine GalA residues that is branched by four highly conserved ordered sidechains, designated A through D. The side chains of RG-II are complex containing several species of rare sugars in up to twenty linkages to form a structure that is highly conserved in the walls of most land plants (ONEILL et al., 2004). RG-II sidechains C and D are disaccharides linked to the C-3 of the backbone GalA residues and [$\leftarrow$ 2- β -D-Dha-5,1- β -L-Ara], respectively. The RG-II sidechains A and B have eight and nine to eleven residues, respectively. Sidechain A is specifically involved in the coordination of boron atoms that mediate RG-II dimerization *in muro* (ONEILL et al., 2004).

The mutants of putative pectin biosynthetic genes, thus far identified, have demonstrated that pectin is involved in many biological processes essential to plants. The *Nicotiana plumbaginifolia* GUT1 (*npgut1*) mutant and the *Arabidopsis thaliana* MUR1 (*mur1*) produce aberrantly synthesized RG-II that yield dwarfed plants with reduced cell adhesion and diminished wall strength (Iwai et al., 2002) (Reuhs et al., 2004). Mutants thought to be involved in HG synthesis (*qua1-1*) (Bouton et al., 2002), HG methylation (*qua2-1*) (Mouille et al., 2007), and pectin localization (*emb30*) (Shevell et al., 2000) are essential for WT-like cell expansion and cell adhesion. Of these, only *NpGUT1* and *AtQUA1* are hypothesized by be glycosyltransferases directly involved in the synthesis of pectin structures. *NpGUT1* is a putative glucuronosyltransferase, related to animal exostosins, that is thought to catalyze the

addition of a β -D-GlcA residue onto sidechain A of RG-II, however this has not been proven. The *gut1* mutant eliminates the disaccharide 1- β -D-GalA-2,1-L-Gal (Iwai et al., 2002). The *qual* mutant is hypothesized to be a galacturonosyltransferase (GalAT) that catalyzes the addition of α -1,4-GalA residues onto a growing chain of HG (Orfila et al., 2005).

In order to further elucidate the role of pectic polysaccharide structure in wall function, the genes involved in pectin biosynthesis must be identified. GAUT1 is a Golgi localized pectin biosynthetic α 1,4-GalAT that synthesizes homogalacturonan in the cell walls of plants (Sterling et al., 2006). The GAUT1-related gene family was identified based on amino acid similarity to GAUT1, the only confirmed HG:GalAT, which was purified from Arabidopsis cell suspension culture in fractions having HG-GalAT activity. The GAUT proteins in the GAUT1-related gene family have between 56-84% amino acid similarity to GAUT1 (Sterling et al., 2006). Recent re-analysis of the phylogenetic relationships between members of the Arabidopsis GAUT1-related gene family also included the known GAUT families of poplar (*Populus trichocarpa*) and rice (*Oryza sativa*). An ortholog of *GAUT2* was not identified in either poplar or rice, and is thought to be a pseudogene. Orthologs of *GAUT8* and *GAUT12* were not apparent in rice and may indicate that these genes have a specialized role in secondary wall synthesis (Caffall Ph.D dissertation, chapter 2). The phylogenetic analysis of GAUT genes in Arabidopsis, poplar and rice suggests that most GAUT genes are conserved in dicots and monocots and thus must be essential to wall polysaccharide biosynthesis in these organisms.

QUA1 and *GAUT8* map to the At3g25140 locus in the Arabidopsis genome (Bouton et al., 2002) that has 57% amino acid identity and 77% amino acid similarity to GAUT1 and that is present in clade B of the GAUT1-related gene family (Caffall Ph.D. dissertation, Chapter 2, 2008). The *qual-1* mutants were compromised in cell adhesion and also displayed differences in

pectin accumulation and glycosyl residue composition upon wall fractionation compared to WT (Leboeuf et al., 2005) suggesting that *QUA1* is likely to function as a pectin biosynthetic GalAT. Microsomal membranes isolated from *qual-1* tissues were shown to have reduced HG-GalAT (α 1,4-GalAT) and xylan biosynthetic (β 1,4-XylT) activity (Orfila et al., 2005), suggesting that the biosynthesis of HG and xylan are simultaneously impaired in the *qual-1* mutant. The *qual-1* mutant phenotype demonstrates that QUA1 plays an important, albeit an unresolved, role in the biosynthesis of HG (Bouton et al., 2002; Leboeuf et al., 2005; Orfila et al., 2005).

The *irx8-1/gaut12-1* and *irx8-5/gaut12-2* mutants (Persson et al., 2007) are members of the GAUT1-related gene family and reside in clade E with GAUT11-GAUT15 (Caffall Ph.D. dissertation, Chapter 2, 2008). GAUT12 has 40% amino acid identity and 61% amino acid similarity to GAUT1 (Sterling et al., 2006). GAUT12 is essential for glucuronoxylan biosynthesis but also has an effect on HG synthesis (Persson et al., 2007). GAUT12, unlike GAUT1 and GAUT8, is a putative GalAT involved in the synthesis of a component of xylan polysaccharides that may serve as a primer or cap for xylan biosynthesis (Pena et al., 2007) or a novel linkage between xylan and pectic polysaccharides (Pena et al., 2007).

GAUT6 has 46%/64% amino acid sequence identity/similarity to GAUT1. The glycosyl residue compositions of *gaut6* mutant walls were significantly different from WT and were also unique, compared to either *gaut8* or *gaut12* mutants. The leaf, silique and inflorescence walls of *gaut6-1* were reduced in GalA composition by 20%, 20% and 27%, but were not decreased in Xyl compared to WT walls (Caffall Ph.D. dissertation, Chapter 2, 2008). The composition data is informative but has limitations with regard to the identity of the specific linkages and parent polysaccharides affected. In addition, composition alone is unable to distinguish the compensatory changes in polysaccharides that may mask a more specific alteration in the walls

of these mutants. Some essential information was lacking in order to develop more detailed hypotheses regarding the underlying cause of the wall composition phenotype observed in the mutants of this member of the GAUT1-related gene family. In order to clarify the role of GAUT6 in plant wall biosynthesis, we sought to carry out a more detailed analysis of GAUT6 mutant growth phenotype and wall polysaccharides compared to WT.

The data obtained from the fractionation and subsequent composition and linkage analyses of *gaut6* walls compared to WT, and presented in this chapter show that GAUT6 plays a role in plant wall biosynthesis. The *gaut6* mutant walls are highly enriched in chelator/buffer soluble pectin and depleted in RG-I as well as in mass % of the hemicellulose-enriched and cellulose-enriched fractions. In addition, *gaut6* mutants have a seed germination phenotype under specific conditions and upon analysis of the seed mucilage reveal that *gaut6* seeds have a reduced total amount of seed mucilage, suggesting that the seed epidermal walls may be compromised by the lesions in GAUT6. The specific changes observed in the walls of aerial tissues and seed mucilage of the *gaut6* mutants suggest that GAUT6 plays a role in an HG structure associated with RG-I.

RESULTS

Genotyping and RNA transcript levels in gaut6 mutants

The homozygous mutants of *GAUT6* were isolated previously and the level of *GAUT6* transcript synthesized in mutant plants was assessed by RT-PCR (Caffall Ph.D. dissertation, Chapter 2). Total RNA was isolated from inflorescence, stem, silique and leaf of WT, *gaut6-1*, *gaut6-2*, *gaut6-3* and *gaut6-4* homozygous mutant plants. Using GAUT specific primers, transcript was not amplified from *gaut6-1* and *gaut6-2* that are knock-out mutants, however

gaut6-3 and *gaut6-4* transcript was able to be amplified indicating that these mutants are not knock-outs of GAUT6.

GAUT6 has mildly reduced stem growth

The previous analysis of *gaut6* mutant walls showed that a severe growth phenotype was not observed (Caffall, Ph.D. dissertation, Chapter 2, 2008). In order to investigate the possibility that the growth of *gaut6* mutants was affected in a more subtle manner, the growth of *gaut6* mutants compared to WT was assessed by quantifying the maximum length of leaves and stems during growth at standard temperatures and at elevated temperatures (Figure 3.1). At standard temperatures, *gaut6-2* stems had reduced elongation compared to WT, however no change in leaf growth compared to WT was detected. The stems of *gaut6-1* and *gaut6-4* were slightly reduced compared to WT, however, a significant statistical difference from WT was not established. The growth of *gaut6* plants was not more readily affected at higher temperatures and the stem height of mature plants was not significantly different from that of WT. The data show that *gaut6* mutants are only very slightly reduced in wall expansion/elongation that causes the height of stems to lag behind that of WT during the bolting of stems.

The seeds of gaut6, but not WT are sensitive to sterilization procedure

A novel phenotype was observed in the GAUT gene mutants. Depending on the seed sterilization procedure used for sowing seeds to sterile media, the germination of *gaut6* mutants was compromised. The original seed sterilization procedure entailed suspending the seeds in a 70% ethanol solution for 10 minutes and then a 25% bleach solution for 10 minutes followed by three consecutive washes with sterile water. In one experiment, the order of ethanol and bleach

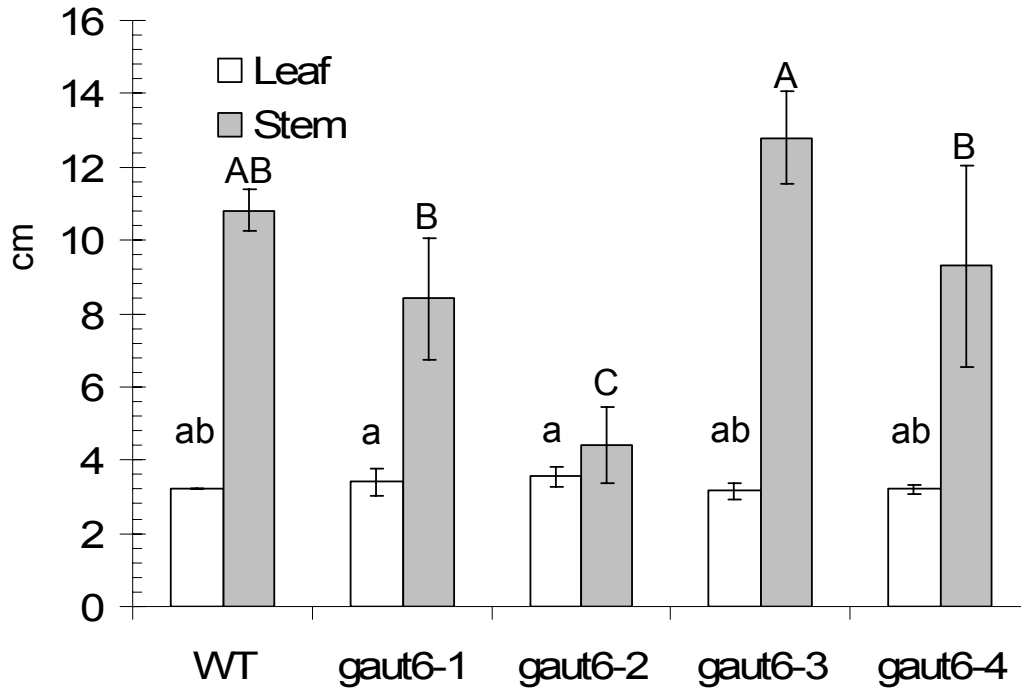


Figure 3.1. The leaf and stem growth of WT and *gaut6*.

The growth of *Arabidopsis* WT and *gaut6* plants were measured at 28 days after sowing under standard growth conditions (see methods). The data are the average in cm ($\pm$ sd) of leaf length (white bars) and stem height (gray bars). The significant difference of the data for different mutants was determined by ANOVA procedures and *a priori* α of 0.05 (95% confidence). Data having similar letters are not significantly different. Lower case corresponds to leaf data and upper case corresponds to stem data.

treatment in the sterilization procedure was accidentally reversed: bleach followed by ethanol instead of ethanol followed by bleach. This resulted in a reduction in the germination rates of seeds of *gaut6-1*, *gaut6-2* and *gaut6-3* while the germination rate of *gaut6-4* and WT was similar to that without pretreatment. It was hypothesized that the bleach treatment may have caused the reduced germination, based on the initial discovery of this phenotype.

The affect of bleach or water on WT and GAUT mutant seed germination was, therefore, directly tested by applying one of the following pretreatments: 70% ethanol, 25% bleach or sterile water (Figure 3.2). The results showed that the germination of WT seeds that had been sterilized by washing in ethanol was 100% and that the germination of GAUT mutants was similar to WT except for germination of *gaut6-2* which was slightly reduced to 96% germination. The germination of WT seeds that had been pre-treated by washing in bleach was slightly reduced to 88%, similar to the germination of *gaut6-3* and *gaut6-4*. The germination rates of *gaut6-1* and *gaut6-2*, however, were reduced to between 18 and 40%, respectively, by the bleach pretreatment. The germination of Arabidopsis WT and mutants when seeds were pretreated by washing in water was comparable to the results of bleach pretreatment. From this result, it was hypothesized that the reduced viability of *gaut* mutant seeds was exacerbated by bleach, but that bleach was not the primary cause of the reduced germination rate since sterile water pretreatment caused a similar reduction in viability. Thus, pretreatment of *gaut6-1*, and *gaut6-2* with an aqueous solution, with or without bleach, greatly reduced germination rates.

Ethanol pretreatment rescues mutant seed sensitivity to aqueous solutions

The sensitivity of *gaut6* mutants to aqueous pretreatment, but not ethanol pre-treatment, prompted an investigation into the effect of ethanol versus water on seed mucilage.

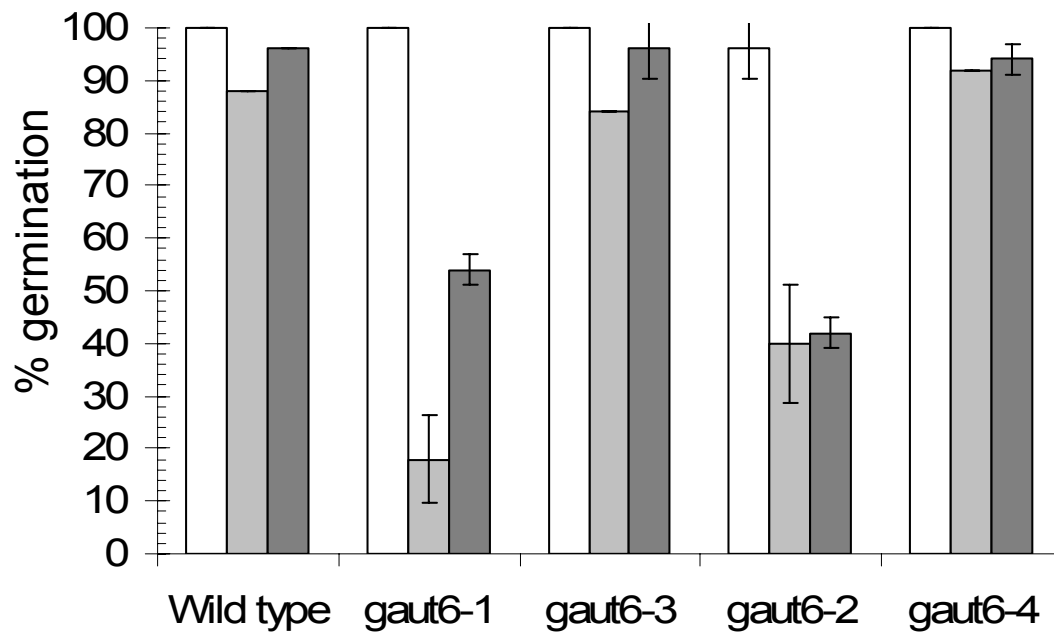


Figure 3.2. The affect of seed sterilization on the germination of WT and *gaut6* seeds.

Data are the average % germination ($\pm$ sd) of WT and *gaut6* seeds from triplicate plates of 25 seeds each. The germination rate was quantified after 10 days following three different pretreatments: 70% ethanol (white bars), 25% bleach (grey bars) or sterile water (dark gray bars).

Polysaccharides are easily solubilized in water or aqueous buffers but not in ethanol solutions. The solubilization of seed polysaccharides by water or the lack of solubilization of the polysaccharides by ethanol was assessed by seed mucilage expansion when pretreated with water or ethanol. The thickness of the mucilage layer was quantified in WT and *gaut6* seeds (Figure 3.3) that were pretreated with sterile water or 70% ethanol, prior to staining with 0.05% aqueous ruthenium red. The mucilage thickness of WT and *gaut6* mutants was measured with a dissecting scope and a slide micrometer. The results showed thicker mucilage in water-treated seeds than 70% ethanol-treated seeds. There was a great deal of variability, such that significant statistical differences in mucilage thickness could not be established between water and ethanol treatments. However, based on the observed trend and that aqueous solutions appear to have detrimental affect on *gaut6* seed germination, it is hypothesize that the ethanol treatment may precipitate the mucilage polysaccharides onto the seed and therefore prevent their full expansion in the aqueous solution, thus, preserving WT-like germination rates in *gaut6* mutant seeds.

GAUT6 leaf wall structure is altered

The walls of *gaut6* have been shown to be reproducibly altered in glycosyl residue composition compared to WT walls (Figure 3.4). The alcohol insoluble wall residue (AIR) or whole cell walls of *gaut6-1* had reduced GalA and Gal composition and increased Ara, Rha and Xyl composition compared to similarly extracted WT walls. The quantity of walls, however, extracted from a given amount of *gaut6* fresh leaf tissue did not appear to be significantly different from WT (Figure 3.5). The wall compositional phenotype of *gaut6* mutants compared to WT presented an interesting opportunity to further explore the causes of the wall compositional changes caused by mutations in GAUT genes.

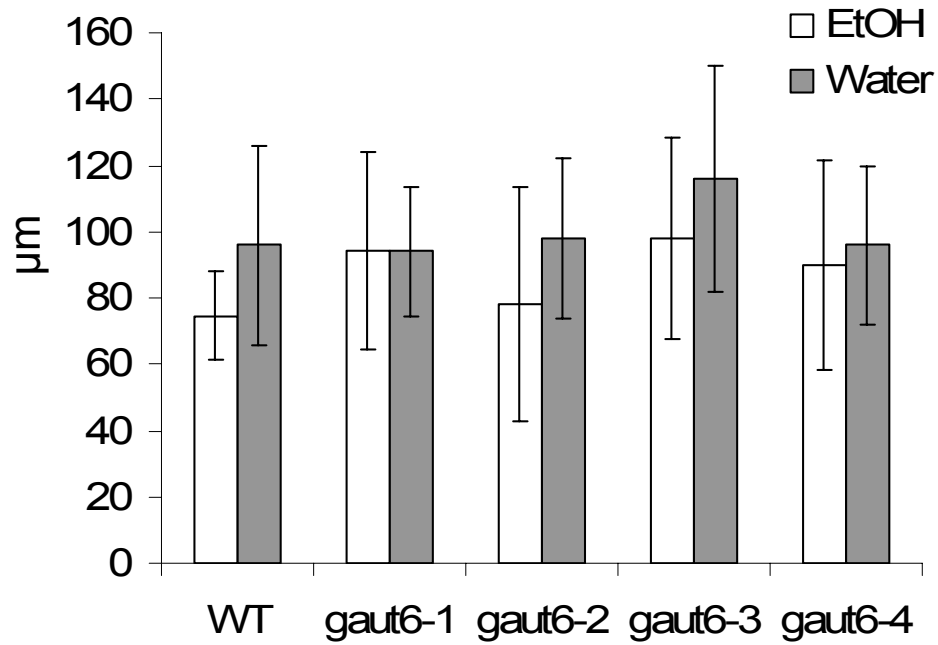


Figure 3.3. The mucilage thickness of WT and *gaud6* seeds after soaking in ethanol or water.

Data are the mucilage thickness (μm , $\pm$ sd) of WT and GAUT6 mutant seed wetted with 70% ethanol (white bars) or water (gray bars) and then stained with 0.05% ruthenium red.

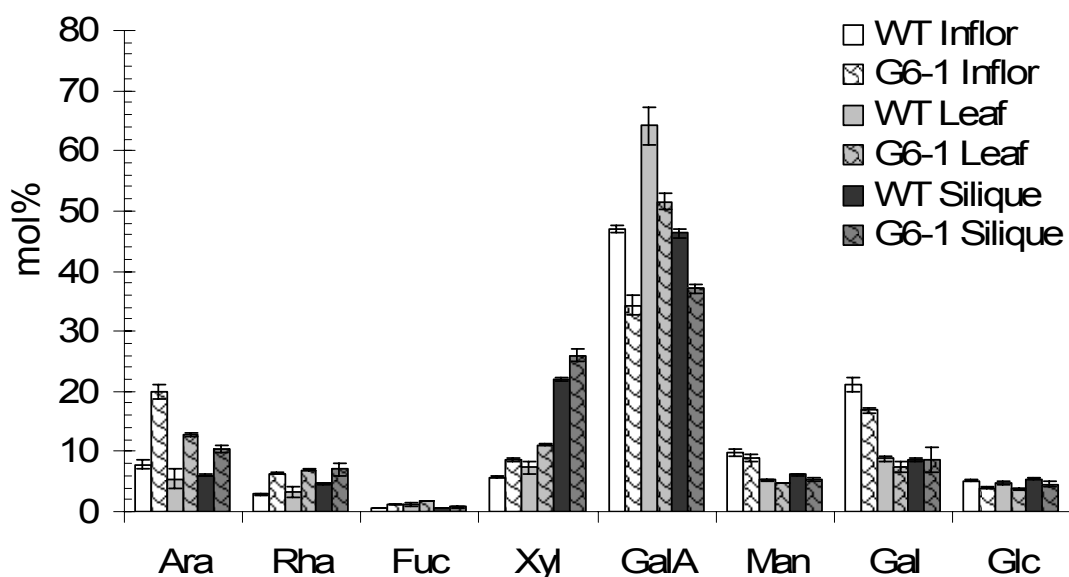


Figure 3.4. The tissue-specific glycosyl residue composition of WT and *gaut6* walls.

The data are the glycosyl residue wall composition of WT (solid bars) and *gaut6-1* (cross-hatched bars). The wall glycosyl residue composition was determined by GC-MS of the TMS derivatives of alcohol insoluble wall residue (AIR) (see methods) from inflorescence (white bars), leaf (medium gray bars) and silique (dark gray bars). The glycosyl residues quantified are arabinose, Ara; rhamnose, Rha; fucose, Fuc; xylose, Xyl; galacturonic acid, GalA; mannose, Man; galactose, Gal; and glucose, Glc.

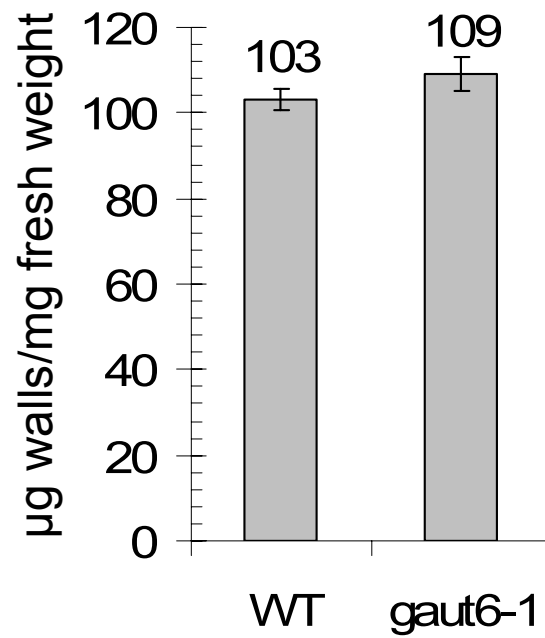


Figure 3.5. The dry mass of total walls extracted from WT and *gaut6* leaf tissue. The data are the average µg walls per mg of fresh leaf tissue $\pm$ standard deviation.

Fractionation of WT and gaut6 mutant walls

To further investigate the specific changes in the wall composition of GAUT mutants, the leaf AIR walls of WT, *gaut6-1* and *gaut6-3* leaf walls were sequentially fractionated by enzymatic hydrolysis and chemical fragmentation using pectinmethylesterase in combination with endopolygalacturonase (PME/EPG), 50 mM sodium carbonate and 5 mM EGTA and 4 N KOH (Figure 3.6). The PME/EPG fraction is the polysaccharide linked to HG with four or more contiguous non-esterified GalA residues that is accessible by the enzymes. The HG in this case may, or may not have been originally methylesterified, in that the PME would remove the methyl-esters. The polysaccharides released into sodium carbonate/EGTA fraction are primarily the pectin released by the mildly alkaline buffer and by chelating of calcium and/or borate ions of the wall. Hemicelluloses were solubilized from the wall residue with strong alkaline 4 N KOH that disrupts the hydrogen bonding of the hemicelluloses to cellulose (4 N KOH sol). The remaining residue of the wall has been stripped of much of the pectin and hemicellulose, leaving a cellulose-enriched pellet (4 N KOH insol).

The fractionation of *gaut6-1* and *gaut6-3* AIR walls yielded the release of less PME/EPG, more sodium carbonate/EGTA-soluble, less KOH-soluble material and less KOH-insoluble material remaining. The data are the μg of each fraction mg^{-1} of the total recovered material (Figure 3.7). The glycosyl residue compositions of *gaut6* mutant wall fractions were altered compared to WT. The PME/EPG-released fraction had reduced Rha. There was increased Xyl in the 4 N KOH soluble fraction, but there was no change in the Xyl composition of total walls. More relative GalA was present in the PME/EPG-released and sodium carbonate/EGTA soluble fraction, but there was less GalA in the KOH soluble and KOH insoluble material. The wall

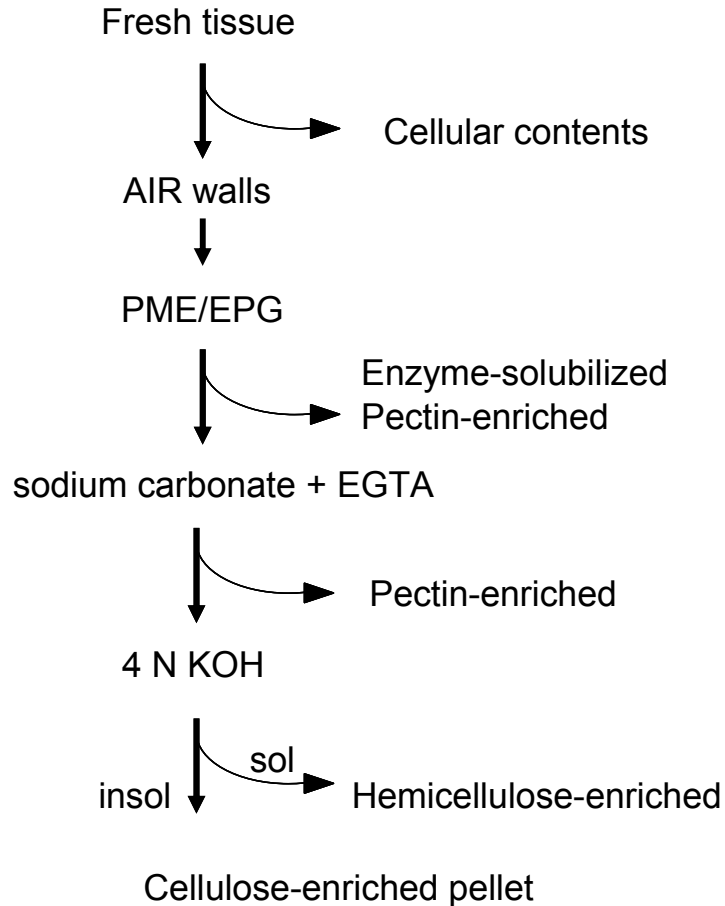


Figure 3.6. The fractionation of WT and leaf AIR walls.

The diagram illustrates the sequential fractionation procedure carried out on alcohol insoluble residue (AIR) of WT and *gaut6-1* and *gaut6-3* walls. The combined treatment of walls with *A. niger* PME and EPG in 50 mM ammonium formate, pH 6.0 (PME/EPG) releases an enzyme accessible pectin-enriched fraction. Sodium carbonate in addition to 5 mM EGTA (sodium carbonate/EGTA) releases a pectin-enriched fraction that may not be enzyme accessible or may have been calcium crosslinked. Concentrated alkali was used to extract the hemicellulose-enriched fraction (4 N KOH sol) leaving a cellulose-enriched residue (4 N KOH insol).

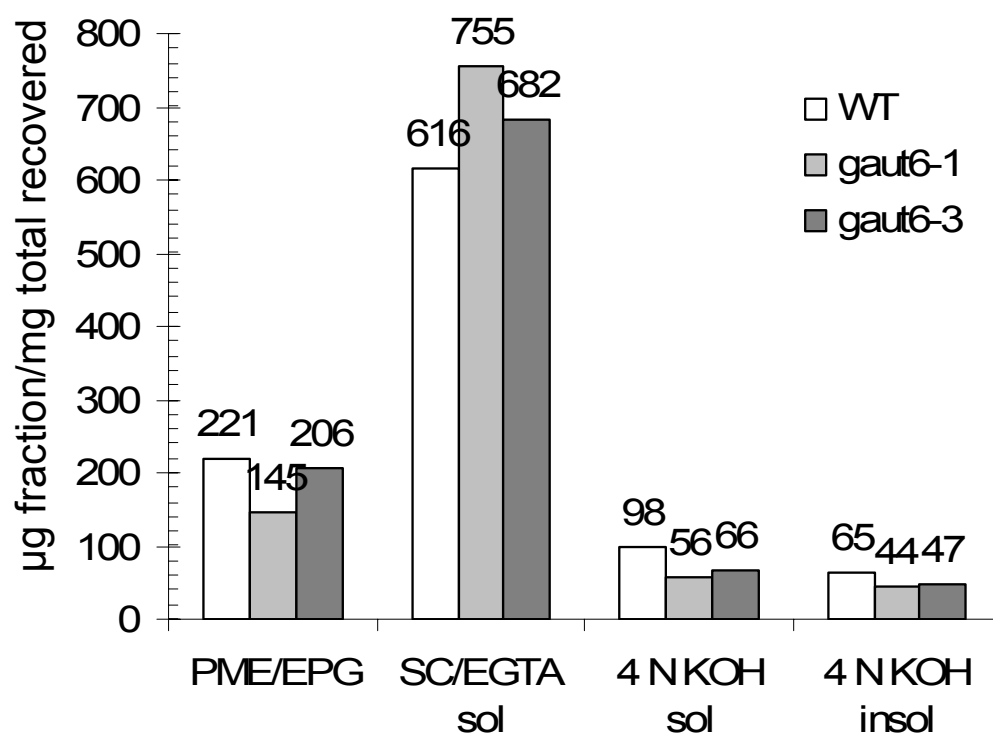


Figure 3.7. The wall fraction weights of WT, *gaut6-1* and *gaut6-3* from leaf de-starched AIR walls. The weights of the residue extracted by pectinmethylesterase and endopolygalacturonase (PME/EPG), sodium carbonate and EGTA (SC/EGTA sol), 4 N KOH (4 N KOH sol) and the remaining pellet (4 N KOH insol) are shown expressed as the $\mu\text{g mg}^{-1}$ of the total recovered fraction weights.

fractions of *gaut6-1* and *gaut6-3* mutants were reduced in the Ara content of all fractions; however there was no significant change in Ara content of total walls compared to WT.

The results of *gaut6-1* and *gaut6-3* wall and wall fraction glycosyl residue composition analysis compared to WT (Table 3.1), suggest that a reduced amount of un-calcium crosslinked pectin is present and released by PME/EPG treatment in *gaut6* walls. A greater amount of material is then released in the EGTA fraction suggesting that the *gaut6* mutation causes a change in pectin structure such that an accessible region of HG is not synthesized in *gaut6* walls and thus leads to the formation of more pectin. Both the PME/EPG released pectin and sodium carbonate/EGTA released pectin contain a higher proportion of HG compared to RG-I, according to the GalA/Rha ratio in these fractions. According to these results, the walls of *gaut6-1*, and to some extent *gaut6-3*, appear to be reduced in HG and may also be reduced in RG-I and RG-I branching by arabinan sidechains, resulting in an overall decrease in GalA rich pectin as observed in the glycosyl residue compositions of *gaut6* AIR walls compared to WT.

PME/EPG wall fractions analyzed by glycosyl residues linkage analysis

The linkage analysis was carried out on the PME/EPG fraction of WT and *gaut6-1* leaf walls because considerably less material was released in this fraction; approximately 66% of that released by WT walls suggesting that either less pectin is produced or that less pectin is available for release by the combined PME and EPG enzyme treatment (Table 3.2). The PME/EPG-released fraction of the leaf walls represents the polysaccharides released by cleavage of the pectic backbone by EPG alone, by EPG after de-methylesterification by PME in ammonium formate buffer (pH 6.0) soluble walls. Less T-Araf, 2-Araf, 5-Araf/4-Arap, 3,5-Araf/3,4-Arap

Table 3.1. The glycosyl residue composition of WT and GAUT6 mutant leaf wall fractions.

(^a) The walls of WT, *gaut6-1* and *gaut6-3* walls were sequentially fractionated by combined treatment with pectinmethylesterase and endopolygalacturonase (PME/EPG), and then solubilized by 50 mM sodium carbonate and 5 mM EGTA, and then solubilized by 4 N KOH (4 N KOH sol). The remaining pellet is enriched in cellulose that is not readily hydrolyzed by the methods used for glycosyl residue composition, and therefore, the composition of the 4 N KOH insoluble (4 N KOH insol) fraction is that of the non-cellulosic wall polysaccharides. In addition the sum of the mol % glycosyl residue composition of the fractions (Total) and the glycosyl residue composition of the WT, *gaut6-1* and *gaut6-2* AIR walls fractionated of walls (AIR walls) are given. (^b)The glycosyl residues quantified are abbreviated as in Figure 3.3. (^c) The GalA/Rha ratio expresses the approximate abundance of GalA residues potentially in RG-I compared to that potentially present in HG.

Cell Wall Fraction ^a	Sample	Ara ^b	Rha	Fuc	Xyl	GalA	Man	Gal	Glc	ug/mg ^c	GalA/Rha ^d
PME/EPG	WT	10.6	7.5	0.3	1.1	76.8	0.4	3.4	0.0	220.6	10.2
	<i>gaut6-1</i>	5.7	4.6	0.4	1.5	78.3	0.5	9.1	0.0	145.5	17.2
	<i>gaut6-3</i>	5.4	3.3	0.6	1.8	77.5	0.7	10.5	0.3	206.1	23.4
EGTA/SC sol	WT	10.6	12.5	0.0	3.7	58.1	0.0	14.9	0.0	616.0	4.6
	<i>gaut6-1</i>	6.6	9.6	0.1	3.0	69.9	0.0	10.8	0.0	755.0	7.3
	<i>gaut6-3</i>	5.3	10.7	0.1	3.4	70.1	0.1	9.9	0.5	681.9	6.6
4 N KOH sol	WT	4.9	2.9	1.2	24.7	9.9	1.1	19.1	36.2	98.5	3.4
	<i>gaut6-1</i>	3.1	2.5	0.5	33.4	5.6	1.3	16.8	36.8	56.0	2.2
	<i>gaut6-3</i>	4.8	4.2	1.0	30.0	12.9	1.4	20.2	25.6	65.8	3.0
4 N KOH insol	WT	8.1	7.5	1.4	11.3	10.5	5.3	16.0	39.7	65.0	1.4
	<i>gaut6-1</i>	4.5	4.9	1.1	8.8	6.5	3.4	16.9	53.9	43.5	1.3
	<i>gaut6-3</i>	6.8	7.3	1.9	13.0	12.4	5.5	14.7	38.4	47.0	1.7
Total cell walls	WT	15.7	11.3	1.9	16.3	32.4	1.7	20.7	0.0		2.9
	<i>gaut6-1</i>	18.6	12.5	2.2	16.5	27.8	2.1	20.2	0.0		2.2
	<i>gaut6-3</i>	15.1	12.5	2.1	14.0	35.6	1.6	19.2	0.0		2.9

Table 3.2. The glycosyl residue linkage analysis of WT and *gaut6-1* leaf wall PME/EPG fractions.

^(a)The data are the glycosyl residue linkage analysis of the WT and *gaut6-1* PME/EPG fraction (compositions listed in Table 3.1). Linkage (determined by a modified Hakamori method (1964)) of interest are shown in bold type. The residues are expressed as the % peak area.

Residue	WT	<i>gaut6-1</i>
T-Araf	6.2	5.2
2-Araf	5.5	4.1
5-Araf	8.6	5.3
3,5-Araf	4.1	3.3
2,3,5-Araf	3.9	2.2
T-Rhap	3.0	2.3
2-Rhap	4.2	7.8
3-Rhap	2.6	2.0
2,3-Rhap	1.3	1.1
2,4-Rhap	1.5	1.2
2,3,4-Rhap	2.9	2.3
T-Xylp	5.4	5.4
3-Xylp	4.4	6.4
4-Xylp	1.8	3.2
2,4-Xylp	2.0	0.0
2,3,4-Xylp	0.8	0.5
T-Manp	2.3	3.1
T-Galp	4.7	6.1
2-Galp	0.4	1.8
4-Galp	5.6	5.3
3,4-Galp	1.1	0.7
2,4-Galp	2.4	2.6
4,6-Galp	1.4	2.2
2,3,4,6-Galp	6.1	5.4
4-Galp A	5.0	6.0
3,4-Galp A	4.2	6.7
2,4-Galp A	6.8	3.2
Total Ara	28.3	20.2
Total Rha	15.4	16.7
Total Xyl	14.5	15.5
Total Man	2.3	3.1
Total Gal	21.7	24.2
Total GalA	16.0	15.9

and 2,3,5-Araf/2,3,4-Arap was observed in *gaut6* PME/EPG released walls, resulting in an overall reduced level of Ara that is likely a component of RG-I sidechains. Similar amounts of 2-linked Rhap, but less of the branched Rhap was found in *gaut6-1* PME/EPG released fraction compared to WT. A slight increase in 4-linked Xylp and total Xylp was observed in this fraction. An increase in T-Galp and overall Galp suggests an increase in the branching of RG-I galactan. Similar amounts of total GalpA and 4-linked GalpA were observed between WT and *gaut6-1* fractions, consistent with the composition of the PME/EPG fraction for *gaut6-1*.

Water extracted polysaccharides are increased in gaut6 compared to WT

It was hypothesized that some wall component may have been lost during the de-starching of AIR walls in aqueous buffer to alter the levels of Ara, Rha and GalA in the remaining wall residue that was subjected to fractionation. In order to investigate the discrepancy in the Ara, Rha and GalA content between the glycosyl residue composition of wall fractions and total AIR walls, total AIR walls not treated with alpha amylase for de-starching were extracted with sterile water twice over 24 hours. The combined supernatants were lyophilized and the resulting residue was subjected to glycosyl residue composition (Figure 3.8). More carbohydrate was obtained from the *gaut6* mutant (349 $\mu\text{g mg}^{-1}$ water extract) compared to WT (291 $\mu\text{g mg}^{-1}$ water extract). This fraction had an RG-I-like glycosyl residue composition that was enriched in Ara and Gal with lower levels of Rha, Xyl, GalA and Glc. The *gaut6* water-extracted fraction had increased levels of Ara and Rha, compared to the WT water-extracted fraction.

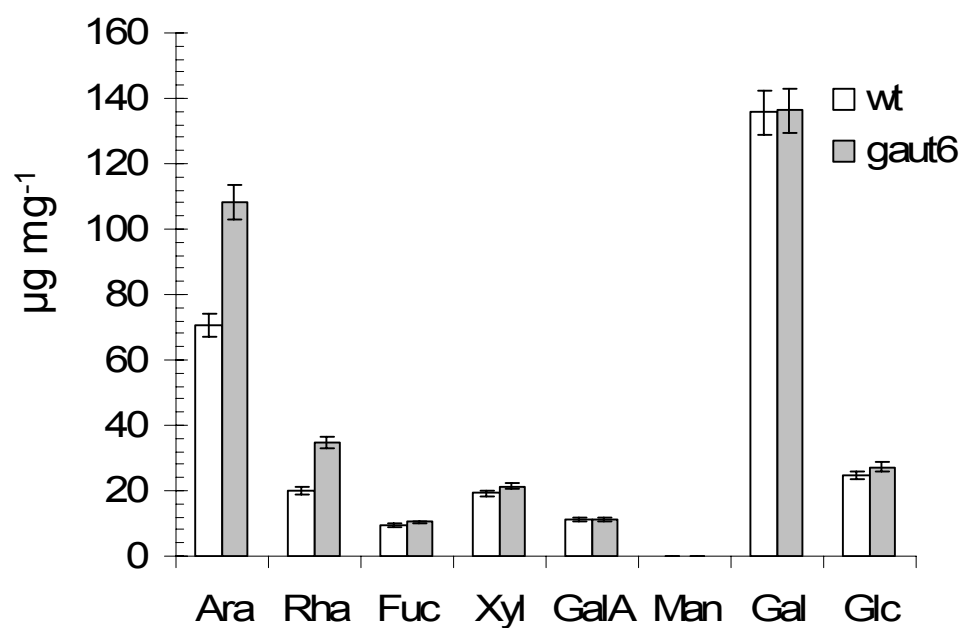


Figure 3.8. The water extracted residue of AIR walls of WT and *gaut6-1* leaves.

The data are glycosyl residue composition, expressed as µg per mg of water extract, from WT and *gaut6-1* leaf AIR walls.

The glycosyl residue composition and linkage analysis of seed-extractable polysaccharides of WT and gaut6.

It is hypothesized that the reduced germination of GAUT mutant seeds compared to WT after aqueous pretreatment is caused by aqueous solutions that may wash away mucilage components or modify the mucilage that consequently affects seed germination. The characterization of the mucilage is an essential first step in attempting to identify the differences in mucilage that may contribute to reduced germination in *gaut6* mutant seeds compared to the germination of WT, and thus also reveal clues as to the function of GAUT6 in plant wall polysaccharide biosynthesis.

The mucilage was extracted with hot water to yield the water-extractable material (WEM) and then with hot buffer to yield the buffer-extractable material (BEM) from ~two grams of WT or *gaut6* seed for the characterization of the released polysaccharides. The water extracted mucilage comprised approximately 62.5 $\mu\text{g mg}^{-1}$ seed of WT, 44.2 $\mu\text{g mg}^{-1}$ seed of *gaut6-1*, 50.4 $\mu\text{g mg}^{-1}$ of *gaut6-2*, 54.4 $\mu\text{g mg}^{-1}$ of *gaut6-3* and 49.6 $\mu\text{g mg}^{-1}$ of *gaut6-4*, demonstrating an overall reduction in mucilage produced in the mutant. The glycosyl residue composition of the WEM, BEM and remaining seed material (RSM) was determined (Table 3.3). The WEM consists of the water soluble pectins that are not crosslinked to other wall components or to the seed. The glycosyl residue composition of the WEM revealed equal parts Rha and GalA with a small amount of Gal, Xyl and Man. The WEM of the *gaut6* mutants had increased Rha and Xyl, and decreased GalA compared to the WEM of WT. This result is similar to the observed results of the glycosyl residue composition of total cell walls of *gaut6* mutants compared to WT. The BEM represents the pectin extractable by calcium chelators and mildly alkaline buffer that was not solubilized previously by hot water,

Table 3.3. The WT and *gaut6* glycosyl residue composition of seed extractable polysaccharides.

(^a) The data are the mol % glycosyl residue composition of polysaccharide fractions of water-extractable material (WEM), buffer-extractable material (BEM) and residual seed material (RSM). (^b) The fractions were extracted from seed of wild type (WT) and *gaut6* mutants. (^c) The glycosyl residue compositions are abbreviated as in Table 3.1. (^d) The Glc content was omitted from the WEM and BEM glycosyl residue composition based on linkage analysis, which determined that the Glc content was derived from starch.

Fraction ^a	Source ^b	Ara ^c	Rha	Fuc	Xyl	GalA	Man	Gal	Glc ^d
WEM	WT	0.0	32.0	0.0	4.0	53.0	0.8	10.2	0.0
	<i>gaut6-1</i>	0.0	40.3	0.0	4.9	44.9	0.9	9.0	0.0
	<i>gaut6-2</i>	0.0	35.8	0.0	4.4	45.5	0.8	13.6	0.0
	<i>gaut6-3</i>	0.0	42.0	0.0	5.4	45.0	0.5	7.1	0.0
	<i>gaut6-4</i>	0.0	39.2	0.0	4.8	44.0	0.7	11.4	0.0
BEM	WT	0.0	18.9	0.0	7.0	68.5	5.5	0.0	0.0
	<i>gaut6-1</i>	0.0	21.7	0.0	6.6	67.5	2.8	1.3	0.0
	<i>gaut6-2</i>	0.0	23.8	0.0	7.2	65.8	3.2	0.0	0.0
	<i>gaut6-3</i>	0.0	28.8	0.0	11.3	53.1	5.7	1.0	0.0
	<i>gaut6-4</i>	0.0	16.1	0.0	8.7	68.6	6.6	0.0	0.0
RSM	WT s	28.1	15.3	2.9	12.1	13.2	1.1	20.2	7.1
	<i>gaut6-1</i>	32.3	16.3	3.1	11.9	10.1	1.0	18.1	7.1
	<i>gaut6-2</i>	29.1	17.9	2.5	11.5	13.0	0.9	17.3	7.8
	<i>gaut6-3</i>	27.1	19.5	2.7	13.8	13.2	1.1	15.1	7.6
	<i>gaut6-4</i>	28.4	21.2	2.8	11.0	13.7	1.3	15.7	5.8

which accounted for a very small amount of material (<10 % of WEM). The buffer extracted mucilage is greater than 70 mol % GalA with approximately equal quantities of Rha, Xyl, Man and Gal. No differences were observed between WT and *gaut6* BEM. The glycosyl residue composition of the RSM was determined by directly subjecting the extracted seeds to TMS without prior crushing or grinding. The RSM represents the remaining mucilage adhering to the seed coat and possibly some seed coat polysaccharides. The composition of this material consists of a large quantity of Ara (27 to 32 mol %) and Gal (15 to 20 mol %) and approximately equal amounts of Rha, GalA, Xyl, Glc with minor amounts of Fuc and Man. The glycosyl residue composition of the RSM showed that *gaut6* seeds were increased in Ara and reduced in GalA composition compared to WT.

The WEM was subjected to glycosyl residue linkage analysis and confirmed that the GalA-containing polysaccharide as RG-I, which is a polymer of 2-linked Rha_p and 4-linked Gal_pA. The results of glycosyl linkage analysis using a modified Hakomori methylation for partially methylated alditol acetates without prior reduction of uronic acids samples yielded samples that consisted largely of 2-linked Rha_p 4-linked Gal_p. The linkage analysis showed that the majority of the Glc of the WEM samples was 3,6-linked, 6-linked and 4,6-linked and 2,3,4,6-linked Glc_p that may originate from starch, amylopectin, cellulose or a mixed linkage glucan, and thus, was not included in the previous compositional analyses or the linkage result.

Gaut6-1 have altered seed extracted phenolic content

A yellow-brown color of the WEM mucilage was observed and a noticeable difference in the color of the WT and *gaut6* mutant mucilage samples was observed. In order to quantify the color differences, the WEM was scanned across broad wavelengths (Figure 3.9). The WEM

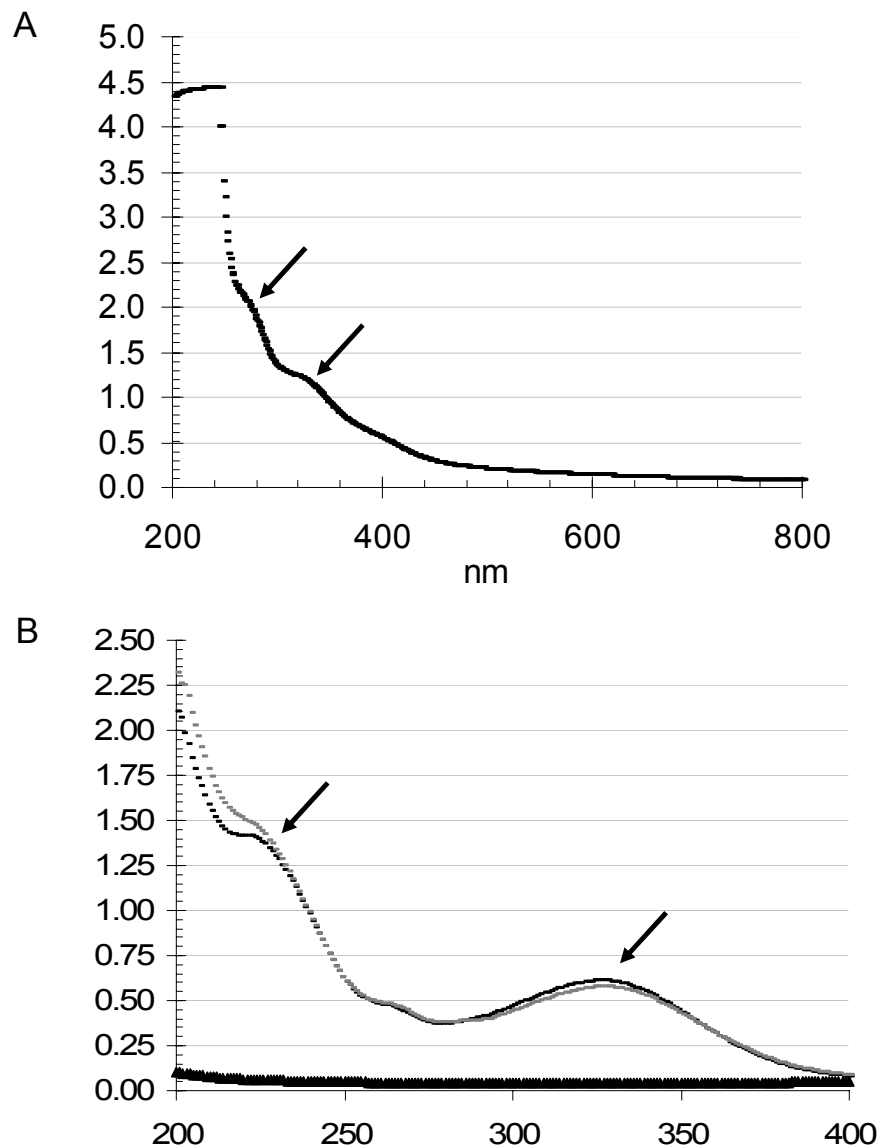


Figure 3.9. The Absorbance maxima of water extracted and 60% methanol seed extract.

(A) The hot water extracted mucilage (WEM) absorbance in WT, as visualized by a wavelength scan; absorption maxima of the peak of interest was at ~330 nm and 230 nm (black arrows). (B) The wavelength scan of the material extracted with 60% methanol from WT (black dashes) and *gaut6-1* (gray dashes) was shown to have similar absorption maxima (330 nm and 230 nm) as the phenolics extracted by hot water.

fraction was diluted to 0.25x and subjected to a wavelength scan. Two shallow peaks were observed at 260-290 nm and at 320-350 nm indicating the presence of at least two UV absorbing components in the sample. The seeds of WT and *gaut6-1* were extracted with methanol in an attempt to enrich for phenolic acids and facilitate analysis of the phenolic constituents. Phenolic compounds may be extracted from plant material by 60% methanol (Cai, 2003). The methanol extracted phenolics include those phenolics of the mucilage as well as the seed coat. The 60% methanol extract was subjected to a wavelength scan similar to that carried out on the WEM (Figure 3.10). The methanol extract of WT showed two peaks with absorbance maxima at ~265 and ~335 nm that correlated well with the peaks observed previously in the WEM. The Folin & Ciocalteu's assay reagent for phenolics was used to quantify the phenolics in the 60% methanol extract. The phenolic content of *gaut6-1* was higher than that of WT, based on phenolic gallic acid equivalent per mg of seed extracted. The increase in phenolics was confirmed by reverse phase chromatography and GC-MS (data not shown).

DISCUSSION

The pectin extracted from gaut6 mutant walls has altered structure compared to WT

There were three pectin-enriched fractions analyzed in the analysis of WT and *gaut6* walls; the water-extract, the PME/EPG-released and the sodium carbonate/EGTA-soluble walls. The AIR walls of *gaut6* mutants are reduced in GalA levels; however, the composition of the PME/EPG-released and sodium carbonate/EGTA-soluble walls had increased GalA levels. Subsequent analysis of the water extract of WT and *gaut6* walls showed that *gaut6* mutants more readily release RG-I-like polysaccharides to aqueous solutions such as that used for the de-starching of AIR walls. It is hypothesized that RG-I was lost to the de-starching buffer and

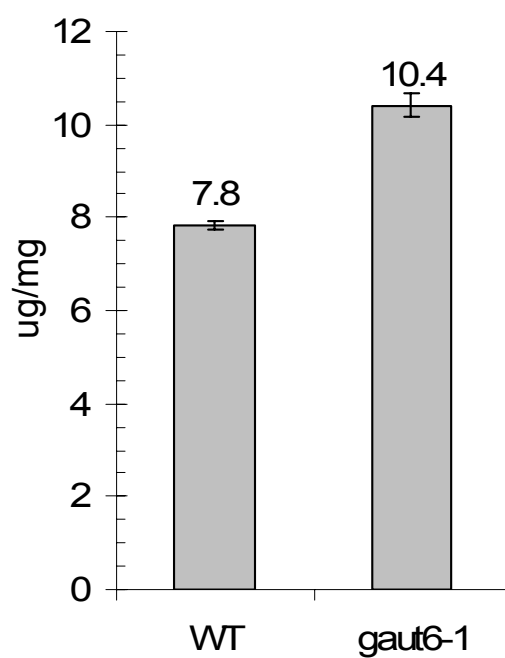


Figure 3.10. Phenolics extracted from Arabidopsis WT and *gaut6* seeds.

The data are the average phenolics ($\pm$ sd) of triplicate samples determined by Folin & Ciocalteu's assay reagent for phenolics (Lowry et al., 1951a) expressed in gallic acid equivalents extracted with 60% methanol from equal weights of seeds.

is the cause of the apparent depletion of RG-I in the PME/EPG-released and sodium carbonate/EGTA-soluble fractions. This is evidenced by the reduced levels of Ara and Rha and increased GalA/Rha ratio in these fractions. Indeed, the glycosyl residue linkage analysis showed an increase in unbranched 2-linked Rha_p and decreased 5-linked Ara_f and T-Ara_f residues in the PME/EPG fraction. The linkage analysis of this fraction suggests that there is less branching of *gaut6-1* RG-I compared to the WT fraction. RG-I and HG are thought to be covalently connected by backbone residues (Keegstra et al., 1973). The overall reduction in pectic polysaccharides in the AIR walls and the excessive loss of RG-I-like polymers to aqueous solutions in *gaut6* mutants suggest that *gaut6* mutants may lack a specific wall crosslinking. More specifically, the loss of an RG-I crosslinking HG may affect the synthesis of RG-I in ways that have not been elucidated in the literature. Because of the unknown consequences of altered HG structure on RG-I, the reduction in RG-I may indicate a role for GAUT6 function in either the synthesis of HG or in the synthesis of stretches of HG linking RG-I domains.

In addition to the crosslinking of HG, RG-I and RG-II through the backbone residues, HG *in vivo* is thought to be crosslinked by calcium in the wall (Willats et al., 2001b). The level of calcium crosslinking depends on the degree of methylation of the HG polymers (Willats et al., 2000). There is an increase in the mass of pectin-enriched material released only by calcium chelators, but not by the PME/EPG treatment or water extraction in *gaut6* mutants compared to WT. An increase in the overall quantity of calcium complexed pectin was observed in apple (*Malus domestica*) overexpressing a native PG that specifically hydrolyses HG polysaccharides, though the molecular weight of those polysaccharides was less than that found in WT apple (Atkinson et al., 2002). Increased calcium complexed pectin has been observed in response to irregular pectin structures caused by wall damage. A mechanism of heightened defense

responses as a result of PG over-expression, which specifically hydrolyzes wall HG polysaccharides, has been demonstrated in tobacco (*Nicotiana tabacum*) and Arabidopsis plants (Ferrari et al., 2007b). Increases in the $\mu\text{g mg}^{-1}$ of sodium carbonate/EGTA soluble walls were also observed in Arabidopsis *gaut12* walls (Persson et al., 2007) affected in xylan and pectin synthesis. Increased levels of pectin were not observed, however, in Arabidopsis GAUT8 (QUA1) mutants (Leboeuf et al., 2005) affected in HG and xylan synthesis, but which is a putative HG:GalAT (Figure 3.11). The *qual* plants, however, have a relatively severe deficiency in HG polymers accounting for the severe phenotype of *qual* plants, while the lesion in GAUT6 mutants causes changes in a different and apparently minor subfraction of HG structure and thus different changes in wall phenotype are observed.

The gaut6 mutants have walls with less hemicellulose

The hemicellulose-enriched and cellulose-enriched fractions were similarly reduced in $\mu\text{g mg}^{-1}$ of the total recovered material: *gaut6-1* and *gaut6-3* had 57% and 67% of WT hemicellulose-enriched walls, respectively, and 67% and 72% of cellulose-enriched walls, respectively. The hemicelluloses extracted from GAUT6 mutants were increased in Xyl and Glc. It is possible that this reflects a dramatic down regulation of hemicellulose in *gaut6* walls. Based on the glycosyl residue composition of the *gaut6* 4 N KOH soluble fraction, xylan does not appear to be affected, potentially ruling out xylan biosynthetic function for GAUT6.

The gaut6 mutants have walls with less cellulose

The leaf walls of both *gaut6-1* and *gaut6-3* mutants had 67-72% cellulose remaining in the cellulose-enriched 4 N KOH insoluble fraction than similar walls of WT. Reduced cellulose

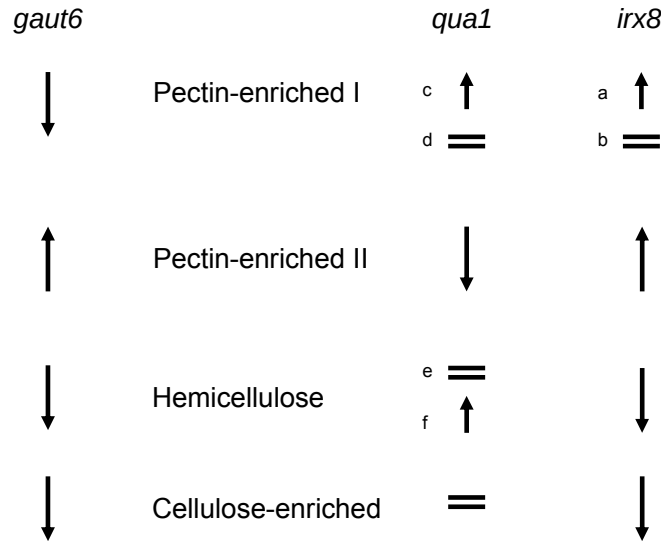


Figure 3.11. The *gaut6*, *qua1-1* and *irx8* wall fractions.

The *gaut6* leaf walls, *qua1* suspension culture walls and *irx8* stem walls were fractionated and compared to similar walls of WT. The pectin-enriched I fraction is that pectin that was extracted without the use of calcium chelators. The pectin-enriched I fraction of *gaut6* walls was obtained by a single step procedure using PME/EPG. The pectin-enriched fraction I of *irx8* walls was obtained by a two-step procedure using EPG (a) and then PME/EPG (b) treatment. The pectin-enriched fraction I of *qua1* walls was obtained by harvesting was released to the medium (c) or extracted from AIR walls with water (d). The pectin-enriched fraction II that was extracted with 50 mM sodium carbonate and 5 mM EGTA (*gaut6* and *irx8*) or 70 mM ammonium oxalate (*qua1*). Hemicellulose polysaccharides are those that were extracted with 4 N KOH (*gaut6* and *irx8*) or those that were extracted with 0.05 N NaOH (e) and 4 N NaOH (f). The cellulose-enriched residue is that material remaining after the sequential extraction of polysaccharides as described above. The mass of mutant wall fractions, compared to the WT control, in each experiment is indicated; no change (==), an increase (↑) or decrease (↓).

content, which is a direct result of the loss of cellulose synthases, results in dwarfing, cell swelling (Arioli et al., 1998; Williamson et al., 2001), induced defense responses (Pilling and Hofte, 2003), increased lignin (Cano-Delgado et al., 2003) and pectin accumulation (Gilmor, 2005). In plants where cellulose synthesis is inhibited by cellulose synthase inhibitors such as isoxaben and 2,6-dichlorobenzonitrile (DCB), the cells have similar symptoms as those of *CesA* mutants (Cano-Delgado et al., 2003). For example, the isoxaben-habituated *Arabidopsis* cells have wall compositions that are decreased in Gal and Glc and are increased in Man and uronic acids (Manfield et al., 2004). Bean cell suspension cultures habituated to DCB had walls that were reduced in cellulose and xyloglucan but were increased in calcium complexed pectin. In the case of *gaut6*, it is hypothesized that altered pectin structure causes an upregulation in pectin, manifested as increased sodium carbonate/EGTA pectins that may lead to a down-regulation in the quantities of cellulose produced.

It is also known that many mutants that are not *CesA* gene mutants also cause reduced cellulose synthesis and therefore show similar phenotypes as *CesA* gene mutants. The role of GAUT genes in cellulose synthesis is undoubtedly an indirect one, but the gene affected must lead to a significant decrease in cellulose content in the walls of *gaut6* mutants. *QUA1/GAUT8*, is hypothesized to be involved in HG biosynthesis, but also has an effect on wall xylan synthesis. Thus far, *qual-1* suspension-cultured walls do not show a decrease in the cellulose-enriched wall fractions however, cellulose does not make up a large proportion of suspension cultured cells (Leboeuf et al., 2005). Reduced cellulose content of cell walls has been observed in *irx8* plants; however, the *irx8-1* and *irx8-5* mutant stems (Persson et al., 2007) are impaired primarily in aspects of glucuronoxylan synthesis. It is possible that the disorganization of wall polymers in the matrix is enough to cause a detrimental affect on the organization of other wall polymers

such that the synthesis of hemicelluloses and celluloses is down regulated or impaired in these walls. Only further work in this area will clarify the causes of such wide reaching affects of wall organization in *gaut6* mutants.

GAUT6 mutants have reduced seed extractable mucilage and phenolic content

Seed germination studies led to the analyses of the seed extractable mucilage and wall polysaccharides and showed that the total amount of mucilage was reduced; a substance that is largely composed of RG-I. Comparable composition and linkage results, however, were found between the mucilage of WT and *gaut6* mutants. In addition, increased amounts of phenolics were extracted from the *gaut6* seeds suggesting that the mucilage or walls are more lignified or crosslinked than the WT seeds. It is known that phenolic compounds are present in plant cell walls and play an important role in wall strength by crosslinking wall components (Jones et al., 2003) and it is possible that phenolic crosslinking may be upregulated in response to the changes in wall architecture caused by the mutation in the GAUT6 gene of these plants.

CONCLUSIONS

The GAUT1-related gene family of Arabidopsis thus far has yielded three members, GAUT1, GAUT8 and GAUT12, which are undoubtedly involved in the synthesis of GalA containing wall polysaccharides. The pectic polysaccharides are abundant in the primary wall. Pectin is essential for proper cell expansion, cell adhesion and development of plants. Both GAUT1 and GAUT8 are implicated specifically in the synthesis of HG. We have demonstrated that GAUT6 are required for the proper formation of the plant cell wall by wall fractionation, composition and linkage analysis of both wall and seed mucilage polysaccharides. Based on

these analyses, GAUT6 may target a sub-component of HG, which secondarily affects RG-I and hemicellulose content.

MATERIALS AND METHODS

Growth and growth measurements

Arabidopsis thaliana var. Columbia S6000 T-DNA insertion mutant seed lines were obtained from the Arabidopsis Biological Resource Center (Alonso et al., 2003) through SIGnal (<http://signal.salk.edu/>). The seeds were sown on pre-moistened soil and grown to maturity with a 14/10 photoperiod at $150 \mu\text{Ei m}^{-2}\text{s}^{-1}$ and temperatures of 19°C/14°C at 60% constant relative humidity. Plants were fertilized with Peters 20/20/20 with micronutrients, as needed. Leaf length was measured from tip to petiole and stem height was measured from meristem to first rosette leaf (cm) on the 24th day after sowing.

Wall and mucilage extraction and fractionation

The walls from leaves were sequentially extracted from frozen ground tissue two times with 100 ml each of 80 % ethanol, 100 ml ethanol, chloroform:methanol (1:1) and acetone over a glass scintered vacuum flask. AIR walls were then de-starched with Sigma Type-Ia porcine alpha amylase in 50 mM ammonium formate, pH 6.5, for 24 hrs.

The AIR walls were sequentially fractionated by enzymatic and chemical means. The enzyme treatments were carried out in ammonium formate, pH 6.0 for 24 hours at room temperature with *Aspergillus niger* EPG and *Aspergillus niger* PME. The walls were then sequentially extracted with 50 mM sodium carbonate (~ pH 10.0) and 5 mM EGTA and then with 4 N KOH. After enzymatic or chemical treatment, the suspension was centrifuged at 8,000

x g for 10 minutes to separate the soluble component from the wall residue. The residue was washed with sterile water, centrifuged and the supernatants were combined. The extracted wall fractions (except for the PME/EPG fraction) were neutralized to pH 7.0, if necessary, and dialyzed (except for EPG and PME/EPG) two times with four liters of filtered water and 0.02% thimerosal at 4°C. All fractions except for the 4 N KOH insolubles were filtered through glass fiber filter to remove particulates.

Glycosyl residue composition

The glycosyl residue composition analysis of cell walls and wall fractions was carried out according to Caffall et al. (2008). Briefly, walls or fractions were air dried or lyophilized to dryness with 20 µg of inositol. The samples were hydrolyzed in 1 N methanolic-HCl for 16 hours at 80°C. Samples were dried under a stream of dry air. TMS (200 µl) was added to the samples and the samples were heated for 20 minutes at 80°C. The residue was dried under a stream of dry air, resuspended in 3 ml of hexane and filtered through packed glass wool in a Pasteur pipette. The residue was again dried and resuspended in 100 to 150 µl hexane; 1 µl was injected into the GC-MS. The GC-MS used was an Agilent Technologies model 6890N Network GC System with Mass Selective Detector (Agilent Technologies, Santa Clara CA, USA) and with Agilent Technologies capillary DB-1ms column of 100% dimethylpolysiloxane was used.

Glycosyl Residue Linkage Analysis

The glycosyl residue linkage analysis of cell walls and wall fractions was adapted from Hakomori et al., (1964). Briefly, 100-200 µl (or 10-20 drops) of dry DMSO was added to the

wall samples and mixed until dissolved. The residue was methylated by addition of 500 μ l potassium hydride-dimsyl anion. The samples were frozen in a dry ice-ethanol bath and 1 ml of iodomethane was added to the frozen samples, while purging head space with N_2 . The samples were stirred overnight. Ultrapure water (1 ml) was added to each sample. The iodomethane was removed by bubbling N_2 through the sample. Next, each sample was passed over a C18 Sep-Pak cartridge. The eluted fraction was dried. As a standard, Myo-inositol was added to each sample. The samples were hydrolyzed by addition of 200 μ l of 2 M TFA followed by incubation at 121°C for 2 hours. The samples were allowed to cool and then dried down. Isopropanol (10 drops) was added to each sample and then dried. A 10 mg/ml solution of sodium borodeuteride in 1 M ammonium hydroxide was added to each sample and then incubated at room temperature for at least 1 hr. The samples were neutralized with 3 drops of glacial acetic acid followed by addition of Methanol:acetic acid (9:1). The samples were dried. Methanol (100%) was added to each sample and then dried down. TFA (250 μ l) was added to each sample and incubated at 50°C for 10 minutes. Isopropanol (1 ml) was added to the samples and then dried down. Sodium carbonate (1 ml of 0.2 M) was added to the dry samples and then vortexed. Methylene chloride (1 ml) was added to the samples, which were then mixed and centrifuged. The aqueous layer (upper) was removed and discarded (repeat with 1 ml of water). Finally, the samples were resuspended in 10-15 drops of methylene chloride and injected (2 μ l) into the gas chromatograph. The GC-MS used is an Agilent Technologies model 6890N Network GC System with Mass Selective Detector (Agilent Technologies, Santa Clara CA, USA) equipped with a Sigma-Aldrich (St. Louis MO, USA) capillary SP-2330 column of 80% biscyanopropyl/20% cyanopropylphenyl siloxane.

Seed sterilization and staining

The seeds were suspended in 1 ml of 70% ethanol for 10 minutes with rocking. The seeds were briefly centrifuged for 1 minute and the liquid was pipetted off. The seeds were similarly sequentially suspended once in 25% bleach and then three times with sterile water. The seeds were plated onto solid sterile media plates containing 1 x Murshige and Skoog basal salts pH 5.7 and 1% sucrose supplemented with 1000x stock vitamin stock (1 ml L⁻¹) of *myo*-inositol (550 mM), nicotinic acid (8 mM), pyridoxine-HCl (5 mM) and thiamine-HCl (3 mM). Seeds were stained with ruthenium red that was applied either directly to dry seeds or to seeds that had been sterilized and the seeds were viewed under a dissecting microscope.

Mucilage extraction

The seed mucilage was extracted for 1 hour six times each at 60°C with excess sterile water (1 ml/100 seeds or 1 ml/1 mg of seeds). The seeds were allowed to settle and the supernatant containing mucilage was pipetted off. The water-extracted mucilage supernatants were combined and lyophilized for analysis. The water-extracted seeds were further extracted with 50 mM sodium bicarbonate and 5 mM EGTA, pH 10. The sodium carbonate/EGTA-extracted polysaccharides were dialyzed and lyophilized.

Phenolic extraction and quantitation

The amount of phenolic acids was calculated by the Folin-Ciocalteu method and the results expressed as gallic acid equivalents (Lowry et al.). The phenolics were extracted from seeds with 60% methanol for 3 hours at 60°C. The 60% methanol extract was injected into the Dionex or aliquoted for TMS analysis. Phenolic compositions were analyzed by TMS

derivatization followed by GC-MS. The 60% methanol extract was passed over a reversed-phase Zorbax C18 (4.6 x 280 mM) column.

CHAPTER 4
THE CLONING AND EXPRESSION OF ATGAUT6 IN *N. BENTHAMIANA* AND *E.*
***COLI*.⁴**

³ Kerry H Caffall and Debra Mohnen. To be submitted to Planta.

ABSTRACT

Pectins are a class of plant cell wall polysaccharides that are rich in galacturonic acid. In dicotyledonous plants, pectin accounts for up to one third of the dry weight of the primary wall. Galacturonosyltransferase6 (GAUT6), which is a member of the Arabidopsis GAUT1-related family, has 46% amino acid identity and 64% amino acid similarity to GAUT1, a homogalacturonan galacturonosyltransferase (HG:GalAT). Like the other GAUT proteins, GAUT6 is predicted to be a type-II membrane GT8 glycosyltransferase that is hypothesized to function in the synthesis of pectic polysaccharides. The putative function of GAUT6 as an HG:GalAT was tested by expressing the native and epitope-tagged GAUT6 protein in *N. benthamiana* leaves and testing for HG:GalAT activity. The expression of GAUT6 in *N. benthamiana* leaves resulted in higher levels of HG:GalAT activity than in untransfected, or vector-only control plants. The increase in HG:GalAT activity was observed by the incorporation of [^{14}C]GalA into α 1,4-linked oligogalacturonides and also by the direct observation of increases in the degree of polymerization of discretely-sized oligogalacturonides using the OGA-PAGE assay. The Arabidopsis GAUT6 protein expressed in *N. benthamiana* was membrane associated and appeared to be Golgi localized, as predicted. In addition, N-terminally truncated GAUT6 was expressed in *E. coli* and the Ni-affinity-purified protein was obtained and also shown to catalyze the incorporation of [^{14}C]GalA onto oligogalacturonides. The optimal GAUT6 acceptor structure and reaction conditions have yet to be determined, however GAUT6 is able to use the oligogalacturonide as an acceptor and the data presented provide evidence that GAUT6 is involved in pectin biosynthesis and that GAUT6 is likely to function as a GalAT in the synthesis of HG.

INTRODUCTION

The plant cell wall is an essential component of the plant. The cell wall is largely comprised of carbohydrate, lignin and protein components that require a large biological investment for the plant to make. The pectic polysaccharides are a class of wall polysaccharides that make up a major proportion of the dry weight of the primary wall of dicot plants. The three classes of pectins have complex and unique structures that are essential to wall function. The pectic polysaccharides are homogalacturonan (HG), rhamnogalacturonan (RG-I) and rhamnogalacturonan-II (RG-II). There are at least 20 different sugar residues that make up the pectic polysaccharides with many more linkages. The structures of HG, RG-I and RG-II are discussed in detail in Chapter 1. Galacturonic acid (GalA) makes up a large proportion of the pectic polysaccharides. There are at least four unique α -1,4-GalA linkages necessary for the synthesis of the pectic polysaccharides. These linkages reside in the backbones of HG, RG-I and RG-II, and in RG-II sidechain A. At least as many galacturonosyltransferases (GalATs) would be needed to synthesize the structures present in pectic polysaccharides, and it is hypothesized that additional isoforms are likely required for initiation and elongation of backbone polymers, as well as for the tissue-specific or developmentally regulated synthesis of pectic polysaccharides.

Pectic polysaccharide function is essential to plant growth and development. More specifically, pectic polysaccharides have been implicated in cell-adhesion and the structural integrity of plant tissues (Marry et al., 2006). Plant cell destiny is determined by the location of each cell through programmed divisions and differentiation. Plants with reduced cell adhesion can result in disorganized meristems and ultimately cell death at the meristematic regions (Ishii et al., 2001). Pectins also function in the signaling pathways that lead to defense responses in plants. The oligosaccharide pectic fragments produced by phytopathogenic fungi and bacteria,

or physical damage are detected by the plant cell, and cause the elicitation of specific defense responses (Ridley et al., 2001). The oligosaccharides that elicit such responses are referred to as oligosaccharins (Shibuya and Minami, 2001) or host-associated molecular patterns (Ferrari et al., 2007b). Phytoalexin-elicitor active oligogalacturonides derived from wall pectins cause changes in gene expression. The induction of genes in a coumarin phytoalexin biosynthesis pathway, demonstrates that pectic elicitors induce defense response pathways (Ferrari et al., 2007a) (Davis and Hahlbrock, 1987). Recent work on pectic elicitors has shown that PG-expressing tobacco plants were constitutively upregulated in their defense responses as a result of wall architectural changes brought about by the fragmentation of HG by EPG enzymes (Ferrari et al., 2007b).

Though knowledge regarding the structure of pectic polysaccharides is continually being refined, and the function of pectic polysaccharides is quickly being elucidated by mutant studies, few genes involved in pectin biosynthesis have been identified by reverse genetic analysis. Considering the estimated number of glycosyltransferases necessary for the complete synthesis of pectic polysaccharides, ~56 (Mohnen, 1999; Ridley et al., 2001) (Mohnen et al., 2008), only few have been identified based on demonstration of proven catalytic activity. The putative glucuronosyltransferase gene (*NpGUT1*) was discovered in a T-DNA insertion line of *Nicotiana glauca* leaf callus tissue (*nolac H-18; non-organogenic callus with loosely attached cells*) that displayed reduced cell adhesion (Iwai et al., 2002). The structural characterization of *gut1* RG-II sidechains suggests that *NpGUT1* catalyzes the addition of a single glucuronic acid residue onto RG-II sidechain A, however, this has not been definitively shown. Recently a putative α 1,5-arabinosyltransferase has been identified that synthesizes arabinan sidechains that are an important component of RG-I structure (Harholt et al., 2005). A xylogalacturonan β 1,4-xylosyltransferase has been identified that catalyzes the addition of xylose residues onto the

GalA backbone residues of HG to yield xylogalacturonan, a substituted form of HG (Jensen et al., 2008). Two α 1,3- XylTs have been identified in the CAZy family GT77 that appear to encode XylTs (RGXT1 and RGXT2) that have an activity compatible with structures found only in RG-II (Egelund et al., 2006). *GAUT1* (galacturonosyltransferase1) was identified in partially purified *Arabidopsis thaliana* suspension culture (Sterling et al., 2006) and shown to be an HG biosynthetic GalAT that catalyzes the addition of GalA residues onto an oligogalacturonide acceptors in an α -1,4 configuration. This discovery was of particular importance because the GAUT1 functions to synthesize the backbone of polysaccharides of HG, and likely the backbone of RG-II.

The identification of GAUT1 enabled the identification of the 15 GAUT and the 10 galacturonosyltransferase-like (GATL) genes of the GAUT1-related gene family, which is a specialized subgrouping of the CAZY database GT8 family. The additional GAUT and GATL proteins have 56 to 84% and 42 to 53% amino acid identity and similarity to GAUT1, respectively. The GAUT family has seven clades (A-G). The phylogenetic analysis of the *Arabidopsis*, poplar and rice GAUT families identified the conservation of most GAUT genes across these species, with some exceptions. *GAUT2*, a potential pseudogene was not identified in poplar or rice. The apparent orthologs of *GAUT8* and *GAUT12* were not identified in the rice GAUT family, although these genes have been shown to have unique essential function in *Arabidopsis*.

The *Arabidopsis QUA1/GAUT8* gene mutant has been characterized and the walls of this mutant were shown to be reduced in the pectic polysaccharide HG and in the hemicellulose xylan. It is hypothesized that QUA1 is an HG:GalAT. The HG biosynthetic α 1,4-GalAT activity of *qua1-1* plants was reduced compared to wild type plants, however the xylan β 1,4-

XylT activity was also reduced, casting doubt as to the *in vivo* activity of QUA1 (Bouton et al., 2002; Orfila et al., 2005). Additional evidence suggesting that QUA1 is involved in pectin synthesis is that the *qual-1* plants have reduced cell adhesion (Bouton et al., 2002), a phenotype associated with altered pectin structure (Iwai et al., 2002) and altered pectin localization (Shevell et al., 2000). Also, *QUA1* transcript expression is upregulated in cells habituated to the cellulose synthase inhibitor, isoxaben, which also have increased wall pectin content (Manfield et al., 2004; Orfila et al., 2005). An additional GAUT gene, *IRX8/GAUT12*, has been implicated in the synthesis of xylan polysaccharides. *Arabidopsis irx8* plants have a phenotype indicative of reduced secondary walls, and more specifically, reduced xylan polysaccharides. Based on the detailed wall structural analysis of *irx8* plants (Pena et al., 2007; Persson et al., 2007), *IRX8* is likely to encode a protein that is involved in the synthesis of an oligosaccharide thought to be a primer of xylan biosynthesis or a novel xylan-associated wall linkage (Pena et al., 2007; Persson et al., 2007). Analysis of the walls of T-DNA insertion mutants of 13 GAUT genes demonstrated that mutation of genes of the GAUT clades A, B, D, F and G yielded plants with reduced wall GalA composition suggesting that these GAUTs are likely to be involved in pectin synthesis, while those of GAUT clade E mutants had reduced Xyl composition and are likely to be involved in xylan synthesis (Caffall Ph.D. dissertation Chapter 2).

GAUT6 and *GAUT5* make up clade G of the GAUT1-related gene family and are hypothesized to function as pectin biosynthetic GalATs involved in plant cell wall biosynthesis (Caffall Ph.D. dissertation Chapter 2). The *Arabidopsis thaliana gaut6* T-DNA mutants have walls with reduced levels HG and hemicellulose. Based on the current opposing hypotheses regarding the function of specific GAUT genes in pectin and xylan biosynthesis, definitive function can only come from biochemical evidence of catalytic activity. To this end, it is the

goal of this study to clarify the function of GAUT6 in wall biosynthesis by demonstrating the catalytic activity of heterologously expressed GAUT6. We have succeeded in demonstrating that GAUT6 expression in *N. benthamiana* increased HG:GalAT activity and that heterologously expressed GAUT6 in *E. coli* is able to catalyze the transfer of GalA residues from UDP-[¹⁴C]GalA onto oligogalacturonide acceptors *in vitro*.

RESULTS

GAUT6 is conserved among dicot and monocot species

The GAUT6 gene is a member of clade G of the *Arabidopsis thaliana* GAUT1-related gene family that has 25 members and has been found to have orthologs in both dicot and monocot species. Based on the amino acid similarity, GAUT6 and GAUT5, are likely to have similar function. Nonetheless, *gaut6* and not *gaut5* Arabidopsis T-DNA insertion mutants were found to have reduced GalA wall compositions. Therefore, *GAUT6* was chosen for biochemical characterization. The amino acid sequence of *AtGAUT6* was searched against several genomes using blastx (amino acid search query against the translated nucleotide sequence of the subject genome, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The full length amino acid sequences of potential GAUT5/GAUT6 orthologs were identified in poplar (*Populus trichocarpa*; estExt_fgenes4_pm.C_LG_V0534 and fgenes4_pg.C_LG_II000411), rice (*Oryza sativa*; Os11g37980) and Sorghum (*Sorghum bicolor* e_gw1.5.15262.1 [Sorbi1:4822212]) (Figure 4.1). In addition, the incomplete sequence of a potential GAUT5/GAUT6 ortholog was identified in tomato (*Lycopersicon esculentum*) that aligned well with the GAUT6 amino acid sequence, but because of the incompleteness of the sequence, the closest aligning GAUT could not be definitively determined and therefore was not included in the alignment of Arabidopsis

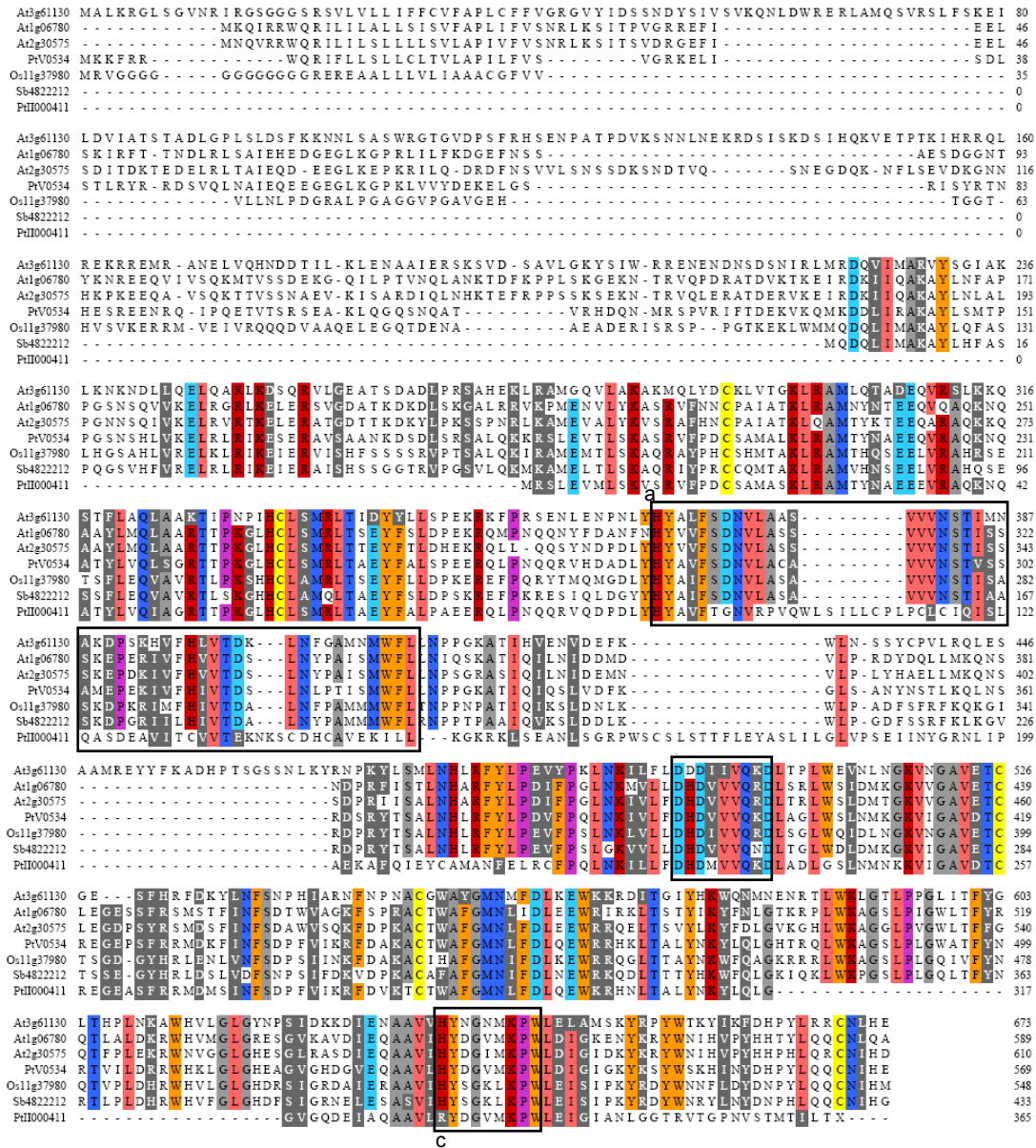


Figure 4.1. The alignment of selected GAUT proteins and putative GAUT6 orthologs.

The GAUT-specific motif (a), the DxD motif (b) and the GAUT1-related gene family motif (c) are boxed. The alignment of Arabidopsis At3g61130 (GAUT1), At1g06780 (GAUT6) and At2g30575 (GAUT5) with *P. trichocarpa* orthologs (PtV0534 and PtII000411), a *Oryza sativa* (rice) ortholog (Os11g37980) and a *Sorghum bicolor* ortholog (Sb4822212). The conserved (colored) amino acids and the similar amino acids (gray) are shown.

GAUT5/GAUT6 orthologs. Based on the complete sequences in poplar, wheat and rice orthologs, we concluded that orthologs of GAUT5 or *GAUT6* are conserved among species of herbaceous dicots, woody dicots and monocot grasses.

Cloning of the native, HIS-tagged and GFP-tagged AtGAUT6

Three pCAMBIA constructs were created with the full length *AtGAUT6* DNA coding sequence for transformation into tobacco plants by *Agrobacterium tumefaciens* mediated transformation: GAUT6, GAUT6-GFP and GAUT6-HIS. The full length *AtGAUT6* was amplified from Arabidopsis wild type (WT) stem with gene specific primers designed to the predicted start and stop codons of the *AtGAUT6* open reading frame (ORF) and was subsequently cloned into the pCAMBIA35-GFP expression vector (Figure 4.2). The *AtGAUT6* ORFs were inserted into pCR 2.1-TOPO vector for sequencing and subcloned into a modified pCAMBIA vector (courtesy of Dr. M. Bar-Peled) having the eGFP sequence and cauliflower mosaic virus 35S promoter. The modified pCAMBIA vector, which will heretofore be referred to as pCAMBIA35, offers high constitutive expression of the transgene in dicot plants, with or without the GFP fusion protein sequence. The pCAMBIA35 vector was further modified to incorporate a polyhistidine tag specifically for the creation of the pCAMBIA:GAUT6-HIS construct. The 3' polyhistidine epitope tag was included for detection of the recombinant protein during Western blotting and as a purification tag. The HIS tag was followed by a stop codon to prevent translation of the GFP.

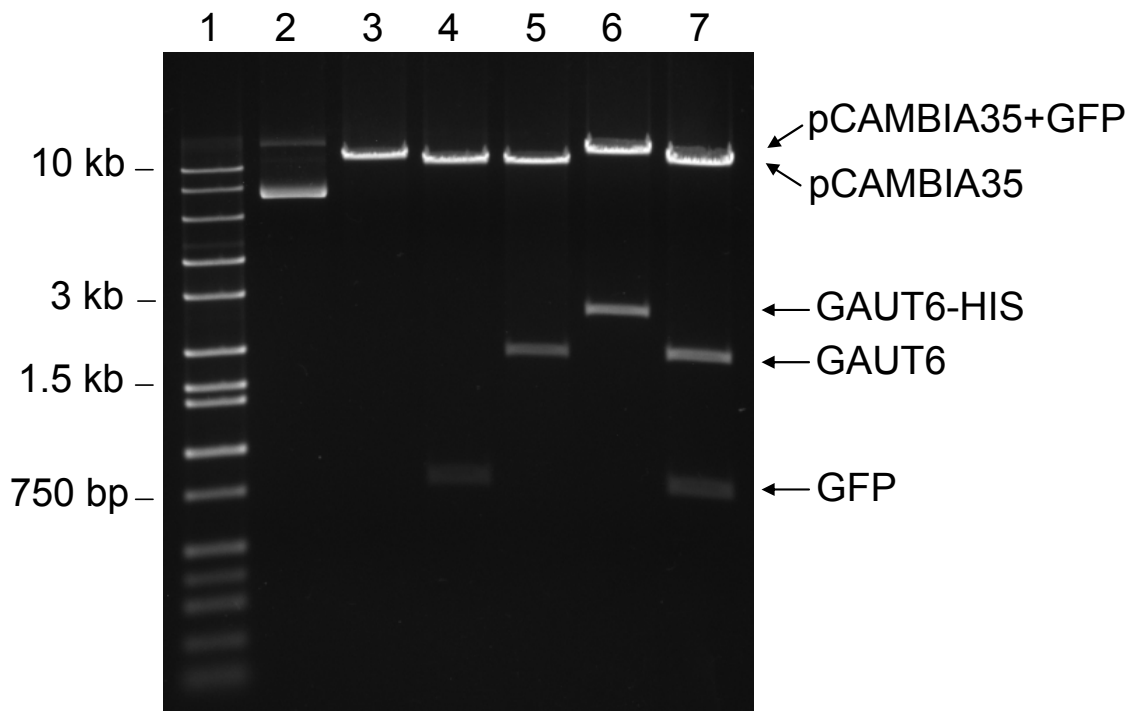


Figure 4.2. Restriction digest of pCambia and pCambia GAUT6 constructs.

The lanes were loaded as follows: (lane 1) Broad range marker, (lane 2) pCambia uncut (10,744 bp), (lane 3) pCambia linearized (10,744 bp), (lane 4) pCambia:GFP cut (KpnI/XbaI cut, expected 10,012 bp and 732 bp), (lane 5) pCambia:GAUT6 cut (KpnI/XbaI cut, expected 10,012 bp and 1,821 bp), (lane 6) pCambia:GAUT6-HIS cut (KpnI/XbaI cut, 10,012 bp and 2,571 bp), (lane 7) pCambia:GAUT6-GFP (KpnI/XbaI cut, 10,012 bp, 1,821 bp and 732 bp).

GAUT6 is detected in tobacco leaves

The GAUT6-GFP and GAUT6-HIS were transiently transformed into *Nicotiana benthamiana* plant leaves, via *Agrobacterium tumefaciens* mediated transformation, to quickly and efficiently produce recombinant GAUT6 protein. The isolated microsomal membrane and soluble extracts of *Nicotiana* expressing the native, GFP-tagged and HIS-tagged GAUT6 were analyzed by Western blot using anti-GFP and anti-HIS commercial antibodies as probes. The presence of GAUT6 in the appropriate tobacco lysates verified that the protein was expressed in the tissues of transformed plants. Two gels similarly loaded with the soluble and membrane fractions of tobacco leaves transiently transformed with pCAMBIA35:GFP, pCAMBIA35:GAUT6-GFP and pCAMBIA35:GAUT6-HIS were transferred to PVDF membrane and probed alternately with anti-GFP and anti-HIS antibodies (Figure 4.3). The GAUT6-GFP and the GAUT6-HIS proteins are predicted membrane proteins of approximately 100 kDa and 75 kDa, respectively. The membrane probed with anti-GFP antibody showed a single band of ~27 kDa in the pCAMBIA35:GFP transformed *Nicotiana* soluble protein sample consistent with the predicted size location and of GFP (27 kDa). The membrane probed with anti-HIS antibody showed a single band of ~100 kDa in the pCAMBIA35:GAUT6-GFP transformed microsomal membrane extracts, consistent with the predicted size location and size of the GAUT6-HIS protein (97 kDa). The size of the protein bands indicate that the bands are likely the proteins of interest and that the GAUT6 proteins were not further processed by cleavage of the signal peptide, at least in *Nicotiana*, and are in fact membrane bound in the samples analyzed.

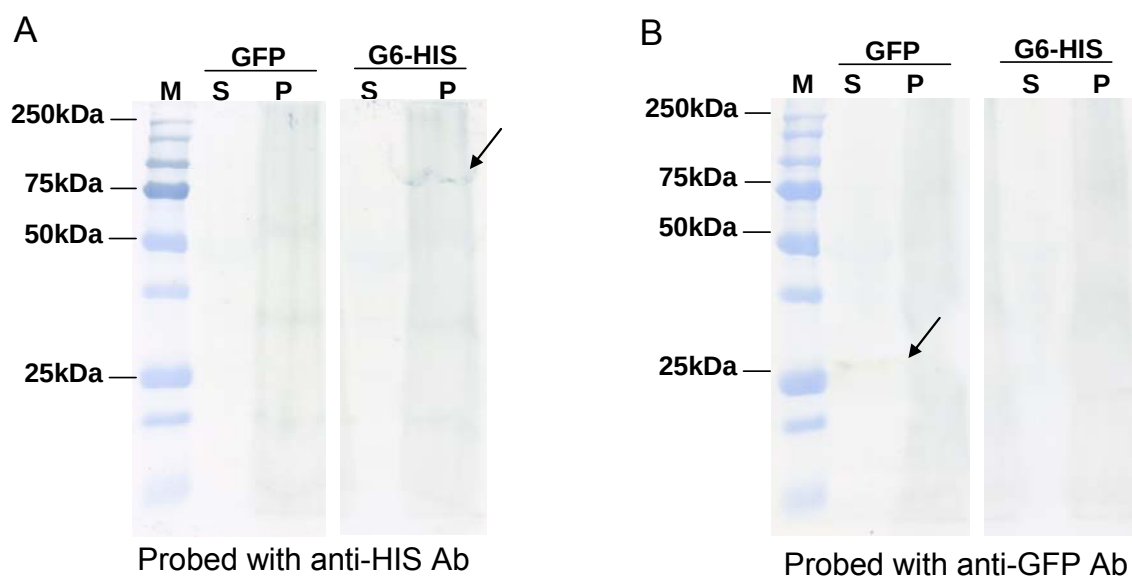


Figure 4.3. Western blot of transiently transformed tobacco leaf lysates.

Western blot showing the soluble and membrane fractions of pCAMBIA:GFP transfected and pCAMBIA:GAUT6-HIS transfected tobacco leaves probed with monoclonal anti-polyhistidine antibodies (A) and anti-GFP antibodies (B). Black arrows indicate the presence of protein bands of the expected sizes for GFP (27 kDa) and GAUT6-HIS (~100 kDa).

HG:GalAT activity and GAUT6 protein are localized to the Golgi apparatus

The subcellular localization of pectin biosynthetic HG:GalAT activity is in the Golgi apparatus (Sterling et al., 2001), which coincides with the localization of the GAUT6-GFP fusion protein, as determined by fluorescent microscopy of *Nicotiana* leaf cells transiently transformed with pCAMBIA35:GAUT6-GFP. The localization of GAUT6 within *Nicotiana* cells was determined based on the comparison of GAUT6-GFP to the tested membrane-bound Golgi localized MUR4-YFP (Zhou et al., 2007) and cytosolic GFP controls of transiently transformed *Nicotiana* leaves (Figure 4.4). The GAUT6-GFP fluorescence in tobacco epidermal cells has a punctate pattern of fluorescence typical of fluorescently labeled proteins localized to the endomembrane of plant cells. The MUR4-YFP fusion protein is expressed in the Golgi apparatus and shows similar punctate patterns as that observed in the cells of *Nicotiana* transformed with Golgi localized GAUT6-GFP.

GAUT6 increases HG:GalAT activity in Nicotiana microsomal membranes

The microsomal membrane preparations of tobacco transiently transformed with GAUT6 or GAUT6-HIS resulted in membrane preparations that had higher HG:GalAT activity than that of similarly prepared microsomal membranes of either un-transformed or empty vector transformed plants. The microsomal membrane preparations were prepared as described previously (Doong et al., 1995) (Doong and Mohnen, 1998), with some modifications, and constitute the detergent-permeabilized membrane protein. HG:GalAT activity may be assayed by the OGA-PAGE assay that entails incubating uniformly sized OGAs with equal amounts of protein from permeabilized microsomal membranes (P60) of GAUT6-expressing or control plants, followed by the analysis of the reaction products on a high-percentage acrylamide PAGE

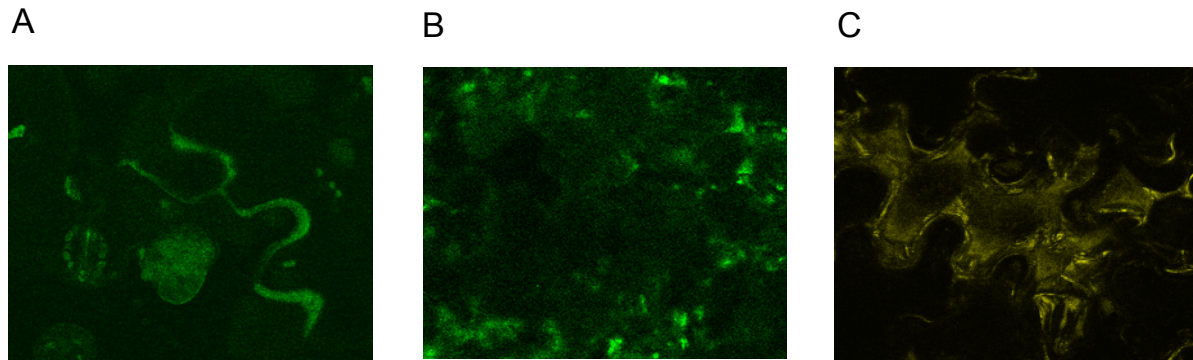


Figure 4.4. Sub-cellular localization of GAUT6 in transiently transformed tobacco.

Shown are the results of transient transformation of *Nicotiana benthamiana* leaves with (A) pCAMBIA:GFP, (B) pCAMBIA:GAUT6-GFP or (C) pCAMBIA:MUR4-YFP.

gel. *Nicotiana* expressing native, un-tagged GAUT6 had greater amounts of OGAs of higher DP than preparations from untransformed plant material (Figure 4.5A). The supernatant did not harbor any detectable GalAT activity (Figure 4.5B), demonstrating that residual activity was not present in the soluble protein fraction. Densitometry scans of OGA-PAGE gels of reaction products from three hour incubations of permeabilized membranes of the control and GAUT6 transiently transformed *Nicotiana* leaves containing OGA DP 11 substrate (Figure 4.5C) reflect data that are the average densitometry scans of reaction products from two independent transformed experiments and show the greater abundance of OGA substrate of DP 12, 13, 14 and 15 in reactions from GAUT6-expressing samples over that of the control samples. In addition, the 10-mer and 11-mer are more greatly depleted in reactions supplied with the membranes of GAUT6 expressing tobacco, over time demonstrating that a greater proportion of the DP 11 OGA used as the substrate was utilized to synthesize OGAs of larger DP.

The pCAMBIA35:GAUT6-HIS construct was created in order to test for HG:GalAT activity in the GAUT6-containing protein from the transformed *Nicotiana* samples. The transiently transformed *Nicotiana* leaves expressing GAUT6-HIS were subjected to a similar experimental series as that carried out with *Nicotiana* transiently transformed with the un-tagged GAUT6 in order to confirm that the observed increase in HG:GalAT activity was due to the expression of GAUT6 protein in these tissues. There was not any GAUT6-HIS detected in the supernatant by Western blotting. The transiently transformed *Nicotiana* leaf membranes expressing GAUT6-HIS also resulted in the synthesis of OGA's of higher DP than that of the empty vector control, indicating that the GAUT6-HIS transformed membrane extracts also had greater HG:GalAT activity than control samples (Figure 4.6). In addition, as expected, the cultures containing the GAUT6-HIS construct mixed at a ratio of 1 to 9 with culture containing a

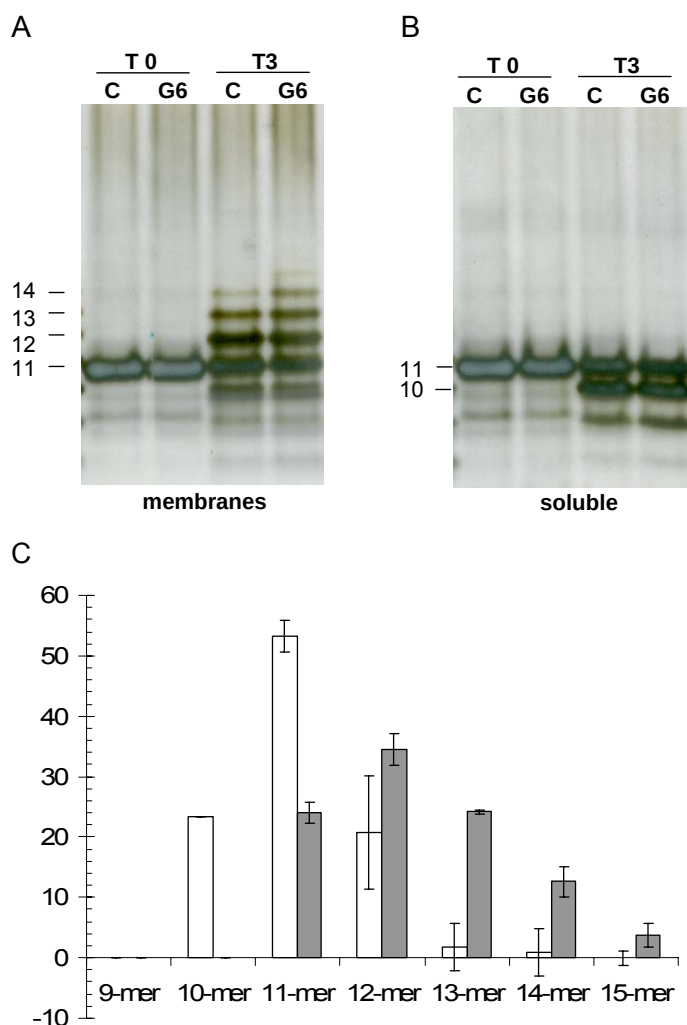


Figure 4.5. GalAT activity in transiently transformed *N. benthamiana* lysates.

The HG:GalAT reaction products separated by OGA-PAGE of *N. benthamiana* microsomal membrane proteins (A) or soluble proteins (B) from untransformed and GAUT6-expressing samples incubated with OGA DP 11. Densitometry of HG:GalAT reaction products from experiments separate from that shown in A and B, of OGA-PAGE assay of (C) microsomal membrane proteins from untransformed (white bars) and GAUT6 transformed (gray bars) from two independent experiments. The data are the average background-subtracted signal of reaction products from two independent infiltrations $\pm$ standard deviation.

plasmid encoding the p19 silencing suppressor protein contained HG:GalAT of even greater activity than that of the GAUT6-HIS alone (Figure 4.6). The increase is most notable at incubation times of 3 and 20 hours. A detectable increase in the HG:GalAT activity by co-infiltration of tobacco leaves with GAUT6-HIS plus p19 over that of the control membranes suggested that GAUT6 is partially silenced in the absence of p19.

To better quantify the increase in activity of GAUT6 membranes of control membranes observed by the OGA-PAGE assay, the HG:GalAT filter assay as previously described (Sterling et al., 2005) was carried out using UDP-[¹⁴C]GalA (Figure 4.7). Equal amounts of GAUT6 transformed or control permeabilized membrane protein of *Nicotiana* plants was incubated with UDP-[¹⁴C]GalA and mixed OGAs of DP of 7-25. The reactions were stopped with base, which destroys activity, and then spotted to cetylpyridinium chloride-coated filter paper squares. The squares were washed with 1.5 N NaCl to release the unincorporated UDP-[¹⁴C]GalA and dried before loading the filter papers into scintillation vials for counting. The data are the T0 subtracted average pmol incorporated per µg protein from three hour reactions loaded with equal amounts of protein. The filter assay results of GAUT6 and pCAMBIA35 vector control, and GAUT6-HIS/p19 and pCAMBIA35/p19 vector control are shown (Figure 4.7). The membranes of GAUT6-HIS and GAUT6-HIS plus p19 expressing *Nicotiana* leaves had greater incorporation of [¹⁴C]GalA onto OGAs compared to that of the empty vector transformed membranes.

Cloning of AtGAUT6 for heterologous expression in E. coli

For expression in *E. coli*, a GAUT6-HIS construct was designed with a deletion of 81 nucleotides to eliminate the cytosolic and transmembrane domain encompassing 27 amino acids (GAUT6Δ1-27). The GAUT6Δ1-27 construct was designed to eliminate the transmembrane

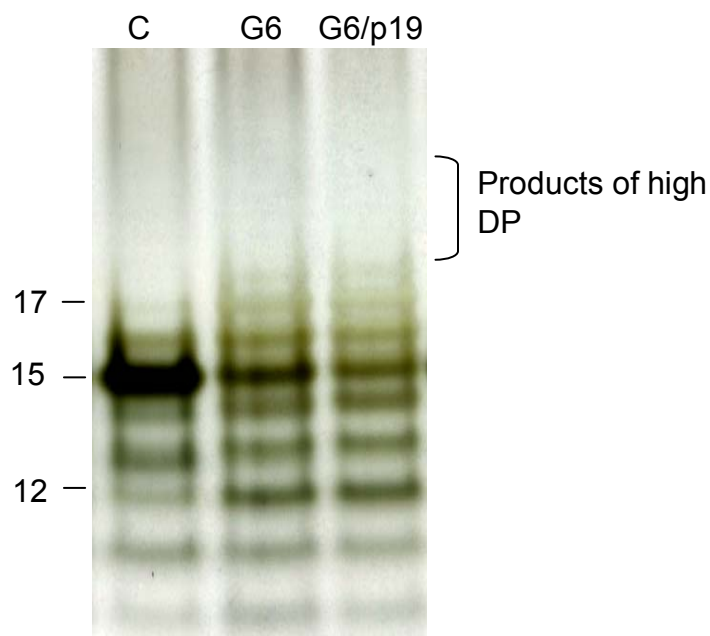


Figure 4.6. The HG:GalAT activity of GAUT6-HIS is enhanced by co-transformation of p19 in *N. benthamiana*.

The HG:GalAT assay reaction products of microsomal membranes of control (vector-only) (C), GAUT6-HIS (G6) and GAUT6-HIS/p19 (G6/p19) infiltrated *N. benthamiana* leaves. A uniformly-sized OGA of DP 15 was used as the acceptor in these reactions. Products of higher DP stain blue and are not individually resolved.

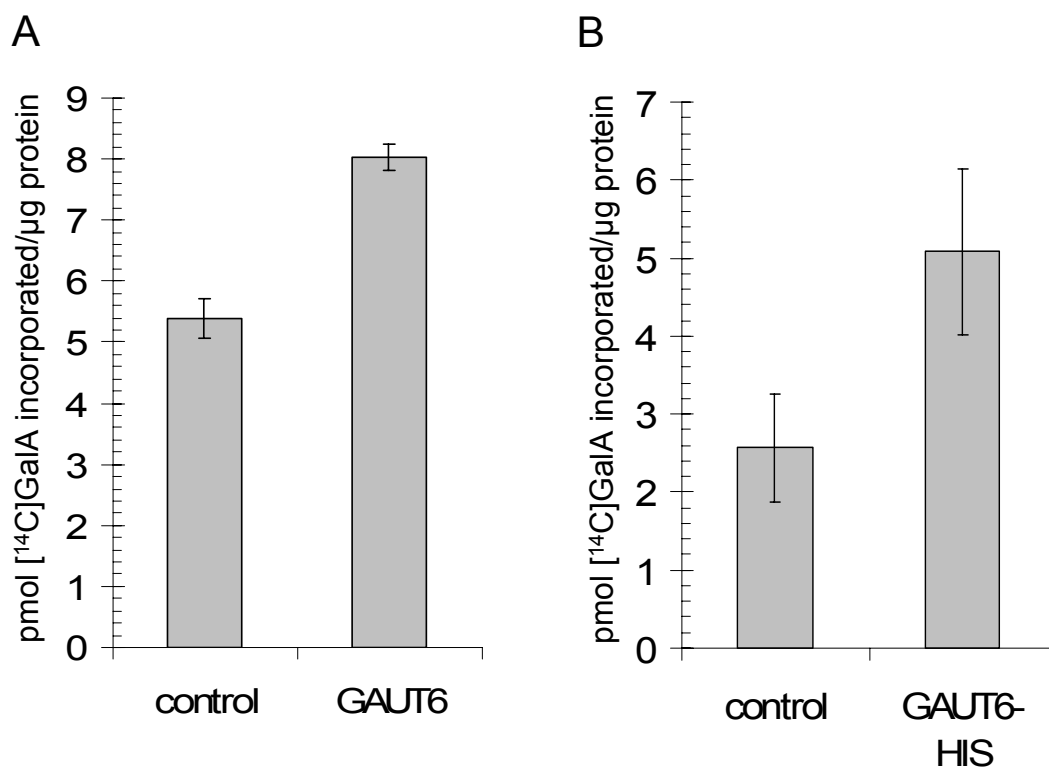


Figure 4.7. UDP-[¹⁴C]GalA GalAT activity assay of transiently transformed *N. benthamiana* leaves.

The HG:GalAT assay using OGA of DP of 7 to 24, UDP-[¹⁴C]GalA and microsomal membrane protein from untransformed and pCAMBIA35:GAUT6 transformed *N. benthamiana* leaves (A) or pCAMBIA:GFP and pCAMBIA:GAUT6-HIS transformed *N. benthamiana* leaves (B). The data in A are the average background-subtracted pmol [¹⁴C]GalA incorporated/μg protein ± standard error of duplicate reactions.

domain and to minimize further modification to the GAUT6 protein. The second construct was designed with a deletion of 441 nucleotides that eliminates the cytosolic and transmembrane domain, as well as, the stem region of the protein. The stem region may function as a mobile tether between the membrane and globular C-terminus that can lead to structural instability, thus, elimination of the stem region may enhance the stability of the protein, which can become freely mobile and structurally problematic when the transmembrane domain is removed (Sasai et al., 2001). Both constructs were engineered with an N-terminal NdeI restriction endonuclease site. The PCR products of the GAUT6 Δ 1-27 and GAUT6 Δ 1-149 deletion constructs of 1,689 bp (563 aa) and 1323 bp (441 aa), respectively, of GAUT6 were inserted into pCR2.1-TOPO (Invitrogen) and further subcloned into pET28a (Novagen) plasmid via the NdeI and NotI site (Figure 4.8). The constructs were in-frame with the N-terminal HIS tag and the GAUT6 stop codon was retained to prevent translation of the optional C-term HIS tag. The predicted molecular weight of the N-terminally HIS tagged GAUT6 Δ 1-27 and GAUT6 Δ 1-149 protein in *E. coli* are 72 kDa and a 52 kDa, respectively.

Expression of AtGAUT6 Δ 1-27 in E. coli

The induction of GAUT6 Δ 1-27 expression in *E. coli* results in some soluble protein of the expected molecular weight; however, a proportion of the protein is aggregated forming inclusion bodies (Figure 4.9). It is not likely that the protein in the soluble fraction is a result of incomplete centrifugation because all preparations were centrifuged at 20,000 x g for 1 hour and care was taken to avoid taking any pellet (p20) into the soluble (s20) fraction. For this construct, there is a great amount of variability in the size of the protein produced. The smaller protein products are unlikely to be the result of protein breakdown, because the cell lysis and isolation of

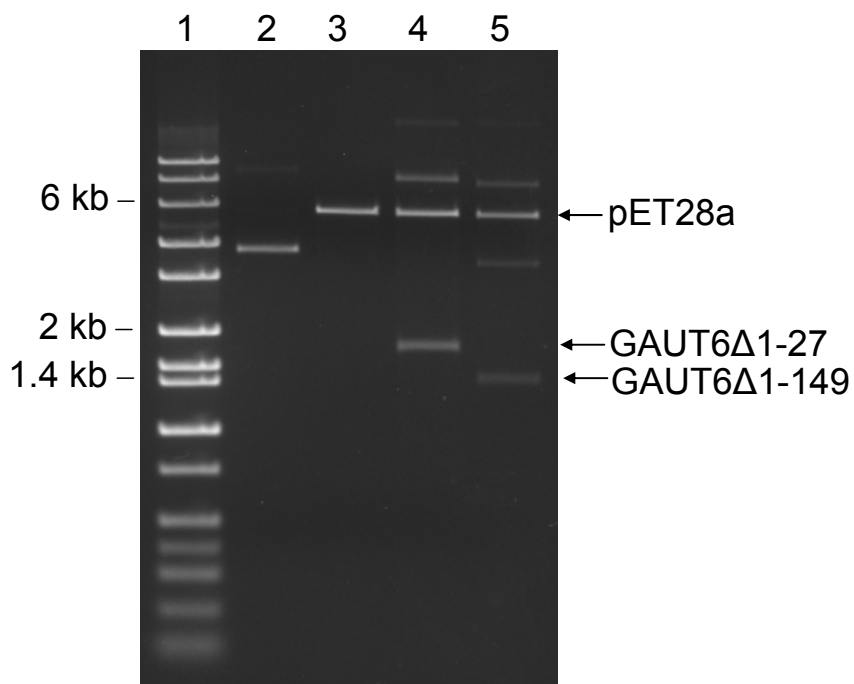


Figure 4.8. Restriction digest of pET28a and pET28a:GAUT6 constructs.

The lanes were loaded as follows: (lane 1) broad range marker, (lane 2) pET28a uncut (5,369 bp), (lane 3) pET28a linearized (5,369 bp), (lane 4) pET28a:GAUT6Δ1-27 cut (NdeI/NotI cut, expected 5,300 bp and 1,728 bp), (lane 5) pET28a:GAUT6Δ1-149 cut (NdeI/NotI cut, expected 5,300 bp and 1,362 bp).

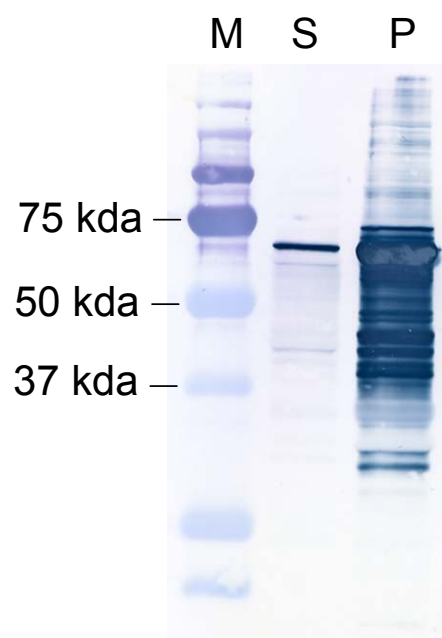


Figure 4.9. Western blot of GAUT6 Δ 1-27 in *E. coli* lysates.

The *E. coli* GAUT6 Δ 1-27 soluble (S) and insoluble (P) protein was subjected to electrophoresis and blotted to PVDF membrane and probed with anti-HIS antibody. The protein produced was observed only after IPTG induction. Significant binding of the antibody was not observed in *E. coli* lysates lacking the pET28a: GAUT6HIS construct (not shown).

soluble and insoluble protein was carried out on ice and with protease inhibitors throughout the experiment. It seems most plausible that the alternate gene products are caused by a cessation of protein translation. Because the protein is N-terminally HIS-tagged, the products are apparent by Western blotting with anti-HIS tag antibodies. The GAUT6 ORF has a high density of arginines that are coded for by rare-codons in *E. coli*, which may be a contributing factor if stop-translation is the cause of the appearance of incomplete translation products.

In lieu of in vitro synthesis of a codon-optimized GAUT6 ORF, the optimization of culture induction conditions was carried out in an attempt to increase the amount of soluble protein obtained and decrease the amount of stop-translation during induction of GAUT6 Δ 1-27 constructs. A variety of cell strains, culture additives and temperature conditions were assayed. The GAUT6 Δ 1-27 was transformed into, and expressed, in BL21(DE3)pLysS, BL21 Star(DE3) and K12 Rosetta-gami2(DE3) strains of *E. coli*. None of the strains tested eliminated or even made a dramatic difference in the appearance of GAUT6 Δ 1-27 in inclusion bodies. The Rosetta-gami strain expresses rare tRNAs for AGG, AGA, AUA, CUA, CCC and GGA codons to facilitate the translation of non-codon-optimized genes in *E. coli* as well as the *trxB* and *gor* mutations for cytoplasmic disulfide bond formation. The Rosetta-gami(DE3) strain of *E. coli* was chosen based on a slight reduction in truncation products.

The expression and aggregation of GAUT6 Δ 1-27 was monitored at IPTG concentrations of 0.5 mM and 0.25 mM in LB medium, with or without 0.2% glucose or 660 mM sorbitol plus 2.5 mM betaine in the LB medium (Figure 4.10). The addition of glucose to protein expression medium reduces expression levels (Novy and Morris, 2002). Sorbitol serves to exert osmotic stress on the bacteria, while betaine is known to protect proteins under stress conditions and promotes the production of correctly folded protein (de Marco et al., 2005; Picaud et al., 2006).

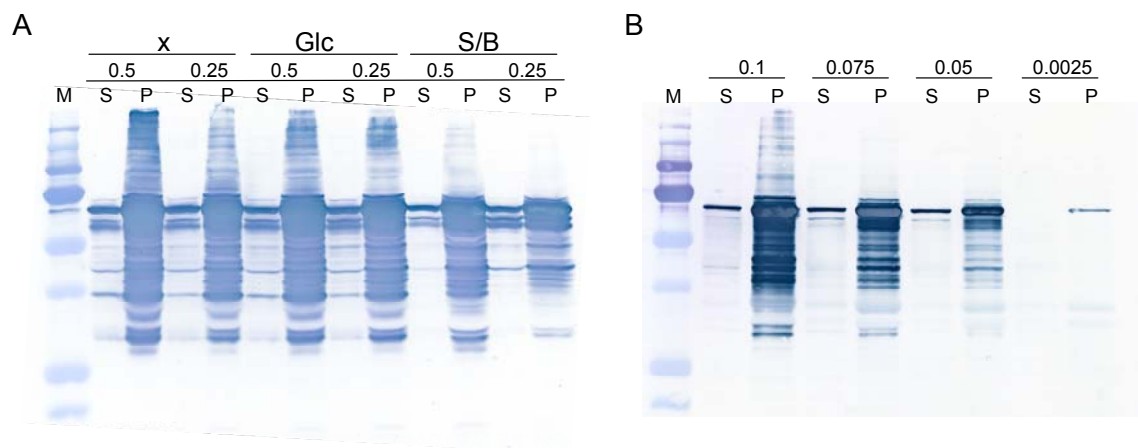


Figure 4.10. The optimization of *E. coli* GAUT6 Δ 1-27 protein expression.

The affect of media additives (A) and IPTG concentration (B) on GAUT6 Δ 1-27 solubility and expression levels in *E. coli* lysates were assessed by Western blot probed with anti-HIS antibody. In blot A, no additives (x), glucose (Glc, final concentration 0.2%) or sorbitol and betaine (S/B, final concentrations 660 mM and 2 mM, respectively) were added to the LB medium and induced with IPTG at a final concentration of 0.5 mM or 0.25 mM. In blot B, the affect of IPTG concentration (mM) on the solubility and expression level of GAUT6 Δ 1-27. The soluble (S) and insoluble (P) proteins are shown with the pre-stained protein marker (M).

Only the addition of sorbitol and betain, at reduced IPTG concentrations, had an appreciable affect on the amount of truncation products. Rough estimates suggest that approximately half of the GAUT Δ 1-27 protein produced in this strain under optimized conditions is soluble. Next, the solubility of GAUT6 Δ 1-27 was tested with decreasing levels of inducing agent (IPTG) with the assumption that lower levels of induction will lead to a greater proportion of soluble and correctly translated protein. In fact, what was discovered was that even with basal expression, the majority of the GAUT6 Δ 1-27 protein is still insoluble, with no detectable soluble GAUT6 Δ 1-27 protein.

Deletion of the stem region of GAUT6 Δ 1-149 restores complete solubility in E. coli

In an attempt to circumvent the poor solubility and the formation of truncation products produced from the GAUT6 Δ 1-27 construct, a GAUT6 Δ 1-149 construct was designed in which the N-terminal cytosolic domain, the transmembrane domain, as well as the stem region, was deleted. Expression of GAUT6 Δ 1-149 in *E. coli* BL21(DE3)pLysS cells yielded soluble protein without detectable truncation products or aggregation of the protein in inclusion bodies (Figure 4.11). Soluble GAUT6 Δ 1-149 was achieved without further optimization of IPTG concentrations and without the addition of solubility enhancers to the medium. The expression of GAUT6 Δ 1-149 in the soluble fraction, like that of the GAUT6 Δ 1-27, was detectable only by Western blotting indicating that relatively low levels of expression in *E. coli* were achieved.

The Ni-affinity purified, truncated GAUT6 protein in E. coli has HG:GalAT activity

The lysate of *E. coli* expressing the HIS-tagged GAUT6 Δ 1-27 proteins (Figure 4.12A) and GAUT6 Δ 1-149 proteins (Figure 4.12B) were passed over a Ni-affinity column for initial

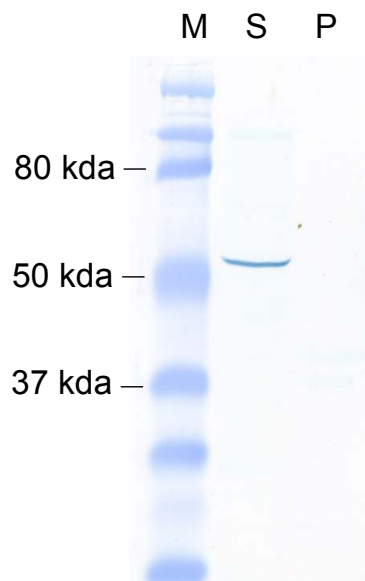


Figure 4.11. Western blot of GAUT6 Δ 1-149 in *E. coli* lysates.

The *E. coli* GAUT6 Δ 1-149 soluble (S) and insoluble (P) protein was subjected to electrophoresis and blotted to PVDF membrane and probed with anti-HIS antibody. The protein produced was observed only after IPTG induction. Significant binding of the antibody was not observed in *E. coli* lysates lacking the pET28a:GAUT6-HIS construct (not shown).

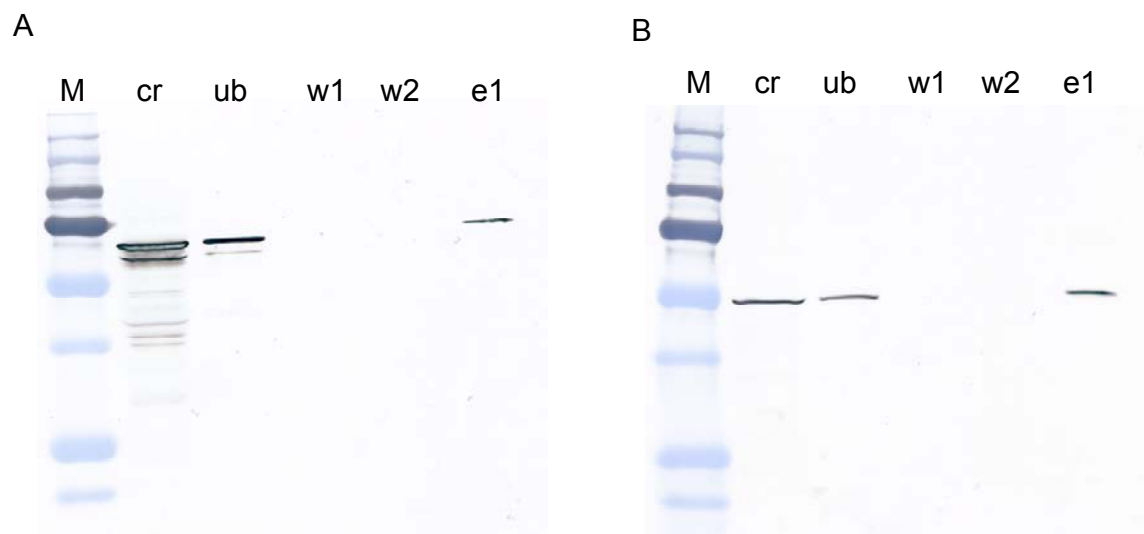


Figure 4.12. The Ni-affinity purifications of GAUT6 Δ 1-27 and GAUT6 Δ 1-149 protein.

Shown are Western blot, probed with anti-HIS antibody (Sigma A7058), of GAUT6 Δ 1-27 (A) and GAUT Δ 1-149 (B) crude soluble protein (cr) and fractions of protein passed over a Ni-affinity (BioRad Profinia) 5 ml column: unbound (ub), wash 1 (w1), wash 2 (w2) and eluted (e1) protein fractions.

purification. The soluble (s20) and insoluble (p20) GAUT6 Δ 1-27, and the semi-purified GAUT6 Δ 1-27 and GAUT6 Δ 1-149 proteins were assayed for GalAT activity. The soluble GAUT6 Δ 1-27 protein contained 79% of the total protein and 77% of the total detectable HG:GalAT activity. Some HG:GalAT activity (22%) was detected in the detergent dispersed p20 fraction of GAUT Δ 1-27 protein. The partially purified GAUT6 Δ 1-149 protein from the soluble *E. coli* fraction had detectable HG:GalAT activity (Figure 4.13). Because no protein was identified in the pellet of *E. coli* expressing GAUT6 Δ 1-149, these fractions were not assayed for HG:GalAT activity.

GAUT6 binds irreversibly to agarose

The heterologously expressed GAUT6 protein expressed in *E. coli* was shown to irreversibly bind to a column containing 6% agarose, but did not bind to a column of polyvinyl base material. The HIS-tagged constructs of GAUT6 were designed for ease of initial purification of the heterologously expressed protein by Ni-affinity purification. Many different matrices exist in the market place for such chromatographic separations and the Profinia matrix was ultimately used for the successful separations shown in Figure 4.14A and B. Attempts were also carried out using a matrix that contained Agarose that was found to strongly bind GAUT6 proteins. The release of GAUT6 from the column was observed in a partially degraded form by denaturing solutions such as 6M guanidine (Figure 4.14A). The binding of GAUT proteins to agarose has been observed previously based on chromatographic separations utilizing UDP-agarose that unpredictably deplete HG:GalAT from Arabidopsis solubilized membrane enzymes, and based on immunoprecipitations utilizing Protein A-Agarose beads, as evidenced by a loss of activity (unpublished data J. D. Sterling and M. A. Atmodjo). Our data suggest that the near-

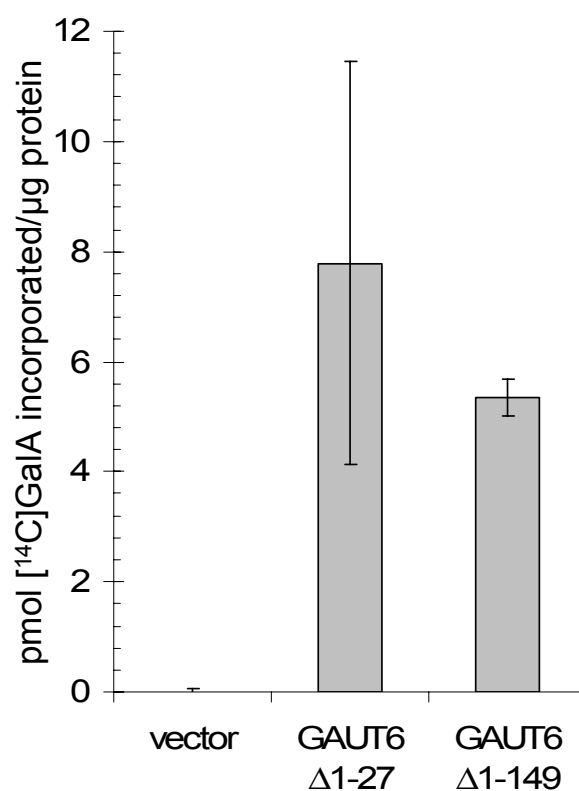


Figure 4.13. The HG:GalAT activity of *E. coli*-expressed truncated *At*GAUT6.

The Ni-affinity column-purified GAUT6Δ1-27 and GAUT6Δ1-149 soluble protein was incubated with UDP-[¹⁴C]GalA and OGA acceptors of DP 7-24 for three hours at 30°C and HG:GalAT activity was measured. The data are T0 subtracted average pmol [¹⁴C]GalA incorporated/μg protein ± standard error of duplicate reactions.

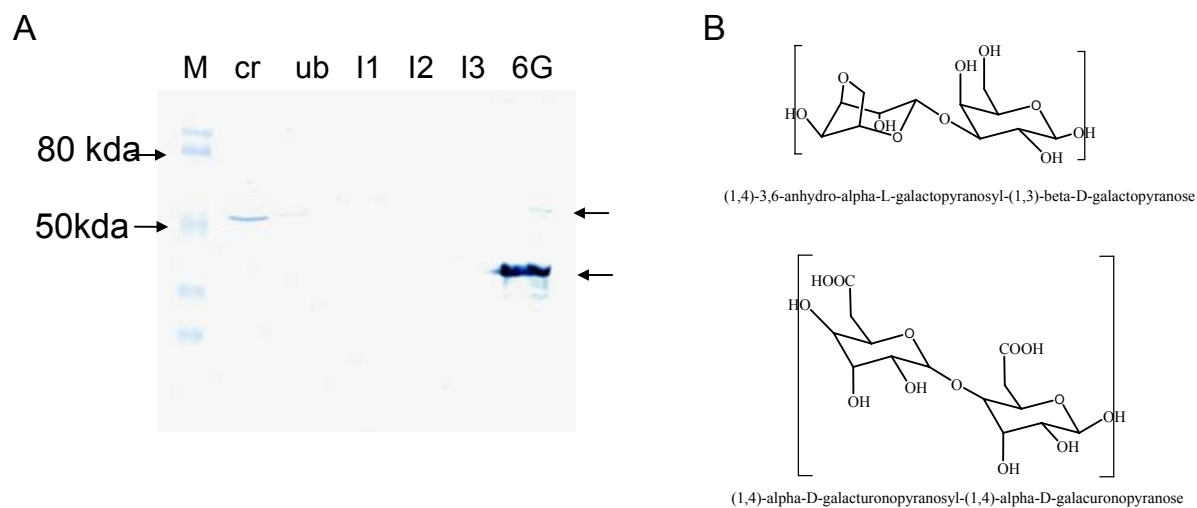


Figure 4.14. Agarose binds irreversibly to GAUT6 Δ 1-149 protein.

The soluble GAUT6 Δ 1-149 crude (cr) protein was passed over a Ni-affinity column of 6% agarose yielding protein fractions of unbound protein (ub) and protein eluted with 125 mM imidazole (I1), 125 mM imidazole and 0.5 M NaCl (I2), 250 mM imidazole and 0.5 M NaCl (I3) and 6 M Guanidine and 0.5 M NaCl (6G) that was analyzed by Western blot probed with anti-HIS antibodies. The structures of agarose (upper) and homogalacturonan (lower) are compared (B).

irreversible binding of GAUT proteins to agarose containing solid supports does occur, and that agarose-containing products should be avoided in manipulations of GAUT proteins. Such strong binding as that observed by the GAUT6 protein to the agarose containing column, may potentially indicate that the agarose molecule is acting as a transition-state analog in the active site of GAUT molecules. The structure of an agarose molecule has some similarity to an OGA molecule and may permit such binding to the active site of a galacturonosyltransferase (SEE Figure 4.14B). The GAUT6 Δ 1-149 is a truncated form of GAUT6 consisting of the C-terminal globular region of the protein that is predicted to harbor the active site of the enzyme and therefore the globular region of the protein must be involved in the binding to the agarose molecule.

DISCUSSION

GAUT6 is an HG:GalAT involved in pectin biosynthesis

The expression of the full-length GAUT6 in transiently transformed tobacco leaves and expression of two truncated forms of GAUT6 in *E. coli* have shown that GAUT6 is able to catalyze the transfer of UDP-[¹⁴C]GalA onto OGA acceptors consistent with HG-GalAT activity and a role for GAUT6 in HG biosynthesis. HG:GalAT enzyme activity is enhanced in tobacco leaves transiently transformed with pCAMBIA35:GAUT6 and pCAMBIA35:GAUT6-HIS. Furthermore, epitope-tagged GAUT6 protein expressed in bacteria has HG:GalAT activity. The results show that GAUT6 protein is an HG:GalAT *in vitro*. The precise *in vivo* role of GAUT6 as an HG:GalAT specifically involved in HG, as opposed to RG-I or RG-II synthesis, will require substrate specificity studies.

GAUT6 is one member of the 25-gene GAUT1-related gene family that encodes proven and putative wall biosynthetic galacturonosyltransferases. Detailed prior biochemical characterization of GAUT6 had not been carried out, however, the *GAUT6* Arabidopsis T-DNA insertion mutants produce plants that have a phenotype indicative of impaired wall polysaccharide biosynthesis (Caffall 2008, Ph.D. dissertation, chapter 2). The *gaut6-1* phenotype included reduced stem growth and reduced wall GalA glycosyl residue composition. More specifically, *gaut6* mutant plants were reduced wall GalA, reduced RG-I crosslinking into the wall and reduced PME/EPG-released wall polysaccharides. In addition, *gaut6* walls were reduced in hemicellulose-enriched and cellulose-enriched fractions, coincident with increased wall phenolic content (Caffall Ph.D. dissertation, Chapter 3, 2008). The depletion in secondary wall components is hypothesized to be a secondary effect of reduced pectin. The growth and wall phenotype of *gaut6* mutant plants is consistent with a role for GAUT6 as an HG:GalAT involved in pectin biosynthesis.

The activity of heterologously expressed GAUT6 in E. coli

The activity of nickel-affinity purified GAUT6-HIS *E. coli* expressed protein is far lower than *in vivo* HG:GalAT activity. The *in vitro* activity was detectable by the radioactive HG:GalAT assay, but not by the OGA-PAGE assay. The reduced rates of activity of GAUT6 protein expressed in *E. coli* may be due to a proportion of mis-folded protein, or incorrect acceptor specificity. Also, protein partners, if necessary, are not available in *E. coli* cell lysates. In addition, the N-terminally truncated versions of GAUT6 may have altered protein structure, lowering rates of activity. The low activity may also be due simply to low protein concentrations. A thorough optimization of GAUT6 protein expression, activity assay

conditions and acceptor specificities may result in the detection of increased activity. In addition, the data suggest that a codon-optimized GAUT6 sequence may be preferred to obtain maximum activity from GAUT6 expressed in *E. coli*. Codon optimization may result in significant increases in the amount of active protein recovered from *E. coli* lysates, thus increasing recovered GalAT activity (Burgess-Brown et al., 2008).

Wall biosynthetic galacturonosyltransferases

It is hypothesized that wall polysaccharide biosynthesis requires protein complexes. By and large the components of the protein complexes necessary for pectin biosynthesis have not been fully identified, but it has been suggested that a large complex of ~150 MDa is required (Ohashi et al., 2007). A complex containing GalAT activity has been partially purified from pea and azuki bean epicotyls of 560 kDa, however; the identities of the individual protein components have not been reported (Ohashi et al., 2007). It is hypothesized that one or more pectinmethyltransferases are required for *in vivo* homogalacturonan synthesis (*QUA2*), based on the correlation of the expression of *QUA2*, and specific *QUA2* isoforms, with specific GAUTs (Mouille et al., 2007). This protein, however, was not among the proteins identified in the HG:GalAT active fractions that identified GAUT1 and GAUT7, suggesting that the association of PMTs with HG-GalAT complexes may be transient and not essential for HG:GalAT activity (Atmodjo and Mohnen, unpublished data).

CONCLUSIONS

Glycosyltransferases have been studied in mammalian systems, however, heterologous expression and/or the subsequent biochemical characterization of active plant wall biosynthetic

glycosyltransferases has been achieved for only a few. Here the analysis of the *Arabidopsis thaliana* GAUT6, has resulted in the identification of GAUT6 as a pectin biosynthesis HG:GalAT. HG:GalAT activity above control samples was detected in tobacco and *E. coli* transformed with GAUT6 constructs showing that GAUT6 has *in vitro* HG:GalAT catalytic activity, a hypothesis that is consistent with the phenotype of *gaut6* plants that have stunted growth and reduced RG-I and HG. Thus GAUT6 is the fifth functionally confirmed pectin biosynthetic glycosyltransferase.

METHODS

Alignment of GAUT6 and orthologs

The alignment of *Arabidopsis* At3g61130 (GAUT1), At1g06780 (GAUT6) and At2g30575 (GAUT5) with *P. trichocarpa* orthologs (estExt_fgenes4_pm.C_LG_V0534 abbreviated PtV0534, and fgenes4_pg.C_LG_II000411 abbreviated PtII000411), a *Oryza sativa* (rice) ortholog (Os11g37980) and a *Sorghum bicolor* ortholog e_gw1.5.15262.1 [Sorbi1:4822212] abbreviated Sb4822212). The alignment was carried out with the M-COFFEE server (<http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee.cgi/index.cgi>) (Wallace et al., 2006) and converted to a colored alignment with The Sequence Manipulation Site (Stothard, 2000).

Cloning of GAUT6

The full length ORF of GAUT6 was amplified from *Arabidopsis* stem total RNA with GAUT6-specific forward primer (At1g06780gsF) (atgaacaaattcgatgagcagagg) and reverse primer (At1g06780gsR) (tcaagctgaagattgcactgttgcaagt) and inserted into the pCR2.1 vector for storage and sequencing. GAUT6 was subcloned from pCR2.1 into pCAMBIA35 for transient

expression experiments in *N. benthamiana* and into pET28a for expression in *E. coli*. GAUT6 DNA was amplified with the gene specific forward primer plus KpnI restriction site (G6F+KpnI(2)) (ggtacccatgaaacaaattcgtcgatggcagagg) and the gene specific reverse primer plus Sall site (G6R+Sall) (gtcgacgcttgaagattgcactgttgcaagt) and ligated via the KpnI and Sall sites to create the pCAMBIA35:GAUT6 construct. The GAUT6 DNA was amplified with G6F+KpnI(2) and GAUT6 gene specific-reverse primer minus the stop codon (G6R ns) (gcttgaagattgcactgttgcaagtagg), and ligated into the pCAMBIA vector via the KpnI site for creation of the pCAMBIA:GAUT6-GFP construct. The GAUT6-HIS construct was generated by amplifying the full length GAUT6 with At1g06780gsF and GAUT6 gene specific reverse primer excluding the stop codon and minus one nucleotide to bring the C-terminal HIS tag into frame (G6Rns fs-1) (cctacttgcaacagtgcattctcaagc). The GAUT6 was amplified from pET28 with G6F+KpnI and a pET28 HIS-tag specific-reverse primer (pET28R HIS) (tcagtgggtgggtgggtgggtgctcgagtgc) and ligated into pCAMBIA at the KpnI site. The constructs destined for transient transformation into tobacco were transformed into *Agrobacterium tumefaciens* strain GV3101 (pMP90) by electroporation and selected with 50 $\mu\text{g ml}^{-1}$ kanamycin, 50 $\mu\text{g ml}^{-1}$ gentamycin and 50 $\mu\text{g ml}^{-1}$ rifampicin. GAUT6 DNA was amplified with gene a specific forward primer plus NdeI restriction endonuclease site at positions 82-109 (catatgtcgaatcggcttaagagcatcactccc) and 448-475 (catatgacagatgtgaaaacgaaggagatcagagac) and ligated into the pET28a vector in frame with the N-terminal HIS-tag via an engineered NdeI and pCR2.1 NotI restriction endonuclease sites. Constructs were transformed into *E. coli* strains BL21(DE3)pLysS (Novagen, Madison WI, USA), BL21 Star(DE3) (Invitrogen, Carlsbad CA, USA) and K12 Rosetta-gami2(DE3) (Novagen) (Novy et al., 2001) by heat shock at 42°C

Transient transformation of N. benthamiana

Four ml of YEP medium (1% w v⁻¹ yeast extract, 1% w v⁻¹ peptone, 0.1% w v⁻¹ NaCl, pH 7.0) and 50 µg ml⁻¹ of kanamycin, gentamycin and rifampicin was inoculated with *A. tumefaciens* containing the appropriate plasmid and incubated at 25°C with shaking in the dark for two days. A sterile 125 ml flask containing 40 ml of LB-MES (1% w v⁻¹ tryptone, 0.5% w v⁻¹ yeast extract, 1% w v⁻¹ NaCl, 10 mM MES, pH 5.7) containing 125 µM acetosyringone (100 µl of 50 mM acetosyringone in DMSO) and the antibiotic was inoculated with the entire 4 ml of overnight culture. The culture was incubated over night at 25°C in the dark. The 40 ml of overnight culture was transferred to a sterile 50 ml tube and centrifuged at 5,000 x g for 20 minutes to pellet the cells. The cell pellet was resuspend in 40 ml of infiltration medium (10 mM MgCl₂, 10 mM MES, pH 5.7) containing 125 µM acetosyringone. The cells were gently resuspended by inverting the tube. The culture was incubated at 25°C in the dark for 24 hours with shaking. The density of *A. tumefaciens* cells was adjusted to OD 600 nm = 1.0 with infiltration medium and injected into the leaf tissue of *Nicotiana benthamiana* tobacco plants that were 4 to 5 weeks old from the underside of the leaf using a 20 ml syringe without a needle. The leaf segments were harvested on the third day after infiltration for analysis.

Microscopy of GFP constructs

The pCAMBIA:GAUT6-GFP construct was created as above and the fusion protein in the infiltrated leaves was visualized by dissecting scope to verify fluorescence of the GFP control and the GAUT6-GFP fusion protein. The subcellular localization was carried out using a Leica TCS SP2 spectral confocal scanning laser microscope (Leica Microsystems Inc. Suite 107 410 Eagleview Blvd. Exton PA 19341) with 63x water immersion objective lens.

Isolation of tobacco microsomal membranes

Equal amounts of leaf tissue from GAUT6-expressing and control plants (~2 to 4 g) were harvested on ice from inoculated plants after three days. The tissues were flash frozen in liquid nitrogen and ground to a fine powder in 50 ml polypropylene (Oakridge) tubes followed by homogenization with an electric hand-held grinder (PowerGen 125 from Fisher Scientific, Foster City CA, USA) in 10 ml of homogenizing buffer (HB) for two minutes (50 mM HEPES pH 7.3, 50% glycerol, 0.25 mM MnCl₂, 25 mM KCl, 0.1% β-mercaptoethanol). The homogenate was centrifuged at 3450 x g for 20 minutes at 4°C. The pellet was discarded and the supernatant was filtered through one layer of miracloth into high speed centrifuge tubes on ice. The supernatant was centrifuged at ~60,000 x g (30,000 rpm) for one hour at 4°C. The supernatant (S60) was carefully decanted and stored at -80°C. The pellet (microsomal membranes, P60) was resuspended in 0.6 ml of solubilization buffer/dilution buffer (SB/DB) with a small glass homogenizer (50 mM HEPES pH 7.3, 0.25 mM MnCl₂, 0.25% v v⁻¹ Triton X-100 and 25% v⁻¹ glycerol). The protein was quantified by the DC protein assay (Bio-Rad, Hercules CA, USA) (Lowry et al., 1951b).

Expression of GAUT6 in E. coli

Fifty ml of LB containing kanamycin (50 µg ml⁻¹) was inoculated with the frozen culture of *E. coli* harboring the pET28a:GAUT6 constructs and shaken at 225 rpm at 37°C overnight. Two 1L flasks containing 250 ml of LB with kanamycin (50 µl ml⁻¹) were inoculated with 12 ml of the overnight culture and shaken until the cell density reached OD₆₀₀ between 0.5 and 0.6. The LB medium for the expression of GAUT6Δ1-27 was supplemented with 660 mM sorbitol

and 2.5 mM betain or 0.2% glucose. After the cultures reached the appropriate cell density, the flasks were placed on ice for 5 minutes and MnCl_2 was added (1.9 mM final concentration) and IPTG (0.5 mM final concentration, unless other wise stated). The induced cultures were grown with shaking (225 rpm) at 17°C for 5 hours for GAUT6 Δ 1-27 or at 37° for 4 hours for GAUT6 Δ 1-149. The cells were placed on ice for 10 minutes followed by centrifugation at 3,200 x g for 10 minutes to pellet the cells. The cells were re-suspended in ~7 ml of Tris-HCl pH 8.0, 20% glycerol and Complete EDTA-free protease inhibitor (Roche Applied Science, Mannheim Germany). PMSF was added to a concentration of 0.5 mM immediately prior to cell lysis. The cells were lysed by sonication (10 minutes total sonication time, using 10 s pulsed sonication). The lysed cell homogenate was centrifuged at 20,000 x g for one hour at 4°C. The supernatant (S20) was carefully removed and stored at -80°C. For some experiments the P20 fraction was resuspended in 1.5 ml of solubilization buffer/dilution buffer with a glass homogenizer and also stored at -80°C.

Antibody detection of GAUT6

Western blots were probed with commercial antibody specific for N-terminally 6HIS tagged proteins and GFP fusion proteins. The anti-HIS antibody was a mouse anti-HIS IgG with horse radish peroxidase conjugate (A7058) (Sigma, St. Louis MO, USA) and the anti-GFP antibody (G6539) (Sigma) was a mouse anti-GFP IgG primary used in combination with a goat anti-mouse-IgG horse radish peroxidase conjugate (A8924) (Sigma) secondary antibody. The antibodies were resuspended in the recommended volumes and applied to blots at 1 to 10,000 dilution for *E. coli* lysates or a 1 to 5,000 dilution for *N. benthamiana* lysates.

OGA-PAGE and UDP-[¹⁴C]GalA filter assays for HG:GalAT activity

The OGA-PAGE reactions were carried out with 40 µg of protein, as determined by the DC protein assay (Lowry et al., 1951b), which was added to the reaction mixture to give a 20 µl total reaction volume. The reaction mixture contained 4 µl of 5x buffer to yield a final concentration of 0.2 M sucrose, 0.05% w v⁻¹ bovine serum albumin, 50 mM HEPES pH 7.3, 25 mM KCl and 1.0 mM MnCl₂. Next, 5 µl of 1 µg µl⁻¹ OGAs, 1 µl of 10 µg µl⁻¹ UDP-GalA, 40 µg protein and water to equal 20 µl were added. The reactions were stopped by boiling for 5 minutes to denature the protein and were then diluted to 50 µl total volume. Two µl of each diluted sample (~0.2 µg of OGA) was mixed with 6 µl of sterile water and 2 µl of 4x phenol red loading dye and loaded onto an acrylamide gel. The PAGE gel was poured as previously described (Goubet et al., 2002) and electrophoresed at 17.5 volts for ~1 hr and 40 minutes. The gel was stained by first rinsing with filtered water for 5 minutes, followed by rocking the gel in a solution of 2% w v⁻¹ alcian blue in 50% v v⁻¹ ethanol for 20 minutes followed by extensive rinsing in filtered water. The de-stained gel was rocked in an aqueous solution of 4 mg ml⁻¹ silver nitrate for 20 minutes followed by three brief rinses with filtered water. The gel stain was developed by addition of 4% w v⁻¹ sodium carbonate and stopped by addition of a 5% v v⁻¹ acetic acid aqueous solution.

For the 30 µl radioactive reaction, 6 µl of 5x Rad Reaction buffer (final 1x concentration was 0.2 M sucrose, 0.05% w v⁻¹ bovine serum albumin, 50 mM HEPES pH 7.3, 25 mM KCl and 1.0 mM MnCl₂). Next, 8 µl of 10 mg ml⁻¹ PGA (DP 7 to 25), 3 µl 18,163 cpm µl⁻¹ UDP-[¹⁴C]GalA, approximately 40 µg of protein (10 µl) and 3 µl of sterile water were added to 1.5 ml tubes. For each sample, duplicate timed reactions plus a T0 reaction were carried out and incubated at 30°C for three hours. The reactions were stopped by addition of 5 µl of 400 mM

NaOH. For T0 controls, the 5 μ l of 400 mM NaOH was added prior to incubation. The stopped reactions were spotted onto cetylpyridinium chloride-coated filter paper squares and allowed to dry for 5 minutes (Sterling et al., 2001). The dry filter papers were washed with 1.5 M NaCl three times for 15 minutes each, to release the unincorporated UDP-[^{14} C]GalA. Washed filters were allowed to dry for 2 hours and then loaded into scintillation vials to which scintaverse (Fisher) scintillation fluid was added for counting.

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APPENDIX A

PEARSON CORRELATION ANALYSIS OF THE GLYCOSYL RESIDUE COMPOSITIONS OF *ARABIDOPSIS* WILD-TYPE AND GAUT MUTANTS.

The wall compositions described in Chapter 2 of this dissertation demonstrated that many GAUT mutants have altered wall glycosyl residue composition compared to the walls of similarly grown WT plants. In order to further investigate the changes in wall composition of the mutants compared to WT, Pearson Correlation Coefficients (PCCs) were generated from the mol % compositions of each mutant and WT. The PCCs describe a linear relationship between two variables (glycosyl residues) expressed as a value ($|r|$) between 1 and -1, depending on the strength and direction of the linear relationship between the two variables. For example, the glycosyl residue compositions of inflorescences tend to have an increase in Rha that is correlated with an increase in Xyl, and thus these two residues have a positive correlation close to +1.0 (inflorescence PCC = 0.8982, $P = <0.0001$).

The glycosyl residue compositions of both WT and GAUT mutants presented a special opportunity to experiment with different methods of data analysis. PCCs were used here to attempt to make correlations between residues that would suggest specific wall polysaccharides or relationships between wall polysaccharides. The data was originally to be included in the publication presented in Chapter 2 of this dissertation. It was not included due to the nature of the data. The data used for the analysis was a mol% value. Because data are based on a

percentage, the values will always skew to some extent when one component is altered creating a preponderance of negative correlations, and thus some of the correlation values will ultimately be the result of this effect. Because we could not differentiate those values that were real trends from those that were a result of the skewing of percentile values, the data was not included. Nonetheless, several trends were identified that have very low P values and high PCC values that appear to suggest real trends among the glycosyl residues of the walls of the tissues analyzed.

Correlation analysis suggests interesting trends in WT wall glycosyl residue composition

The glycosyl residue composition used to test the analysis was that of WT inflorescence (n=34), silique (n=38), stem (n=24) and leaf (n=38) walls (Table A1) that correspond to data presented in Figure 2.3. The residues compared were Ara, Rha, Xyl, GalA, Gal and Glc. The compositions of walls from different tissues suggest that wall compositions are tissue-specific. The mass of walls extracted from a given amount of fresh tissue was quantified (Figure A1). The analysis of wall composition and the dry weight of walls per fresh weight tissue suggest that wall structure, and thus function, is tissue-specific.

The PCCs generated solely from WT data showed several trends (Table A2). The PCCs that have a P value less than 0.0001 are shown in bold font. There were many correlations of glycosyl residues in inflorescence but they were complex. Ara was positively correlated with Rha and Xyl, but negatively with GalA and Glc. Rha and Xyl were both negatively correlated with Gal, and GalA was positively correlated with Glc. The analysis of leaf glycosyl residue composition was less complicated in that GalA was negatively correlated with Ara, Rha and Xyl while Ara, Rha and Xyl are all positively correlated with each other. Fewer correlations of high

Table A-1. WT tissue-specific glycosyl residue composition.

(^a) The Arabidopsis WT glycosyl residue composition of multiple tissues is listed for inflorescence, leaf, silique and stem. The average (mean), minimum value (min), maximum value (max) and difference between the minimum and maximum value (range) are listed as mol %.

(^b) The glycosyl residue quantified are listed as arabinose, Ara; rhamnose, Rha; fucose, Fuc; xylose, Xyl; galacturonic acid, GalA; mannose, Man; galactose, Ga; and glucose, Glc. The values are expressed as mol %.

Inflorescence ^a	Ara ^b	Rha	Fuc	Xyl	GalA	Man	Gal	Glc
mean	19.4	6.3	1.1	8.8	35.9	7.1	17.3	4.3
min	7.0	2.7	0.5	5.1	11.4	2.1	11.6	2.3
max	35.5	9.6	1.7	13.5	49.2	10.3	38.9	7.0
range	28.5	6.9	1.2	8.3	37.7	8.2	27.3	4.7
Leaf								
mean	8.4	4.8	1.4	9.3	53.1	4.3	11.0	7.6
min	2.1	1.8	0.5	5.1	27.6	2.1	5.3	2.5
max	18.0	9.0	4.6	15.9	66.9	5.5	29.0	16.8
range	15.9	7.2	4.0	10.8	39.3	3.4	23.8	14.4
Silique								
mean	11.0	5.9	1.0	20.3	39.4	5.2	11.8	5.4
min	4.4	3.7	0.6	9.9	22.8	1.7	6.8	3.2
max	18.9	10.9	3.1	29.8	48.5	8.6	35.2	12.0
range	14.5	7.2	2.5	19.9	25.7	6.9	28.4	8.8
Stem								
mean	8.5	4.4	0.8	27.6	38.9	4.8	10.7	4.4
min	2.9	2.5	0.5	11.1	17.4	1.1	5.8	1.7
max	21.3	6.9	1.2	38.5	48.6	5.9	33.7	6.0
range	18.5	4.4	0.8	27.4	31.2	4.8	27.8	4.3

Table A-2. The residue to residue Pearson Correlation Coefficients of WT Arabidopsis tissue-specific wall glycosyl residue composition.

(^a) The residue to residue Pearson Correlation Coefficients (PCCs) were calculated using WT mol% tissue-specific wall glycosyl residue composition determined by GC-MS of TMS derivatized AIR walls of inflorescence, silique, leaf and stem. (^b) The glycosyl residues quantified are listed as arabinose, Ara; rhamnose, Rha; fucose, Fuc; xylose, Xyl; galacturonic acid, GalA; mannose, Man; galactose, Gal and glucose, Glc. The values in bold type are those that have P values less than 0.0001. PCCs were calculated using SAS 9.1.3 software (© 2002-2004 produced by the SAS Institute Inc. Cary N.C. USA.

Inflorescence ^a	Ara ^b	Rha	Xyl	GalA	Gal	Glc	Silique	Ara	Rha	Xyl	GalA	Gal	Glc
Ara	1	0.70228 <.0001	0.54528 0.0009	-0.95241 <.0001	-0.09963 0.5751	-0.62645 <.0001	Ara	1	0.44846 0.0047	-0.37982 0.0187	-0.68103 <.0001	0.47261 0.0027	-0.35211 0.0302
Rha	0.70228 <.0001	1	0.8982 <.0001	-0.50779 0.0022	-0.58567 0.0003	-0.39932 0.0193	Rha	0.44846 0.0047	1	0.28234 0.0859	-0.42175 0.0084	-0.40057 0.0127	0.09991 0.5506
Xyl	0.54528 0.0009	0.8982 <.0001	1	-0.36921 0.0316	-0.71094 <.0001	-0.0699 0.6945	Xyl	-0.37982 0.0187	0.28234 0.0084	1	-0.26292 0.1108	-0.55758 0.0003	0.59491 <.0001
GalA	-0.95241 <.0001	-0.50779 0.0022	-0.36921 0.0316	1	-0.3517 0.0414	0.5783 0.0003	GalA	-0.68103 <.0001	-0.42175 0.0084	-0.26292 0.1108	1	-0.40545 0.0116	-0.23976 0.1471
Gal	-0.09963 0.5751	-0.58567 0.0003	-0.71094 <.0001	-0.3517 0.0414	1	-0.25503 0.1455	Gal	0.47261 0.0027	-0.40057 0.0127	-0.55758 0.0003	-0.40545 0.0116	1	-0.28765 0.0799
Glc	-0.62645 <.0001	-0.39932 0.0193	-0.0699 0.6945	0.5783 0.0003	-0.25503 0.1455	1	Glc	-0.35211 0.0302	0.09991 0.5506	0.59491 <.0001	-0.23976 0.1471	-0.28765 0.0799	1
Leaf	Ara	Rha	Xyl	GalA	Gal	Glc	Stem	Ara	Rha	Xyl	GalA	Gal	Glc
Ara	1	0.89761 <.0001	0.77947 <.0001	-0.91723 <.0001	0.09827 0.5572	0.06257 0.709	Ara	1	0.46395 0.0224	-0.38757 0.0613	-0.66117 0.0004	0.88273 <.0001	-0.81604 <.0001
Rha	0.89761 <.0001	1	0.83409 <.0001	-0.84773 <.0001	-0.07406 0.6586	0.10008 0.5499	Rha	0.46395 0.0224	1	-0.02365 0.9126	-0.22702 0.2861	0.04895 0.8203	-0.74504 <.0001
Xyl	0.77947 <.0001	0.83409 <.0001	1	-0.69984 <.0001	-0.22579 0.1729	-0.15754 0.3449	Xyl	-0.38757 0.0613	-0.02365 0.9126	1	-0.39855 0.0537	-0.55691 0.0047	0.13607 0.5261
GalA	-0.91723 <.0001	-0.84773 <.0001	-0.69984 <.0001	1	-0.33841 0.0377	-0.24866 0.1322	GalA	-0.66117 0.0004	-0.22702 0.2861	-0.39855 0.0537	1	-0.50976 0.0109	0.58329 0.0028
Gal	0.09827 0.5572	-0.07406 0.6586	-0.22579 0.1729	-0.33841 0.0377	1	-0.05696 0.7341	Gal	0.88273 <.0001	0.04895 0.8203	-0.55691 0.0047	-0.50976 0.0109	1	-0.57803 0.0031
Glc	0.06257 0.709	0.10008 0.5499	-0.15754 0.3449	-0.24866 0.1322	-0.05696 0.7341	1	Glc	-0.81604 <.0001	-0.74504 <.0001	0.13607 0.5261	0.58329 0.0028	-0.57803 0.0031	1

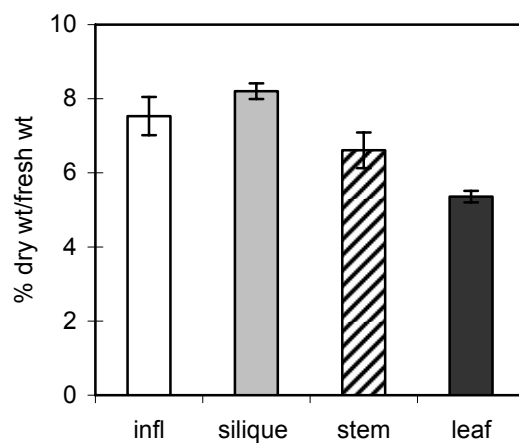


Figure A-1. The wall % dry weight per fresh weight of Arabidopsis tissues. The data are the average wall % dry weight per fresh weight $\pm$ standard deviation of inflorescence (infl), silique, stem and leaf tissues.

confidence were observed in silique walls. GalA content was negatively correlated with Ara, while Xyl was positively correlated with Glc but negatively correlated with Gal.

The trends in the PCCs of the WT glycosyl residue mol% composition data suggest that specific residues are associated with particular structures in the wall. For instance, GalA is negatively correlated with Rha in each tissue. The Rha content is largely due to the RG-I content. An increase in RG-I effectively reduces the relative mol % GalA content from HG because RG-I has half the number of GalA residues as HG. In silique and stem tissues, the Ara and Gal are strongly positively correlated, but have a correlation close to zero in inflorescence and leaf, suggesting that the Gal in these tissues may be due to an arabinogalactan. In stem, Rha and Gal are not correlated suggesting that the Gal of stem is in an arabinogalactan not associated with RG-I. In siliques, Xyl and Glc are strongly positively correlated suggesting that XG polysaccharides that are known to function as a storage polysaccharide are abundant in the developing seeds of siliques. Xyl and Glc were not correlated strongly in any other tissue. Ara and Rha are positively correlated in all tissues, but less strongly in silique and stem tissues, where AGPs may constitute a significant proportion of the Ara.

Most GAUT mutants that had altered wall composition had similar residue to residue correlations as WT

PCCs were also generated for the mutant wall composition data in order to identify trends that were not in accordance with those established by the analysis of WT glycosyl residues. The trends in mutant composition correlations were similar to WT in the majority of cases. This indicates that the response of the plant to fluctuations in wall

composition, caused by the T-DNA insertion, were normal in nature but to an extent outside of that typical of WT *Arabidopsis*. In other words, many of the compositional changes detected may be brought about by compensatory mechanisms in the plant to deal with changes that are detrimental to the plant growth and development. The correlations of GAUT mutant wall compositions in some cases were different than those observed in WT walls of the corresponding tissues. Mutants in GAUT 12 had non-WT-like trends in stem wall composition. In WT stem Xyl is positively correlated with Ara and Rha but negatively with GalA; the trend is the opposite for GAUT 12 mutants; Xyl is negatively correlated with Ara and Rha, and positively with GalA.

APPENDIX B

THE FRACTIONATION OF GAUT12/IRX8 STEM WALLS

(data reprinted with permission from the publisher).

The GAUT12 gene is one of 15 genes in the GAUT1-related gene family of Arabidopsis. The *gaut12* mutant is allelic to *irx8* (*irregular xylem8*) originally identified based on the altered morphology of xylem elements in the inflorescence stems of Arabidopsis T-DNA insertion mutant plants (Turner and Somerville, 1997). WT xylem elements have heavily thickened secondary walls and are generally round in cross-sections of the stem. The irregular xylem phenotype is identified by the appearance of collapsing xylem elements and a thin secondary wall of xylem elements. The growth and wall phenotype of several *irx* mutants were analyzed and showed that *irx7*, *irx8* and *irx9* had significantly reduced wall Xyl content in addition to significantly reduced wall cellulose content (Brown et al., 2005). The genes affected in *irx7*, *irx8* and *irx9* mutants correspond to predicted glycosyltransferases in the families of GT47, GT8 and GT43 respectively (Brown et al., 2005), and have subsequently been shown to be putative GTs involved in the same xylan biosynthesis pathway (Brown et al., 2007). An additional GT8 (PARVUS) is also involved in this xylan biosynthesis pathway (Lee et al., 2007).

The Arabidopsis *irx8* mutants produced severely dwarfed and semi-sterile plants and have walls that are reduced in Xyl wall glycosyl residue composition. In

collaboration with the Dr. C. Somerville group, a detailed investigation of the glycosyl residue composition of *irx8* mutant walls and wall fractions, compared to WT, was carried out (Persson et al., 2008). The composition of total walls of the *irx8* mutants is listed in Table 3 of Chapter 2 of this dissertation and shows that the *irx8* walls of stem were increased in GalA, Gal and Fuc composition and severely reduced in Xyl composition. In order to further investigate the wall structures contributing to the wall composition phenotype, the walls of *irx8* mutants and WT were fractionated by sequential enzymatic digest and chemical solubilization. First the stem AIR walls were digested by EPG alone (EPG) and then with a combination of PME and EPG (PME/EPG). The remaining residue was sequentially extracted with 50 mM sodium carbonate plus 5 mM EGTA (SC/EGTA), followed by 4 N potassium hydroxide (4N KOH soluble and 4 N KOH insoluble). After each treatment the aqueous fraction was separated by centrifugation and decanted for glycosyl residue composition and linkage analyses.

For preliminary analysis of the wall fractions of *irx8* mutants and WT, the total sugar content of the individual fractions was assayed by the phenol-sulfuric acid assay. The quantification of the total sugar in each fraction (expressed as $\mu\text{g mg}^{-1}$ total sugar recovered) showed that more carbohydrate was released from *irx8* walls in the EPG, PME/EPG, SC/EGTA and 4 N KOH soluble fractions and that *irx8* walls had less cellulosic residue remaining in the 4 N KOH insoluble fraction, compared to WT (Table B-1). The mass % of material recovered was determined from the weights of the fractions (Table B-2). In *irx8* walls, more material was released in the EPG fraction, while a similar amount of material was released in the PME/EPG fraction, compared to

Table B-1. The total sugar of *irx8* and WT wall fractions.

(^a) Cell wall fraction refers to the fraction of wall material released by endopolygalacturonase (EPG), a combination of pectinmethylesterase and EPG (PME/EPG), a combination of sodium carbonate and EGTA (SC/EGTA) and 4 N KOH (4 N KOH sol) and the final 4 N KOH insoluble residue (4 N KOH insol). (^b) The samples analyzed were those from Arabidopsis stems of WT, *irx8-5/gaut12-1* and *irx8-5/gaut12-2* walls. (^c) The total sugar was quantified by the phenol-sulfuric acid assay and is expressed as the percent total sugar detected in all fractions.

Cell Wall Fraction ^a	Sample ^b	Total Sugar ^c
EPG	WT	6.7
	<i>irx8-1/gaut12-1</i>	36.5
	<i>irx8-5/gaut12-2</i>	45.2
PME/EPG	WT	7.1
	<i>irx8-1/gaut12-1</i>	10.2
	<i>irx8-5/gaut12-2</i>	29.8
SC/EGTA	WT	50.6
	<i>irx8-1/gaut12-1</i>	127.7
	<i>irx8-5/gaut12-2</i>	241.3
4 N KOH sol	WT	165.1
	<i>irx8-1/gaut12-1</i>	241.8
	<i>irx8-5/gaut12-2</i>	371.0
4 N KOH insol	WT	770.5
	<i>irx8-1/gaut12-1</i>	583.7
	<i>irx8-5/gaut12-2</i>	312.7

WT. The wall material extracted by sodium carbonate in combination with EGTA was greater in the *irx8* mutants. The EPG, PME/EPG and SC/EGTA fractions constitute different fractions of pectic polysaccharides in the wall. It is evident that the *irx8* mutants had a greater proportion of pectin-rich fractions than the comparable fractions of WT stem walls. The 4 N KOH soluble fraction extracts from the *irx8* walls, however, were reduced to approximately 52% that of WT, while the cellulosic residue was reduced to approximately 54% that of WT. These results are in accordance with the hypothesis that IRX8/GAUT12 functions in secondary wall synthesis. The glycosyl residue composition of the wall fractions of *irx8* and WT walls was also determined (Table B2). The glycosyl residue composition of EPG-released material showed that the mutants were virtually devoid of Xyl and GalA containing polysaccharides in this fraction, despite having released approximately 2.5 times the amount of material in this fraction compared to WT. The PME/EPG fraction of *irx8* mutants was also severely reduced in GalA, and had increased, Ara, Rha, Xyl and Gal. The SC/EGTA fraction of *irx8* mutants was similar to WT except that the mutants were also reduced in GalA composition. The 4 N KOH soluble fraction, that contains the bulk of the hemicellulose, was severely reduced in Xyl content and Glc content. The 4 N KOH insoluble fractions that are comprised mostly of cellulose were similar between WT and mutant, except that the *irx8* walls were depleted in Glc composition.

The fractionation of the walls of *irx8* mutants and WT, has confirmed that the walls are depleted in a Xyl-containing wall component that is released by alkaline extraction. This suggests that the wall component is a xylan. In addition, the walls of *irx8* mutants are depleted in a GalA containing component that is released by EPG, PME

Table B-2. The glycosyl residue composition of Arabidopsis inflorescence stem walls from WT, *irx8-1/gaut12-1* and *irx8-5/gaut12-2*.

(^a) The cell wall fractions are the material released from total walls (AIR) by endopolygalacturonase (EPG), a combination of pectinmethylesterase and EPG (PME/EPG), a combination of sodium carbonate and EGTA (SC/EGTA), 4 N KOH (4 N KOH sol) and the remaining 4 N KOH-insoluble residue (4 N KOH insol) and alcohol insoluble wall residue (Total cell walls). (^b) The glycosyl residue quantified by GC-MS of TMS derivatized walls are abbreviated: arabinose, Ara; rhamnose, Rha; fucose, Fuc; xylose, Xyl; galacturonic acid, GalA; mannose, Man; galactose, Gal; and glucose, Glc. (^c) The mass % of the wall fractions indicates the percentage of the total walls that each fraction makes up, by weight. (^d) The 4 N KOH insoluble fraction was analyzed by Saemann Hydrolysis (Selvendran et al., 1979; Zhong et al., 2005) followed by TMS to determine the approximate quantity of Glc attributed to cellulose polymers.

Cell Wall Fraction ^a		Ara ^b	Rha	Fuc	Xyl	GalA	Man	Gal	Glc	mass% ^c
EPG	WT	0.0	0.0	0.0	19.3	28.3	10.7	41.7	*	8.0
	<i>irx8-1/gaut12-1</i>	9.9	3.0	0.2	3.6	1.6	9.7	72.1	*	21.0
	<i>irx8-5/gaut12-2</i>	15.9	6.6	0.4	0.0	1.6	8.6	66.9	*	20.0
PME/EPG	WT	0.0	0.0	0.0	3.9	66.6	1.5	27.9	*	8.2
	<i>irx8-1/gaut12-1</i>	6.7	5.1	0.2	8.9	3.9	9.6	65.6	*	7.6
	<i>irx8-5/gaut12-2</i>	7.5	8.5	0.4	11.7	6.5	8.9	56.4	*	8.7
SC/EGTA	WT	6.7	6.6	0.3	1.6	81.0	1.4	2.4	0.0	33.0
	<i>irx8-1/gaut12-1</i>	9.0	6.7	0.2	2.7	75.5	0.3	5.7	0.0	42.0
	<i>irx8-5/gaut12-2</i>	14.3	10.4	0.1	4.7	67.4	n.d.	3.1	0.0	48.0
4 N KOH sol	WT	2.2	2.1	0.4	72.8	7.9	n.d.	7.9	6.8	25.0
	<i>irx8-1/gaut12-1</i>	4.5	2.8	0.7	30.9	12.2	6.5	25.9	16.5	16.0
	<i>irx8-5/gaut12-2</i>	4.7	2.7	0.8	30.5	11.1	6.0	29.9	14.4	10.0
4 N KOH insol	WT	6.8	4.2	0.2	14.5	21.0	3.6	15.4	34.2	25.0
	<i>irx8-1/gaut12-1</i>	4.4	4.3	0.3	14.9	13.3	4.9	25.1	32.9	14.0
	<i>irx8-5/gaut12-2</i>	2.6	3.4	0.0	8.8	21.6	3.6	21.1	38.8	13.0
4 N KOH insol-SH ^d	WT	2.8	0.8	0.3	3.7	1.6	10.0	2.8	78.0	25.0
	<i>irx8-1/gaut12-1</i>	3.3	0.7	0.2	3.8	2.8	8.9	5.8	74.6	14.0
	<i>irx8-5/gaut12-2</i>	3.5	1.6	0.1	3.9	2.9	6.8	5.8	75.4	13.0
Total cell walls	WT	6.5	6.2	0.0	41.7	28.6	2.2	10.8	4.0	100.0
	<i>irx8-1/gaut12-1</i>	11.9	6.8	0.7	22.0	22.5	3.6	23.1	9.4	100.0
	<i>irx8-5/gaut12-2</i>	2.3	1.3	0.1	7.2	37.4	0.3	38.2	13.3	100.0

and EPG and/or sodium carbonate plus EGTA. This suggests that the GalA component is pectin. Further analyses carried out by S. Persson and colleagues confirmed that IRX8/GAUT12 was involved in xylan synthesis. However, the underlying cause of decreased pectic polysaccharides was not determined.

APPENDIX C

THE CHARACTERIZATION OF GAUT14 MUTANT LEAF WALLS

The role of GAUT genes has proven to be of importance in plant growth and development as has been demonstrated in the literature and within the pages of this thesis. The analyses of GAUT T-DNA mutant cell walls in Chapter 2 of this dissertation showed that GAUT genes 12, 13 and 14 showed similar glycosyl residue compositional profiles. For example, the Xylose was reduced and the GalA was increased in the tissues of these mutants. Based on these results the further analyses of GAUT14 mutants was undertaken to attempt to understand the changes in the cell wall in relation to that which is known about IRX8/GAUT12, a GalAT involved in the synthesis of a xylan primer sequence, and also in relation to GAUT6, which is an HG biosynthetic HG:GalAT.

GAUT14 growth and seed germination phenotypes

RT-PCR isolated from the total RNA of *gaut14-1* and *gaut14-2* resulted in the inability to amplify *GAUT14* transcript, indicating that both mutants are knockouts. Previous analyses showed that *gaut14* mutants did not show a severe growth phenotype, however, a more detailed investigation of *gaut14* growth compared to WT by measuring the length of stems and leaves at standard and elevated temperatures showed some changes. The length of *gaut14-1* and *gaut14-2* stem height and leaf length was reduced

compared to WT at standard temperatures (Figure C-1). In addition, the final height of stems and the final length of the leaves of *gaut14-1* was more dramatically reduced compared to WT at increased temperatures (data not shown). The growth of *gaut14-1* and *gaut14-2* plants is reduced showing that cell expansion might be affected in *gaut14* plants.

Fractionation of WT and gaut14 mutant walls

The glycosyl residue composition of AIR walls of *gaut14-1* and *gaut14-2* had increased GalA and Gal composition and reduced Ara, Rha and Xyl composition compared to WT (Caffall Ph.D. dissertation, Chapter 2, 2008). It was also observed that the mass of walls extracted from a given amount of fresh tissue was reduced in *gaut14* mutants compared to WT (Figure C-2).

In order to further investigate the changes observed in the wall compositions of *gaut14* mutants compared to WT, the walls of WT, *gaut14-1* and *gaut14-2* were fractionated by enzymatic and chemical means (Table C-1). The walls were sequentially treated with EPG alone (EPG), and then a combination of PME and EPG (PME/EPG). The majority of the remaining pectin was extracted with 50 mM sodium carbonate and 5 mM EGTA. The hemicelluloses-enriched fraction was extracted with 4 N KOH (4 N KOH sol) resulting in a cellulose-enriched pellet (4 N KOH insol). The *gaut14* mutants released a reduced $\mu\text{g mg}^{-1}$ of material in the EPG, PME/EPG and cellulosic fractions, but unchanged from WT in mass of the sodium carbonate/EGTA fraction. Interestingly, the mutant walls were increased in the hemicellulose-enriched 4 N KOH soluble fraction.

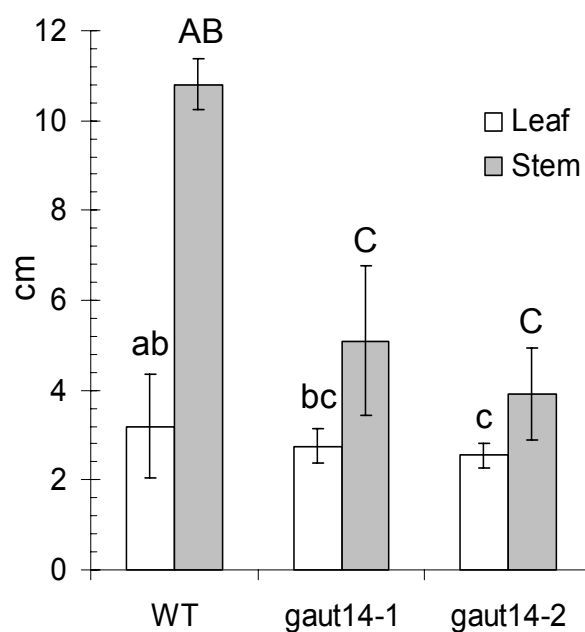


Figure C-1. The growth and seed germination of *gaut14* mutant plants compared to WT. The data are the average length of leaf (white bars) and stems (gray bars) $\pm$ standard deviation of the of WT and *gaut14* mutants. released fraction compared to WT. A slight decrease was observed in the Xyl content present in the EPG-released and 4 N KOH soluble fractions and there was also an increase observed in the Glc content of the 4 N KOH soluble fractions compared to WT.

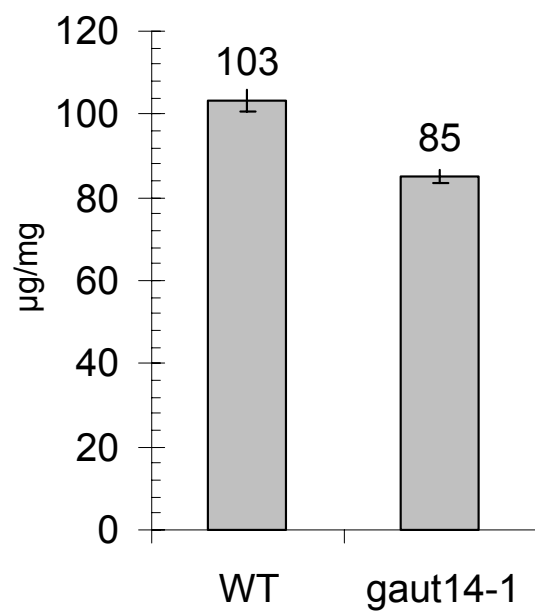


Figure C-2. The mass of AIR walls per fresh weight in WT and *gaut14-1* leaf tissue.

The data are the μg of walls mg^{-1} of fresh leaf tissue $\pm$ standard deviation.

Table C-1. The glycosyl residue composition of leaf AIR walls and wall fractions of WT and *gaut14* T-DNA mutant plants. The data show (^a) the glycosyl residue composition of the EPG-released (EPG), PME/EPG-released, sodium carbonate and EGTA soluble (sod. carb./EGTA soluble), 4 N KOH soluble (4 N KOH sol), 4 N KOH insoluble (4 N KOH insol) wall fractions and total starting AIR walls (Total cell walls). The glycosyl residue composition (^b) is expressed as the mol % of arabinose (Ara), rhamnose (Rha), fucose (fuc), xylose (Xyl), galacturonic acid (Gala), mannose (Man), galactose (Gal) and glucose (Glc). The weights of each fraction (^c) is expressed as the μg fraction mg^{-1} of total recovered material. The GalA to Rha ratio (^d) is shown.

Cell Wall Fraction ^a	Sample	Ara ^b	Rha	Fuc	Xyl	GalA	Man	Gal	Glc	ug/mg ^c	GalA/Rha ^d
EPG	WT	28.6	7.8	1.4	2.3	35.3	1.7	22.8	0.0	195.4	4.5
	<i>gaut14-1</i>	33.9	11.8	1.8	2.6	7.2	3.7	39.0	0.0	185.3	0.6
	<i>gaut14-2</i>	30.7	9.2	1.7	2.5	8.8	4.0	43.1	0.0	170.8	1.0
PME/EPG	WT	7.2	9.8	1.8	10.4	57.8	4.3	8.7	0.0	142.4	5.9
	<i>gaut14-1</i>	7.4	5.6	1.8	9.9	63.4	2.9	9.0	0.0	55.0	11.2
	<i>gaut14-2</i>	6.6	10.0	2.1	9.9	60.9	3.6	6.9	0.0	63.2	6.1
sod. carb./EGTA	WT	6.2	9.3	2.0	6.4	65.4	0.7	4.6	5.6	290.2	7.0
	<i>gaut14-1</i>	5.9	10.5	2.4	8.1	65.2	0.9	4.9	2.2	298.8	6.2
	<i>gaut14-2</i>	6.9	10.9	2.7	7.5	65.0	0.8	4.9	1.2	287.4	5.9
4 N KOH sol	WT	13.6	3.7	1.6	12.4	5.4	2.0	14.2	47.0	119.9	1.5
	<i>gaut14-1</i>	12.7	3.2	1.5	11.3	3.4	1.7	12.0	54.1	177.7	1.1
	<i>gaut14-2</i>	12.2	3.3	1.5	9.4	4.8	1.7	12.4	54.7	198.8	1.4
4 N KOH insol	WT	19.6	6.7	0.6	6.5	13.5	3.8	20.8	28.4	133.4	2.0
	<i>gaut14-1</i>	23.4	6.9	0.8	7.3	11.8	3.6	18.8	27.3	115.7	1.7
	<i>gaut14-2</i>	19.1	7.5	0.7	7.0	15.7	4.3	17.7	28.0	115.3	2.1
Total cell walls	WT	8.1	4.9	5.3	14.4	54.3	4.8	8.2	0.0		11.0
	<i>gaut14-1</i>	6.8	8.1	4.7	12.0	56.2	3.1	9.1	0.0		7.0
	<i>gaut14-2</i>	6.5	8.0	3.9	11.2	60.0	2.6	7.9	0.0		7.5

The fractionation and glycosyl residue compositions of *gaut14-1* and *gaut14-2* fractions showed that there was a slightly reduced amount of the EPG-released fraction that was also more highly enriched in RG-I and arabinose and depleted in HG than the WT EPG-released fraction. The reduced un-calcium complexed pectin of the EPG-released fraction in addition to the increased GalA/Rha ratio and Ara and Rha composition, suggest that there may be a decrease in the un-calcium complexed HG polysaccharides of *gaut14* mutants compared to WT.

The EPG-released fraction of WT and *gaut14-1* walls were subjected to linkage analysis because the *gaut14* fraction showed a slight decrease in mass of material released from *gaut14-1* and *gaut14-2* walls (Table C-2). The *gaut14* walls of this fraction also showed a dramatic decrease in GalA content and an increase in Rha and Ara that may potentially indicate an increase in RG-I polysaccharides. The linkage analysis showed an increase in T-Araf, 5-Araf and 2,3,5-Araf and an increase in 2-linked Rhap of *gaut14* walls compared to WT. There was also an increase in 4-linked Xylp and total Xyl. The 4-linked Galp was elevated but the total Galp was reduced. In addition, the 3,4-linked GalpA was reduced and the total GalpA was reduced.

The water soluble walls of gaut14 mutants is reduced and depleted of GalA residues

The water-extracted of WT and *gaut14-1* walls was obtained and subjected to glycosyl residue composition (Figure C-3). The water extract of *gaut14-1* was similar to that of WT, but with significantly less GalA composition.

Table C-2. The glycosyl residue linkage analysis of the PME/EPG fraction extracted from leaf AIR walls of WT and *gaut14-1*. The data are the % peak area of residues detected for the PME/EPG fraction of leaf AIR walls of WT and the *gaut14-1* mutant.

Residue	WT	<i>gaut14-1</i>
T-Araf	10.2	13.7
5-Araf	5.8	6.9
2,3,5-Araf	4.7	8.3
T-Rhap	1.7	1.2
2-Rhap	5.4	7.7
T-Fucp	2.9	1.5
4-Xylp	3.6	6.8
3,4-Xylp	3.4	2.6
2,3,4-Xylp	3.8	4.2
4-Manp	3.4	5.3
4-Galp	0.9	4.3
4,6-Galp	4.5	6.3
2,3,4,6-Galp	28.7	13.3
3,4-Galp A	11.8	6.3
2,4-Galp A	9.1	11.5
Total Ara	20.7	29.0
Total Rha	7.1	8.9
Total Xyl	10.8	13.6
Total Gal	34.2	23.9
Total GalA	20.9	17.8

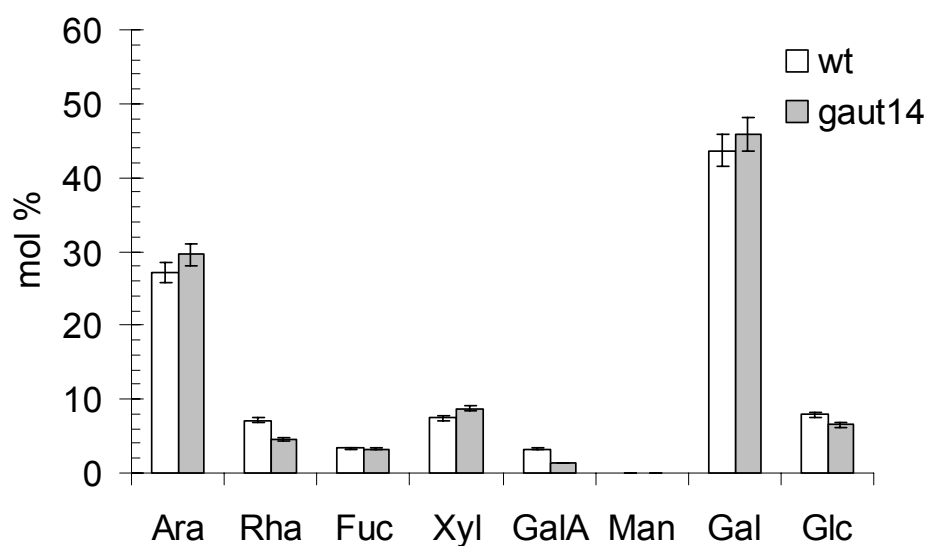


Figure C-3. The water soluble polysaccharide extracted from leaf AIR walls of WT and *gaut14-1*. The data are the mol % glycosyl residue composition $\pm$ error of the water extract of leaf AIR walls of WT and *gaut14-1* walls.

The walls of gaut14 mutants have less PME/EPG-released pectin and more hemicellulose

The walls of *gaut14* mutants have reduced un-calcium crosslinked, buffer soluble pectin (EPG +PME/EPG). The EPG released and the PME/EPG released fraction GalA composition of *gaut14* mutants was somewhat increased compared to WT. This result signifies that *gaut14-1* and *gaut14-2* are reduced in the un-calcium complexed pectin that generally is lower in level of methylesterification, and that the quantity of HG in this fraction is further reduced to <25% of WT GalA. There is no observed change in the mass% of calcium crosslinked pectin (NC/EGTA) or in the composition of this fraction compared to WT. The PME/EPG fraction released from *gaut14* walls was also increased in RG-I arabinan and 2-linked Rha suggesting an increase in arabinosylated RG-I.

The walls of *gaut14-1* and *gaut14-2* were reduced in the mass% of wall residue released by PME/EPG treatment, which constitutes the un-calcium complexed portion of pectin enriched walls. The EPG-released wall fraction of *irx8-1* and *irx8-5* putative xylan biosynthetic mutants was increased and the composition of this fraction was similar to that of *gaut14-1* and *gaut14-2*, except that the Xyl composition was reduced in the *irx8* but not *gaut14* fraction. *Gaut14-1* and *gaut14-2* do not show a reduction in xylan in the EPG fraction; however Xyl composition is reduced in the PME/EPG, 4 M KOH soluble fractions and in total walls. It is plausible that the lesion in GAUT14 may affect xylan synthesis, based on the composition result. *Irx8*, however, has a distinct reduction in the amount of material released by concentrated alkaline treatment reflecting a dramatic reduction in xylan content of these walls. *Gaut14* plants did not show a reduction in the hemicellulose material released by concentrated alkaline treatment and therefore does not appear to have a major affect on xylans in the wall.

Qua1-1, on the other hand, showed detectable decrease in xylan synthase activity (Orfila et al., 2005), however *qua1-1* suspension cultures produced a larger mass% of hemicellulose-enriched walls that were released by concentrated alkali (Leboeuf et al., 2005). If the affect of *gaut14-1* and *gaut14-2* originates from a similar biological issue as that of *qua1-1*, then we would argue for the reduced Xyl as a compensatory change in wall composition due to the loss of GAUT14. Without further work however, it is impossible to discount the possibility that GAUT14 may share a role in xylan synthesis similar to that of GAUT12 that is thought to synthesize a GalA containing oligosaccharide essential for xylan synthesis. The overwhelming evidence, however, points to GAUT14 having a role in HG biosynthesis. In addition *gaut14* has reduced HG primarily in the uncalcium complexed fraction and not the calcium complexed fraction such as that observed in the walls of *qua1*, indicating that these two genes potentially operate in a functionally distinct manner.

The changes observed in *gaut14* walls and seed polysaccharides suggest that GAUT14 may target (1) HG or (2) hemicellulose.