Running head: Galacturonosyltransferase-like gene family in Arabidopsis

Corresponding Author:
Dr. Michael G. Hahn
Complex Carbohydrate Research Center
University of Georgia
Athens, GA 30602
Telephone number: 706-542-4457
Fax Number: 706-542-4412
Email address: hahn@ccrc.uga.edu

Research Category: Biochemical Processes and Macromolecular Structures
Molecular Analysis of a Family of *Arabidopsis* Genes Related to Galacturonosyltransferases

Yingzhen Kong\textsuperscript{1a}, Gongke Zhou\textsuperscript{1ab}, Yanbin Yin\textsuperscript{2}, Ying Xu\textsuperscript{2}, Sivakumar Pattathil\textsuperscript{1}, Michael G. Hahn\textsuperscript{1,3*}

\textsuperscript{1}Complex Carbohydrate Research Center, University of Georgia, 315 Riverbend Road, Athens, GA 30602–4712, USA

\textsuperscript{2}Computational System Biology Lab and Institute of Bioinformatics, University of Georgia, Athens, GA 30602, USA

\textsuperscript{3}Department of Plant Biology, University of Georgia, Athens, GA 30602, USA
Financial Source: This work was supported by a grant from the National Science Foundation (MCB-0646109).

These authors contributed equally to this work.

Current Address:
Qingdao Institute of Bioenergy and Bioprocess Technology
No.189 Songling Road
Laoshan District
Qingdao 266101
People’s Republic of China
Summary

We are studying a GA\textit{lacturonosylTransferase-Like (GATL)} gene family in \textit{Arabidopsis thaliana} that was identified bioinformatically as being closely related to a group of 15 genes (\textit{GAUT1-15}), one of which (\textit{GAUT1}) has been shown to encode a functional galacturonosyltransferase. Here we describe the phylogeny, gene structure, evolutionary history, genomic organization, protein topology, and expression pattern of this gene family in \textit{Arabidopsis}. Expression studies (RT-PCR) demonstrate that all ten \textit{AtGATL} genes are transcribed, albeit to varying degrees, in \textit{Arabidopsis} tissues. Promoter::β-glucuronidase expression studies show that individual \textit{AtGATL} gene family members have both overlapping and unique expression patterns. Nine of the ten \textit{AtGATL} genes are expressed in all major plant organs, although not always in all cell types of those organs. \textit{AtGATL4} expression appears to be confined to pollen grains. Most of the \textit{AtGATL} genes are expressed strongly in vascular tissue in both the stem and hypocotyl. Sub-cellular localization studies of several GATL proteins using yellow fluorescent protein (YFP) tagging provide evidence supporting the Golgi localization of these proteins. Plants carrying T-DNA insertions in three \textit{AtGATL} genes (\textit{atgatl3}, \textit{atgatl6}, and \textit{atgatl9}) have reduced amounts of GalA in their stem cell walls. The xylose content increased in \textit{atgatl3} and \textit{atgatl6} stem walls. Glycome profiling of cell wall fractions from these mutants using a toolkit of diverse plant glycan-directed monoclonal antibodies showed that the mutations affect both pectins and hemicelluloses, and alter overall wall structure as indicated by altered epitope extractability patterns. The data presented suggest that the \textit{AtGATL} genes encode proteins involved in cell wall biosynthesis, but their precise roles in wall biosynthesis remain to be substantiated.

Introduction

Plant cell walls are composed mostly of networks of polysaccharides, primarily cellulose, pectins and hemicelluloses. The synthesis of these polysaccharides requires a significant commitment of the plant’s genomic resources; perhaps as many as 10% of genes in \textit{Arabidopsis} have been estimated to be involved in plant cell wall synthesis, maintenance, modification and degradation (Carpita et al., 2001). Many of these genes belong to multi-gene families (Henrissat et al., 2001) whose individual members have distinct patterns of expression among plant cells and tissues (Taylor et al., 1999; Fagard et al., 2000; Peng et al., 2000; Taylor et al., 2000; Sarria et al., 2001; Williamson et al., 2001; Orfila et al., 2005; Harholt et al., 2006; Burton et al., 2006; Persson et al., 2007; Peña et al., 2007). At least 50 glycosyltransferases (GTs) are predicted to be required for pectin synthesis (Ridley et al., 2001). Recently, several putative glycosyltransferases that may be involved in the biosynthesis of different pectins have been identified using mutational and/or biochemical approaches. These include three members from CAZy (Cantarel et al., 2009) family GT47, two members from family GT77 and two members
from family GT8. These include: *ARAD1* (GT47), that is believed to encode an α-L-arabinosyltransferase involved in the synthesis of arabinan side chains of RG-I (Harholt et al., 2006); *XGD1* (GT47) encodes a β-(1,3)-xylosyltransferase possibly involved in XGA synthesis (Jensen et al., 2008); *NpGUT1* (GT47), involved in RG-II side chain synthesis in tobacco as a putative glucuronosyltransferase (Iwai et al., 2002), although mutants in orthologous genes in *Arabidopsis* evidence xylan defects (Brown et al., 2009; Wu et al., 2009; Séveno et al., 2009); *RGXT1* and *RGXT2* genes (GT77), that may participate in the synthesis of side chain A of RG-II as (1,3)-α-D-xylosyltransferases (Egelund et al., 2006); *QUA1* (GT8), the mutation of which results in a dwarf phenotype, reduced cell adhesion, a 25% reduction in the amounts of GalA in the leaves, and slightly lower levels of Xyl and xylosyltransferase activity (Bouton et al., 2002; Orfila et al., 2005); and *GAUT1* (GT8) gene that is involved in HG synthesis as a (1,4)-α-D-galacturonosyltransferase (Sterling et al., 2006). Other studies have linked at least two other members of the GAUT1-related gene family (*IRX8/GAUT12* and *PARVUS/AtGATL1*) to the synthesis of a specific sub-fraction of pectin (Persson et al., 2007) and/or to the synthesis of the GalA-containing tetrasaccharide sequence located at the reducing end of dicot and gymnosperm xylans (Brown et al., 2007; Peña et al., 2007; Persson et al., 2007; Brown et al., 2009).

Among these previously identified genes, however, *GAUT1* is the only galacturonosyltransferase that has been functionally determined to be involved in pectin synthesis (Mohnen, 2008). Bioinformatic analysis of the *Arabidopsis* genome identified 24 other genes with high sequence similarity to *GAUT1* (Sterling et al., 2006). Sequence alignment and phylogenetic analysis of the *Arabidopsis* GAUT1-related gene family separates them into the GAUT (GAlactUronosyl Transferase) and GATL (GAlacturonosylTransferase-Like) families. There are 15 GAUT genes in *Arabidopsis* that encode proteins predicted to be 61-78 kDa, whereas the 10 *Arabidopsis* GATL genes encode proteins that have molecular masses between 39 and 44 kDa (Sterling et al., 2006).

The absence of functional characterization of most of the members of the GAUT1-related gene family leaves open the question of the roles of these genes and their encoded proteins in cell wall synthesis. A recent analysis of cell walls isolated from homozygous mutants of twelve members of the GAUT family demonstrated that mutations in eight of these genes resulted in discernable changes in cell wall monosaccharide compositions. Mutations in AtGAUT6 result in a reduction in GalA that coincides with higher levels of Xyl and Rha in the wall and preliminary
results suggested a role for AtGAUT6 in HG synthesis. Mutations in AtGAUTs 9, 10, 11 and 12 resulted in significant reductions in GalA content without decreases in Xyl content. Mutations in AtGAUT13 and AtGAUT14 resulted in increased GalA and Gal content coinciding with reduced Xyl and Rha content compared to WT. The results of this study reinforces the hypothesis that the proteins encoded by the AtGAUT genes are involved in pectin and/or xylan synthesis, although further work is necessary to validate this hypothesis (Caffall et al., 2009). We report here on a study of the GATL family in Arabidopsis to lay the foundation for functional characterization of these putative glycosyltransferases and identification of their role(s) in plant cell wall biosynthesis.

Results

Gene Structure and Phylogenetic Relationship of GATL family

A previous study had identified ten GATL genes within the Arabidopsis GAUT1-related gene family that have high sequence similarity (Sterling et al., 2006). Pair-wise comparisons of the amino acid sequences of the whole coding regions of these proteins showed between 56% and 84% identity and between 71% and 89% similarity (Table 1). The predicted molecular masses of GATL proteins range between 39 and 44 kDa. The initial analysis of the GATL phylogeny in Arabidopsis did not yield a statistically robust sub-clade structure for this GT8 sub-family (Sterling et al., 2006). Subsequent inclusion of additional GATL protein sequences from eight additional fully sequenced plant genomes resolved the GATL sub-family into six sub-clades with robust statistical support (Yin et al., 2010). This sub-structure of the GATL tree remains largely unchanged by the addition of sequences from three additional sequenced plant genomes (maize, Medicago truncatula and papaya). In the current analysis; an additional clade (GATL-c) that contains only monocot genes was resolved with high statistical support (Figure 1A). The phylogram shows a basal clade (GATL-g) of GATLs from Physcomitrella patens and Selaginella mollendorfii, and six clades of angiosperm GATLs. The functional significance of the GATL sub-clades remains to be determined. All but one of the angiosperm GATL sub-clades have both monocot and dicot representatives, suggesting that the divergence of the GATL family occurred before the evolutionary split between monocots and dicots.

As several of the AtGATL proteins appeared as pairs of paralogs in the phylogenetic tree (Figure 1A), we investigated whether traceable genome duplication events contributed to the
complexity of the GATL family in Arabidopsis. The chromosomal locations of AtGATL genes in relation to the segmental duplication history of these regions were analyzed (Blanc and Wolfe, 2004). The resulting chromosome map (Figure 1B) shows that AtGATL genes are only present on chromosomes I, III and IV. According to the map, AtGATL8/AtGATL9 and AtGATL5/AtGATL6 constitute pairs of paralogous genes evolved from recent segmental duplication events. The AtGATL1/AtGATL2, AtGATL9/AtGATL10, and AtGATL5/AtGATL7 pairs evolved, respectively, from older segmental duplication events with good statistical significance. The duplication history of AtGATL genes supports the observed close phylogenetic associations between some members of the AtGATL family. For AtGATL3 and AtGATL4, no traceable duplication history could be found, even though their genome positions are close to duplicated blocks.

The AtGATL genes are similar not only in terms of their primary sequence and the size of their encoded proteins, but also in their intron/exon organization (Figure 1C). Of the 10 AtGATL genes, only AtGATL5 and AtGATL6, which appear as paralogs, contain one intron at the same position in their 3'UTR. No introns are present in any of the other AtGATLs. The common intron/exon organization shared by these AtGATL genes supports the results from the phylogenetic analysis and the duplication history.

Expression profiles of the AtGATL gene family by RT-PCR

To understand the roles of the AtGATL genes in plant development, we conducted semi-quantitative RT-PCR analysis to determine the tissue-specific expression patterns for all AtGATL genes in different tissues taken from 7-week-old plants (Figure 2). Analyzing different parts of the inflorescence stem makes it possible to monitor primary cell wall and secondary cell wall synthesis in a single stem simply by sectioning at different distances from the apical meristem because secondary cell wall formation increases from the top to the base of the stem (Turner and Somerville, 1997). An ACTIN gene served as a positive control because it is expressed ubiquitously in all organs.

All 10 AtGATL genes are expressed, but at different levels and with different tissue distribution patterns (Figure 2). The majority of the AtGATL genes are expressed at various levels in all of the different organs analyzed. However, AtGATL4 is expressed only in flowers. AtGATL2 expression is primarily limited to leaves, upper and lower stems, and, at a low level, in
roots. \textit{AtGATL6} is highly expressed in upper and middle stems, but not in lower stems, suggesting that this gene may be involved in primary cell wall synthesis or the initiation of secondary cell wall synthesis.

**Expression profiles of the \textit{AtGATL} proteins**

To extend the observations made with the RT-PCR analyses, we generated \textit{promoter::GUS} reporter lines in a wild-type Columbia background for each of the \textit{AtGATL} genes by fusing the β-glucuronidase (\textit{GUS}) gene (Jefferson et al., 1986; Jefferson, 1987) to upstream (promoter and 5’-untranslated regions) and downstream (3’-untranslated regions) sequences of each of the \textit{AtGATL} genes (\textit{AtGATL::GUS}). Histochemical staining analyses of GUS activity in the transgenic plants revealed expression patterns that were consistent with the outcome of the RT-PCR analyses done at the whole tissue level, but with higher resolution (Figures 3 and 4). For example, expression of the GUS reporter driven by the \textit{AtGATL4} promoter was restricted to flowers, in agreement with the RT-PCR analysis, but was confined to pollen grains and elongating pollen tubes. The tissue/organ-specific \textit{GUS} expression profiles of all \textit{AtGATLs} are summarized below.

10 day old \textit{Seedlings}: Expression of the \textit{GUS} reporter gene constructs in 10-day-old seedlings was detected in the cotyledons, primary leaves, shoot apex, and roots, depending on the \textit{AtGATL} gene examined, though frequently only in specific cell types within those tissues. As shown in Figure 3, \textit{AtGATL1}, 3, 7, 8 and 10 are expressed in cotyledons and primary leaves, whereas \textit{AtGATL5} and 9 are only detected in primary leaves. A close examination of primary leaves shows that \textit{AtGATL1} and 5 are only expressed in the vasculature of primary leaves. Most \textit{AtGATLs}, except for \textit{AtGATL3}, 4, 5 and 9, are also expressed in the trichomes, which form complex secondary walls (Marks et al., 2008). Interestingly, \textit{AtGATL1}, 2, 3, 5, 6 and 8 are also expressed in the shoot meristem, where only primary wall synthesis would be expected. All \textit{AtGATL} reporters, with the exception of \textit{AtGATL4}, are expressed in roots, but with distinct expression patterns for each gene. \textit{AtGATL2} is expressed in root tips, developing lateral root meristems, and at the base of extended lateral roots. \textit{AtGATL1} and \textit{AtGATL3} are expressed in root tips and portions of older roots. The restricted expression pattern of these two GATLs might explain why no expression of these genes could be detected in RT-PCR analyses of whole roots. Both \textit{AtGATL6} and \textit{AtGATL9} are only expressed in root tips. In addition, \textit{AtGATL5}, 7, 8
and 10 are strongly expressed throughout the root, but only AtGATL8 and AtGATL10 are expressed in root hairs.

**Developing Flowers:** Quite diverse patterns of expression were observed in developing flowers among the various AtGATL gene family members (Figure 3). All AtGATL family members, except for AtGATL2, are expressed in a variety of floral organs, which is consistent with the RT-PCR analysis. AtGATL5 expression in flowers can be only detected in filaments, where AtGATL1 and AtGATL10 expression can also be detected. AtGATL1, 4 and 7 are expressed in the anthers. Expression of AtGATL4 was also observed in elongating pollen tubes (Figure 3; inset). Carpel tissue expresses AtGATL3, 4, 6 and 10 in the stigma and AtGATL3 in the style. AtGATL3, 8, 9 and 10 are expressed in the sepals of the flower, whereas expression of the AtGATL3, 9 and 10 genes is detected in petals.

**Siliques:** All ten AtGATL genes except AtGATL2, 4 and 5 are expressed in senescing siliques (Figure 3). Many AtGATL members, such as AtGATL1, 3, 7, 8 and 10, are expressed in the abscission zone, and AtGATL7, 8, 9 and 10 are expressed in stigmatic tissue. AtGATL7 shows a unique expression pattern, being expressed in the pedicel of the silique. The overall expression patterns in young seedlings and flowers are summarized in Supplemental Table S2.

**Stem and Hypocotyl:** The RT-PCR data documented that all AtGATL gene family members, except for AtGATL4, are highly expressed in inflorescence stems (Figure 2). To determine in which cells within the stems these genes are expressed, hypocotyls and upper, middle and lower parts of inflorescence stems were sectioned, stained and analyzed. Overall, AtGATLs show overlapping, but not identical, expression patterns in stem and hypocotyl. Figure 4 shows that expression of many of the AtGATL genes is restricted primarily to the vascular tissues, though the patterns of expression vary depending on the AtGATL gene. The simplest expression pattern was observed for AtGATL5 and AtGATL10, whose expression could be detected only in xylem and phloem, respectively, in different parts of stems and hypocotyls. AtGATL6 is expressed in the phloem of the upper stem and in the phloem and developing secondary xylem of the hypocotyl (Figure 4). In contrast, AtGATL8 expression varies a lot in different parts of stems and is expressed in almost all vascular tissues except for fibers and secondary xylem. It is noteworthy that AtGATL1 was specifically expressed in cells undergoing secondary wall thickening, including interfascicular fibers and metaxylem in stems and secondary xylem in hypocotyls. The AtGATL1 expression pattern observed here is consistent with the finding that
this gene is specifically associated with secondary wall thickening in fibers and vessels (Brown et al., 2007; Lee et al., 2007). Interestingly, \textit{AtGATL2} shows an overlapping expression pattern with \textit{AtGATL1} in all parts of the stem, except that there is no expression in fibers. In hypocotyls, \textit{AtGATL2} can only be detected in the developing secondary xylem. \textit{AtGATL3} is specifically expressed in the epidermis, cortex and phloem of stems and hypocotyls. \textit{AtGATL7} is detected in the phloem of lower stems, the cortex of hypocotyls, upper and middle stems, and the epidermis of upper and middle stems. Finally, \textit{AtGATL9} is highly expressed in the protoxylem of all parts of the stem, and the cortex of upper stems and hypocotyls. The overall expression patterns in young seedlings are summarized in Supplemental Table S3.

**Subcellular Localization of the AtGATL proteins**

The topology of AtGATL proteins were analyzed using the plant membrane protein database, Aramemnon (http://aramemnon.botanik.uni-koeln.de/index.ep). The overall results of these analyses are shown in Supplemental Table S4. All AtGATLs were predicted to be soluble proteins by this analysis. However, the Aramemnon database strongly predicts that all GATL proteins are targeted to secretory pathway, with at least half of the 18 programs used in Aramemnon predicting an N-terminal hydrophobic signal peptide domain for all of the GATL proteins.

To establish the sub-cellular localization of the AtGATL proteins experimentally, we chose four AtGATL isoforms, AtGATL2, AtGATL3, AtGATL7 and AtGATL9, belonging to four different GATL sub-clades (GATL-d, GATL-e, GATL-b, GATL-a; Figure 1A) and generated fluorescently-tagged fusion proteins by fusing EYFP to the C-terminus of the full-length AtGATL proteins. The recombinant constructs were transiently co-expressed in tobacco leaf epidermal cells with Gmct–ECFP [Golgi marker; (Saint-Jore-Dupas et al., 2006; Nelson et al., 2007)] or ECFP-WAK2-HDEL [ER marker; (Nelson et al., 2007)] constructs, respectively. Confocal microscopy was used to determine the sub-cellular localization of the recombinant AtGATLs. EYFP-tagged AtGATL3 showed a punctate localization pattern when expressed in tobacco leaf epidermal cells (Figure 5C). Co-localization experiments revealed that the localization pattern of AtGATL3-EYFP is identical to that of Gmct–ECFP (Figure 5B and D), which was previously shown to be localized in the Golgi (Saint-Jore-Dupas et al., 2006; Nelson et al., 2007). Together, these results demonstrate that AtGATL3 is a Golgi-localized protein.
Similar sub-cellular localizations were also observed for AtGATL2, AtGATL7 and AtGATL9 proteins (data not shown). The Golgi localization of the AtGATL proteins is consistent with their possible role(s) in the biosynthesis of non-cellulosic polysaccharides, which occurs in the Golgi (Nebenführ and Staehelin, 2001).

Identification of T-DNA-Tagged Mutants

To obtain homozygous mutant plants with disruptions in the AtGATL genes, we screened T-DNA insertion lines available from the SALK Institute (http://signal.salk.edu/cgi-bin/tdnaexpress) through the Arabidopsis Biological Resource Center (see Methods for details). Homozygous lines with an insertion in the exon or within the 5’ or 3’ non-coding regions were obtained for AtGATL3, 5, 6, 8 and 9 (see Figure 6A). RT-PCR of total RNAs isolated from homozygous atgatl mutant lines allowed us to identify four knockout mutants (atgatl5, atgatl6, atgatl8 and atgatl9) and 1 knockdown mutant (atgatl3) (Figure 6B). No discernible phenotypic changes were observed in the growth or morphology of any of the mutant plants compared with the wild type under normal growth conditions.

Cell Wall Composition of Five atgatl Mutants

The amino acid sequences of the AtGATLs contain domains characteristic of glycosyltransferases (Sterling et al., 2006; Yin et al., 2010), suggesting that these proteins may be involved in cell wall polysaccharide biosynthesis. To determine whether disruptions of the five AtGATL genes caused alterations in the monosaccharide composition of total cell wall material, the relative amounts of neutral monosaccharides and GalA were determined for wide-type and five atgatl mutant plants. Stems from 10-week-old plants were chosen for this analysis because this tissue can be harvested in large quantities and all four of the disrupted AtGATL genes are highly expressed in stems, based both on RT-PCR (Figure 2) and promoter::GUS results (Figure 4). Compared with wide-type plants, atgatl3, atgatl6 and atgatl9 mutants showed 23.7%, 16.4%, and 20.5% reduction in GalA content, respectively, which was counterbalanced by increases in Xyl in atgatl3 and atgatl6 (Table 2). The decrease in GalA content of atgatl3, atgatl6, and atgatl9 support the argument that like GAUT1 and QUA1, two other members of the GT8 family, the three AtGATL genes function as putative GalATs involved in pectin synthesis. For atgatl5 and atgatl8, no significant changes in sugar content were observed relative to wild-type plants.
To obtain a more complete picture of possible changes in cell wall composition and structure resulting from mutations in the five GATL genes, we analyzed stem cell walls from the mutants using glycome profiling. This method (Pattathil et al., in preparation) involves ELISA-based screening of sequential extracts prepared from the plant cell walls using a toolkit of ~150 plant cell wall glycan-directed monoclonal antibodies that recognize diverse epitopes present on most major classes of plant cell wall polysaccharides, including xyloglucans, xylans, pectins, and arabinogalactans (Pattathil et al., 2010). Glycome profiling gives information about changes both in the nature of the epitopes present in the cell walls and in the extractability of those epitopes from the walls (Zhu et al., 2010); the latter providing some information about larger-scale changes in wall structure. Glycome profiles of the five atgatl mutant walls show subtle differences, primarily in epitope extractability patterns, when compared to the glycome profile of w.t. cell walls (Figure 7). For example, the oxalate extracts of atgatl5 and atgatl6 stems contain easily detectable levels of xyloglucan epitopes, whereas the oxalate extract of w.t. walls show no detectable xyloglucan. There are also subtle differences in the levels of arabinogalactan epitopes in the 1 M KOH extracts of atgatl3, atgatl5 and atgatl9 stem walls compared with the equivalent extract of w.t. stem walls. Lastly, subtle differences in the extractability patterns of xylan epitopes are observable in the glycome profiles of all five atgatl mutant stem walls compared with the profile of w.t. stem walls.

Discussion

The GAUT1-related gene family in Arabidopsis consists of 25 genes, all of which encode proteins belonging to CAZy glycosyltransferase family 8 (GT8). Phylogenetically, this gene family splits into two clades of related genes, the GAlactUronosyl Transferase (GAUT) clade of 15 genes, one of which, AtGAUT1, has been shown to encode a functional homogalacturonan galacturonosyltransferase (Sterling et al., 2006), and the GAlacturonosyl Transferase-Like (GATL) clade of 10 genes. The proteins encoded by both clades contain several conserved amino acid sequences that are unique to these clades within the larger GT8 family (Yin et al., 2010). However, AtGATLs and AtGAUTs also differ from one another in a couple of ways. These differing characteristics raise questions about the possible role(s) of AtGATL proteins in cell wall glycan synthesis.

AtGATL proteins are smaller and lack an obvious transmembrane domain such as that which is present in the AtGAUTs. Nonetheless, AtGATL proteins do appear to contain a
hydrophobic signal peptide at their N-termini, which would be expected to target AtGATL proteins to the endomembrane system. Previous studies had shown that AtGATL1 is retained within the endomembrane system, specifically in the ER and Golgi compartments (Kong et al., 2009; Lee et al., 2009). We demonstrate here that four other AtGATL proteins, belonging to four different sub-clades within the GATL sub-family co-localize with a Golgi marker (Figure 5). Thus, the localization of these AtGATL proteins places them in a position within the cell to participate in plant cell wall matrix polysaccharide synthesis, which is thought to take place in the Golgi (Scheible and Pauly, 2004; Sandhu et al., 2009). However, AtGATL proteins contain no trans-membrane domain, nor do they contain any other known signal sequences that would retain them within the Golgi. Thus, these AtGATL proteins must be retained within the Golgi by another mechanism. There is precedence for transmembrane domain-independent Golgi localization. For example, reversibly glycosylated polypeptide (RGP) showed cytoplasmic and transient association with Golgi, yet it does not possess any signal sequence (Sagi et al., 2005; Drakakaki et al., 2006). We hypothesize that the AtGATL proteins are synthesized in the cytosol and retained in Golgi by virtue of their interaction with proteins that are membrane-embedded or anchored, and that carry Golgi retention signals. This hypothesis awaits further experimental investigation, particularly with respect to possible protein-protein interactions involving AtGAUT proteins, which have transmembrane domains and are localized to the Golgi (Sterling et al., 2001).

The AtGATL proteins show a significantly higher sequence identity/similarity (Table I) than do the AtGAUTs (Sterling et al., 2006; Caffall et al., 2009). For example, AtGATL5 and AtGATL6 share 77% identity to each other, and AtGATL8 and AtGATL9 are 84% identical in their coding regions. Such high sequence identity might be indicative of functional redundancy among these sets of AtGATL family members. The phylogenetic analysis of the GATL protein family also suggests such functional redundancy, particularly among the AtGATL5/AtGATL6 and AtGATL8/AtGATL9 pairs, which appear to have arisen by relatively recent partial genome duplication events in Arabidopsis.

However, the gene expression data presented here, as examined using transgenic pGATL::GUS expression, argues against functional redundancy of these proteins. The constructs used for these studies included the 3’ untranslated regions of the GATL genes to better assure that any GUS expression observed accurately reflects AtGATL gene expression, in light of previous
reports that DNA elements in the 3’ regions of plant genes can regulate gene expression (Dean et al., 1989; Dietrich et al., 1992; Larkin et al., 1993; Fu et al., 1995a; Fu et al., 1995b; Chen et al., 1998). As indicated by GUS expression, each of the AtGATL genes shows a unique expression pattern, though there is some overlap, particularly in the vasculature. For example, AtGATL5 is expressed in secondary xylem of stems and in filaments of flowers, while AtGATL6 is expressed in phloem of upper stem and in stigma and style of the flower. Different expression patterns were also observed for the AtGATL8 and AtGATL9 genes. Furthermore, recent research on two poplar isoforms of AtGATL1 also suggests functional specialization of GATL proteins (Kong et al., 2009). The differing tissue-specific expression patterns between such duplicated genes suggests sub-functionalization or neo-functionalization has happened, a process that occurs mainly through mutations in regulatory sequences instead of via mutations in the coding sequence (Blanc and Wolfe, 2004; Haberer et al., 2004; Langham et al., 2004).

The GATL proteins also share high sequence identity/similarity among species. For example, PdGATL1.1 and PdGATL1.2 share about 80% identity at the amino acid level with AtGATL1. Transformation of either poplar GATL1 ortholog into the Arabidopsis atgatl1 mutant background complemented several phenotypes of the atgatl1 mutant, including decreased xylose content, tissue morphology and growth habit (Kong et al., 2009). Thus, the functions of GATL genes may be largely conserved between different species.

In order to gain insight into the biochemical function of AtGATL family members, we analyzed the cell walls isolated from stems of T-DNA insertion mutants of AtGATL3, 5, 6, 8 and 9 using both glycosyl composition analysis and glycome profiling. Both analyses revealed only subtle changes in wall composition and structure, and thus did not yield definitive information about the functions of these five AtGATL in cell wall biogenesis. For example, the glycosyl composition analyses showed significant decreases in the GalA content of stem walls in atgatl3, atgatl6 and atgatl9, but also showed changes in other glycosyl residues (Table II). Glycome profiling of the stem cell walls did not reveal the absence of any particular polysaccharide component recognized by the diverse suite of plant glycan-directed monoclonal antibodies used in any of the five atgatl mutants analyzed (Figure 7) and therefore also did not provide direct evidence of AtGATL function in these plants. Nonetheless, glycome profiling did demonstrate changes in overall wall structure in each of the five mutants, as evidenced by altered patterns of epitope extractability. For example, some xyloglucan epitopes were more easily extracted from
the walls of *atgatl5* and *atgatl6* stems compared with w.t. walls. Changes in the extractability patterns of pectic arabinogalactan epitopes were also observed. Our results suggest that cell wall analyses carried out at whole plant or whole tissue level resolution are unlikely to document dramatic changes in cell wall composition and structure in mutants when expression of the affected gene is highly localized to specific cell types in w.t. plants, as is the case for most of the *GATL* genes in *Arabidopsis* (Figures 3 and 4). Thus, it will likely be more informative to examine specific cell types that express a *GATL* gene of interest for changes in wall structure in order to infer the function of that GATL protein in cell wall synthesis. For example, we have found that *atgatl5* shows defects in seed coat mucilage production, suggesting that AtGATL5 plays a role in synthesizing the pectic polysaccharides that are the principal components of this mucilage (Kong et al., in preparation). This phenotype would not be observed in analyses carried out at the whole plant or whole tissue level.

Several pieces of data, in addition to the decreased GalA content mentioned above, implicate AtGATL3, AtGATL6 and AtGATL9 in pectic polysaccharide synthesis, which occurs in primary wall synthesis. The *AtGATL3 promoter::GUS* expression results, which show that *AtGATL3* expression in stem is mainly localized in primary cell wall-rich cells, such as epidermis and cortex supports the hypothesis that *AtGATL3* is a primary cell wall-associated gene. The RT-PCR data showed that *AtGATL6* expression is lower at the base of the stem compared with the top, indicating that *AtGATL6* is preferentially expressed in younger stems, where primary cell wall synthesis predominates. Given the fact that *AtGATL6 promoter::GUS* is expressed only in the phloem of upper stems, we suggest that *AtGATL6* may be involved in primary cell wall synthesis in developing phloem. Transcriptional profiling of genes differentially expressed during *in vitro* xylem differentiation in *Arabidopsis* suspension cells showed that *AtGATL6* and *AtGATL9* expression levels decreased rapidly before xylem vessel element formation (Kubo et al., 2005), further supporting a role for these two genes in primary cell wall synthesis. However, the hypothesis that *AtGATL3*, *6*, and *9* are involved in pectin synthesis will require further experimental substantiation.

It is noteworthy that all *AtGATL* genes except *AtGATL4* are expressed in vascular tissue, with several family members showing overlapping expression patterns in different vascular cell types. This is true for the phloem (*AtGATL3, 6, 7, 8* and *10*), protoxylem (*AtGATL8* and *9*), metaxylem (*AtGATL1, 2*, and *5*), secondary xylem (*AtGATL1, 2, 5, 6* and *8*) and cortex
(AtGATL3, 7, 8, and 9). Such overlapping expression patterns for many family members during vascular development suggest a potential for combinational AtGATL action in vascular cell wall synthesis, or alternatively that some functional redundancy may occur among GATL proteins, at least in some tissues. The latter could explain the absence of dramatic phenotypes in the five mutant lines that were examined in this study.

The involvement of AtGATL1 in xylan synthesis during secondary wall formation has been reported previously (Brown et al., 2007; Lee et al., 2007) and a close correlation between AtGATL1 expression and expression of AtCESA (cellulose synthase) genes associated with secondary cell wall formation has also been reported (Mutwil et al., 2009). Although AtGATL1 has been linked to xylan synthesis, it is not clear whether this GATL is directly involved in xylan biosynthesis, possibly in connection with the synthesis of the GalA-containing reducing end oligosaccharide (Peña et al., 2007), or indirectly through the synthesis of another polysaccharide that establishes a foundation for xylan synthesis (Mohnen, 2008). In this context, it is interesting to note the very strong AtGATL1 expression observed in the root apical meristem and elongation zones (Figure 3), tissues where xylan synthesis is not known to occur in Arabidopsis.

Furthermore, four AtGATLs, including AtGATL3, 6, 7 and 8, have been shown to be co-expressed with AtCESA genes that are involved in primary cell wall synthesis (Mutwil et al., 2009). These data suggest that the majority of AtGATL genes expressed in vascular tissues are involved in primary cell wall synthesis in these tissues. It is also notable that five of the AtGATL members are expressed in the abscission zone of silique, and six are expressed in trichomes. Both trichomes and abscission zones are rich in pectins (Henderson et al., 2001; Marks et al., 2008), polysaccharides characteristic of primary cell walls, further suggesting that these AtGATL genes are involved in pectin synthesis in these tissues.

AtGATL4 stands out among the AtGATL family because it is expressed exclusively in the flower. The GUS reporter-based expression analyses localized AtGATL4 expression specifically to the pollen grain and elongating pollen tube. Pectic polysaccharides are a major component of pollen grain and pollen tube walls, where they help maintain the cylindrical shape of the pollen tube and act as adhesion molecules during the fertilization process (Lord and Russell, 2002; Bosch et al., 2005). AtGATL1 and 7 are also expressed in pollen, although not specifically, implicating a potentially specialized function of some AtGATL family members in deposition of pollen cell wall pectin components.
In summary, the unique and overlapping cell type-specific expression of each \textit{AtGATL} family member provides useful information and a platform for understanding their functions. The Golgi localization of the AtGATLs, their expression patterns, the available microarray data and co-expression analyses, as well as the cell wall compositional analyses strongly implicate at least some of the \textit{AtGATL} family members, like \textit{AtGATL3, 6 and 9}, in the biosynthesis of primary cell walls in diverse organs and tissues of \textit{Arabidopsis} by contributing to pectin synthesis. However, given the partial overlapping gene expression patterns for several \textit{GATL}s in some \textit{Arabidopsis} tissues, more extensive gene knockout analyses, either simultaneously using RNAi or by piling up multiple insertional mutations, and biochemical studies in specific cell- and tissue-types will be required to address the biological functions of the \textit{AtGATL} genes.

**Material and Methods**

**Bioinformatic Analyses of GATL Protein Sequences**

The twelve draft plant genomes, predicted genes and translated protein data used for bioinformatic analysis of the GATL family were downloaded from various sources, as specified in Table S1 (see Supplementary Data). GATL proteins were identified from these genomes using an HMMER search (Eddy, 1998) for the Pfam (Finn et al., 2006) Glyco\_transf\_8 (PF01501, 345 aa long) domain as the query. This HMMER search, using an E-value cutoff \(\leq 1e-2\), identified 99 GATL proteins from the 12 genomes. Four protein sequences were removed due to the fact that they were truncated or lacked key amino acid motifs characteristic of GT Family 8 (Yin et al., 2010). A maximum likelihood phylogenetic tree was constructed using PhyML v2.4.4 (Guindon and Gascuel, 2003) for the 95 full length proteins based on their multiple sequence alignment generated using MAFFT v6.603 (Katoh et al., 2005) with the L-INS-I method. Specifically, PhyML analyses were conducted with the JTT model, 100 replicates of bootstrap analyses, estimated proportion of invariable sites, four rate categories, estimated gamma distribution parameter, and an optimized starting BIONJ tree. A clade of six genes (five from \textit{Physcomitrella patens} and one from \textit{Selaginella moellendorfii}) that are basal to all other plant GATL proteins examined was selected as the outgroup to root the phylogeny. A rectangular phylogram of the GATL protein sequences was generated using the Interactive Tree of Life (iTOL) web server (Letunic and Bork, 2007).
**Plant Material and Growth Conditions**

*Arabidopsis* plants were grown on soil in a controlled-environment chamber under a 14-h light/10-h dark cycle at 19°C during the light period and 15°C during the dark period. The light intensity was 150 µEi m⁻² s⁻¹, and the relative humidity was maintained at 60-70%. *Arabidopsis* plants of the Columbia ecotype were used for transformation and isolation of DNA and RNA. T-DNA-mutagenized seeds were obtained from the SALK Institute (http://signal.salk.edu/cgi-bin/tdnaexpress) through the *Arabidopsis* Biological Resource Center (Supplemental Table S5). T-DNA insertion mutants for *gatl3*, 5, 6, 8, and 9 were identified using the flanking primers (LP and RP) generated by the SIGnal T-DNA verification primer design website (http://signal.salk.edu/tdnaprimers.html) and primers from the T-DNA left border LBa1 (5’-GCGTGGACCCTTGCAACT-3’) or LBb1 (5’-TCAAACAGGATTTTCGCCTGCT-3’). The sequences of the flanking primers for *AtGATL* genes are provided in Supplemental Table S6.

**RNA Extraction and RT-PCR**

For semi-quantitative RT-PCR analysis of *AtGATL* gene expression, siliques, flowers, leaves, upper stems, middle stems and lower stems were harvested from 7-week-old *Arabidopsis* plants and frozen immediately in liquid nitrogen. Roots for RT-PCR were obtained from plants grown hydroponically for 14 days under sterile conditions in B5 vitamin, with 1% (w/v) sucrose, pH 5.8, at 22°C in constant white light. Approximately 100 mg of tissue samples was ground in liquid nitrogen, and total RNAs were extracted with the RNeasy plant mini kit (Qiagen, Valencia, CA) and treated with RNase-free DNase (Qiagen) to remove contaminating genomic DNA. One µg of total RNA was reverse-transcribed using Superscript® III Reverse Transcriptase (Invitrogen) in a 20 µl RT-first strand synthesis reaction that contained oligo(dT) primers. RT–PCR products were generated using primer sequences unique to each of the 10 *AtGATL* genes (Supplemental Table S7). Semi-quantitative RT-PCR was performed using the following program: 95°C, 3 min; 30 to 35 cycles of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C, then hold at 72°C for 5 min. To determine whether comparable amounts of RNA had been used for RT-PCR from the different tissues, the ACTIN2 gene (*At3g18780*) was used as a control.

To determine *AtGATL* transcript levels in their respective homozygous mutant lines, total RNAs were isolated from stems of 7-week-old wild type and homozygous *atgatl* mutant lines and the gene transcript levels were analyzed according to the method described above.
Knockouts were defined as mutants with RT–PCR reactions that yielded no detectable PCR product using gene-specific primers. Knockdown mutants were those that yielded significantly less, but detectable PCR product compared to the wild type plants.

**Plasmid Construction and plant transformation**

The cell-specific expression pattern of the *AtGATL* genes was studied using the β-glucuronidase (GUS) reporter gene. For each *AtGATL promoter::GUS* reporter gene construct, approximately 2.5 kb upstream of the predicted ATG start codons (including 5’-untranslated regions) and 1-1.5 kb downstream of the predicted stop codons (including 3’-untranslated regions) were PCR amplified using Pfx50 TAQ Polymerase (Invitrogen) with gene-specific primers containing appropriate restriction sites. Then the two amplified genomic DNA fragments were fused with GUS gene in the binary vector pBI101 to create the *AtGATL promoter::GUS* reporter gene construct. Sequences of the individual primers used are described in the Supplemental Table S8. Each of the *AtGATL promoter::GUS* reporter gene constructs was sequenced to verify their construction. The gene fusions were first electroporated into *Agrobacterium* strain GV3101, and then introduced into *Arabidopsis* wild-type plants (Col ecotype) via a floral dip method (Clough and Bent, 1998). Transgenic plants were selected on plates containing kanamycin (50 mg/L).

**Histochemical GUS Assays**

Expression of *AtGATL::GUS* transgenes was visualized by staining for GUS activity as described (Jefferson et al., 1987). Briefly, transgenic plants or excised tissues were stained in GUS staining solution [100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 1 mM ferricyanide, 1 mM ferrocyanide, 0.1% Triton X-100 (v/v), and 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid] at 37°C. The staining buffer was removed, and the samples were cleared in 70% (v/v) ethanol until the blue color became visible. For each construct, plants from five to ten independent transgenic lines were examined. Patterns of gene expression for each construct were consistent across multiple transgenic lines and representative plants were photographed under a stereoscopic microscope (Olympus SZH-ILLD) equipped with a Nikon DS-Ril camera head using NIS-Elements Basic Research software.
Sub-Cellular Localization of AtGATL Proteins

The coding regions for selected AtGATL genes were cloned in frame with an enhanced yellow fluorescent protein (EYFP) gene under the control of the 35S promoter in a pCAMBIA-based binary vector (Kong et al., 2009) to generate the fusion constructs (35S-AtGATL-EYFP). Primers used for creating the EYFP fusions are listed in Supplemental Table 9. The AtGATL constructs were sequenced to verify their construction and then transformed individually into Agrobacterium tumefaciens strain GV3101. The constructs were individually co-transformed into fully expanded leaves of Nicotiana benthamiana plants together with the enhanced cyan fluorescent protein (ECFP)-tagged Golgi marker Gmct–ECFP (Saint-Jore-Dupas et al., 2006; Nelson et al., 2007). Co-transformation and signal observation were done as described previously (Kong et al., 2009).

Cell Wall Extraction and Sugar Analysis

Cell walls were prepared as alcohol-insoluble residues (AIR) as described previously (Persson et al., 2007). In brief, stems from 10-week-old Arabidopsis wild type and mutants were harvested on ice, flash frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle. The ground materials were consecutively extracted with 2 vol. of 100 mL of ice-cold 80% (v/v) ethanol, 100% ethanol, chloroform:methanol (1:1; v/v), and 100% acetone. Starch was removed from the walls by treatment with Type-I porcine α-amylase (Sigma-Aldrich; 47 units/100 mg cell wall) in 100 mM ammonium formate (pH 6.0) for 48 h at room temperature with constant rotation. De-starched walls were centrifuged, washed three times with sterile water, twice with 100% acetone, and air dried. Sugar composition analyses were carried out on three independently prepared cell wall preparations using trimethylsilyl (TMS) ethers of methyl glycosides as described (Caffall et al., 2009).

Cell Wall Fractionation

Sequential extraction of cell walls (AIR) isolated from wild type and atgatl mutant plants were done on 10 mg ml⁻¹ suspensions. First, the AIR samples were suspended in 50 mM ammonium oxalate (pH 5.0). The suspension was incubated overnight at room temperature with constant mixing. After the incubation, the suspension was centrifuged at 3400g, and the supernatant was decanted and saved, and the pellet was washed three times with deionized water.
before subsequent extraction steps. The washed pellet was then sequentially extracted in the same manner using 50 mM sodium carbonate (pH 10), 1 M KOH and 4 M KOH. In each step, the supernatants were individually decanted and saved. The 1 M KOH and 4 M KOH extracts were neutralized with glacial acetic acid. All cell wall extracts were dialyzed against four changes of 20 L of deionized water (sample:water ~1:60) at room temperature for a total of 48 hours and then lyophilized.

**Total Sugar Estimation and ELISA**

Cell wall extracts were dissolved in de-ionized water (0.2 mg mL$^{-1}$) and total sugar contents of cell wall extracts were estimated using the phenol-sulfuric acid method (Masuko et al., 2005). Cell wall extracts (60 μg sugar mL$^{-1}$) were applied to the wells of ELISA plates (Costar 3598) at 50 μL per well and allowed to evaporate to dryness overnight at 37°C. A Biotek robotic system (Biotek, Burlington, VT) was used to perform fully automated ELISA using a series of 150 monoclonal antibodies directed against plant cell wall carbohydrate epitopes (Pattathil et al., 2010). ELISA data are presented as a heat map in which the antibody order is based on a hierarchical clustering analysis of the antibody collection that groups the antibodies according to their binding patterns to a panel of diverse plant glycans (Pattathil et al., 2010).

**Monoclonal Antibodies**

Monoclonal antibodies were obtained as hybridoma cell culture supernatants either from laboratory stocks at the Complex Carbohydrate Research Center [CCRC, JIM and MAC series; available from CarboSource Services (http://www.carbosource.net)], or from Plant Probes [LM series, PAM1; (http://www.plantprobes.net)].

**Supplemental Data.**

Supplemental Table S1: Sources for the twelve genomes used for bioinformatic analysis of the GATL family.

Supplemental Table S2: Summary of whole plant _AtGATL_ expression patterns based on Figure 3.

Supplemental Table S3: Summary of whole plant _AtGATL_ expression patterns based on Figure 4.
Supplemental Table S4: Analysis of the topology of AtGATLs using the plant membrane protein database, Aramemnon.

Supplemental Table S5: T-DNA insertion lines used in this study.

Supplemental Table S6: Flanking primer sequences for insertions in AtGATL genes.

Supplemental Table S7: Primers used for RT-PCR.

Supplemental Table S8: Primers used for construction of AtGATL promoter::GUS fusions.

Supplemental Table S9: Primers used for construction of AtGATL::GFP fusions.

Acknowledgements

The authors gratefully acknowledge the assistance of Malcolm O’Neill (CCRC, Univ. of Georgia) with the glycosyl composition analyses.

LITERATURE CITED


synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of Arabidopsis. Plant Cell **12**: 2409-2423


**Fu H, Kim SY, Park WD** (1995a) A potato *Sus3* sucrose synthase gene contains a context-dependent 3’ element and a leader intron with both positive and negative tissue-specific effects. Plant Cell **7**: 1395-1403

**Fu H, Kim SY, Park WD** (1995b) High-level tuber expression and sucrose inducibility of a potato *Sus4* sucrose synthase gene require 5’ and 3’ flanking sequences and the leader intron. Plant Cell **7**: 1387-1394


Sterling JD, Quigley HF, Orellana A, Mohnen D (2001) The catalytic site of the pectin biosynthetic enzyme α-1,4-galacturonosyltransferase is located in the lumen of the Golgi. Plant Physiol 127: 360-371


Figure Legends

Figure 1. Phylogenetic analysis, chromosomal location, and gene structure of AtGATLs.
(A) Phylogenetic tree of 95 GATL proteins from 12 plant species whose genomes have been sequenced (see Supplemental Table S1). The phylogenetic reconstruction was carried out using PhyML as described in Materials and Methods. The six genes in GATL-g were selected as the outgroup to root the phylogeny. Selected supporting values for nodes > 70% are shown. The phylogeny is displayed using the Interactive Tree of Life (iTOL) web server (Letunic and Bork, 2007).

(B) Chromosomal positions and duplication events for AtGATL genes in the Arabidopsis genome (http://wolfe.gen.tcd.ie/athal/dup). Black and purple boxes depict recent and ancient duplicated segments in chromosomes (Chr) I, III and IV, respectively. Blue solid lines between boxes link the recently duplicated regions. Dashed red lines link genes from ancient duplications and are of lower statistical significance than the others.

(C) Gene structure analysis of AtGATL genes using GSDS (http://gsds.cbi.pku.edu.cn/).

Figure 2. Expression profiles of AtGATL genes in different Arabidopsis tissues.
Semi-quantitative RT–PCR of total RNA isolated from siliques (S), flowers (F), leaves (L), roots (R), upper stems (Us), middle stems (Ms) and lower stems (Ls) was used to assess AtGATL transcript levels in tissues of 7-week-old Arabidopsis plants. The ACTIN2 gene (At3g18780) was used as a control. All AtGATL gene amplification reactions were carried out for 35 PCR cycles and the actin gene was amplified for 30 cycles.

Figure 3. Histochemical analysis of the expression patterns of AtGATLpr::GUS fusions in Arabidopsis roots and seedlings of 2-week-old plants, and in the flowers and siliques of 8-week-old plants.
Whole seedlings were cleared and stained for GUS activity (blue coloring). The insets in the AtGATL1, 2, 7, 8 and 10 seedling panels show intense staining of trichomes, and the inset in the AtGATL4 single flower panel shows staining in pollen grains and elongating pollen tubes. A summary of the observed AtGATL expression patterns is provided in Supplemental Table S2.

**Figure 4. Histochemical analysis of expression of AtGATLpr::GUS fusions in Arabidopsis inflorescence stems and hypocotyls of transgenic plants.**

Transverse hand-cut sections from lower, middle, and upper parts of 8-week-old inflorescence stems and 8-week-old hypocotyls were cleared and stained for GUS activity (blue coloring). A summary of the observed AtGATL expression patterns is provided in Supplemental Table S3.

**Figure 5. Sub-cellular localization of EYFP-tagged AtGATL proteins.**

EYFP-tagged AtGATL proteins were transiently expressed in leaf epidermal cells of Nicotiana benthamiana plants, and their sub-cellular locations were examined with a laser scanning confocal microscope. Scale bars represent 20 µm.

(A) Fluorescent signals of tobacco leaf epidermal cells expressing EGFP alone. Note that the EGFP protein yielded signal throughout the cytoplasm and the nucleus.

(B) Localization of Gmct–ECFP Golgi marker protein (green).

(C) Localization of AtGATL3–EYFP protein (red) in the same cell as in (B).

(D) Merged image of (B) and (C), showing co-localization of AtGATL3–EYFP and Gmct–ECFP.

**Figure 6. Insertional mutants in AtGATL genes.**
(A) Schematic diagrams of the locations of T-DNA inserts in the coding regions (large white rectangles) or UTRs (smaller black rectangles) of the AtGATL3, AtGATL5, AtGATL6, AtGATL8 and AtGATL9 genes.

(B) AtGATL transcription level in homozygous mutants of atgatl3, atgatl5, atgatl6, atgatl8 and atgatl9. RT-PCR of total RNAs isolated from stems of 7-week-old homozygous atgatl mutant lines was performed using gene-specific primers (see Supplemental Table S6). The ACTIN2 gene (At3g18780) was used as a control. All AtGATL gene amplification reactions were carried out for 35 PCR cycles and the actin gene was amplified for 30 cycles.

Figure 7. Glycome profiling of sequential stem wall extracts prepared from five atgatl mutants and wild type plants.

Sequential stem cell wall extracts of 10-week old atgatl and wild-type plants were generated as described in Methods. The presence of cell wall glycan epitopes in each extract was determined by ELISAs using 150 glycan-directed monoclonal antibodies (Pattathil et al., 2010) and the data presented as heat maps. Reagents used for extracting stem materials are indicated on the top of each column. The panel on the right lists the array of antibodies used (left-hand side) grouped according to the principal cell wall glycan (right-hand side) recognized by the antibodies. The colored outlines highlight changes in glycome profiles in the mutant walls compared with the w.t.: green outlines – xyloglucan epitopes; blue outlines – pectin and arabinogalactan epitopes; red outlines – xylan epitopes. The yellow-black scale indicates the strength of the ELISA signal: bright yellow color depicts strongest binding and black color indicates no binding.
Table I. Amino acid sequence comparisons between predicted full-length sequences of AtGATL proteins

Pair-wise BLAST alignments were done to obtain the identities (above slash) and similarities (below slash), as percentages, over the alignment lengths.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>AtGATL1 (361 aa)</th>
<th>AtGATL2 (345 aa)</th>
<th>AtGATL3 (345 aa)</th>
<th>AtGATL4 (351 aa)</th>
<th>AtGATL5 (361 aa)</th>
<th>AtGATL6 (346 aa)</th>
<th>AtGATL7 (361 aa)</th>
<th>AtGATL8 (393 aa)</th>
<th>AtGATL9 (390 aa)</th>
<th>AtGATL10 (365 aa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtGATL1</td>
<td>69/83</td>
<td>62/73</td>
<td>56/72</td>
<td>59/74</td>
<td>61/74</td>
<td>66/77</td>
<td>59/74</td>
<td>59/73</td>
<td>60/72</td>
<td></td>
</tr>
<tr>
<td>AtGATL2</td>
<td>58/71</td>
<td>58/73</td>
<td>58/74</td>
<td>60/77</td>
<td>59/75</td>
<td>57/74</td>
<td>57/74</td>
<td>58/75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtGATL3</td>
<td>65/76</td>
<td>62/75</td>
<td>63/75</td>
<td>63/76</td>
<td>55/69</td>
<td>57/72</td>
<td>60/74</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtGATL4</td>
<td>60/74</td>
<td>55/72</td>
<td>60/74</td>
<td>53/71</td>
<td>53/71</td>
<td>58/76</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtGATL5</td>
<td>77/85</td>
<td>75/84</td>
<td>62/76</td>
<td>65/78</td>
<td>60/77</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtGATL6</td>
<td>76/83</td>
<td>64/77</td>
<td>67/78</td>
<td>60/75</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtGATL7</td>
<td>65/76</td>
<td>68/77</td>
<td>64/78</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtGATL8</td>
<td>84/89</td>
<td>65/77</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtGATL9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>66/78</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table II. Glycosyl residue compositions of cell walls isolated from stems of wild-type and *atgatl* plants.

The glycosyl residue compositions of cell walls isolate from inflorescence stems of 10-week-old *Arabidopsis* plants as determined by GC–MS of TMS derivatives as described in Materials and Methods. Data are mol% ± SE of three independent analyses. Glycosyl residues are abbreviated as arabinose (Ara), rhamnose (Rha), fucose (Fuc), xylose (Xyl), mannose (Man), galactose (Gal), glucose (Glc), and galacturonic acid (GalA).

<table>
<thead>
<tr>
<th>Mol%</th>
<th>Ara</th>
<th>Rha</th>
<th>Fuc</th>
<th>Xyl</th>
<th>Man</th>
<th>Gal</th>
<th>Glc</th>
<th>GalA</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>9.6±0.9</td>
<td>5.9±1.1</td>
<td>1.4±0.1</td>
<td>46.6±3.3</td>
<td>4.4±0.5</td>
<td>8.2±0.5</td>
<td>2.7±0.4</td>
<td>21.3±1.6</td>
</tr>
<tr>
<td><em>atgatl</em>3</td>
<td>8.5±0.4</td>
<td>5.1±0.3</td>
<td>1.1±0.1</td>
<td>55.1±0.3</td>
<td>3.7±0.1</td>
<td>7.86±0.2</td>
<td>2.4±0.1</td>
<td>16.2±0.7</td>
</tr>
<tr>
<td><em>atgatl</em>5</td>
<td>10.8±0.9</td>
<td>6.2±1.1</td>
<td>1.5±0.1</td>
<td>43.2±2.4</td>
<td>4.6±0.5</td>
<td>8.7±0.6</td>
<td>2.4±0.5</td>
<td>22.8±2.7</td>
</tr>
<tr>
<td><em>atgatl</em>6</td>
<td>8.6±0.4</td>
<td>5.8±1.0</td>
<td>1.4±0.1</td>
<td>52.1±1.6</td>
<td>4.1±0.4</td>
<td>7.62±0.8</td>
<td>2.6±0.5</td>
<td>17.8±0.1</td>
</tr>
<tr>
<td><em>atgatl</em>8</td>
<td>8.4±0.2</td>
<td>5.9±0.2</td>
<td>1.3±0.1</td>
<td>51.3±0.4</td>
<td>4.6±0.3</td>
<td>7.3±0.5</td>
<td>2.6±0.1</td>
<td>18.8±3.0</td>
</tr>
<tr>
<td><em>atgatl</em>9</td>
<td>9.7±0.4</td>
<td>6.7±1.0</td>
<td>1.6±0.1</td>
<td>48.5±1.7</td>
<td>4.6±0.5</td>
<td>9.0±0.3</td>
<td>3.0±0.6</td>
<td>16.9±0.7</td>
</tr>
</tbody>
</table>