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Arabidopsis thaliana T-DNA Mutants Implicate *GAUT* Genes in the Biosynthesis of Pectin and Xylan in Cell Walls and Seed Testa

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ABSTRACT **Galacturonosyltransferase 1 (GAUT1) is an α1,4-D-galacturonosyltransferase that transfers galacturonic acid from uridine 5′-diphosphogalacturonic acid onto the pectic polysaccharide homogalacturonan (Sterling et al., 2006). The 25-member *Arabidopsis thaliana GAUT1*-related gene family encodes 15 GAUT and 10 GAUT-like (GATL) proteins with, respectively, 56–84 and 42–53% amino acid sequence similarity to GAUT1. Previous phylogenetic analyses of AtGAUTs indicated three clades: A through C. A comparative phylogenetic analysis of the *Arabidopsis*, poplar and rice GAUT families has sub-classified the *GAUT*s into seven clades: clade A-1 (*GAUT*s *1* to *3*); A-2 (*GAUT4*); A-3 (*GAUT*s *5* and *6*); A-4 (*GAUT7*); B-1 (*GAUT*s *8* and *9*); B-2 (*GAUT*s *10* and *11*); and clade C (*GAUT*s *12* to *15*). The *Arabidopsis GAUT*s have a distribution comparable to the poplar orthologs, with the exception of *GAUT2*, which is absent in poplar. Rice, however, has no orthologs of *GAUT*s *2* and *12* and has multiple apparent orthologs of *GAUT*s *1*, *4*, and *7* compared with either *Arabidopsis* or poplar. The cell wall glycosyl residue compositions of 26 homozygous T-DNA insertion mutants for 13 of 15 *Arabidopsis GAUT* genes reveal significantly and reproducibly different cell walls in specific tissues of *gaut* mutants *6*, *8*, *9*, *10*, *11*, *12*, *13*, and *14* from that of wild-type *Arabidopsis* walls. Pectin and xylan polysaccharides are affected by the loss of GAUT function, as demonstrated by the altered galacturonic acid, xylose, rhamnose, galactose, and arabinose composition of distinct *gaut* mutant walls. The wall glycosyl residue compositional phenotypes observed among the *gaut* mutants suggest 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 for GAUT11 in seed mucilage expansion and in seed wall and mucilage composition.**

Key words: **Carbohydrate metabolism; cell walls; *Arabidopsis*; biosynthesis; mutant; pectin; mucilage.**

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 can hydrogen bond with hemicelluloses, such as xyloglucan and xylan, to make up the cellulose–hemicellulose network (Herth, 1983; Nishiyama et al., 2002). Hemicelluloses may also play a role in the organization of cellulose microfibrils, their crystallinity, as well as the ratio of cellulose Iα to Iβ (McCann et al., 1990; Jarvis, 2000). Proteins such as wall glycosyl hydrolyases, extensins, expansins, arabinogalactan proteins, hydroxyproline-rich glycoproteins, proline-rich proteins,

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wall-associated kinases, and others contribute to wall structural integrity and the regulation of wall structural components (reviewed in Cosgrove, 2000; Showalter, 2001). The pectic polysaccharides form the wall matrix by interacting in known, as well as unidentified, ways with other pectins and with hemicelluloses, wall proteins, and phenolic compounds to yield the complex 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 (Mohnen, 2008; Scheller et al., 2007). 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. HG is a polymer of α-(1,4)-linked-D-GalA that is largely unbranched, but may be modified by acetyl groups at the O-2 and O-3, and methylesters at the C-6 of many GalA residues (Mort et al., 1993). The tricellular junctions, middle lamellae, and primary wall are rich in HG, which may constitute up to 69% of primary wall pectin (Ridley et al., 2001). The galacturonan of some plant species may be substituted by terminal xylose residues, or by a disaccharide (Xyl*p*-(1,2)-Xyl*p*→) 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). The substituted galacturonan RG-II represents approximately 10% of pectin (Scheller et al., 2007; Mohnen et al., 2008). An RG-II structural unit, which is thought to be embedded within a larger HG region, consists of four highly complex side chains (A–D) attached to an HG backbone of seven to nine GalA residues: a structure highly conserved across species. 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 O'Neill et al., 2004). RG-I has a repeating disaccharide backbone of $[\rightarrow$α-D-Gal*p*A-1,2-α-L-Rha*p*-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 various arabinogalactan structures (Carpita and Gibeaut, 1993; Mohnen et al., 2008). 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).

Despite the increasing detail with which primary wall structure is understood, few pectin biosynthetic genes have been identified. Thus far, only a single gene, *AtGAUT1*, has been definitively shown to encode a protein having HG-galacturonosyltransferase (GalAT) activity (Sterling et al., 2006). AtGAUT1 is a Golgi localized type-II membrane protein that catalyzes the elongation of HG oligosaccharides (oligogalacturonides) in an α1,4-configuration, consistent with HG-GalAT function. A BLAST search (www.ncbi.nlm.nih.gov/) of the *Arabidopsis* genome identified 25 members of the *GAUT1*-related gene family. The *Arabidopsis GAUT1*-related gene family has 15 putative galacturonosyltransferase (*GAUT*) and 10 galacturonosyltransferase-like (*GATL*) members (Sterling et al., 2006). All the *GAUT1*-related genes are members of the CAZy GT8 family (Cantarel et al., 2009; www.cazy.org/).

Thus far, three mutants in three *GAUT1*-related family genes, *irx8*, *parvus*, and *quasimodo*, have been studied and the observed phenotypes suggest that these GAUT proteins, in addition to GAUT1, are likely to have GalAT activity and are involved in the synthesis of cell wall polysaccharides. The *Arabidopsis* IRX8 and PARVUS 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 are lacking the reducing end pentasaccharide [→4)-β-D-Xyl*p*-(1,4)-β-D-Xyl*p*-(1,3)-α-L-Rha*p*-(1,2)-α-D-Gal*p*A-(1,4)-D-Xyl*p*], purified from a sub-fraction of xylan (Peña et al., 2007). It has been suggested that the GalA in this xylan reducing-end pentasaccharide may be inserted by IRX8/GAUT12 (Peña et al., 2007). Alternatively, it has been hypothesized that IRX8/GAUT12 may synthesize a sub-fraction of HG to which xylan is attached (Persson et al., 2007). Recent studies of *parvus/gatl1* have demonstrated that *parvus* and *irx8* have a similar reduction in Xyl and β1,4-XylT activity, and, therefore, may be involved in a similar xylan biosynthetic pathway (Brown et al., 2007; Lee et al., 2007). The data provide descriptive evidence for possible functions of IRX8/GAUT12 and PARVUS/GATL1. However, biochemical evidence of enzymatic activity has not been obtained to confirm these hypotheses.

QUA1/GAUT8 has 77% amino acid similarity to GAUT1. *Qua1-1* 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 *qua1-1* stem tissue are reduced in both α1,4-GalAT and β1,4-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 α1,4-GalAT involved in HG biosynthesis (Orfila et al., 2005). However, the observed reductions in xylan and XylT activity of the *qua1-1* 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. Analysis of the cell wall glycosyl residue

compositions of 26 *gaut* mutants described in this work demonstrates aberrant wall composition among mutants of *GAUT*s *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 (WT), have distinct patterns, suggesting that these GAUTs 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 testa cell wall and mucilage.

RESULTS

The *GAUT* Family of *Arabidopsis*, Poplar, and Rice

The *Arabidopsis GAUT1*-related gene family encodes 15 GAUT 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 resulted in the designation of three *GAUT* clades, clades A through C, 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, was not included in the study reported here. It was previously determined that some *Arabidopsis GAUT* genes had conserved orthologs among species of both vascular and non-vascular plants (Sterling et al., 2006). The genomes of rice (*Oryza sativa*) and poplar (*Populus trichocarpa*) have now been sequenced and a BLAST search of *Arabidopsis* GAUT motifs against the poplar and rice genomes revealed *GAUT1*-related gene families of 21 members in poplar and 22 members in rice (Figure 1). Due to a recent genome duplication event in *Populus* (Tuskan et al., 2006), there are one to two apparent poplar orthologs for each *Arabidopsis GAUT*. A similar distribution of *GAUT*s in poplar and *Arabidopsis* is observed, except for the absence of a *GAUT2* ortholog in poplar. In contrast, rice has major distinctions from *Arabidopsis* and poplar in the distribution of *GAUT* gene orthologs. Rice does not have apparent orthologs of *GAUT2* or *GAUT12*. In addition, there are multiple apparent isoforms of *GAUT*s *1*, *4*, *7*, and *9*, suggesting an expansion of the role of these *GAUT* genes in rice.

The rice and poplar genes included in this comparative phylogenetic analysis resolved the *GAUT* genes 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-1, A-2, A-3, and A-4; *GAUT* clade B is subdivided into clades B-1 and B-2; and *GAUT* clade C remains undivided. The corresponding *GAUT*s in each clade are: A-1 (1 to 3); A-2 (4), A-3 (5 and 6) and A-4 (7); B-1 (8 and 9), B-2 (10 and 11) and C (12 to 15).

GAUT Gene Transcript Expression in *Arabidopsis* Tissues

Available transcript expression of *AtGAUT*s compiled from the Whole Genome Array, Massively Parallel Signature Sequence, and Genevestigator bioinformatic databases (Table 1) was used to select tissues used for the cell wall analyses reported here. In addition, total RNA from 8-week-old *Arabidopsis* WT inflorescence, silique, stem, and leaf tissues was used for qualitative and semi-quantitative RT–PCR using *GAUT* gene-specific primers. PCR products corresponding to the transcripts of 14 *GAUT* genes, excluding *GAUT2*, were detected in the WT inflorescence, leaf, stem, silique, and root tissues tested. *GAUT2* may be expressed at a very low level or at different stages of development that have not yet been tested (Figure 2). Qualitative RT–PCR results partially agree with the published transcript expression data (see Table 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 *GAUT*s *7*, *10*, *11*, and *12* in leaf, *GAUT*s *1*, *3*, and *7* in stem, and *GAUT*s *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 under-reported *GAUT* gene transcript expression. The relative transcript expression of the *GAUT* genes, however, more closely agrees with that reported by Genevestigator (Zimmermann et al., 2004). Genevestigator does not list a probe for *GAUT5*, and therefore has no expression data for this gene, while the MPSS database reports low to moderate expression of *GAUT5*, in agreement with the result reported here.

In general, RT–PCR indicated that relative transcript expression in *Arabidopsis* was highest for *GAUT*s *1*, *4*, *8*, *9*, and *12*, moderate for *GAUT*s *3*, *5*, *6*, *10*, *14*, and *15*, and low for *GAUT*s *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. Whether the smaller band represents a splice variant has not been investigated. The RT–PCR data indicated that the *GAUT* genes were expressed at some level in all tissues tested; therefore, inflorescence, silique, leaf, and stems were used for the chemical and biochemical studies of the *GAUT* mutants.

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-bin/tdnaexpress) through the Arabidopsis Biological Resource Center (Alonso et al., 2003). Mutant seed lines were preferentially selected with the T-DNA insertion site in an exon, 5′ UTR, or intron of the *GAUT* gene, if such lines were available. SALK insertion seed lines of *GAUT1* were not available and neither homozygous nor heterozygous mutants were recovered from the SALK insertion seed lines for *GAUT4*.

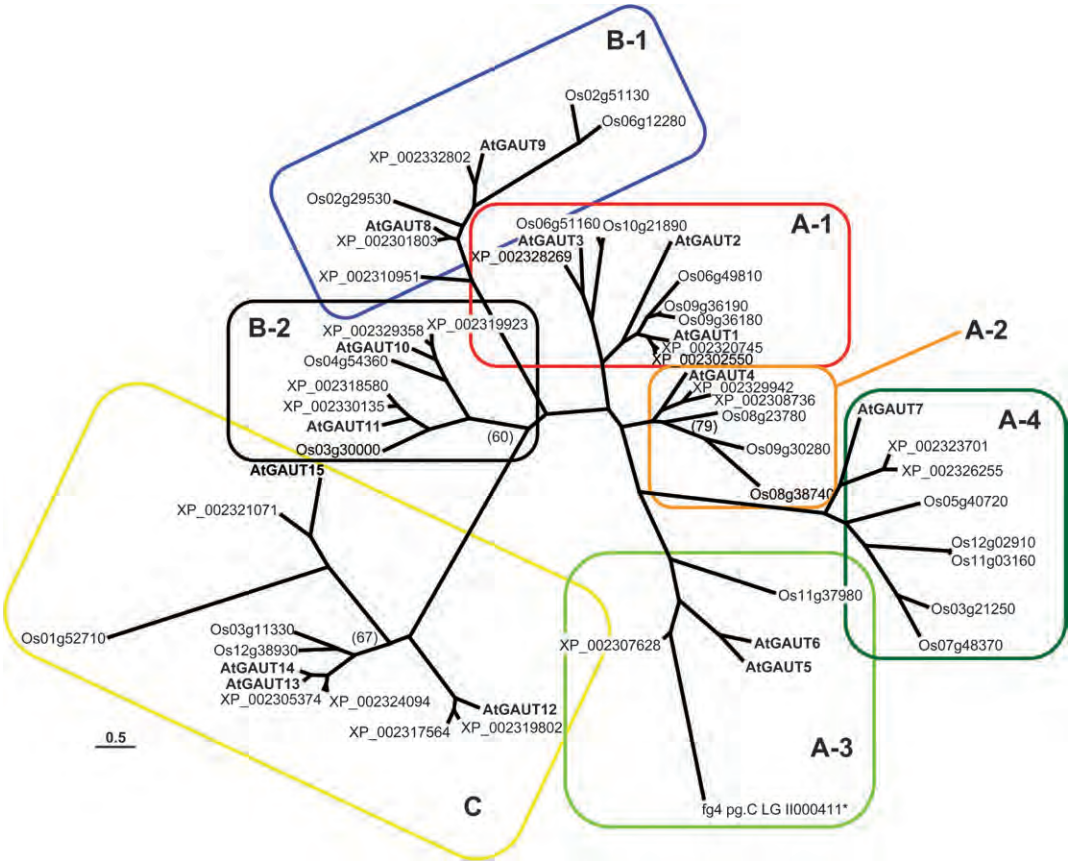

Figure 1. The GAUT Protein Family of *Arabidopsis*, Poplar, and Rice.

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 500 000 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). *P. trichocarpa* GAUT protein sequences are identified by their NCBI RefSeq accessions (www.ncbi.nlm.nih.gov/RefSeq/), except one (designated with *) where the Joint Genome Institute locus identifier was used (no RefSeq accession available).

RT–PCR of total RNA isolated from homozygous *gaut* mutant lines identified 10 knockout mutants and 10 knockdown mutants (Table 2).

Growth Phenotypes of *gaut* Mutants

The *gaut* mutants plants were initially inspected visually for obvious growth phenotypes, such as dwarfing and/or organ malformation, compared to WT plants. Major abnormalities were not observed in plant growth or morphology for most *gaut* mutants isolated in this study, with the exception of *gaut8* and *gaut12*. The presence of subtle growth phenotypes may require more sensitive methods than those applied here. Functional redundancy among the GAUT proteins may contribute to the lack of severe phenotypes observed among *gaut* mutants. Estimates put forth by Østergaard and Yanofsky (2004) predict that mutations in only approximately 10% of genes may result in detectable mutant phenotypes due to gene redundancy among large gene families in higher organisms. Thus far, two out of 13 *GAUT* genes (~15%) have yielded mutants with severe growth phenotypes, which is in line with the predicted outcome (Østergaard and Yanofsky, 2004).

Previously analyzed *qua1-1* insertion mutants (insertion in the 5′UTR) had severe dwarfing, sterility, and bumpy epidermal surfaces as a result of reduced cell adhesion (Bouton et al., 2002). Mutants allelic to *qua1-1* (*gaut8-2*, *gaut8-3*, and *gaut8-4*) produced only heterozygous and WT progeny, suggesting an embryo-lethal phenotype. A single homozygous mutant was isolated, *gaut8-1*, with a predicted insertion in the 3′UTR that did not show the expected *qua1-1* phenotype and was experimentally determined to have detectable *GAUT8* transcript by RT–PCR, which may account for the WT-like phenotype of these plants.

The *irx8-1*/*gaut12-1* and irx8-5/*gaut12-2* mutant plants were severely dwarfed and sterile, which necessitated recovery of

Table 1. Bioinformatic *Arabidopsis GAUT* Gene Transcript Expression Data.

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	14 093
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	18 061
At2g30575	*GAUT5*	–	11	19	1	14	7	18	5	20	–
At1g06780	*GAUT6*	+	0	4	0	0	0	0	0	0	11 224
At2g38650	*GAUT7*	+	68	69	111	62	40	218	53	236	7126
At3g25140	*GAUT8*	+	405	125	72	230	285	664	117	329	27 875
At3g02350	*GAUT9*	+	74	78	28	450	249	106	93	69	15 384
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	12 028
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

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 in different tissues, available through the Massively Parallel Signature Sequences (MPSS) website (http://mpss.udel.edu/at/) (Meyers et al., 2004): INF (Inflorescence—mixed stage, immature buds, classic MPSS), LEF (Leaves—21 d, untreated, classic MPSS), LES (Leaves—21 d, untreated), ROF (Root—21 d, untreated, classic MPSS), SIF (Silique—24–48 h post-fertilization, classic MPSS), SIS (Silique—24–48 h 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).

homozygous plants from the progeny of heterozygous parental plants, as previously reported (Persson et al., 2007). The phenotype of *irx8-1/gaut12-1* and *irx8-5/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. In addition, *gaut12-5* heterozygous mutants were dwarfed compared to WT, and more severely dwarfed compared to the *irx8-1/gaut12-1* or *irx8-5/gaut12-2* heterozygotes. RT–PCR of RNA from homozygous *irx8-1/gaut12-1* and *irx8-5/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. 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 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 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 detects 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 3), the TMS method was chosen to analyze *gaut* mutant walls. A statistical assessment of the TMS method showed that at least four independent TMS analyses per wall sample are necessary to detect a 15% difference between the glycosyl residue composition of different wall samples with 90% or greater statistical confidence (Supplemental Table 1). 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. Thus, for example, rhamnosyl compositions would be normalized according to the following formula:

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

Normalization of mutant glycosyl residue composition to WT controls allowed 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 *GAUT*s in WT tissues according to the Whole Genome Array (Yamada et al., 2003) and Massively Parallel Signature Sequences (Meyers et al., 2004) databases (see Table 1). To identify *gaut* mutant

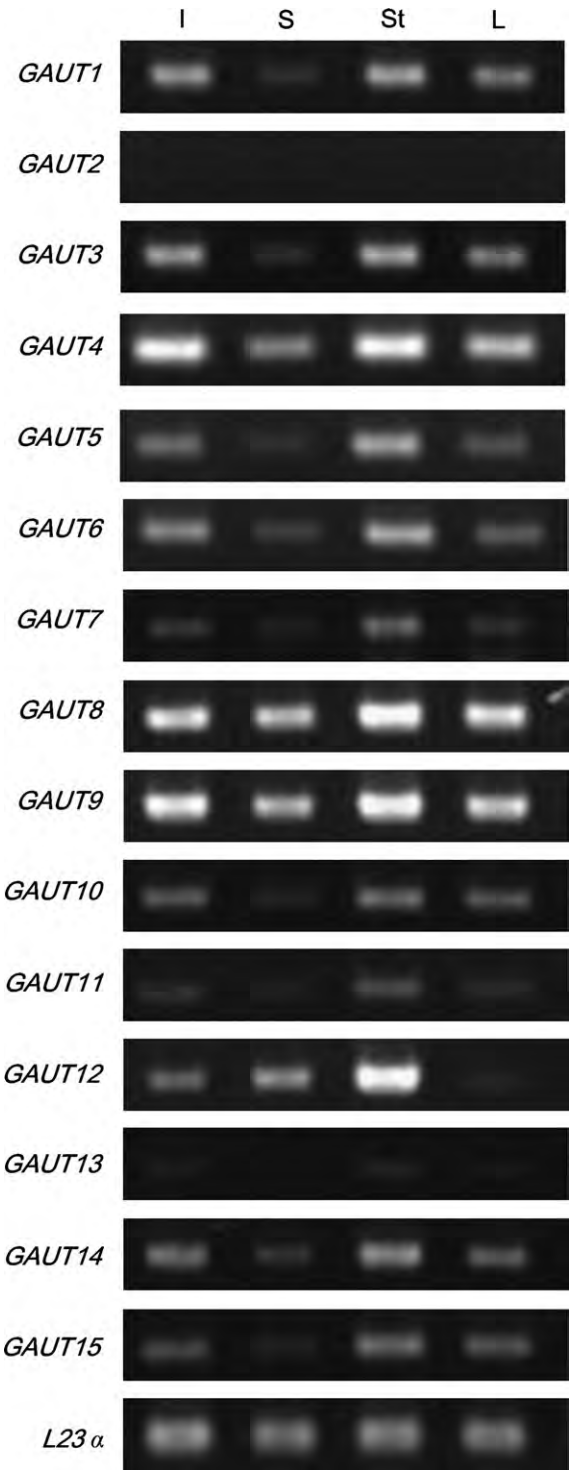

Figure 2. Transcript Levels of *GAUT* Genes in WT *Arabidopsis* Tissues.

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 (Supplemental Table 2). All reactions were carried out using 2 μg total RNA amplified for 26 PCR cycles. Similar results were obtained in three independent experiments. Control: RT–PCR using primers to L23α small ribosomal protein.

wall glycosyl residue compositions that were statistically different from those of WT walls, the normalized compositions were evaluated by ANOVA procedures ($t_{\alpha(2)} = 0.1$). As an extra measure of stringency, a 15% point or greater departure from the normalized WT mean, in addition to a statistically different outcome by ANOVA, was required for declaration of a real difference 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 mutant lines, representing 13 *GAUT* genes, revealed that specific *gaut* mutants have unique wall composition changes, which include *increases* and *decreases* in GalA, as well as significant changes in other glycosyl residues (Table 3). The wall glycosyl residue compositions that were statistically different in the *gaut* mutants compared to WT are shown in bold italics in Table 3. Reproducible mutant phenotypes were identified by comparing the natural log transformed data for all mutants that had statistically different mol% GalA, Xyl, Rha, Gal, and Ara levels compared to WT in at least two mutant alleles of the same gene or in at least two tissues of the same mutant allele (Supplemental Figure 1).

Eight *gaut* mutants 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, resulting in distinguishable patterns of glycosyl residue composition changes in the walls of *gaut* mutants (summarized in Table 4). The silique tissues of *gaut6-1* and *gaut6-3* were consistently reduced in GalA, increased in Xyl, Rha, and Fuc, and similar to WT in Gal and Ara wall composition. Viable *gaut8* homozygous knockout mutants were not isolatable, and, therefore, the wall composition of *qua1-1* is used to establish a phenotype grouping for *gaut8* mutants. The leaves of *qua1-1* that were previously analyzed (Bouton et al., 2002) were decreased in GalA and Xyl, but were not changed in Rha or other sugars. The *gaut9-1* stems were reduced in wall GalA and increased in Xyl and Fuc. The *gaut10-1*, *gaut10-2*, and *gaut11-1* were consistently reduced in silique GalA only. The *irx8-1*/*gaut12-1* and *irx8-5*/*gaut12-2* mutant stems were severely reduced in Xyl, coincident with elevated Ara, Rha, and Gal content. The *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; Peña et al., 2007; Persson et al., 2007). *Gaut13-1*, *gaut14-1*, and *gaut14-2* had increased GalA and Gal and reduced Xyl, Rha, Ara, and Fuc, with greater mol% changes in *gaut14-1* (T-DNA insertion in an exon) than *gaut14-2* (T-DNA insertion in the 3′ region). 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*,

Table 2. The *Arabidopsis GAUT* Family and T-DNA Insertion Seed Lines.

Locus	Gene	Clade[a]	I / S[b]	SALK	Mutant Name	L[c]	KO/KD/W[d]
At3g61130	*GAUT1*	A-1	100/100				Not available
At2g46480	*GAUT2*	A-1	65/78	122209	*gaut2-1*	P	Not detected
At4g38270	*GAUT3*	A-1	68/84	001920	*gaut3-1*	I	KO
				113167	*gaut3-2*	5′	KD
At5g47780	*GAUT4*	A-2	66/83	034472	*gaut4-1*	5′	Not recovered
				001026	*gaut4-2*	5′	Not recovered
At2g30575	*GAUT5*	A-3	45/67	050186	*gaut5-1*	E	KO
				058223	*gaut5-2*	P	KD
At1g06780	*GAUT6*	A-3	46/64	007987	*gaut6-1*	E	KO
				056646	*gaut6-2*	E	KO
				073484	*gaut6-3*	5′	KD
At2g38650	*GAUT7*	A-4	36/59	015189	*gaut7-1*	E	KD
				046348	*gaut7-2*	P	KD
At3g25140	*GAUT8*	B-1	58/77	030075	*gaut8-1*	3′	KD
				039214	*gaut8-2*	E	HM lethal
				041919	*gaut8-3*	I	HM lethal
				102380	*gaut8-4*	I	HM lethal
At3g02350	*GAUT9*	B-1	57/76	135312	*gaut9-1*	E	W
				115588	*gaut9-2*	E	W
				040287	*gaut9-3*	E	KD
At2g20810	*GAUT10*	B-2	50/72	029319	*gaut10-1*	E	KO
				082273	*gaut10-2*	E	KD
At1g18580	*GAUT11*	B-2	51/71	104761	*gaut11-1*	5′	KD
				148781	*gaut11-2*	3′	KD
At5g54690	*GAUT12*	C	40/61	044387	*gaut12-1*	I	KO
				014026	*gaut12-2*	E	KO
				038620	*gaut12-5*	P	HM lethal
At3g01040	*GAUT13*	C	43/62	122602	*gaut13-1*	E	W
At5g15470	*GAUT14*	C	43/62	000091	*gaut14-1*	E	KO
				029525	*gaut14-2*	3′	KO
At3g58790	*GAUT15*	C	37/56	113194	*gaut15-1*	I	W
				117272	*gaut15-2*	P	W
				070957	*gaut15-3*	I	KO

a *GAUT* clades based on phylogenetic analysis (Sterling et al., 2006).
b The amino acid sequence identity and similarity (I/S) of each *GAUT* gene to *GAUT1* (Sterling et al., 2006).
c The tentative location of the T-DNA insertion site is in one of the following gene structures; exon (E), 5′ untranslated region (5′), intron (I), promoter (P), or 3′ untranslated region (3′).
d Transcript levels of *GAUT* T-DNA insertion mutant lines: Knockout, KO; Knockdown, KD; WT-like, W. Transcript for *GAUT2* was not detectable in WT; therefore, the status of the mutant transcript was not able to be determined.

gaut3-2, and *gaut6-2*; and decreased Glc in mutants of *gaut5-1*, *gaut5-2*, and *gaut10-2*. Few significant changes were found in the walls of *gaut*s *2*, *3*, *5*, *7*, and *15*, and those that did occur were not consistent between two or more mutants or in more than one tissue of a single mutant.

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 to aid in seed dispersal. The mucilage of WT and *gaut* mutant seeds was investigated by ruthenium red staining as a facile method to determine whether 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 efficiently binds ruthenium red stain due to the negative charge on the GalA residues in mucilage. This method has been successfully employed to identify mucilage or testa polysaccharide biosynthesis mutants (Western et al., 2001). The seed mucilage was evaluated by observing the staining intensity of mucilage and measuring

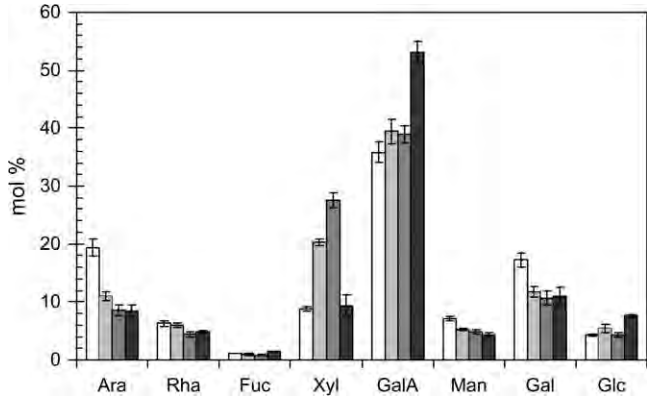

Figure 3. Glycosyl Residue Composition of *Arabidopsis* WT Cell Walls.

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; $n \geqslant 18$. Glycosyl residues are abbreviated as arabinose (Ara), rhamnose (Rha), fucose (Fuc), xylose (Xyl), galacturonic acid (GalA), mannose (Man), galactose (Gal), and glucose (Glc).

the mucilage thickness under a dissecting microscope 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 4A–4C) revealed that ~68% of *gaut11-2* seeds had little extruded mucilage, while the remaining *gaut11-2* seeds (~32%) had reduced thickness of the mucilage layer to approximately half that of WT. Samples of WT and *gaut11-2* seed were tested three separate times independently, with similar results obtained in seed derived from different parental plants (Table 5). Analysis of the uronic acid content of the hot water-extracted mucilage (WEM) of *gaut11-2* and WT seed indicated that WEM of WT had 59 μg uronic acid per 200 extracted seeds, while *gaut11-2* mucilage had 48 μg uronic acid per 200 extracted seeds (see Table 4). The total carbohydrate extracted, as detected by a phenol sulfuric acid assay, 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 (Figure 4) and 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 *gaut11-2* 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.

Table 3. Percent Cell Wall Glycosyl Residue Composition of *Arabidopsis gaut* Mutants Compared to Wild-Type.[a]

		(mutant mol%/WT mol%*100)							
Mutant	Tissue[b]	Ara	Rha	Fuc	Xyl	GalUA	Man	Gal	Glc
gaut2-1	S	***160***[c]	116	108	114	***84***	103	103	***77***
	L	152	***173***	98	***124***	91	89	112	123
gaut3-1	I	74	***64***	***74***	111	104	108	***125***	***125***
	S	90	62	72	***82***	110	104	126	***118***
gaut3-2	I	99	102	181	***118***	99	89	97	***131***
	S	132	112	109	118	87	98	86	***120***
gaut5-1	I	117	112	110	105	85	***131***	102	73
	S	109	132	117	130	94	***148***	112	***61***
gaut5-2	I	98	99	97	97	106	103	93	97
	S	102	118	43	95	105	***141***	78	***71***
gaut6-1	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
gaut6-2	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
gaut6-3	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
gaut7-1	I	91	113	104	110	102	89	93	126
	L	114	130	117	90	96	107	93	114
gaut7-2	I	100	96	87	98	114	89	96	105
	L	112	102	100	110	113	102	108	51
gaut8-1	I	***65***	***67***	***72***	81	111	106	119	***116***
	S	59	***55***	***35***	137	102	102	95	111
gaut9-1	I	130	***167***	***156***	***154***	99	159	***82***	***139***
	S	89	113	118	122	92	***154***	99	136
	ST	101	131	***153***	***148***	***80***	146	***72***	127
gaut9-2	I	***77***	***79***	***70***	100	106	100	***119***	99
	S	82	72	***207***	103	104	282	99	85
	ST	100	90	96	105	81	58	***129***	106
gaut9-3	I	139	130	151	***137***	108	102	91	114
	S	147	137	***178***	***128***	***82***	100	98	112
	ST	100	100	100	100	100	100	100	100
gaut10-1	I	103	98	***93***	107	89	120	112	86
	S	103	103	110	116	***83***	113	92	108
gaut10-2	I	152	***154***	128	115	87	94	92	***75***
	S	***131***	104	85	103	***83***	85	110	***78***
gaut11-1	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
gaut11-2	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
gaut12-1	I	148	120	97	101	***82***	89	102	142
	S	147	115	112	***33***	114	100	***127***	121
	ST	179	124	130	***55***	103	66	***168***	132

Table 3. Continued

		(mutant mol%/WT mol%*100)							
Mutant	Tissue[b]	Ara	Rha	Fuc	Xyl	GalUA	Man	Gal	Glc
gaut12-2	I	163	137	105	130	82	80	91	115
	S	65	67	***176***	***25***	***129***	102	***126***	169
	ST	198	154	126	***58***	117	60	148	109
gaut13-1	I	62	***58***	***63***	***68***	***125***	111	120	123
	S	***24***	***26***	117	89	***137***	99	110	***159***
gaut14-1	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
gaut14-2	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
gaut15-1	I	87	***83***	89	***76***	104	105	***119***	***166***
	S	***171***	107	117	118	85	86	96	***84***
gaut15-2	I	111	***161***	67	134	99	213	72	***81***
	S	98	147	90	***190***	***82***	156	60	112
gaut15-3	I	***77***	***78***	***71***	109	111	109	***117***	98
	S	130	***84***	95	112	93	103	109	84

a Data represent four independent TMS GC–MS reactions from four independent wall extractions. Residues are abbreviated according to Figure 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 (ST).
c Bold highlighted italicized values indicate mutant glycosyl residue compositions that were statistically and ±15% different from the WT mean.

Table 4. Phenotypic Grouping of *gaut* Mutants.[a]

gaut	GalA	Xyl	Rha	Gal	Ara
6	Down	Up	Up	Down	No change
8[b]	Down	Down	No change	No change	No change
9	Down	Up	Variable	Variable	No change
10	Down	No change	No change	No change	No change
11	Down	No change	Variable	Variable	Variable
12	Up[c]	Down	No change	Up	No change
13	Up	Down	Down	Up	Down
14	Up	Down	Down	Up	Down

a Changes in the relative amount of the designated glycosyl residues compared to WT.
b Due to the lethality of *gaut8* homozygous mutants, the *qua1-1* leaf compositions were used for the phenotypic grouping of *gaut8* (Bouton et al., 2002).
c The GalA composition of *gaut12* stems and siliques was increased, but was reduced in inflorescences.

DISCUSSION

Newly Resolved *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 *GAUT* clades (Figure 1), instead of three, as previously proposed by Sterling et al. (2006). The previous *Arabidopsis GAUT* clade A that included *AtGAUT1–GAUT7* has been subdivided into four clades; *GAUT* clade A-1 (*AtGAUT* 1 through 3), *GAUT* clade A-2 (*AtGAUT4*), clade A-3 (*AtGAUT5* and *AtGAUT6*), and *GAUT* clade (*AtGAUT7*). The former *Arabidopsis* clade B has been subdivided into *GAUT* clade B-1 (*AtGAUT8* and *AtGAUT9*) and *GAUT* clade B-2 (*AtGAUT10* and *AtGAUT11*). The former *Arabidopsis GAUT* clade C has not been subdivided and contains *AtGAUT12* through *AtGAUT15*.

GAUT2 does not appear to have a direct ortholog in either rice or poplar. It is possible that *GAUT2* may not be a complete copy of a *GAUT1* duplication event, based on a shorter N-terminus compared to *GAUT*s 1–7; however, its length is comparable to the other *GAUT*s. *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 nonfunctional truncated homolog. It cannot be ruled out, however, that *GAUT2* may have a very low abundance transcript and a unique function in *Arabidopsis* alone, although this seems unlikely based on the current data.

The *Arabidopsis* and poplar genomes have one (At2g38650) and two (XP_002323701, XP_002326255) copies of *GAUT7*, respectively, while the rice genome contains five *GAUT7*-like sequences. There is considerable evidence that the AtGAUT7 protein resides in a complex with AtGAUT1, a complex that has homogalacturonan α1,4-GalAT activity (M.A. Atmodjo, 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 function as an 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.

Poplar and rice each have putative orthologs of *GAUT9*: XP_002332802 (poplar), Os06g12280 (rice), and Os02g51130 (rice). Poplar also has at least one putative ortholog of *GAUT8* (XP_002301803). There is not an obvious ortholog of *GAUT8* in rice, although there is one rice gene (Os02g29530) positioned between *GAUT8* and *GAUT9*. Phylogenic analyses using additional sequenced plant genomes may clarify the relatedness of the latter gene to *GAUT8* and *GAUT9*.

GAUT12 has two poplar orthologs but no orthologs in rice (Figure 1). *GAUT12* has been linked xylan synthesis. The

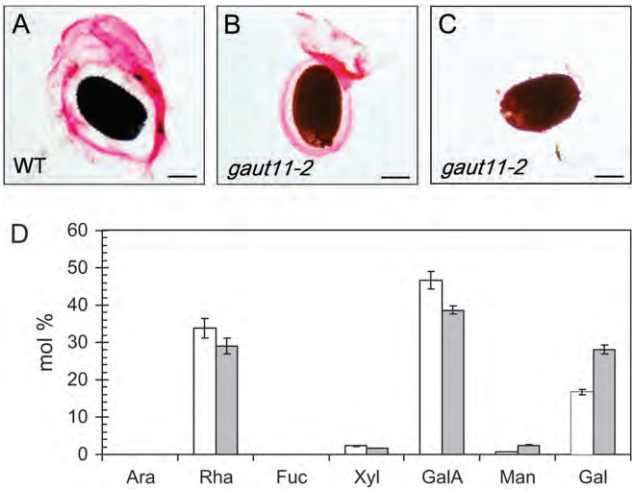

Figure 4. Staining and Glycosyl Residue Composition of WT and *gaut11-2* Seed Mucilage.

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, 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 from the WT mucilage in panel (A). Inset bar = 100 μm. The composition **(D)** of WT (white bars) and *gaut11-2* (gray bars) hot water-extracted mucilage was determined by GC–MS.

Table 5. WT and *gaut11.2* Mucilage Expansion and Uronic Acid Content.

	Mucilage (% seeds)[a]		UA (ug UA/200 seeds)[b]	
Experiment	WT	*gaut11-2*	WT	*gaut11-2*
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
	$P = 2.3^{-3}$		$P = 2.2^{-4}$	

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*-hydroxylbiphenyl reagent assay.

putative functions that have been hypothesized for GAUT12 include an α1,4-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; Peña et al., 2007; Persson et al., 2007). *GAUT12* has been shown to be essential for normal growth and more specifically for the synthesis of secondary wall glucuronoxylan and/or wall HG synthesis. Rice does not have an apparent homolog of *GAUT12*, and appears to produce secondary wall xylan and glucuronoarabinoxylan, but not 4-*O*-methylglucuronoxylan (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 the synthesis of 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 profile, role in secondary wall 4-*O*-methylglucuronoxylan synthesis, and exclusivity among the dicot species suggest that *GAUT12* has undergone a differentiation that has rendered it essential in dicots and 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 (Orfila et al., 2005), however, did reveal prominent expression in that tissue, which is more closely aligned with our results. 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 expression (Mouille et al., 2007).

The Wall Compositions of Multiple *gaut* Mutants are Altered Compared to WT

Analysis of the walls of *gaut* mutants using the TMS method (Doco et al., 2001) allowed the GalA content of the walls to be quantified. An accurate quantification of wall GalA content is important when attempting to identify mutants of putative pectin biosynthesis genes, because GalA is a major component of the pectic polysaccharides (Ridley et al., 2001). Mutants of *GAUTs 6*, *9*, *10*, and *11* had statistically significant reductions in GalA content in more than one mutant sampling. Two other *gaut* mutants, *gaut13* and *gaut14*, had statistically significant increased wall GalA content. The wall compositional phenotypes of the *gaut* mutants are discussed below.

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 signifies 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. thesis, 2008).

GAUTs 8, 9, 10 and 11 have been placed in two separate subclades (B-1 and B-2). However, all mutants in the two B clades show marked reductions in wall GalA content. *Qua1-1* mutant plants have walls with both reduced GalA and Xyl, and microsomal membrane protein preparations from *qua1-1* stems had reduced GalAT and xylan synthase activity compared to WT (Orfila et al., 2005; Brown et al., 2007). The QUA1 cumulative experimental evidence argues in favor of a putative pectin biosynthetic GalAT, based on the significant reduction in homogalacturonan and the 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 pectinmethyltranserfases is strongly correlated with *QUA1*/*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 these *gaut* plants, 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; Peña et al., 2007; Persson et al., 2007). Based on the evidence, a role for the genes in *GAUT* clades A as well as a role for the genes in clade B and C in pectin biosynthesis is proposed.

In contrast to QUA1/GAUT8, IRX8/GAUT12 is believed to function in glucuronoxylan synthesis essential for secondary wall function. The *irx8-1*/*gaut12-1* and *irx8-5*/*gaut12-2* mutant plants have reduced Xyl content with increases in the GalA content in stem and silique walls, consistent with previous reports and consistent with the proposed function of IRX8/GAUT12 in the synthesis of an oligosaccharide essential for xylan synthesis. Mutants of *IRX8*/*GAUT12* and other putative xylan biosynthetic genes, *IRX7*, *IRX8*, *IRX9*, *IRX14*, and *PARVUS*, have similar wall compositional phenotypes (Peña et al., 2007; Persson et al., 2007). IRX8/GAUT12 may play a specialized role, among the GAUTs, 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 *IRX8*/*GAUT12* is also expressed. In inflorescences, *irx8*/*gaut12* mutants show a reduction in GalA to 82% that of WT. Thus, the changes brought about by the lesion in *GAUT12* additionally impact the pectin component of the wall. The underlying causes for the reduced GalA content in the inflorescence may be of significance to understand how pectin and xylan synthesis are regulated and connected.

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 is increased in the walls of *gaut13* and *gaut14* (and also *gaut12*). Pectic galactans have been associated with wall strengthening (McCartney et al., 2000) and are also increased in *irx8*/*gaut12* 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 mutants of these genes is also a xylan and that GAUT13 and GAUT14 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*, which 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 composition and linkage analysis of *gaut11-2* mucilage suggests a minor reduction in RG-I-like extractable polysaccharides. The *gaut11-2* mutant has reduced mucilage expansion and reduced GalA content of extracted mucilage and testa, suggesting a role in the synthesis of mucilage polysaccharides. The *gaut11-2* mutant 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 visible phenotype of *gaut11-1* is similar in character to the *mucilage modified* (*mum*) mutants (Western et al., 2001, 2004). Three types of *mum* mutants have been described: mutants of pectin modification (*mum2* and *mum1*), mutants affecting cytoplasmic rearrangement (*transparent testa glabra-1*; *ttg1*, *glabra-2*; *gl2*), and mutants of mucilage biosynthesis (*mum3*, *mum5*, and *mum4*) (Western et al., 2001). Preliminary data suggest 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+} cross-linking of HG in the wall (Jones et al., 2003), 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). In addition, *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 and Table 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* SAIL mutant yielded only heterozygous and WT progeny; homozygotes were not obtained (M.A. Atmodjo and I. Petrascu, 2008, unpublished data). More vigorous attempts to isolate and characterize *GAUT1* and *GAUT4* and their respective mutants will undoubtedly aid in the clarification of their roles in pectin and wall biosynthesis. A degree of lethality has also been demonstrated in *gaut8* and *gaut12* mutants, both in this report and elsewhere (Bouton et al., 2002; Persson et al., 2007). *Qua1-1*, *irx8-1*, and *irx8-5* mutants are severely dwarfed and semi-sterile (Brown et al., 2005; Orfila et al., 2005).

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 pectin and wall biosynthetic pathways. The wall compositional phenotypes support the proposition that (1) GAUT proteins play a role in wall biosynthesis, (2) GAUTs 6, 9, 10, and 11, which have the highest amino acid similarity to GAUT1, have putative functions in pectin biosynthesis, and (3) GAUTs 13 and 14 are likely to have putative functions in xylan biosynthesis like GAUT12, or in pectin RG-I biosynthesis. The mutant wall composition phenotypes presented here are not sufficient to prove *GAUT* function, but serve to support hypotheses regarding *GAUT* function. The data demonstrate that mutants corresponding to more than half of the *gaut* mutants have significantly altered wall polysaccharides and strongly support a role for the family in pectin and/or xylan synthesis and function. Potential gene redundancy could explain the lack of wall phenotypic changes in some of the *gaut* mutants, and the generation of double mutants might uncover phenotypes masked by such potential redundancy.

METHODS

Sequence Alignment of GAUT Family Proteins and Phylogenetic Analysis

Protein sequences were identified by BLAST search of *Arabidopsis thaliana* (www.Arabidopsis.org/index.jsp), *Oryza sativa* (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 (www.biosci.ohio-state.edu/pcmb/Facilities/abrc/abrchome.htm). *Arabidopsis* WT and *gaut* mutant seeds were sown on pre-moistened soil and grown to maturity under 60% constant relative humidity with a 14/10 light/dark cycle (14 h (19°C; 150 μEi m^{-2} s^{-1})/10 h (15°C)). The plants were fertilized (Peters 20/20/20 with micronutrients) once a week or as needed. WT and T-DNA seeds were sown in 'growth sets' of 20 plants. Walls were harvested from multiple 8-week-old WT and PCR-genotyped mutant plants and pooled, respectively, together for wall glycosyl residue composition analysis. The following tissues were harvested for the wall analyses: the apical inflorescence excluding the young siliques; the young fully expanded leaves approximately 3 cm long; green siliques; and the top 8 cm of actively growing stem minus the inflorescence and siliques.

DNA Extraction and Mutant Genotyping

Fresh, flash-frozen leaf tissue (100–200 mg) was ground with a 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 μg ml^{-1} proteinase K and 1% (w/v) n-lauroylsarcosine) and extracted with an equal volume of phenol:chloroform: isoamyl alcohol (49:50:1, v/v). RNA was degraded by addition of 2 μl of DNase-free RNase A (10 mg ml^{-1}) for 20 min at 37°C. The DNA was precipitated twice with 70% (v/v) ethanol and suspended in a final volume of 50 μl. Primers used for mutant genotyping were designed by ISECT tools (http://signal.salk.edu/isects.html). 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 (see Supplemental Table 2).

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 2 ml of 80% (v/v) ethanol, 100% ethanol, chloroform:methanol (1:1, v/v), and 100% acetone. Centrifugation in a table-top centrifuge at 6000 *g* for 10 min was used to pellet the sample 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 resulting pellet was washed three times with sterile water, twice with acetone, and dried in a rotary speed-vac overnight at 40°C and weighed.

Mucilage Extraction

Mucilage was extracted from 200 *Arabidopsis* seeds incubated with sterile water at 60°C over the course of 6 h as follows. Each hour during the 6-h period, the seeds were centrifuged and the supernatant was transferred to a sterile tube. The combined supernatants were lyophilized and re-suspended in 600 μl of sterile water. Phenol-sulfuric (Dubois et al., 1956) and *m*-hydroxybiphenyl (Blumenkrantz and Asboe-Hansen, 1973) assays, to quantify total sugars and uronic acids, respectively, were carried out using 100 μl of the mucilage extracts. Duplicate 200 μl aliquots of the mucilage extract were used for glycosyl residue composition analyses. To analyze the seed coat material remaining after extraction, the water-extracted seeds were aliquoted in water to glass tubes and 20 μg 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) as acetone suspensions to individual tubes and allowed to air dry. Inositol (20 μg) was added to each tube and the samples were lyophilized and analyzed for glycosyl residue composition by combined gas chromatography–mass spectrometry (GC–MS) of the per-O-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acid methanolysis basically as described by York et al. (1985). 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 min. The cooled samples were evaporated under a stream of dry air, re-suspended in 3 ml of hexane, and filtered through packed glass wool. The dried samples were re-suspended in 150 μl of hexane and 1 μl of sample was injected onto an HP 5890 gas chromatograph interfaced to a 5970 MSD using a Supelco DB1 fused silica capillary column.

Statistical Analyses

The variance ratio test (α = 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, NC, 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, p. 105 of Biostatistical Analysis, 4th edn (Zar, 1999) (Supplemental Table 1).

RNA Extraction and RT–PCR

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% (w/v) SDS in 50 mM Tris-HCl pH 7.8 and 40% water-saturated phenol) and shaken for 15 min at 25°C. Tissue samples were centrifuged for 10 min at 8000 *g* and 4°C, and the supernatant removed to a clean tube. The samples were extracted two times with phenol:chloroform:isoamyl alcohol (25:24:01, v/v) and the aqueous phases were pooled. RNA was precipitated overnight with 0.1 vol. of 3 M sodium acetate and 2.5 vol. 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 (see Supplemental Table 2). 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 alongside *GAUT*-specific reactions, utilizing primers designed to the small ribosomal protein L23 alpha, wherein the primers do not produce a product in genomic DNA (Volkov et al., 2003). Qualitative RT–PCR was carried out using 5 μg of total RNA in a 20-μl RT first-strand synthesis reaction that contained oligo(dT) primers. The RT first-strand reaction (2 μl) was added to a PCR reaction mix containing the respective *GAUT* gene-specific primers and amplified for 30 cycles. Semi-quantitative RT–PCR was done using 2 μg of total RNA in a 20-μl RT first-strand synthesis reaction containing oligo(dT) primers. An aliquot (1.5 μl) of the RT first-strand reaction was amplified through 26 cycles of PCR using *GAUT* gene-specific primers. The PCR parameters were: Step 1: 95°C for 5 min; Step 2: 95°C for 0.5 min; Step 3: 55°C for 0.5 min; Step 4: 72°C for 1.5 min; Step 5: Return to step 2 (29 or 25) times; Step 6: 72°C for 2 min; and Step 7: 4°C forever.

Mutant transcript levels were assessed as follows: knockouts (KO) were defined as mutants with RT–PCR reactions that yielded no detectable PCR product using gene-specific primers. Knockdown (KD) mutants were those that yielded a PCR product with significantly decreased intensity compared to the WT.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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