

# ARABINAN DEFICIENT 1 Is a Putative Arabinosyltransferase Involved in Biosynthesis of Pectic Arabinan in Arabidopsis<sup>1[W]</sup>

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The function of a putative glycosyltransferase (At2g35100) was investigated in Arabidopsis (*Arabidopsis thaliana*). The protein is predicted to be a type 2 membrane protein with a signal anchor. Two independent mutant lines with T-DNA insertion in the *ARABINAN DEFICIENT 1* (*ARAD1*) gene were analyzed. The gene was shown to be expressed in all tissues but particularly in vascular tissues of leaves and stems. Analysis of cell wall polysaccharides isolated from leaves and stems showed that arabinose content was reduced to about 75% and 46%, respectively, of wild-type levels. Immunohistochemical analysis indicated a specific decrease in arabinan with no change in other pectic domains or in glycoproteins. The cellular structure of the stem was also not altered. Isolated rhamnogalacturonan I from mutant tissues contained only about 30% of the wild-type amount of arabinose, confirming the specific deficiency in arabinan. Linkage analysis showed that the small amount of arabinan present in mutant tissue was structurally similar to that of the wild type. Transformation of mutant plants with the *ARAD1* gene driven by the 35S promoter led to full complementation of the phenotype, but none of the transformants had more arabinan than the wild-type level. The data suggest that *ARAD1* is an arabinan  $\alpha$ -1,5-arabinosyltransferase. To our knowledge, the identification of other L-arabinosyltransferases has not been published.

Expanding plant cells are surrounded by a primary cell wall composed of cellulose microfibrils interwoven and cross-linked with hemicellulose. The cellulose-hemicellulose network is embedded in a hydrated matrix of polysaccharides and proteins, where the major polysaccharide is pectin. Pectin consists mainly of homogalacturonan and rhamnogalacturonan I (RG I), while minor components include rhamnogalacturonan II (RG II) and xylogalacturonan (Ridley et al., 2001; Willats et al., 2001). RG I is a structurally complex molecule, consisting of a backbone of [-4- $\alpha$ -D-GalUAp-1,2- $\alpha$ -L-Rhap-1-] repeating units. The rhamnose residues often carry side chains of neutral sugars attached at the O-4 position. These side chains typically contain  $\beta$ -1,4-D-galactans,  $\alpha$ -1,5-L-arabinans, or branched type I arabinogalactans (Carpita and Gibeau, 1993; Ridley et al., 2001). Arabin-

ans may carry substitutions at C-2 and/or C-3 consisting of single  $\alpha$ -linked L-arabinofuranosyl residues or small arabinan oligomers. Arabinan side chains can either be directly attached to the RG I backbone or to short galactan chains. Experimental evidence suggests that RG I is covalently linked to homogalacturonan and substituted galacturonan since these polymers can be co-extracted from cell walls using endopolygalacturonase treatment (Schols et al., 1995; Sørensen et al., 2000). However, the exact molecular arrangement of the different pectic molecules relative to one another is not certain (Vincken et al., 2003).

The plant cell wall and its complex carbohydrate structure require intricate biochemical machinery for biosynthesis and assembly. Furthermore, the cell wall is not a static structure but develops according to developmental and environmental signals (Ridley et al., 2001). Pectin and hemicellulosic polysaccharides are synthesized from nucleotide sugars by glycosyltransferase enzymes located in the Golgi apparatus, and a large number of glycosyltransferases must be needed to catalyze the formation of all the various types of glycosidic linkage. For example, it has been proposed that at least 53 glycosyltransferases are required for the biosynthesis of pectin alone (Mohnen, 1999). Very few of the glycosyltransferases have been identified (for a recent review, see Scheible and Pauly, 2004). Assays for the measurement of some of the glycosyltransferase activities involved in pectin biosynthesis have been described, including homogalacturonan

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galacturonosyltransferase (Villemez et al., 1965; Doong et al., 1995), galactan galactosyltransferase (Geshi et al., 2002), and arabinan arabinosyltransferase (Nunan and Scheller, 2003). However, none of the pectin synthesizing glycosyltransferases have been isolated. Genetic approaches have led to the identification of three glycosyltransferases involved in RG II side chain biosynthesis: a putative glucuronosyltransferase (Iwai et al., 2002) and two homologous xylosyltransferases (Egelund et al., 2004). Only the xylosyltransferase activity has been confirmed biochemically using heterologously expressed enzyme (N. Geshi and P. Ulvskov, personal communication). A glycosyltransferase encoded by the *Qual* gene has been suggested to be involved in homogalacturonan biosynthesis, but mutants in this gene show many pleiotropic effects, preventing conclusive functional determination (Bouton et al., 2002; Orfila et al., 2005).

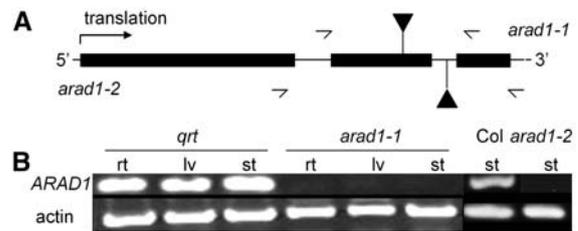
Based on PSI-BLAST sequence similarities, the Carbohydrate Active Enzymes database (CAZY; <http://afmb.cnrs-mrs.fr/CAZY>; Coutinho and Henrissat, 1999) has been constructed dividing putative and characterized glycosyltransferases into currently 78 families, 40 of which are represented in *Arabidopsis thaliana*. Putative glycosyltransferases are still present outside the CAZY database (Egelund et al., 2004; Manfield et al., 2004), but we believe that the majority of all glycosyltransferases are catalogued in the database. Family 47 contains sequences with the pfam03016 motif representing the glucuronosyltransferase domain of mammalian exostosins, which have inverting transfer activities (Lind et al., 1998). Family 47 is particularly interesting because of a large number of unique plant sequences (39 in *Arabidopsis*) and because three enzymes involved in cell wall biosynthesis have already been identified in this family: two xyloglucan galactosyltransferases (Madson et al., 2003; Li et al., 2004; X. Li and W.-D. Reiter, personal communication) and the previously mentioned RG II glucuronosyltransferase (Iwai et al., 2002).

We have used a reverse genetic approach in *Arabidopsis* for the functional characterization of putative glycosyltransferases from CAZY Family 47. Homozygous T-DNA insertion lines of the gene At2g35100, a member of Family 47, were obtained. Biochemical and immunochemical analysis of the walls in the T-DNA knockout lines clearly show a reduction in pectic arabinan. The cell wall changes indicate that At2g35100 encodes an arabinan  $\alpha$ -1,5-arabinosyltransferase.

## RESULTS

### At2g35100 Encodes a Putative Glycosyltransferase

The locus At2g35100 has an open reading frame made up of three exons (Fig. 1). The gene structure has been confirmed by isolation of full-length cDNA clones (e.g. GenBank accession BT015854.1 originating



**Figure 1.** Schematic structure and transcript analysis of the At2g35100 locus. A, Illustration of the intron-exon structure of the At2g35100 locus. The positions of the T-DNA insertions in *arad1-1* and *arad1-2* are indicated (triangles), as well as the position of the primers (arrows) used for screening by PCR. B, RT-PCR analysis of transcript in tissues from the wild type (*qrt* and *Col-0*) and the two homozygous mutant lines. RNA was isolated from mature roots (rt), leaves (lv), or inflorescence stems (st) as indicated. In the mutants no transcript was detected in any of the analyzed tissues. Actin-specific primers were used as controls.

from the Salk Institute Genomic Analysis Laboratory, La Jolla, CA). The encoded protein is calculated to have a polypeptide molecular mass of 52.8 kD. The protein is predicted to be targeted to the secretory pathway and to have a single transmembrane helix near the N terminus, hence, the protein has the features expected for a type II membrane protein targeted to the Golgi vesicles. The protein has four potential sites for N-glycosylation, but one is placed in the predicted transmembrane region. The protein is predicted to be an inverting glycosyltransferase by sequence similarity to other CAZY Family 47 proteins.

### Inactivation of the *ARABINAN DEFICIENT 1* Gene Does Not Cause a Clear Effect on Visual Phenotype

Two independent lines with T-DNA insertion in At2g35100 were identified in the Syngenta SAIL collection and in the Salk collection (Fig. 1). We have designated the mutants *arabinan deficient 1-1* (*arad1-1*) and *arad1-2*, respectively. Homozygous lines were identified by PCR, which yielded products of expected size. Heterozygous sister lines segregated the resistance marker in good agreement with a 3:1 ratio (for *arad1-1*: 65 Basta-resistant and 23 sensitive plants [ $\chi^2$  test,  $P = 0.8$ ]; for *arad1-2*: 607 kanamycin-resistant and 226 sensitive plants [ $\chi^2$  test,  $P = 0.2$ ]), suggesting that both lines contained only one T-DNA insertion. In the *arad1-1* and *arad1-2* lines, we could not detect any transcript by reverse transcription (RT)-PCR (Fig. 1). The *arad1-2* parent line is reported in the Salk database (<http://signal.salk.edu/cgi-bin/tdnaexpress>) to contain a second T-DNA on chromosome 5 in an intron of At5g02440. We confirmed by PCR that this insertion was not present in the homozygous *arad1-2* line (data not shown).

The most clear phenotypic changes were related to cell wall composition (see below). During vegetative growth the mutants showed no differences compared to wild type. The mutant inflorescence would occasionally show slight differences such as thicker stems

and larger cauline leaves, but often the mutant could not be distinguished from the wild type.

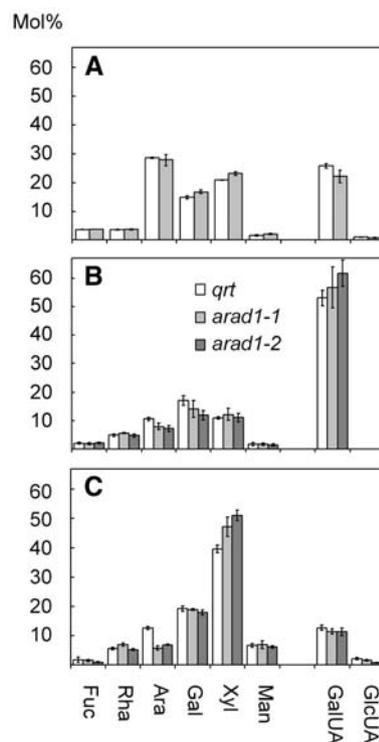
Since radial cell expansion appeared to be increased in stem tissue under certain conditions, we decided to investigate possible modifications to the cellulose-glucan load-bearing network by growing seedlings on medium containing the herbicide isoxaben. This compound inhibits cellulose biosynthesis (Heim et al., 1990) and causes a compensatory increase in biosynthesis of noncellulosic polysaccharides, particularly pectin (Shedletzky et al., 1990; Manfield et al., 2004). Hence, a defect in noncellulosic polysaccharides can be expected to result in a stronger phenotype in the presence of isoxaben due to the inability to compensate for the loss of cellulose. However, the mutant plants did not respond differently from the wild type to isoxaben treatment (data not shown).

### T-DNA Insertion in *ARAD1* Causes a Decrease in Cell Wall Arabinose

To investigate which cell wall polymer may be affected by the mutations, cell wall monosaccharide composition analyses were carried out on alcohol insoluble residue (AIR) obtained from *arad1-1*, *arad1-2*, and wild-type leaf, inflorescence stem, and mature root tissues. AIR prepared from mutant leaves and stems showed a statistically significant reduction of 25% and 54%, respectively, in levels of Ara when compared to wild-type levels (ANOVA,  $P < 0.0001$  for leaves and  $P < 0.000001$  for stem; Fig. 2). A small reduction in Gal could also be observed in leaf AIR, but no reduction in Gal could be observed in stem AIR. No significant changes were observed in monosaccharide composition in root samples (Fig. 2). Since both homozygous mutants *arad1-1* and *arad1-2* showed identical (ANOVA,  $P > 0.3$  for leaves and  $P > 0.9$  for stems) and significant decreases in leaf and stem Ara, we conclude that these alterations are due to the mutation of the *ARAD1* gene. By crossing the homozygous mutants, we confirmed that the *arad1-1* and *arad1-2* mutations are allelic and recessive (Table I).

### Transformation with p35S::*ARAD1* Leads to Complementation of the Mutant Phenotype

To further investigate the cell wall phenotype described above, the *ARAD1* gene driven by the cauliflower mosaic virus (CaMV) 35S promoter was transformed into *arad1-2* and wild-type plants, and the monosaccharide composition of total cell wall AIR from the inflorescence stem of the transformants was determined. In the *arad1-2* background, transformation with the *ARAD1* gene restored the Ara content to the wild-type level (ANOVA,  $P > 0.2$ ; Fig. 3), whereas in the wild-type background the high expression of *ARAD1* characteristic for the CaMV 35S promoter had no effect on the monosaccharide composition (ANOVA,  $P > 0.6$ ). These experiments show that the *ARAD1* gene can complement the *arad1* T-DNA mutant phenotype.



**Figure 2.** Monosaccharide composition of AIR isolated from wild type and *arad1* mutants. Monosaccharide content in AIR from root (A), leaf (B), and stem (C) tissue is expressed as mol% ( $n = 3$ ,  $\pm$ SD). A reduction in Ara content was observed in leaf and stem. Comparable monosaccharide compositions were observed in *arad1-1* and *arad1-2* leaves and stems. Col-0 and *qrt* wild types had indistinguishable monosaccharide compositions (data not shown).

### Expression Analysis of *ARAD1*

RT-PCR analysis of root, leaf, and stem tissue showed that the *ARAD1* gene is expressed in all three tissues (Fig. 1). Expression analysis using the GENEVESTIGATOR database confirmed that At2g35100 is expressed in the whole plant at similar levels. To investigate the expression pattern at the cellular level, we fused the promoter region of *ARAD1* with a gene for  $\beta$ -glucuronidase (GUS) and transformed plants with the  $P_{ARAD1}$ :GUS construct. In young seedlings (2 weeks old) GUS expression was observed in the stele of the root and in the vascular tissues of root and leaves (data not shown). In older seedlings (3 weeks old) the staining increased in the stele of the root and the primary leaves to eventually include all cells in these tissues (Fig. 4B). As the plants matured, GUS staining was observed in all aerial parts (Fig. 4A). Older leaves would occasionally develop a patchy staining pattern with staining at the base of or the full trichome (data not shown). Cross sections of the different parts of the stem showed a process of concentrated GUS staining throughout young stem tissue (Fig. 4C), whereas the staining decreased in the more mature parts of the stem, leading to a relatively weak staining in only the vascular cambium and secondary phloem (Fig. 4D). In the flowers staining was observed in the vascular tissue of

**Table 1.** Genetic analysis of the phenotype of *arad1* mutants

Monosaccharide composition of stem AIR was determined in wild-type (both Col-0 and *qrt*), *arad1-1*, *arad1-2*, and F<sub>1</sub> plants of crosses between *arad1-1* and wild type (*qrt*) and *arad1-1* and *arad1-2*. The female parent was *arad1-1* in both crosses. Genotype of the F<sub>1</sub> plants was confirmed by PCR. The data (mean  $\pm$  SD,  $n = 5$ ) are expressed as mol% of Ara relative to mol% of Ara in Col-0. Numbers followed by the same letter are not significantly different at the 5% level (*t* test).

Genotype	Relative Ara Content
Col-0	1 $\pm$ 0.04 a
<i>qrt</i>	0.97 $\pm$ 0.09 a
<i>arad1-1/arad1-1</i> $\times$ <i>qrt/qrt</i>	0.90 $\pm$ 0.11 a
<i>arad1-1/arad1-1</i> selfed	0.53 $\pm$ 0.04 b
<i>arad1-2/arad1-2</i> selfed	0.54 $\pm$ 0.02 b
<i>arad1-1/arad1-1</i> $\times$ <i>arad1-2/arad1-2</i>	0.52 $\pm$ 0.04 b

sepals, petals, and stamens and in pollen (Fig. 4, E to G). As seen in Figure 4E, GUS activity increased in the siliques as they matured starting from no staining in the early developing siliques to a strong staining in the fully elongated organ. The abscission region of seeds stained strongly, whereas the seed coat stained weakly (data not shown).

#### Decrease in Arabinose Is Due to a Decreased Content of Pectic Arabinan

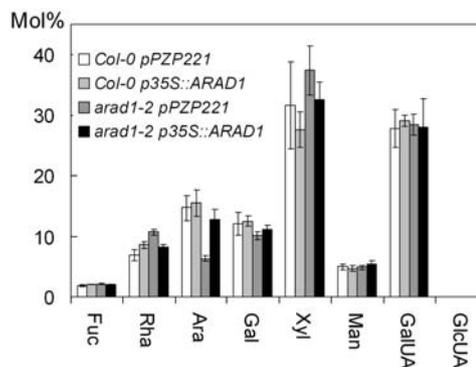
Arabinose in the cell wall is found as side chains of RG I, arabinoxylan, arabinogalactan proteins, and extensins. To identify the polymer affected in *arad1-1*, immunochemical analysis of the leaf and inflorescence stem tissue was carried out. Hand-cut leaf and stem sections were labeled with the LM6 (anti-arabinan), LM5 (anti-galactan), and LM2 (anti-arabinogalactan protein carbohydrate) antibodies (Fig. 5). A clear reduction in the LM6 labeling was observed in leaf and inflorescence stem, corresponding to a reduction in the pectic  $\alpha$ -1,5-arabinan content in these tissues. No differences were observed with the LM5 or LM2 antibodies (Fig. 5). The LM6 epitope was observed in the entire stem section, and the *arad1-1* mutation caused a decrease in all cell types but particularly in pith parenchyma. Some labeling remained in the vascular bundle of *arad1-1* leaves and stems. The specific loss of the LM6 epitope between the wild type and the *arad1-1* mutant is in good agreement with the GUS expression pattern in the stem (Fig. 4, C and D).

The LM6 antibody has previously been shown to bind to glycoproteins that can enter an SDS-PAGE gel. Immunoblot analysis was performed using protein extracted from wild-type and *arad1-1* stem tissue. No differences in the abundance of LM6, LM1 (anti-extensin), or LM2 glycoprotein epitopes were observed between wild type and *arad1-1*, indicating that there is no change to cell wall glycoproteins in *arad1-1*, including the LM6 reactive glycoproteins (Supplemental Fig. 1).

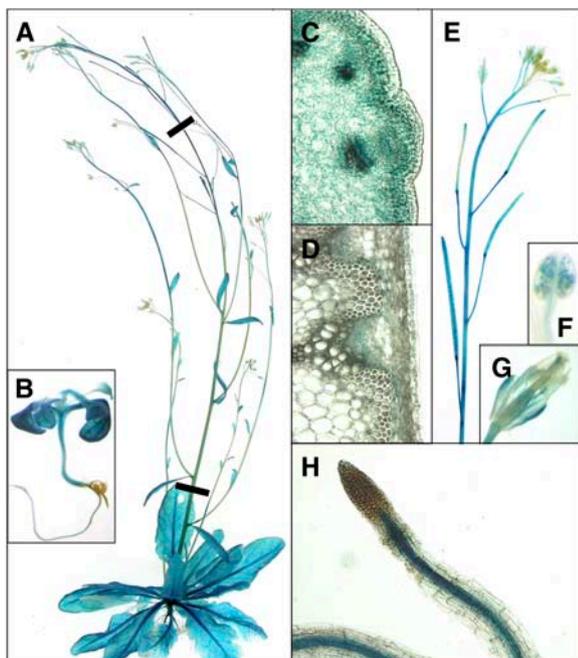
Since the immunochemical analysis indicated a specific effect on pectic arabinan, this component was

further analyzed. RG I was prepared by treating phenol-extracted AIR obtained from inflorescence stems with pectin methyl esterase and endopolygalacturonase followed by purification by size exclusion chromatography. The sugar composition of the purified RG I was analyzed and showed a larger reduction in Ara content (68% reduction) compared to total cell wall AIR (Table II), confirming the specific decrease in arabinan side chains of RG I. To further analyze the structure of RG I in the mutant, we performed linkage analysis of the purified RG I sample (Table II). The data confirmed a large reduction of arabinosyl species, in particular 5-linked arabinofuranose (5-Araf, 71% reduction; 2,5-Araf, 67% reduction; 2,3,5-Araf, 70% reduction). In addition, terminal Araf was reduced (*t*-Araf reduction by 44%), which is in agreement with the loss of 2,5-Araf and 2,3,5-Araf branch points in the arabinan. The ratio of unsubstituted rhamnose (2-Rha) to substituted rhamnose (2,4-Rha) was equal in both plant types (1: 0.8), indicating that the number of RG I side chains was not reduced. Thus, the arabinan side chains in the mutant can be considered shorter but of similar number as in the wild type.

Arabinans have been reported to be involved in the opening and closing of stomata (Jones et al., 2003). We performed a dehydration experiment by placing detached leaves in a flow bench and measuring the weight loss over time and in addition investigated the opening and closing response of stomata in light and dark conditions by microscopy. However, no alterations to stomata function were observed in *arad1-1* mutant tissue, suggesting that the decrease in Ara in the mutant does not affect stomata function (data not shown). No differences in autofluorescence of the guard cells could be observed either. This is supported by the promoter-GUS analysis, which showed that *arad1* is not highly expressed in epidermis and stomata (data not shown).



**Figure 3.** Complementation analysis. Monosaccharide composition of AIR from inflorescence stems is expressed in mol% ( $n = 5$ ,  $\pm$ SD). AIR was isolated from wild type transformed with empty vector (Col-0 pPZP221), wild type transformed with 35S::ARAD1 (Col-0 35S::ARAD1), *arad1-2* transformed with empty vector (*arad1-2* pPZP221), and *arad1-2* transformed with 35S::ARAD1 (*arad1-2* 35S::ARAD1). Full complementation of the reduced Ara content in *arad1-2* could be observed in *arad1-2* when transformed with 35S::ARAD1.



**Figure 4.** GUS staining activity of *Arabidopsis* plants transformed with a promoter-GUS fusion for ARAD1. Three-week-old (B) and 9-week-old (A and C–H) transformants were stained and photographed. Locations of the cross sections of the stem viewed in C and D are indicated in A by black bars, corresponding to the top bar (C) and to the lower bar (D).

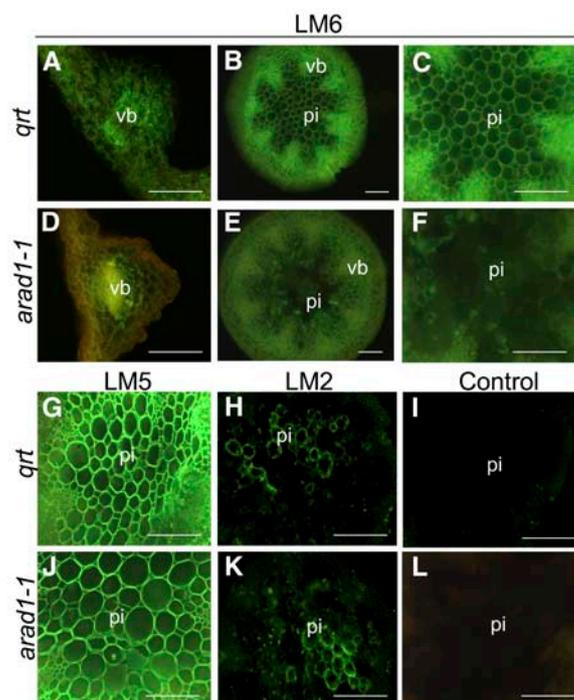
## DISCUSSION

### Mutation in ARAD1 Causes a Specific Reduction in Arabinan Content

Several lines of evidence show that the phenotype of the mutants is caused by mutation in the ARAD1 gene. First, both mutants have a T-DNA insertion in the gene and lack transcript. Second, they show identical phenotype with respect to cell wall sugar composition. Third, while it cannot be excluded that other unknown mutations are present in the genomes of *arad1-1* and *arad1-2*, they do not appear to contain any additional T-DNA insertions. Finally, when expressing ARAD1 in the *arad1-2* mutant, the cell wall sugar composition is restored to wild type.

The ARAD1 protein appears to be involved in the biosynthesis of pectin and specifically in the biosynthesis of arabinan side chains of RG I. The mutation in ARAD1 does not appear to result in changes to other Ara-containing polymers, including arabinogalactan and extensin proteins. Mutations that cause a change in cell wall composition may often have pleiotropic effects. As an example, the *qua1-1* mutation is associated with decreased homogalacturonan, but the tissues are highly distorted and other polymers are also affected, e.g. xylan (Bouton et al., 2002; Orfila et al., 2005). In contrast, the *arad1* mutations are much more subtle and specific. Sometimes a small increment in stem width arising from increased cell size in the pith

parenchyma and slightly larger cauline leaves could be observed in *arad1*, but apart from this we did not observe any change in the structure of the stem or leaf tissues. Furthermore, we have investigated several different polymers and found no significant changes in any polysaccharide besides arabinan. Therefore, we find it very likely that the decreased arabinan content is a direct result of the mutation. Linkage analysis of RG I isolated from the *arad1-1* mutant showed the presence of small amounts of arabinan with linkage very similar to wild type. Immunolabeling of stem and leaf sections with the LM6 anti-arabinan antibody supports this observation. The mutant tissue contained some LM6 reactive glycoprotein material, which may be associated with the vascular bundle. This material was not affected by the *arad1-1* mutation, suggesting that other glycosyltransferase activities may be responsible



**Figure 5.** Immunofluorescence labeling of hand sections of wild-type (*qrt*) and *arad1-1* tissue with the LM6, LM5, and LM2 antibodies. Immunofluorescence labeling of transverse sections of mature leaf and elongating stems with the LM6 anti- $\alpha$ -1,5-arabinan antibody revealed a significant reduction in the  $\alpha$ -1,5-arabinan epitope in *arad1-1* leaf (D) and stem (E and F) compared to wild-type leaf (A) and stem (B and C). Pith parenchyma cell walls were particularly devoid of labeling in *arad1-1* (C) compared to wild type (F), even though the cellular structure appeared to be similar in *arad1-1* and wild-type tissue. Labeling with the LM5 anti- $\beta$ -1,4-galactan antibody showed similar levels of the epitope in wild-type (G) and *arad1-1* (J) stems. Labeling with the LM2 anti-arabinogalactan protein carbohydrate antibody showed that the epitope was of equally low abundance in both *arad1-1* (K) and wild-type (H) stems, and was restricted to some cells of the pith parenchyma. In control experiments, no primary antibody was used (I and L). Scale bar = 100  $\mu$ m. vb, Vascular bundle; pi, pith parenchyma. The experiment was carried out with two different batches of plants, and each time two plants were analyzed with essentially the same results.

**Table II.** Monosaccharide composition and linkage analysis of *RG I* isolated from inflorescence stems from wild type (*qrt*) and *arad1-1*

Data are expressed as mol%.

	<i>qrt</i>	<i>arad1-1</i>
Monosaccharide composition		
Fuc	0.4	0.8
Rha	15	19
Ara	37	12
Gal	27	36
Xyl	0.7	1.6
GalUA	20	31
Total	100	100
Linkage analysis		
2-Rha	10	16
2,4-Rha	8.1	14
<i>t</i> -Xyl	0.6	0.5
<i>t</i> -Gal	7.1	12
4-Gal	18	20
3-Gal	3.2	3.7
6-Gal	2.9	3.4
3,6-Gal	4.1	5.3
<i>t</i> -Araf	1.6	0.9
5-Araf	25	7.3
2,5-Araf	6.0	2.0
2,3,5-Araf	4.8	1.4
4,6-Hexp <sup>a</sup>	1.7	2.8
Total	93.0	88.8

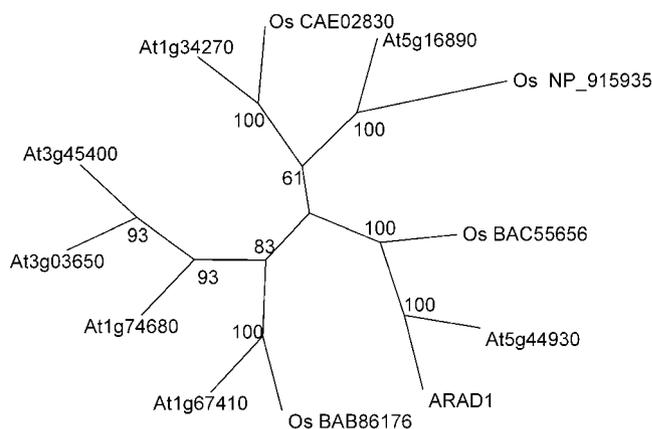
<sup>a</sup>The mass spectrometry fragmentation pattern indicated that this sugar is a hexose, but its identity could not be established.

for its biosynthesis. Since the *arad1-1* T-DNA insertion is in exon 2 and no transcript could be detected, it is highly unlikely that any functional ARAD1 protein is expressed in the mutant. However, the Arabidopsis genome encodes one relatively close homolog to the ARAD1 protein (At5g44930; Fig. 6 and supplemental material). The two Arabidopsis proteins are 65% identical. At5g44930 is not highly expressed in any tissue in Columbia (Col)-0 background. However, it is conceivable that the lacking biochemical phenotype observed in the roots of the *arad1-1* mutant and the small amount of arabinan still present in other tissues, as indicated by the 1,5-Ara in the linkage analysis, could be due to expression of At5g44930. Also, we cannot exclude that expression of At5g44930 is up-regulated in *arad1* mutants. Rice (*Oryza sativa*) appears to encode only one protein (accession no. BAC55656) that is a close homolog to Arabidopsis ARAD1 and At5g44930. The rice sequence is 58% identical to ARAD1. The other Arabidopsis proteins in subgroup B of Family 47 (Li et al., 2004) are more distantly related to ARAD1 with levels of identity below 40%. This comparison suggests that ARAD1 and At5g44930 represent a recent duplication. Therefore, the residual arabinan present in the absence of ARAD1 activity is likely to result from activity of somewhat redundant homologs. Future analysis of mutants in the homologous genes will be required to determine the extent of overlapping function.

Complementation of the mutant phenotype was achieved by expression of *ARAD1* driven by the 35S promoter, but no transgenic plants were recovered that had an elevated amount of Ara in the cell wall and transformation of the wild type with the same construct caused no detectable change in wall composition. This finding indicates that regulation of arabinan biosynthesis is not primarily at the level of transcription of the glycosyltransferase genes. Possibly, arabinan synthesis is limited by the supply of nucleotide sugar substrates or other factors are limiting, e.g. proteins interacting with the ARAD1 protein in a complex or in a metabolon, i.e. an assembly of more loosely associated proteins.

### ARAD1 Is a Member of Glycosyltransferase Family 47

The *ARAD1* gene (At2g35100) encodes a protein ARAD1, which is predicted to be an inverting glycosyltransferase belonging to CAZy Family 47. This family encodes 39 proteins and putative proteins in Arabidopsis, including two xyloglucan galactosyltransferases (Madson et al., 2003; Li et al., 2004; X. Li and W.-D. Reiter, personal communication). Tomato (*Lycopersicon esculentum*) contains xyloglucan with Ara rather than Gal, and the presence of a related galactosyltransferase gene in tomato has therefore caused speculation that it might encode an arabinosyltransferase (Madson et al., 2003). Indeed,  $\alpha$ -L-arabinosyltransferases and  $\beta$ -D-galactosyltransferases are expected to be inverting enzymes and the substrates UDP- $\beta$ -L-Arap and UDP- $\alpha$ -D-Galp have the same configurations except for the C6 hydroxymethyl group in UDP-Gal. Hence, identification of an arabinosyltransferase as being one of the members in Family 47 is not unexpected.



**Figure 6.** Phylogenetic analysis of ARAD1 homologs in Arabidopsis and rice. The analysis includes subgroup B of CAZy Family 47. A multiple alignment of the protein sequences (see supplemental data) was used to construct the tree using the PHYLIP software.

## ARAD1 Is a Putative L-Arabinosyltransferase

Based on the evidence presented here, we expect the ARAD1 protein to encode an arabinan  $\alpha$ -L-arabinosyltransferase. L-Ara is abundant in plants but has furthermore been reported in the actinomycete *Ampullariella digitata* (Fan and Feingold, 1972), the purple sulfur bacterium *Chromatium vinosum* (Hurlbert et al., 1976), and in bovine brain tissue (Wardi et al., 1966; Varma et al., 1977). None of the glycosyltransferases involved in the transfer of L-Ara in the above mentioned organisms have been identified. Final confirmation of the biochemical activity will require heterologous expression of the enzyme and determination of the activity in vitro. The presence of both L-arabinosyltransferases and D-galactosyltransferases in the same protein family also indicates that these proteins may use the same basic biochemical mechanism. However, in spite of the similarity of UDP-L-arabinopyranose and UDP-D-galactopyranose, the products are quite different. In most of the polymers where Ara is found, including arabinan, it is present in the furanose ring configuration, not in the pyranose configuration. This could suggest that the substrate for arabinosyltransferases might be UDP-L-arabinofuranose, but this compound has not been found in plants. Many bacteria contain D-galactofuranose in the coat, and this sugar is incorporated from UDP-galactofuranose, which is formed from UDP-galactopyranose by a mutase. Interestingly, bacterial UDP-galactopyranose mutase has been shown to be active on UDP-L-Ara (Zhang and Liu, 2001). This would suggest a possible mechanism for incorporation of arabinofuranose into plant polymers, but unfortunately no protein in *Arabidopsis* appears to be even remotely similar to bacterial UDP-galactopyranose mutase. In microsomal or Golgi membranes, we have detected incorporation of arabinofuranose from UDP-arabinopyranose in both wheat (*Triticum aestivum*; Porchia et al., 2002) and mung bean (*Vigna radiata*; Nunan and Scheller, 2003). However, after solubilization we could never detect any incorporation of arabinofuranose but only of arabinopyranose and the activity was very low (Nunan and Scheller, 2003). We interpret these findings as evidence that the membranes contain a mutase activity that is closely coupled with the transferase activity.

## The Function of Arabinan

Arabinans are part of pectin in all plants, but their amount varies between species. Few studies have been carried out that may link arabinans to any specific function. Potato (*Solanum tuberosum*) plants expressing a Golgi-localized arabinanase had highly reduced arabinan content (approximately 30% pectic arabinan compared to wild type) but did not show any particular phenotype (Skjøt et al., 2002). This is not surprising in view of the present finding since a similar reduction in arabinan caused a subtle phe-

notype that could only be scored with certainty by biochemical characterization. Arabinans have been implicated in stomatal function since treatment with arabinanase was observed to prevent proper function of stomata (Jones et al., 2003). However, in this investigation we did not find any deficiency in stomatal function. Phenolic compounds associated with pectin and especially arabinan have been suggested to be major components in the stomatal guard cell functionality (Jones et al., 2005). We did not observe any change in autofluorescence of guard cells in *arad1* compared to wild type. It is possible that the homolog(s) of ARAD1 is highly expressed in stomata. Another explanation may be that the mutants studied here have made compensations that allow stomatal function to be maintained in spite of low arabinan content. Arabinans can obviously be decreased to a large extent without detrimental effects on plants grown under constant and optimal conditions. Future experiments with plants grown under more fluctuating and adverse conditions may be required to reveal the biological significance of arabinans.

## MATERIALS AND METHODS

### Plant Material

*Arabidopsis* (*Arabidopsis thaliana* L. Heyn.) ecotype Col-0 was used for all experiments. Seed of T-DNA insertion line SAIL 189\_F10 (*arad1-1*; insert placed in exon 2) and the corresponding background strain (*qrt*) were obtained from Syngenta. T-DNA insertion line SALK\_029831 (*arad1-2*; insert placed 237 bp downstream of start in an intron) was obtained from the Salk Institute (Alonso et al., 2003; Fig. 1).

Plants were grown in peat at an 8-h photoperiod at 100 to 120  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 20°C, 70% relative humidity and watered using tap water as necessary. Fertilizer was not used. To initiate bolting and synchronize stem growth, plants were shifted to a 16-h photoperiod at 100 to 120  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  after 8 weeks growth in the 8-h photoperiod. Inflorescence stems were harvested when they were approximately 15 cm high. Secondary stems and flowers were removed before preparation of cell walls and RG I. Roots were obtained from plants grown hydroponically (Husted et al., 2002).

### Identification of Homozygous Plants

Genomic DNA was prepared as described by Edwards et al. (1991). Homozygosity was verified by PCR using primers suggested by Syngenta for *arad1-1* (gene-specific primers 5'-TATGTGTTTCAGGGTGGAAAAGT-3' and 5'-GGGAGACTTGACGCCAGATT-3', insert-specific primer 5'-TAGCATCTGAATGTCATAACCAAT-3') and the Salk Institute for *arad1-2* (gene-specific primers 5'-GTAGTTATGCCACGGGAGGGG-3' and 5'-GGAGTTGAGGATCGCAACACATT-3', insert-specific primer 5'-GCGTGGACCCCTTGCTGCAACT-3'; Fig. 1).

### Bioinformatics

Protein targeting was predicted using TargetP (Emanuelsson et al., 2000). Transmembrane helix prediction was performed using TMHMM (Sonnhammer et al., 1998). N-glycosylation sites were predicted using NetNGlyc (<http://www.cbs.dtu.dk/services/NetNGlyc/>). In silico expression analysis was performed using GENEVESTIGATOR (Zimmermann et al., 2004), which is based on 1,561 publicly available microarray experiments. Sequence alignments were performed using ClustalX (version 1.83) with the Gonnet series matrix, gap opening penalty of 10, gap extension penalty of 0.2, and a delay of divergent sequences of 30%. Both residue-specific and hydrophobic residue

penalties were on. Phylogenetic tree was constructed with PHYLIP (version 3.6a3; J. Felsenstein, University of Washington, <http://evolution.genetics.washington.edu/phylip.html>) and visualized using Treeview (version 1.6.6).

## Transformation of Plants with 35S::ARAD1

The coding region of *ARAD1* was amplified from the cDNA clone OAO233 (TAIR accession 2337050, originating from B. Lescure, Centre National de la Recherche Scientifique/Institut National de la Recherche Agronomique Laboratoire de Biologie Moléculaire) with primers 5'-GTCCGGAGCTC-ATGGCGCGTAAATCTTC-3' (*SacI*) and 5'-GACATGCATGCTTAAATGG-AAGTGATAAGACCG-3' (*SphI*) using Phusion polymerase (Finnzymes). The PCR product was cloned as a *SacI/SphI* fragment under the control of the 35S promoter and terminator in pP548 (Kay et al., 1987), resulting in pJJ20. Insert and vector-insert junctions were sequenced, and, subsequently, the *XbaI* fragment of pJJ20 containing the CaMV 35S promoter, *ARAD1*, and the CaMV 35S terminator was transferred to pPZP221 (Hajdukiewicz et al., 1994). Col-0 and *arad1-2* plants were transformed with the empty pPZP221 vector or with the vector containing the *p35S::ARAD1* cassette by *Agrobacterium tumefaciens*-mediated transformation using the *A. tumefaciens* strain PGV3850. Seeds were selected for 2 weeks on Murashige and Skoog medium containing 100 µg/mL gentamycin sulfate and subsequently transferred to soil. After 8 weeks on soil, the plants were transferred to 16 h light to induce bolting. Primary stems of five primary transformants from each transformation were harvested when 15 to 20 cm tall and used to prepare total cell wall AIR for determination of monosaccharide composition.

## Cell Wall Preparation

AIR was prepared as described by Fry (1988) with adaptations. Tissue of interest was boiled in 96% ethanol for 30 min. The supernatant was removed after centrifugation at 10,000g for 5 min. The pellet was washed with 70% ethanol with subsequent centrifugation until it appeared free of chlorophyll. A final wash with 100% acetone was performed, and the pellet was dried under vacuum.

Prior to RG I extraction, AIR was treated with enzymes for the removal of residual starch. Approximately 200 mg of AIR was suspended in 50 mL of 10 mM potassium phosphate buffer, pH 6.5, 1 mM CaCl<sub>2</sub>, 0.05% NaN<sub>3</sub> that had been preheated to 95°C. Starch was allowed to gelatinize for 30 s before 1 unit/mL of thermostable α-amylase (Megazyme) was added and the suspension was incubated at 85°C for 15 min. After the incubation the sample was cooled to 25°C and amyloglucosidase and pullulanase (1 unit/mL of each, both from Megazyme) were added and the suspension was incubated for 16 h at 25°C, with continuous shaking at 500 rpm. The suspension was centrifuged for 10 min at 6,000g. The pellet was washed with 50 mL of 10 mM potassium phosphate, pH 6.5, 1 mM CaCl<sub>2</sub>, 0.05% NaN<sub>3</sub>, centrifuged again at 6,000g for 10 min, and finally dried under vacuum.

## RG I Isolation

Isolation of RG I was performed by washing destarched AIR with phenol:acetic acid:water 2:1:1 (v/v/v) for 3 h (1:10 [w/v] ratio between AIR and phenol:acetic acid:water 2:1:1) at room temperature, followed by centrifugation at 6,000g for 5 min. The pellet was washed with water three times to remove phenol and extracted proteins. The pellet was incubated in buffer (50 mM cyclohexane-trans-1,2-diaminetetra-acetate, 50 mM ammonium formate, 0.05% sodium azide, pH 4.5) to a concentration of 10 mg/mL. One unit/milliliter of endopolygalacturonase (Sigma) and pectin methyl esterase (Christensen et al., 1998) was added and incubated for 16 h at room temperature. Both enzymes were homogeneous according to SDS-PAGE. After centrifugation at 6,000g for 5 min, the supernatant was collected. The pellet was resuspended in buffer and centrifuged; the supernatant was pooled with the previously obtained supernatant. The sample was dried under vacuum and resuspended in water. RG I was purified by size exclusion chromatography on a Superose 12 column (1 × 30 cm; Amersham Pharmacia) equilibrated in 0.05 M ammonium formate. Up to 1 mL of sample was applied to the column and eluted with 0.05 M ammonium formate at a flow rate of 0.8 mL/min. The eluent was monitored using a refractive index detector (model 131; Gilson). The columns were calibrated using dextran molecular mass standards (Fluka) and Glc. RG I was obtained as the high molecular mass fractions (>25 kD).

## High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection

Samples were hydrolyzed in 2 M trifluoroacetic acid for 1 h at 120°C. Trifluoroacetic acid was removed by drying under vacuum. Monosaccharide composition was subsequently determined by high performance anion exchange chromatography with pulsed amperometric detection of hydrolyzed material using a PA20 column (Dionex) as described previously (Øbro et al., 2004). Monosaccharide standards were from Sigma and included L-Fuc, L-Rha, L-Ara, D-Gal, D-Glc, D-Xyl, D-Man, D-GalUA, and D-GlcUA. For verification of the response factors, a standard calibration was performed before analysis of each batch of samples.

## Glycosidic Linkage Analysis

For linkage analysis RG I samples were per-O-methylated essentially as described by Hakamori (1964) using NaOH in dimethyl sulfoxide (Anumula and Taylor, 1992). Subsequently, the permethylated glycans were further derivatized to their corresponding partially methylated alditol acetates by trifluoroacetic acid hydrolysis, reduction, O-acetylation, and extraction as described by Anumula and Taylor (1992). The mixture of derivatized monosaccharides was separated on a 30-cm × 0.25-mm SP-2380 fused-silica capillary column (Sigma-Aldrich) using gas-liquid chromatography (6890N GC system; Agilent). The temperature profile commenced from 160°C to 200°C at 20°C per min, then the temperature was held for 5 min and finally increased to 245°C at 20°C per min. The eluting gas stream was analyzed with a quadrupole mass analyzer (5973 mass analyzer; Agilent). Sugars and linkage types were determined based upon both retention time and fragmentation pattern.

## Immunolabeling of Stem Sections and Microscopy

Transverse hand sections (approximately 0.5 mm width) of Arabidopsis stems and leaves were made using a scalpel. The sections were fixed in 4% (w/v) formaldehyde in buffer (50 mM PIPES, pH 6.9, 5 mM MgSO<sub>4</sub>, 5 mM EGTA) for 2 h at 4°C. The sections were then washed with the same buffer and labeled with anti-pectin antibodies as described by Willats et al. (2001). Antibodies used included LM5, which recognizes a short epitope of β-1,4-D-galactan (Jones et al., 1997), and LM6, which recognizes short (5–6 residues) stretches of α-1,5-arabinan (Willats et al., 1998). Labeled sections were observed with a microscope equipped with epifluorescence illumination (Olympus UK). Images were photographed using a digital camera using fixed exposure settings (Nikon UK).

For investigation of stomata, epidermal strips were peeled from 8-week-old leaves and treated as in Jones et al. (2003) for functional experiments or placed in water for autofluorescence experiments.

## Immunochemical Analysis of Proteins in Stem Tissue

Stem tissue was frozen in liquid N<sub>2</sub> and homogenized to a fine powder. Equal volumes of tissue powder and buffer (50 mM EDTA, pH 8.0, 0.25 M NaCl, 1 mM dithiothreitol, 0.75% SDS, and complete protease inhibitors [Roche Diagnostics]) were mixed vigorously and incubated for 10 min at 68°C. The samples were centrifuged at 10,000g for 10 min and the supernatants collected. Protein content was determined using Bradford reagent (Bradford, 1976), and equal amounts of protein (20 µg) were electrophoresed on 8% to 25% gradient gels prepared according to Fling and Gregerson (1986). Immunoblotting was carried out by transferring electrophoresed proteins to nitrocellulose membranes, followed by incubation with antibodies LM1 (anti-extensin; Smallwood et al., 1995), LM2 (Smallwood et al., 1996), LM5 (Jones et al., 1997), and LM6 (Willats et al., 1998) as described by Orfila et al. (2001). Aliquots of 1 µL containing 1 µg of protein were directly assayed for antibody binding by immunodot assays as described by Willats and Knox (1999).

## Promoter-GUS Analysis

A 403-bp fragment upstream of the predicted start codon of *ARAD1* was amplified with the primers 5'-ACCGGAATTCAACAACACTCCACAT-TCTAC-3' (*EcoRI*) and 5'-ACATGCCATGTGGAGATTGAAGAAGGTT-AGG-3' (*NcoI*) using Phusion polymerase (Finnzymes). This fragment covers the region from the stop codon of the upstream open reading frame

(At2g35110) to the start codon of *ARAD1*. The PCR product was cloned as an *EcoRI/NcoI* fragment in pCAMBIA1301 (CAMBIA, Canberra, Australia). To verify the integrity of the construct, the insert and vector-insert junctions were sequenced before transformation into *Arabidopsis*. Primers used for sequencing were a gene-specific antisense primer 5'-GGTGAGAGATTGAACAAC-3' and a GUS-specific antisense primer 5'-CACCAACGCTGATCAATTCCAC-3'. Plants of the Col-0 ecotype were transformed by *A. tumefaciens*-mediated transformation using the *A. tumefaciens* strain PGV3850 and selected by screening for T-DNA-encoded hygromycin resistance. Approximately 50 transformants were obtained. The transformants were selected for 2 weeks on hygromycin, transferred to soil, and grown at 20°C with 8 h light. After 6 weeks on soil, the plants were transferred to 16 h light. GUS staining was performed by overnight incubation at 37°C in GUS staining solution (50 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 1 mg/mL X-Glucuronoside). After staining the plant tissue was cleared with several washes of 96% ethanol. Older tissues were vacuum infiltrated with the GUS staining solution prior to incubation. For each developmental stage, similar staining patterns were observed for at least five individual transformants.

## RT-PCR

Total RNA was isolated according to Logermann et al. (1987), except that guanidine hydrochloride was exchanged with phenol and Tris buffer, pH 9.0. cDNA was generated with 2 µg of RNA using Iscrip (Bio-Rad). PCR was carried out using 5'-GCTCTCCACAGTCCAAAAG-3' as forward primer, 5'-ACGAGCTGCTACGAAAGGAA-3' as reverse primer, and 0.25 µg of cDNA. Primers specific for actin, 5'-GGTCGTAACCCGGTATTGTGCT-3' as forward primer and 5'-TGACAATTCACGCTCTGCT-3' as reverse primer, were used as control.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AC004667.

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