Mutants of *Arabidopsis thaliana* with altered cell wall polysaccharide composition

Wolf-Dieter Reiter\(^1\)*, Clint Chapple\(^2\) and
Chris R. Somerville\(^3\)

\(^1\)Department of Molecular and Cell Biology, and Institute of Materials Science, University of Connecticut, 75 North Eagleville Rd, Storrs, CT 06269,
\(^2\)Department of Biochemistry, Purdue University, West Lafayette, IN 47907, and
\(^3\)Department of Plant Biology, Carnegie Institution of Washington, Stanford, CA 94305, USA

**Summary**

To analyze the synthesis, structure and function of the plant cell wall by a genetic approach, 5200 chemically mutagenized *Arabidopsis* plants were screened for changes in the monosaccharide composition of hydrolyzed cell wall material by gas chromatography of alditol acetates. This screening procedure identified 23 mutant lines representing 11 different loci designated *mur*1 to *mur*11. The *mur* lines fall into essentially three groups: (1) complete absence of a monosaccharide, (2) significant reduction in the amount of a single monosaccharide, and (3) complex alterations in the relative amounts of several monosaccharides. All mutants in the first category represent alleles of the *mur*1 locus, and are deficient in the *de novo* synthesis of fucose. Mutants with reductions in a single monosaccharide have been identified for fucose (*mur*2, *mur*3), arabinose (*mur*4, *mur*5, *mur*6, *mur*7), and rhamnose (*mur*8). Mutants with complex changes in monosaccharide composition are represented by the *mur*9, *mur*10 and *mur*11 loci. Most of the mutant lines did not show obvious morphological or physiological alterations; however, lines *mur*1, *mur*9 and *mur*10 co-segregated with reduced vigor or dwarfishm of the plants. These results demonstrate the feasibility of identifying plants with altered cell wall compositions via a biochemical screening procedure. The availability of these mutants provides novel opportunities to study the functions of cell wall polysaccharides, gain insight into the biosynthesis of cell wall material, and clone cell wall-related genes.

**Introduction**

The cell walls of higher plants represent a unique type of extracellular matrix with both structural and growth-regulating functions. The presence of a rigid cell wall correlates with a mode of development where planes of cell divisions and the dimensions of subsequent cell expansion determine the sizes and shapes of individual cells and the complex tissues and organs of which they are a part (see Carpita and Gibeaut, 1993; McCann and Roberts, 1991; and Reiter, 1994 for review). It is well established that growing plant cells expand by insertion of cell wall material into primary walls which yield to the turgor pressure of the protoplasts; however, it is far from clear how the synthesis of new wall material and expansion of the existing wall are regulated, or how neighboring cells in a growing tissue coordinate the synthesis of their respective walls which are firmly connected to each other via the middle lamella. It is also not known how synthesis of the primary cell wall is terminated, or how some cell types switch to the production of a thick secondary wall after they have reached their final size.

Plant cell walls are primarily composed of polysaccharides encompassing cellulose microfibrils and matrix components (Bacic *et al.*, 1988; McNeil *et al.*, 1984). Cellulose (1,4-β-D-glucan) is synthesized at the plasma membrane from UDP-D-glucose, and released into the apoplast where it associates with other cell wall components (Delfner and Amor, 1995). With the exception of grasses which have an unusual cell wall composition, the matrix polysaccharides can be further subdivided into hemicelluloses which bind to the cellulose microfibrils, and pectic material which is highly negatively charged and tends to form gel-like structures *in vitro* (Jarvis, 1984). The predominant hemicellulose of most plant cell walls is xyloglucan, which consists of a 1,4-β-D-glucan backbone heavily substituted by mono- or trisaccharide side chains (Hayashi, 1989). Pectic polysaccharides are usually classified as homogalacturonans, rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Aspinall, 1980; McNeil *et al.*, 1980; Stevenson *et al.*, 1988). The latter two polysaccharides are structurally highly complex, and appear to be covalently connected to homogalacturonans (Bacic *et al.*, 1988); however, the precise nature of the linkages between pectic components remains elusive. Unlike bacterial polysaccharides, plant cell wall glycanas are not composed of strictly defined repeat units, leading to some ambiguity in the structure of individual building blocks. *Arabidopsis* has previously been shown to have a type I wall typical of most higher plants (Zablickis *et al.*, 1995) making it a good genetic model for understanding cell wall synthesis, structure and function.

All matrix polysaccharides are synthesized from nucleoside-diphospho (NDP)-sugars at the endomembrane.
system (Darvill et al., 1980). Although a considerable amount of biochemical data has been obtained on the synthesis of NDP-sugars in higher plants (Feingold and Avigad, 1980), plant genes encoding nucleotide sugar interconversion enzymes have only recently been identified (Bonin et al., 1997; Dörmann and Benning, 1996; Tenhaken and Thulke, 1996). Very little is known about the translocation of NDP-sugars across the endoplasmic reticulum or Golgi membranes, and the polymerization of NDP-sugars into cell wall polysaccharides. Genes encoding membrane carriers for cell wall precursors or glycosyltransferases in cell wall synthesis have not been described.

In addition to cell wall polysaccharides, the apoplastic space contains a substantial number of proteins, glycoproteins and proteoglycans. One well-characterized class of cell wall glycoproteins are the extensins, which are heavily arabinosylated and believed to serve structural roles (Showalter, 1953; Showalter and Varner, 1989). An abundant class of wall-resident proteoglycans are the arabino-galactan-proteins (AGPs) which contain up to 98% carbohydrate, mainly D-galactose and L-arabinose (Chasan, 1994; Clarke et al., 1979; Fincher and Stone, 1983). Although a wealth of information has been obtained on the expression patterns of extensins and AGPs, the precise functions of these glycoconjugates are unclear. Two AGPs specifically expressed in the transmitting tissue of tobacco flowers have recently been shown to play an important role in pollen tube growth (Cheung et al., 1995; Wu et al., 1995).

To gain insight into the synthesis and function of plant cell wall polysaccharides, and to clone genes involved in the many aspects of cell wall synthesis, it would be useful to have mutants altered in the carbohydrate moieties of cell wall constituents. Although the complete elimination of major cell wall polysaccharides is expected to be lethal, leaky mutants or mutants defective in one of several isofunctional genes may be identifiable. Similarly, mutations that affect the synthesis of small polysaccharide side chains such as those that decorate the xyloglucan backbone may not be lethal, and could be instructive in establishing the importance and function of these polymer branches. Since it is not possible to predict visible phenotypes of mutations affecting the amount or composition of cell wall polysaccharides or cell wall-resident glycoconjugates, we used a non-biased biochemical approach to identify mutants in the synthesis of cell wall glycans. Here we report the results of a mutant screen based on the quantification of cell wall-derived monosaccharides in Arabidopsis.

**Results**

**Monosaccharide content of polysaccharides from Arabidopsis leaves**

To aid in the development of a simple screening procedure for mutants in cell wall composition, we determined the distribution of individual monosaccharides between soluble cell components (e.g. glycolipids, sugar phosphates, and nucleotide sugars), water-soluble polymers (e.g. N-linked glycans and AGPs), cell wall matrix polysaccharides, and the cellulose fraction of the wall. Leaf material was fractionated into these four components, and the neutral monosaccharides rhamnose, fucose, arabinose, xylose, mannone, galactose and glucose were quantified by gas chromatography of their respective alditol acetates following acid-catalyzed hydrolysis. As summarized in Table 1, all monosaccharides except glucose were primarily derived from cell wall matrix components. The cellulose fraction remaining after acid-catalyzed hydrolysis of matrix polysaccharides accounted for more than 75% of total glucose. Substantial percentages of galactose and arabinose were derived from water-soluble polymers, presumably representing AGPs (Table 1). Based on these data, we used the screening method outlined in Figure 1 and previously described by Reiter et al. (1993). This method permits quantification of neutral monosaccharides from cell wall matrix components together with ethanol-precipitable polymers, but excludes low-molecular-weight components and the cellulose fraction. Under these analysis conditions, the monosaccharides rhamnose, fucose, xylose and mannone were more than 75% derived from the cell wall matrix as summarized in Figure 2. Accordingly, mutants with quantitative changes in these four monosaccharides were expected to have an altered wall. The remaining two monosaccharides (arabinose and galactose) were more than 30% derived from water-soluble glycans, leading to the possibility of identifying AGP mutants in addition to mutants in arabinose- and galactose-rich cell wall polysaccharides. To avoid complications in data evaluation caused by leaf starch, glucose was disregarded in subsequent experiments, including the mutant screen.

**Physiological variability of sugar composition values**

It is commonly observed that quantitative measurements of individual cell components will reveal some variability within a genetically homogeneous population. Therefore, in designing a genetic screen, it is necessary to define a range of values that is considered wild-type, and can be used as an objective standard to identify mutant plants. In this respect, we evaluated three different sets of reference values or ‘windows’ of wild-type cell wall monosaccharide composition values: the first one was based on different developmental stages of Arabidopsis leaves (Columbia ecotype), the second on leaves from known mutants of Arabidopsis with severe biochemical or morphological aberrations, and the third on leaves from different geographical isolates (France, Germany, Russia and the Cape Verde Islands). The first and second ‘windows’ were intended to identify plants with unusual monosaccharide compositions.
Table 1. Amounts of neutral monosaccharides (in µg g⁻¹ fresh weight) in different fractions of leaf material.

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Ethanol extract</th>
<th>Soluble polymers</th>
<th>Cell wall matrix</th>
<th>Cellulose fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnose</td>
<td>75.3 ± 2.2</td>
<td>15.2 ± 0.12</td>
<td>682 ± 3.5</td>
<td>ND</td>
</tr>
<tr>
<td>Fucose</td>
<td>6.57 ± 0.14</td>
<td>37.1 ± 0.13</td>
<td>126 ± 0.18</td>
<td>ND</td>
</tr>
<tr>
<td>Arabinose</td>
<td>47.9 ± 1.1</td>
<td>200 ± 4.9</td>
<td>435 ± 1.7</td>
<td>ND</td>
</tr>
<tr>
<td>Xylose</td>
<td>29.9 ± 0.30</td>
<td>44.0 ± 1.4</td>
<td>834 ± 2.7</td>
<td>29.3 ± 0.49</td>
</tr>
<tr>
<td>Mannose</td>
<td>41.6 ± 1.2</td>
<td>54.2 ± 0.53</td>
<td>229 ± 2.8</td>
<td>87.3 ± 1.5</td>
</tr>
<tr>
<td>Galactose</td>
<td>629 ± 18</td>
<td>422 ± 4.6</td>
<td>749 ± 4.9</td>
<td>ND</td>
</tr>
<tr>
<td>Glucose</td>
<td>566 ± 20</td>
<td>13.9 ± 0.23</td>
<td>518 ± 4.6</td>
<td>4090 ± 56</td>
</tr>
</tbody>
</table>

Data represent means of four determinations ± standard deviations. ND, not detectable.

Figure 1. Overview of the screening procedure for mutants in cell wall glycans.

Indirectly caused by developmental or biochemical defects unrelated to cell wall synthesis, and the third window was intended to disregard putative mutants that were still within the range of cell wall compositions observed in naturally occurring populations. The highest and lowest values found in any of these sets of experiments were used to define upper and lower thresholds for mutant screen evaluation purposes.

To determine the influence of the developmental stage on monosaccharide compositions, newly emerging rosette leaves from 3-week-old plants were compared with fully developed leaves harvested from plants of different ages. As shown in Figure 3(a), the relative amounts of fucose, xylose and mannose increased with the age of fully expanded leaves, while the amount of galactose decreased. The relative amounts of rhamnose and arabinose were not significantly affected by the age of the leaves. The sugar composition of newly emerging leaves was significantly different from their fully expanded counterparts, most notably for rhamnose and arabinose (Figure 3a). As summarized in Table 2, the differences in sugar composition observed in this set of experiments defined the upper threshold for fucose, arabinose, xylose and mannose, and the lower threshold for galactose.

To determine the variability in cell wall monosaccharide composition between different geographic isolates, fully expanded rosette leaves from ten different ecotypes of *Arabidopsis* were analyzed (Figure 3b). The differences in monosaccharide compositions observed in this experiment defined the upper threshold for rhamnose and the lower
Figure 3. Monosaccharide composition of (a) leaves in different stages of development, (b) leaves from different ecotypes, and (c) leaves from selected mutant lines. Error bars represent standard deviations with a sample size of four.
Table 2. Ranges of sugar composition values considered ‘wild-type’ for mutant screen evaluation purposes.

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Lower threshold</th>
<th>Upper threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnose</td>
<td>14.9 (ecotypes)</td>
<td>21.7 (ecotypes)</td>
</tr>
<tr>
<td>Fucose</td>
<td>2.9 (ecotypes)</td>
<td>6.0 (developmental stages)</td>
</tr>
<tr>
<td>Arabinose</td>
<td>15.9 (ecotypes)</td>
<td>23.7 (developmental stages)</td>
</tr>
<tr>
<td>Xylose</td>
<td>12.6 (ecotypes)</td>
<td>22.1 (developmental stages)</td>
</tr>
<tr>
<td>Mannose</td>
<td>4.3 (mutant lines)</td>
<td>8.7 (developmental stages)</td>
</tr>
<tr>
<td>Galactose</td>
<td>27.4 (developmental stages)</td>
<td>44.4 (mutant lines)</td>
</tr>
</tbody>
</table>

The values (in weight percentage of total) represent the mean of four determinations ±3 standard deviations in case of the upper threshold, or ±3 standard deviations in case of the lower threshold. The data set used for threshold determination is shown in parentheses.

thresholds for rhamnose, fucose, arabinose and xylose (Table 2).

Finally, we compared the monosaccharide composition from four known mutant lines of Arabidopsis with that of wild-type Columbia (Figure 3c). Two of the mutant lines were auxin-resistant dwarfs (axr1-12 and axr-2), one line was chlorotic (ch1-3), and the fourth line (CS2224) represented a marker strain carrying a total of nine mutations. Sugar composition data obtained for marker line CS2224 defined the lower threshold for mannose and the upper threshold for galactose. The other mutants remained within the boundaries set by the analysis of developmental stages and ecotypes.

Mutant screen

Single leaves from 5200 ethyl methanesulfate (EMS)-mutagenized M3 plants (ecotype Columbia) were screened for the relative amounts of the neutral sugars rhamnose, fucose, arabinose, xylose, mannose and galactose as outlined in Figure 1. Histograms containing the data from at least 100 plants were evaluated for samples with at least one monosaccharide outside the Gaussian distribution, or at least two monosaccharides at the edges of the distributions. A total of 166 putative mutants designated CW1–CW166 were identified during this primary screen. At least 20 M3 progeny per line were re-screened, yielding 70 lines with heritable changes in cell wall composition. Since these changes could have been caused by the combined action of several mutated genes, all 70 lines except for the mur1 line CW149 were back-crossed to wild-type plants, and at least 60 F2 progeny per line were analyzed for their inheritance patterns. Thirty-seven lines were found to be reselectable from F2 progeny with segregation ratios close to 3:1, suggesting single recessive Mendelian traits. Leaf sugar composition values were then determined in a single experiment for all lines, and the data were compared with the threshold values established previously (Table 2). Twenty-three lines showed sugar composition values which were consistently outside the thresholds for at least one monosaccharide, and were kept as the final set of

Table 3. Mutants with changes in cell wall monosaccharide composition.

<table>
<thead>
<tr>
<th>Locus /alleles</th>
<th>Phenotype</th>
<th>Highest number of back-crosses</th>
</tr>
</thead>
<tbody>
<tr>
<td>mur1-1 to mur1-8</td>
<td>-fuc</td>
<td>6</td>
</tr>
<tr>
<td>mur2</td>
<td>↓ fuc</td>
<td>4</td>
</tr>
<tr>
<td>mur3-1, mur3-2</td>
<td>↓ fuc</td>
<td>5</td>
</tr>
<tr>
<td>mur4-1 to mur4-4</td>
<td>↓ ara</td>
<td>6</td>
</tr>
<tr>
<td>mur5</td>
<td>↓ ara</td>
<td>4</td>
</tr>
<tr>
<td>mur6</td>
<td>↓ ara</td>
<td>4</td>
</tr>
<tr>
<td>mur7</td>
<td>↓ ara</td>
<td>4</td>
</tr>
<tr>
<td>mur8</td>
<td>↓ rha</td>
<td>3</td>
</tr>
<tr>
<td>mur9</td>
<td>↓ fuc ↓ xyl</td>
<td>5</td>
</tr>
<tr>
<td>mur10-1, mur10-2</td>
<td>↓ fuc ↑ ara ↓ xyl</td>
<td>5</td>
</tr>
<tr>
<td>mur11</td>
<td>↓ rha ↓ fuc + xyl ↑ man</td>
<td>2</td>
</tr>
</tbody>
</table>

- without; ↓, decreased; ↑, increased.

fuc, fucose; ara, arabinose; xyl, xylose; rha, rhamnose; man, mannose.

Since most of these plants were expected to have altered cell walls, they were designated ‘mur’ lines (from Latin murus, the wall).

Complementation analysis

The 23 mutant lines identified above were placed into six phenotypic groups: 11 lines completely or partly deficient in fucose, seven lines partly deficient in arabinose, one line partly deficient in rhamnose, two lines with a considerable increase in arabinose content, one line partly deficient in fucose and xylose, and one line with partial deficiencies in rhamnose, fucose and xylose and a considerable increase in its mannose content. The lines with deficiencies in fucose were crossed to each other, and monosaccharide composition values were determined in the F1 generation. This analysis yielded three complementation groups (mur1, mur2 and mur3) with eight, one and two alleles, respectively (Table 3). Six of the mur1 lines contained less than 2% of the wild-type amount of fucose, and were considered tight alleles. The remaining two mur1 lines (mur1-3 and mur1-7) contained approximately one-third and one-tenth
of the wild-type amount of fucose, respectively, and were considered leaky alleles. Plants carrying the mur2 and mur3 mutations were approximately 50% deficient in their fucose content. The three loci mapped to different regions of the Arabidopsis genome confirming the results of complementation experiments (J. Li and W.-D. Reiter, unpublished results).

The seven lines with reductions in arabinose content fell into four complementation groups (mur4, mur5, mur6 and mur7) with four alleles at the mur4 locus, and one allele each at the other three loci (Table 3). The mur4, mur5 and mur6 mutations have been mapped to different locations in the Arabidopsis genome confirming the results of complementation experiments (E. Burget, R. Verma, and W.-D. Reiter, unpublished results). The two lines with an increased arabinose content (lines CW133 and CW157) showed reduced vigor and low self-fertility and could not be crossed to each other. To circumvent this problem, plants from line CW133 as the maternal parent were crossed to a CW157 heterozygote as the pollen donor. This cross yielded five plants of which three were similar to the homozygous mutant lines in their cell wall composition and visible phenotype. The remaining two plants were wild-type in both respects. These results indicate that lines CW133 and CW157 are allelic at a locus designated mur10. The remaining three lines were assigned the locus designations mur8, mur9 and mur11. The monosaccharide composition values for representative lines from the 11 complementation groups are summarized in Figure 4.

Visible phenotypes of mur lines

Mutant lines mur2–mur8 and mutant line mur11 did not show obvious alterations in their growth habit or physiology, indicating that the observed alterations in monosaccharide composition were not indirectly caused by a major developmental or physiological aberration. Lines carrying tight mur1 alleles were slightly dwarfed, and showed considerable brittleness in elongating inflorescence stems (Reiter et al., 1993). As shown in Figure 5, plants carrying the mur9 mutation grew very slowly during their rosette stage. Most of the seedlings died early during development.
but plants surviving to the bolting stage developed normal inflorescences. The two mur10 lines were characterized by slow growth, dark-green leaves (Figure 5) and very low seed-set. The morphological phenotypes observed in mutant lines mur1, mur9 and mur10 co-segregated with the cell wall-related phenotypes in F2 populations; however, a close linkage between unrelated genes cannot be ruled out except in the case of mur1 where independent alleles are available, and changes in growth habit and wall strength are clearly caused by a defect in fucose synthesis (Bonin et al., 1997; Reiter et al., 1993).

**Interactions between mur loci**

Since most of the mur mutations were not associated with a visible phenotype, we examined whether the effects of a cell wall mutation would become more obvious by combining it with another mutation known to affect the structure of the wall. A line carrying the mur4 mutation was chosen as having a significant change in monosaccharide composition but no change in its growth habit, and a mur1 line was chosen as having well-defined changes in wall properties and growth habit (brittle walls and slight dwarfism). Plants carrying both the mur1-1 and mur4-1 mutations were identified in segregating F2 populations by screening for plants completely deficient in fucose and partly deficient in arabinose. All of the double mutants were extreme dwarfs (Figure 6) with main inflorescences reaching heights of only a few millimeters. To verify that this phenotype was indeed caused by the combined action of the mur1 and mur4 mutations, construction of a mur1 mur4 double mutant was repeated using the independent alleles mur1-2 and mur4-2. All double mutants obtained in this second screen were again extremely dwarfed, and many of their progeny died before setting seed. This result confirms that the mur1 and mur4 mutations act synergistically leading to a severe morphological phenotype that was not observed in the parental lines.

**Discussion**

Although substantial information is available concerning the chemical composition of the polysaccharide components of plant cell walls, relatively little is known about the enzymes that participate in the biosynthesis of the polysaccharides, or the functions of the various polymers. The goal of the work described here was to evaluate the feasibility of identifying mutations that specifically affect the polysaccharide composition. Our working hypothesis was that, at the very least, it should be possible to identify mutations in some of the regulatory factors that govern the relative amounts of the various polysaccharides, or mutants that were partly or completely defective in the de novo synthesis of nucleotide sugars, their transport across Golgi membranes, or the incorporation of sugar moieties into polymers via glycosyl transfer. Mutations in hypothetical primers for polysaccharide synthesis or in cell wall turnover processes represent additional possibilities. Although the goal of this screen was to identify mutants defective in aspects of polysaccharide synthesis, the direct analysis of polysaccharides, or large fragments thereof, is cumbersome. Instead we chose to quantify the hydrolysis products of polysaccharides via gas chromatography as a
more robust method for characterizing the cell walls of an M2 population. The screen was based on the assumption that some mutations would cause changes in polysaccharide composition that were large enough to be detected as quantitative changes in the monosaccharide profile of the hydrolyzed cell walls.

The results presented here indicate that considerable numbers of mutations affecting cell wall biosynthesis can be identified by screening random individuals from a mutagenized population. Twenty-three mutant lines representing 11 complementation groups were identified in a population of 5200 EMS-mutagenized plants. An additional 47 lines exhibited heritable variation in monosaccharide composition but were not characterized in detail because the magnitude of the change in monosaccharide composition was considered too small to be reliably scored on individual plants. It is possible that these lines may prove useful when higher-resolution screening methods are employed. For instance, the mutant phenotype may be made more pronounced by first separating the major classes of polysaccharides before performing monosaccharide analysis.

Based on the observed changes in monosaccharide composition, the 23 characterized mutant lines fell into three classes representing 11 loci: (1) complete absence of a monosaccharide, (2) reductions in the amounts of a single monosaccharide, and (3) complex changes in the relative amounts of several monosaccharides. Lines of the first type were only obtained for fucose, and all of them mapped to the same locus, mur1. The mur1 mutation has been shown to affect the de novo synthesis of fucose (Bonin et al., 1997; Reiter et al., 1993). Mutations of the second class were identified for rhamnose, fucose and arabinose, representing one, two and four loci, respectively. Since all sugar composition values were computed as weight percentages, reductions in the relative amounts of a single monosaccharide were expected to lead to compensatory increases in the relative amounts of all other sugars. Accordingly, all monosaccharides except arabinose were slightly elevated in mur4 plants to compensate for the 50% arabinose deficiency in these lines; however, this effect was too small to cause any monosaccharide to exceed the upper evaluation threshold. Mutations of the third type (more than one monosaccharide outside the wild-type range) were observed in four lines representing three loci (mur9, mur10 and mur11). Plants carrying mutations at the mur1, mur9 and mur10 loci showed visible phenotypes while the other lines were essentially wild-type in their growth habit.

Polysaccharide mutants of Arabidopsis are affected in cell wall matrix components or glycoproteins

All of the neutral monosaccharides assayed during the mutant screen were primarily derived from cell wall poly-

saccharides. Therefore, most of the mutant lines with major changes in monosaccharide composition are expected to show structural changes in their walls. More than 80% of polymer-bound mannose was derived from cell wall polysaccharides rather than glycoproteins, although mannose represents the major monosaccharide in the carbohydrate part of N-linked glycans (Faye et al., 1989). Arabinose and galactose were sufficiently abundant in the water-soluble polymer fraction of leaf material to allow the identification of mutants in AGPs. Since the hydroxyproline content of Arabidopsis walls is very low (Zablackis et al., 1995), mutants specifically affected in the hydroxyproline-rich extensins (Kieliszewski and Lamport, 1994; Showalter, 1993) were unlikely to be identified by our screening procedure. Mutant lines mur4, mur5, mur6 and mur7 showed significant reductions in the relative amount of arabinose, making them candidates for mutations in the carbohydrate part of AGPs or the pectic component RG-I. The 50% decrease in arabinose content in the mur4 lines is too pronounced to affect AGPs exclusively. Therefore, these plants must be altered in the amounts of arabinose-rich cell wall polysaccharides.

Correlation between visible phenotypes and changes in monosaccharide compositions

During the initial mutant screen, about 20–30% of the M2 plants showed developmental or physiological abnormalities such as reduced vigor, late or early flowering, sterility, dwarfism or chlorosis. Plants with these visible phenotypes were not obviously over-represented in the initial population of putative cell wall mutants, suggesting that the monosaccharide composition of leaf polymers is not significantly affected by disturbances in the development or physiology of the plants. However, the synergistically deleterious phenotype of the mur1 mur4 double mutant suggests that mutations which cause large changes in wall composition may be expected to have large effects on growth and development.

With the exception of mutant lines mur1, mur9 and mur10, visible phenotypes could be separated from the alterations in cell wall composition by back-crossing procedures, and the visible phenotype of mur9 and mur10 plants may still be unrelated to the observed changes in monosaccharide composition since close linkage between these loci and other mutations cannot be ruled out. This possibility appears remote in case of the mur10 mutation for which two alleles with the same changes in growth habit have been isolated. All of the mur lines with visible phenotypes were either slightly dwarfed (mur1) or showed a general reduction in growth rate and vigor (mur9 and mur10). None of the mutant lines identified during this screen showed phenotypes indicative of a cell- or tissue-specific disruption of development. This result is not surprising since total leaf material was used for screening purposes leading to a selection for mutants
with general rather than cell type-specific changes in cell wall composition.

Genetic complexity of cell wall synthesis in higher plants

The screening procedure for changes in monosaccharide compositions identified only a single locus (mur) representing nearly complete loss of function in the synthesis of a monosaccharide. The mur mutation affects one of at least two isoforms of GDP-α-mannose-4,6-dehydratase (Bonin et al., 1997), and was easily identifiable since other isoform(s) appear to be confined to root tissues. The mur phenotype is still scorable in root material since our screening procedure assesses a quantitative trait allowing for detection of the 40% reduction in fucose content in mur roots (Reiter et al., 1993). This suggests that, in some cases, mutations affecting cell wall components can be identified in the presence of genetic redundancy.

In view of the fact that only one mutant allele was obtained for seven of the 11 mur loci, it is apparent that the screen was not saturating. The fact that eight independent alleles of the mur locus were recovered suggests that the seeds used for the screen were heavily mutagenized. The high number of mur mutations relative to the number of alleles at the other ten loci also presumably indicates that this gene is atypically susceptible to chemical mutagenesis. Although it would be premature to speculate on how many different kinds of mutations can be isolated by the approach described here, it seems likely that screening another 10,000 lines by similar methods would yield dozens rather than hundreds of mutations. Thus, if hundreds of genes are required for synthesis of the polysaccharide components of the cell wall, the methods used here appear to be too insensitive to identify mutations affecting the majority of the genes involved. Assuming that many different enzymes are responsible for the complex side-chain modifications of many cell wall polymers, elimination of the corresponding genes would often lead to changes in cell wall composition that are too small to be detected by our screening procedure. Since the method used does not resolve acidic sugars, potentially productive extension of the screen would be to perform a similar mutant hunt under conditions that permit quantification of acidic components of pectic material.

The mur lines described here represent a starting point for the genetic dissection of cell wall synthesis in higher plants. Concurrent progress in the mapping and complete sequencing of the Arabidopsis genome (Somerville and Somerville, 1996) will facilitate the eventual identification of the genes corresponding to the mur loci by map-based cloning methods. The identification of cell wall mutants without obvious growth deficiencies suggests that it may be possible to expand the collection of mutants with altered cell wall compositions substantially without compromising the viability of the plants.

Experimental procedures

Plant material

All of the mutant lines described here were from the Columbia ecotype. Mutagenesis of Arabidopsis seeds with ethyl methanesulfonate was as described by Haughn and Somerville (1986). Plants were grown on Pro-Mix ‘BX’ under continuous fluorescent light (60-70 μmol m-2 sec-1) at 23°C and 70% relative humidity. The following lines were obtained from the Arabidopsis Biological Resource Center at Ohio State University: CS3076 (axr1-12), CS3077 (axr2), CS3121 (ch1-3), CS2224 (ap1-1, cer2-1), hy2-1, py201, tt3-1, g1-1, b2-1, an-1, er-1), CS8901 (Ag-0), CS902 (Cvi-0), CS900 (Aa-0), CS910 (Dijon-G), CS320 (Landsberg erecta), CS2223 (WS), and CS911 (Est). The Niederzoni and RLD plants were derived from laboratory stocks maintained at the Michigan State University—Department of Energy Plant Research Laboratory (East Lansing, Michigan).

Determination of cell wall composition

To determine the cell wall composition of different ecotypes and mutant lines, plants were grown until they started to bolt, and all mature leaves from at least 10 plants per line were pooled, and extracted three times with 30 ml each of 80% ethanol for 30 min. The residue was mixed with small scissors, and equal aliquots were distributed into four microfuge tubes per line. Most lines bolted between 3 and 4 weeks after planting; however, some ecotypes showed a late-flowering phenotype, and mature leaves from these plants were harvested 31 days after planting.

The cell wall composition of leaves in different developmental stages was determined similarly by pooling plant material and dividing it into four aliquots. Leaves from 3-week-old plants were divided into two categories: fully expanded leaves from the outer parts of the rosette, and expanding leaves from the inner part of the rosette with a length of less than 6 mm. In the case of older plants, care was taken to exclude leaves showing signs of senescence.

Hydrolysis of leaf material and quantitative of monosaccharides via gas–liquid chromatography of alditol acetates were carried out essentially as described previously by Reiter et al. (1993). For calibration purposes, a mixture of 50 μg each of l-rhamnose, l-fucose, l-arabinose, d-xylose, d-mannose, d-galactose and d-glucose was autoclaved in 250 μl of 1 M H2SO4 containing 200 μg of myo-inositol as an internal standard. Relative sugar composition values were calculated on a weight percentage basis.

The determination of monosaccharide compositions as a function of developmental stage was done in an experiment separate from the analysis of ecotypes and mutant lines. The values obtained for fully expanded leaves of 3-week-old wild-type Columbia in the first experiment were used to calibrate the composition data for wild-type Columbia in the second experiment to permit direct comparison between the two data sets.

Fractionation of leaf material

To determine the amounts of monosaccharides in different fractions of leaf material, seedlings were grown for 3 weeks under continuous light as described above, then placed in the dark for 30 h to deplete starch reserves. Absence of starch was verified by iodine staining. Leaves were harvested, ground to a fine powder in a mortar cooled with liquid nitrogen, and stored at ~80°C. A 2.4 g sample of this material was extracted with 30 ml of 80% ethanol at 80°C for 30 min, centrifuged at 2000 g for 5 min, and
the pellet re-extracted with 80% ethanol as above. The combined extracts were dried in a vortex evaporator (Labconco), and the residue was redissolved in 8.5 ml of hot 80% ethanol. Four samples of 0.5 ml each were dried in vacuo in screw-cap vials, and their monosaccharide content was determined as described above. The ethanol extract contained some endogenous myo-inositol presumed to be derived from membrane lipids; however, its amount was too low to interfere with the sugar quantification procedure (4% of the myo-inositol added as an internal standard).

To fractionate leaf material into water-soluble and water-insoluble polymers, 12.75 g of pulverized leaf material were suspended in 50 ml of ice-cold buffer A (100 mM MOPS, pH 7.0, 1.5% SDS, 5 mM sodium bisulfite), and sonicated in an ice-water bath until microscopic inspection indicated >95% cell disruption. Cell wall material was pelleted by centrifugation for 10 min and 4°C in an Sorvall SS34 rotor at 15 000 rpm. The cell wall material was then washed with 25 ml of ice-cold buffer B (100 mM MOPS, pH 7.0, 0.5% SDS, 3 mM sodium bisulfite), and centrifuged as above. The two supernatants were combined as fraction S1. The cell wall pellet was washed with 50 ml of buffer B, and the supernatant discarded following centrifugation. Following several washes with water, the cell wall material was resuspended in 16 ml of water, and stored at −20°C.

Ammonium acetate was added to a final concentration of 1 M to fraction S1, and polymers were precipitated by addition of five volumes of a 1:1 mixture of ethanol and acetone. After 24 h at room temperature, the precipitate was recovered by centrifugation at 2000 g for 15 min. Addition of more acetone to the supernatant did not cause any turbidity suggesting that polymers had been efficiently removed. The pellet was washed extensively with 80% ethanol, and stored at 4°C as a suspension in 40 ml of 80% ethanol. To assay the sugar composition of fraction S1, four samples of 800 μl each were dried and hydrolyzed as described above.

To determine the monosaccharide composition of cell wall matrix components, four 160 μl aliquots of purified cell wall material were hydrolyzed for 1 h at 121°C in 250 μl of 1 M H2SO4 containing 200 μg myo-inositol. Cellulose microfibrils are expected to remain essentially intact under these conditions (Fry, 1988) and were pelleted via centrifugation. Aliquots of 200 μl of the supernatants were removed for monosaccharide quantification, and the insoluble material was washed once with 1 ml of 1 M H2SO4, followed by a wash with 1 ml of water. The residue was dried, and vigorously shaken on a vortex mixer for 2 h in 25 μl 72% (w/w) H2SO4 to dissolve the cellulose. Water was added to dilute the sulfuric acid to 1 M, and the samples were hydrolyzed for 1 h at 121°C in the presence of 200 μg of myo-inositol as an internal standard.

Acknowledgments

We wish to thank the Arabidopsis Biological Resource Center for providing seeds from different ecotypes and mutant lines. This work was supported in part by grants from the USDA National Research Initiative, the National Science Foundation, and the US Department of Energy.

References


