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TECHNICAL ADVANCE

A rapid method to screen for cell-wall mutants using discriminant analysis of Fourier transform infrared spectra

Limei Chen[1], Nicholas C. Carpita[2], Wolf-Dieter Reiter[3], Reginald H. Wilson[4], Charles Jeffries[5] and Maureen C. McCann[5,*]

[1]*School of Physics, University of East Anglia, Norwich, NR4 7TJ, UK,*
[2]*Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907, USA,*
[3]*Department of Molecular and Cell Biology, and Institute of Materials Science, University of Connecticut, Storrs, Connecticut 06269–3125, USA,*
[4]*Department of Food Biophysics, Institute of Food Research, Colney, Norwich NR4 7UA, UK, and*
[5]*Department of Cell Biology, John Innes Centre, Colney, Norwich, NR4 7UH, UK*

Summary

We have developed a rapid method to screen large numbers of mutant plants for a broad range of cell wall phenotypes using Fourier transform infrared (FTIR) microspectroscopy of leaves. We established and validated a model that can discriminate between the leaves of wild-type and a previously defined set of cell-wall mutants of *Arabidopsis*. Exploratory principal component analysis indicated that mutants deficient in different cell-wall sugars can be distinguished from each other. Discrimination of cell-wall mutants from wild-type was independent of variability in starch content or additional unrelated mutations that might be present in a heavily mutagenised population. We then developed an analysis of FTIR spectra of leaves obtained from over 1000 mutagenised flax plants, and selected 59 plants whose spectral variation from wild-type was significantly out of the range of a wild-type population, determined by Mahalanobis distance. Cell wall sugars from the leaves of selected putative mutants were assayed by gas chromatography-mass spectrometry and 42 showed significant differences in neutral sugar composition. The FTIR spectra indicated that six of the remaining 17 plants have altered ester or protein content. We conclude that linear discriminant analysis of FTIR spectra is a robust method to identify a broad range of structural and architectural alterations in cell walls, appearing as a consequence of developmental regulation, environmental adaptation or genetic modification.

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*For correspondence (fax +44 1603 501771; e-mail maureen.mccann@bbsrc.ac.uk).

Introduction

The cell walls of plant cells are complex composites of cellulose, cross-linking glycans, proteins and pectic substances. Substantial progress has been made in the isolation and structural characterization of the many non-cellulosic polysaccharides of the primary wall and the dynamic changes they undergo during cell division, expansion and differentiation (Carpita and Gibeaut, 1993; McCann and Roberts, 1991). However, the specific functions of these molecules have been difficult to ascribe. By studying cell walls with altered compositions, either as a consequence of developmental regulation, environmental adaptation or genetic modification, we can assess the effect of such modifications on cell wall properties and plant development. The selection of mutant plants with altered cell wall compositions and architectures is particularly useful because of the wide range of potential modifications and the possibility of uncovering novel genes that encode enzymes which participate in biosynthetic pathways, wall assembly, or in modifications to polymers in muro (Reiter, 1998). Mutants with altered cellulose deposition have been identified directly by birefringence (Potikha and Delmer, 1995). Other cell-wall mutations have appeared serendipitously in the course of screens for developmental phenotypes, such as a temperature-sensitive radial cell swelling mutant that was cellulose-deficient (Arioli *et al.*, 1998; Baskin *et al.*, 1992) or a collapsed xylem phenotype (Turner and Somerville, 1997).

Mutations in non-cellulosic polysaccharides may not always have visually abnormal or otherwise predictable phenotypes, and hence a general method is necessary to select for a broad range of potential modifications. One method that has been successfully applied is to screen directly for neutral sugars of cell-wall polymers that are over- or under-represented when compared to their distribution in the walls of a wild-type population (Reiter *et al.*, 1997). This screen employs derivatisation of the cell-wall non-cellulosic sugars from leaves to alditol acetates and their quantitation by gas-chromatography. Sugar composition can be assayed with great precision using this method, but the large numbers of plants involved in a mutant screen make it labour-intensive. Fourier transform infrared

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(FTIR) microspectroscopy is an extremely rapid, non-invasive vibrational spectroscopic method that can quantitatively detect a range of functional groups including carboxylic esters, phenolic esters, protein amides and carboxylic acids, and can provide a complex 'fingerprint' of carbohydrate constituents and their organization (McCann *et al.*, 1992, 1997; Séné *et al.*, 1994). Multivariate statistical methods including linear discriminant analysis (LDA) (Massart *et al.*, 1988) can be used to classify an unknown sample as a member of a particular group. For example, Kemsley *et al.* (1994) were able to discriminate between different fruits and vegetables on the basis of spectral differences of their parenchymal cell walls. In a mutant screen, hundreds of spectra must be compared with a wild-type collection and any subtle spectral changes are difficult to evaluate by simple inspection. By the use of a data reduction technique such as principal component analysis (PCA) (Massart *et al.*, 1988), a quantitative measurement of the spectral variation of a wild-type population can be determined and individuals with variability greater than this in an unknown population can be identified.

The advantages of FTIR spectroscopy for a mutant screen are many-fold. The time taken to acquire a spectrum of a sample is approximately 30 sec. It is non-destructive and does not require derivatization of the sample, so further assays can be applied to the same sample once potential mutants are identified. Several functional groups absorb infrared radiation at characteristic frequencies, making assignments of some specific wall components possible. In particular, frequencies corresponding to carboxylic and phenolic esters, the amide stretching bonds of proteins, and the carboxylate stretches of uronic acids are clearly resolved in the spectrum. Alterations in polymer orientation and conformation can also be detected. In this paper, we describe the use of FTIR spectroscopy in conjunction with PCA and LDA as tools for detecting a broad range of cell-wall mutants from amongst a large mutagenised population. Using the collection of cell-wall mutants in *Arabidopsis* (Reiter *et al.*, 1997), we established the conditions for sample preparation and generated the specific parameters that optimize statistical analyses of cell-wall components. We demonstrated that PCA of the spectra and LDA can discriminate between wild-type and mutants. We then applied these techniques to a mutagenised population of flax to identify several potential cell-wall mutants, and we confirmed that 70% of our selections had altered sugar compositions. Our studies show that LDA of FTIR spectra provides a suitable method to identify a broad range of alterations in cell wall structure and architecture.

Results

Parameters for spectral collection

To evaluate the utility of FTIR in screening for cell-wall mutants, we first used the collection of neutral-sugar mutants of *Arabidopsis* which were selected by gas-chromatographic analysis of the cell-wall sugars of leaves from an M2 population of EMS-mutagenised seeds (Reiter *et al.*, 1997). These mutants represent 23 lines falling into groups that show complete absence or significant reduction in amounts of a single monosaccharide or more complex alterations in the relative amounts of several monosaccharides. As our intention was to develop a screen that would be sufficiently rapid to survey large numbers of plants, we harvested a set of leaves from each plant and minimised sample preparation to ethanol extraction of chlorophyll, sugars and other small molecules from the intact leaves. These extracted leaves were freeze-dried to remove water, which has an interfering absorbance in the spectrum, and then used directly. Clearing the leaves in this manner allowed us to collect spectral information by transmission rather than reflectance, as the leaves have poor infrared reflectance characteristics. As acquisition times were under 1 min per spectrum, we averaged three spectra taken from different 100×100 μm areas of each leaf (excluding midribs and minor veins) to obtain a more representative sampling. The spectra obtained from all leaves were predominantly from absorbances of cell-wall components, but with some contamination from cytoplasmic proteins and starch. Representative spectra from *Arabidopsis* wild-type leaves in the region of 1800–850 cm^{-1} are shown in Figure 1(a,b). Absorbance intensity varies as a function of leaf thickness, and all subsequent data sets have been baseline-corrected and area-normalised to compensate for these differences before application of statistical methods (Kemsley *et al.*, 1994).

Principal component analysis allows groups of spectra to be compared

An individual spectrum of an *Arabidopsis* leaf (Figure 1a) is a composite of all infrared-absorbing components in the sample. In a given population of samples there will be an inherent variability in several of the components. The complexity and variability of these spectra is such that it is difficult to extract the relevant information from them by visual inspection, and data reduction techniques such as PCA and LDA must be used. Spectra are not analog, but are constructed from datapoints sampled at intervals of 8 cm^{-1}. PCA is a widely used mathematical technique that is used to reduce the dimensionality of the data from the several hundred datapoints in the original spectra to a fewer number of dimensions (Briandet *et al.*, 1996; Defernez *et al.*, 1995; Kemsley *et al.*, 1994). The variability in each individual spectrum relative to the mean of the population can then be represented as a smaller set of values (axes) termed principal components (PCs). The effect of this process is to concentrate the sources of variability in the data into the first few PCs. Plots of the

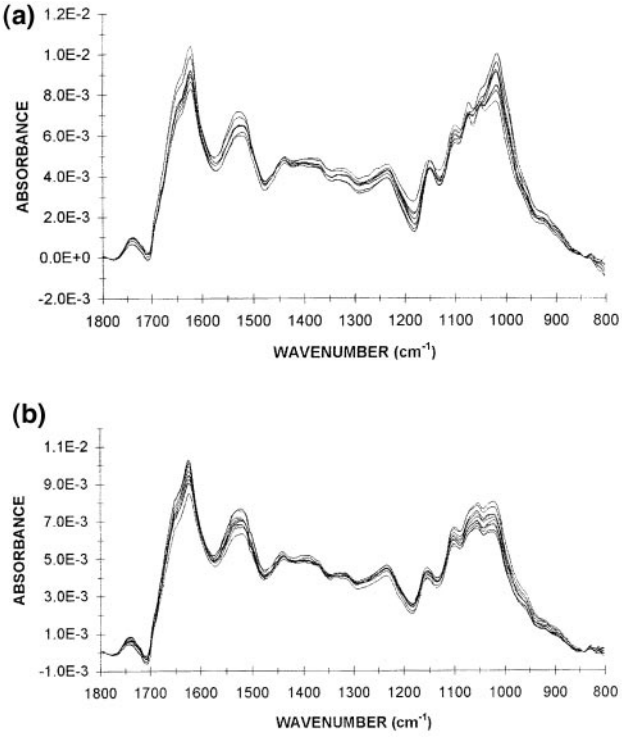

Figure 1. Area-normalized and baseline-corrected FTIR spectra in the range 1800–850 cm^{-1} obtained from leaves of eight light-grown *Arabidopsis* plants (spectra super-imposed) (a) and leaves from 10 plants grown in darkness for 2 days (b).

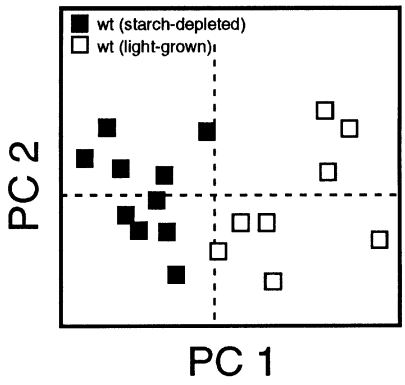

Figure 2. A plot of the first two PCs shows that the two groups of light-grown and starch-depleted *Arabidopsis* plants can be separated by the first PC score. Dashed lines indicate the mean PC scores for the entire population.

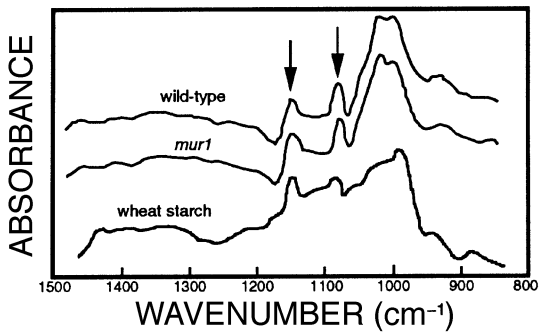

Figure 3. The loading for the first principal component (top) resembles a spectrum of wheat starch with characteristic peaks for starch indicated by arrows.
The loading for PC1 using a group of light-grown and starch-depleted *mur1* plants (middle) is identical to a loading for PC1 derived from the group of light-grown and starch-depleted wild-type plants (top).

so-called PC scores (projection on to PC axes) against one another can reveal clustering or structure in the data set.

For example, our principal question was how many of the known neutral-sugar mutants of *Arabidopsis* could be distinguished from the wild-type population on the basis of PC scores alone. As we were using whole leaves rather than isolated walls to obtain FTIR spectra, we first had to address the problem of the contribution of starch to the spectrum. We compared two sets of *Arabidopsis* wild-type plants, one of which had been grown in continuous light in a growth chamber before the leaves were harvested (Figure 1a), and another which had been starch-depleted following 2 days in darkness before the leaves were harvested (Figure 1b). A single PC score that accounted for over 90% of the total spectral variation could separate the two groups (Figure 2). It is also possible to mathematically derive a spectrum, related to the PC score (termed a PC loading), that represents an independent source of spectral variability in the data set. Examination of PC loadings can sometimes be used to identify the important molecular factors underlying clustering or discrimination (Figure 3). In this instance, the corresponding PC loading for the first PC resembles a spectrum obtained from purified wheat starch (Figure 3). The experiment was repeated using *mur1* plants with the same result (Figure 3).

Comparison of mutant and wild-type Arabidopsis *leaves*

To evaluate if starch would interfere with the identification of a mutant from a wild-type *Arabidopsis* leaf, we compared the collection of 11 different mutant plants compared to the *Arabidopsis* wild-type population, each grown with a daily 16 h photoperiod. Figure 4(a) shows 20 representative spectra collected from wild-type leaves, and Figure 4(b) shows 27 spectra collected from leaves of cell-wall mutants. After normalization, the variance in the IR frequency range 1800–800 cm^{-1} is many times greater in the mutant group than in the wild-type group (Figure 4c). The first PC loading resembled a spectrum of starch but, since the quantity of starch varied randomly in both groups, the first PC score did not separate the two groups. However, the second PC score could be used to separate wild-type and mutant leaves independent of their starch content (Figure 5). Unlike the 'starch' loading, the second PC loading is complex and cannot be interpreted as a simple change in one component (data not shown). However, in this set of known cell-wall mutants, exploratory PCA indicated that mutants can be separated from the wild-type population.

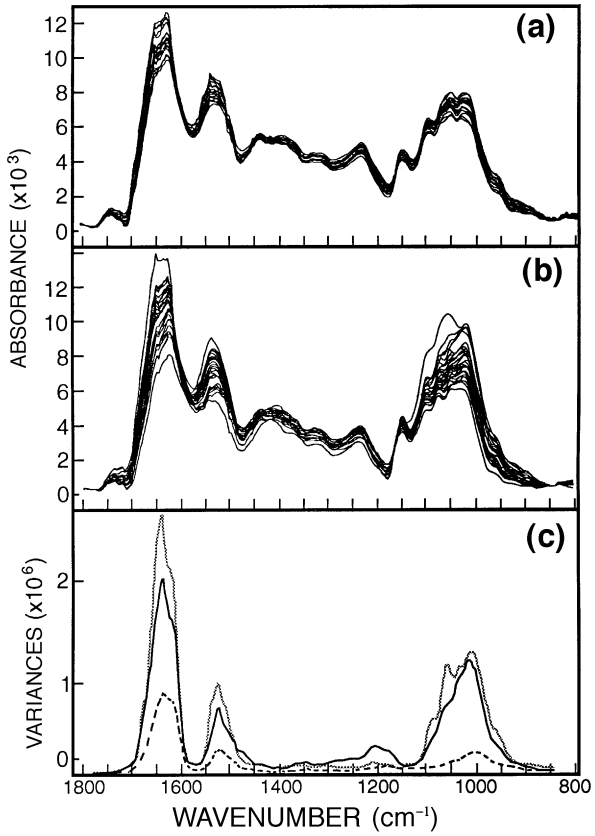

Figure 4. Representative area-normalized and baseline-corrected FTIR spectra from wild-type (a) and mutant (b) *Arabidopsis* leaves, and the calculated variance in the frequency range of 1800–800 cm^{-1} (c).

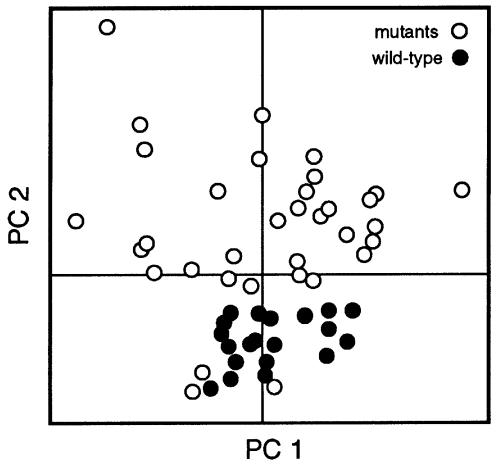

Figure 5. An exploratory principal component analysis shows separation between the groups of wild-type and mutant *Arabidopsis* leaves using the second PC score.

This approach is an example of what is termed 'unsupervised' or exploratory analysis. Although this method can be used to detect samples that are compositionally different, PCA is usually used as a pretreatment of the data before LDA. In contrast to unsupervised analysis, the LDA is termed a 'supervised' or modelling method because the mean observation for each of a number of predefined groups is calculated and new observations assigned to the group whose mean is closest. An observation is the set of PC scores derived from the several hundred datapoints in an individual spectrum. The definition of groups can be very specific or more broadly defined, as in this application where the two groups are broadly defined as 'mutant' and 'non-mutant'. An LDA was performed using the Mahalanobis distance metric, which is a measure of the distance of the individual spectrum from the calculated mean of the two populations in *n*-dimensional space, where *n* is the number of PC scores. Using just two PC scores, 54 out of 57 observations were correctly assigned as either mutant or non-mutant. This data set was then split into a set of 28 observations used to establish a model by LDA, and a validation set of 29 observations, 27 of which were correctly assigned using two PC scores.

The first PC, which accounted for 50% of spectral variability, was attributed to starch. Whilst the LDA model is still very successful in separating variability because of the starch from that due to cell-wall structure, it would clearly be advantageous to screen starch-depleted plants, thus concentrating more of the variation from mutation in the lower PCs.

PCA of wild-type Arabidopsis *and* mur1 *and* mur4 *mutants*

As we were able to correctly identify almost all of the set of characterised cell-wall mutants, we then tested whether the differences observed between wild-type plants and specific mutant lines were caused by a change in cell wall composition, and not some other mutation present in the original M_2 lines. We used populations of *mur1*, a fucose-deficient mutant, and *mur4*, a reduced arabinose mutant, which had been backcrossed up to six times to isolate the mutation from others that may have been in the original heavily mutagenised population. Using starch-depleted plants of the six-times backcrossed populations of these mutants, we can broadly separate these two mutants from each other as well as from the wild-type cluster using the first two PC scores (Figure 6). If the discrimination *mur1* and *mur4* was based on a serendipitous selection of a mutation unrelated to the fucose or arabinose deficiency, respectively, we would expect the discrimination based on cluster analysis to disappear in an extensively backcrossed line. However, a six-times backcrossed line remained clustered and distinguishable from the wild-type population (Figure 7, data shown for *mur1*). Indeed, discrimination using fewer PCs is improved with the number of backcrosses, as extraneous sources of variation are removed (data not shown).

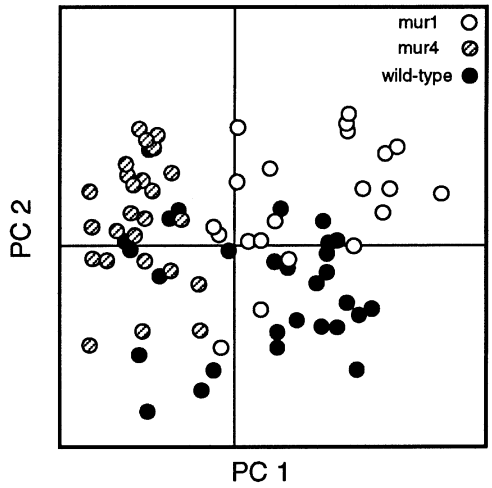

Figure 6. Exploratory PCA indicates that it is possible to broadly discriminate wild-type, the fucose-deficient *mur1*, and the arabinose-deficient *mur4* *Arabidopsis* plants.

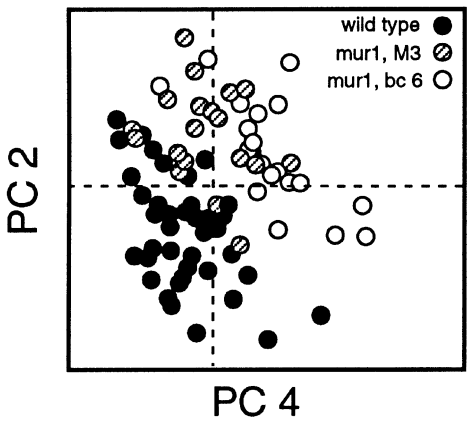

Figure 7. Exploratory PCA discriminates both un-backcrossed and the six-times backcrossed *mur1–1 Arabidopsis* mutant from wild-type, using the second and fourth PC scores.

Selection of potential cell-wall mutants of flax

To be of wide and practical use, an FTIR-based screen must be able to select putative cell-wall mutants directly from a mutagenised population. We had initiated concurrently a program to identify cell-wall mutants in flax, and 1000 EMS-mutagenised plants were sampled for FTIR screening. In addition to alterations in neutral sugars, the primary screen of the mutagenised flax population includes detection of pectin mutants from changes in peak intensities at 1740, 1600 and 1414 cm^{-1}. Spectra were collected from each of three leaves from each flax plant, and PCA was used to identify plants where all three of the selected leaves had a less than 5% probability of belonging to the population (in many cases under 1%). A PCA was performed but, rather than forcing each sample into a class (as LDA does), Mahalanobis distance was used as the criterion for class membership. From the frequency distribution of Mahalanobis distances for a control wild-type population of flax and the EMS-mutagenised population, a total of 59 potential mutants were selected (Figure 8).

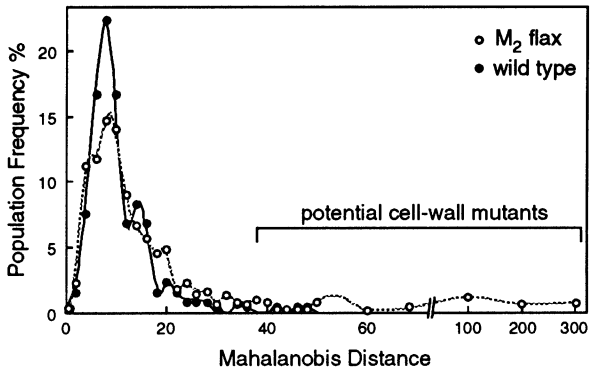

Figure 8. Mahalanobis distance metric identifies potential mutants of flax as outliers from the wild-type population.

Whereas with the known set of *Arabidopsis* mutants and wild-type, the vast majority could be separated on the basis of one or two PC scores (Figure 5), the mutants that were not backcrossed required more PCs to discriminate them from wild-type (Figure 7). For the primary screen of the mutagenised flax, we used five PCs to calculate Mahalanobis distances. The first five PCs account for over 95% of the variability in the entire population. Both wild-type and mutagenised populations exhibited a peak in Mahalanobis distance of about 8, indicating that a large proportion of the mutagenised population was unaltered. However, the mutagenised population also showed small numbers of outliers extending out to Mahalanobis distances up to 300 (Figure 8).

The mole percentage of the non-cellulosic sugars was determined, following TFA hydrolysis, for 24 control flax leaves that were close to the average Mahalanobis distance values for the wild-type population, and these were compared to an additional 24 samples from the flax mutagenized population with similar values. As glucose content was highly variable because of differences in starch content when the leaves were harvested, the mole percentage of each sugar was initially determined in the absence of glucose. The mole percentage of the non-glucose sugars were similar between the two control populations, with the exception of Rha, which was only about one-half that of the wild-type in the mutagenised population (Table 1). The decrease in Rha was offset by selective compensatory increases in the mole percentage of Ara and Gal.

Of the 59 samples whose Mahalanobis distances were outside the wild-type population, 36 had a mole percentage of one or more of its cell-wall sugars that fell outside the range of both of the control populations (Table 1). When Glc was included in the determination of mole percentage, an additional six plants were found to have either exceptionally high or low Glc content, and may have altered

Table 1. Neutral sugar composition of potential flax mutants identified by Mahalanobis distance metric as outliers from the wild-type population. Three samples of leaves from 59 potential mutants were hydrolyzed by TFA and alditol acetate derivatives were separated and quantified by GC-MS. Twenty-four leaves from 24 individual plants were sampled from both the wild-type population and from mutagenised plants with Mahalanobis distances within the wild-type population. All mole percentage values listed below were calculated with the omission of glucose, and 36 samples with over- or under-represented cell-wall sugars were identified, that lie outside the range of both wild-type and mutagenised control populations. The mole percentage of all samples with no apparent defect in non-glucose wall sugars were then recalculated including glucose to yield from the remaining 23 plants another population of six plants with alterations in glucose well beyond the expected range. Values of glucose in recalculations are given in parentheses. Other sugars under-represented (lower case) or over-represented (upper case) are also noted

Sample	Rha	Fuc	Ara	Xyl	Man	Gal	
Wild-type	16.4±2.5	5.0±0.9	21.5±5.3	21.4±4.2	6.1±1.4	29.5±3.3	
Mutagenised control	8.7±2.1	3.9±1.2	26.8±4.9	20.8±3.7	7.0±2.2	32.8±5.9	
							other affected sugars
Rha-deficient:							
70–17	4.6±1.4	2.7±0.1	13.0±0.6	28.4±1.9	8.3±0.3	43.0±1.5	ara, GAL
Ara-deficient:							
41–02	14.9±5.6	4.2±1.2	6.7±3.5	27.0±16.5	14.5±5.8	32.7±10.0	
71–22	8.9±0.1	10.4±1.0	8.9±1.6	36.7±1.0	12.2±3.8	22.9±2.2	FUC, XYL,gal
41–01	21.8±2.5	5.6±2.1	9.2±0.6	23.4±4.7	8.5±3.4	31.5±5.2	RHA
41–14	12.9±1.0	6.2±1.1	9.2±2.6	21.9±1.9	14.7±4.7	33.3±4.8	
71–16	16.0±13.2	2.7±2.4	10.6±1.2	36.4±8.4	14.9±1.0	19.3±5.7	fuc, XYL
71–30	15.3±1.3	5.2±0.8	10.8±0.4	35.2±3.3	5.8±0.3	27.7±2.5	
71–09	15.9±1.0	10.4±0.5	12.8±2.8	22.4±1.9	9.4±1.6	29.2±2.5	FUC
Xyl-deficient:							
45–08	10.4±0.4	4.4±0.2	36.7±0.6	11.9±0.7	6.1±0.4	30.4±1.7	ARA
43–15	8.9±0.8	3.8±0.1	27.8±0.8	12.1±0.4	5.3±0.6	42.1±1.4	GAL,GLU(67.6±1.2)
45–05	8.5±1.0	3.5±0.7	38.2±6.9	12.3±1.3	6.0±0.9	31.5±3.2	ARA
45–01	11.9±2.3	4.6±0.4	34.1±3.0	12.8±1.4	6.0±0.2	30.5±2.2	ARA
44–04	10.0±0.6	4.3±0.7	30.4±3.4	12.9±2.4	6.8±3.1	35.6±1.9	
80–02	8.4±2.7	5.0±1.1	32.6±8.9	13.0±1.7	5.4±0.8	35.5±2.5	ARA
43–24	9.1±0.8	4.2±0.4	31.2±2.8	13.1±0.6	5.6±0.8	36.7±2.7	
80–17	10.5±1.1	4.7±0.5	31.0±3.8	13.3±1.1	5.4±0.5	35.0±3.0	
43–27	9.8±2.0	4.3±0.6	31.7±3.1	14.0±2.4	5.7±1.5	34.4±4.4	ARA
104–05	12.9±0.6	7.9±0.8	30.5±4.5	14.3±2.3	5.0±0.9	33.4±1.7	
104–06	12.2±0.8	7.7±1.1	30.5±4.5	14.0±0.7	4.7±0.6	30.9±1.4	
80–08	11.0±1.2	6.1±0.5	28.8±2.0	15.4±0.9	5.8±0.4	33. 0±0.8	
Gal-deficient							
47–14	6.8±2.3	3.2±1.7	23.5±1.9	38.3±8.2	11.0±4.6	17.3±2.4	rha
Fuc-overexpressed:							
98–12	15.2±2.4	12.0±2.4	19.8±1.5	19.4±3.6	8.1±5.1	25.6±4.6	
70–11	12.2±3.8	10.7±2.0	13.0±5.3	18.9±6.3	16.7±8.1	28.5±7.1	
71–29	14.7±0.9	10.6±2.2	22.8±4.5	17.3±1.7	4.4±0.4	30.2±1.5	
70–06	13.7±2.2	9.6±0.9	18.8±2.1	20.9±0.7	6.3±0.3	30.6±1.6	
70–07	13.7±1.5	8.9±1.0	22.4±7.1	18.8±0.9	5.4±0.6	30.8±4.5	
71–26	19.6±0.9	8.5±0.6	22.2±0.9	14.9±1.2	4.8±0.9	29.9±1.2	
70–08	11.7±1.9	8.4±2.2	25.6±7.2	19.2±1.0	8.4±1.1	26.7±3.3	
49–13	9.9±0.9	6.6±0.3	20.9±1.0	22.4±4.0	5.2±0.7	35.0±4.9	
Ara-overexpressed:							
45–06	9.1±1.5	4.3±0.1	35.0±1.9	17.4±2.9	7.3±1.3	27.0±3.4	
43–23	9.9±1.8	4.3±0.9	34.4±1.1	13.8±2.1	4.7±1.1	32.9±2.8	XYL
43–06	11.0±0.3	6.2±0.8	32.3±0.4	17.1±1.7	4.4±0.2	29.0±2.4	GLU(65.1±0.7)
Xyl-overexpressed:							
41–15	5.5±3.8	1.9±1.0	20.4±2.9	41.3±3.2	11.9±3.8	19.1±5.1	rha, gal, glu
41–12	9.1±3.0	4.1±0.7	10.8±1.1	36.0±5.0	7.3±0.7	32.8±1.2	
41–05	10.7±0.9	4.5±0.2	17.6±0.8	31.3±3.6	7.8±0.4	28.1+2.9	
41–19	11.7±2.4	4.2±0.4	24.3±1.2	26.4±0.3	5.0±1.0	28.4±3.0	
Glucose deficiency:							
48–10	9.5±5.0	4.4±0.8	32.6±3.5	24.3±0.6	5.5±1.3	23.7±5.7	(7.2±1.5)
40–10	16.5±1.6	6.3±0.3	22.3±2.7	15.3±4.0	5.3±0.5	34.4±6.4	(11.8)
42–20	9.9±0.7	7.4±0.4	21.7±1.6	18.8±1.0	6.7±0.9	35.5±0.2	(14.9±2.0)
41–26	19.1±0.3	5.5±0.9	16.1±2.3	16.1±3.5	8.4±1.4	31.7±1.0	(20.5±3.6)
Glucose overexpression:							
102–10	17.5±1.0	5.6±0.1	18.7±2.7	16.1±1.6	3.8±0.2	38.3±4.4	(73.5±5.7)
43–11	9.1±1.1	5.8±0.9	29.5±6.8	17.3±1.0	5.3±1.3	33.0±7.5	(61.5±3.5)

starch content. The remaining 17 plants had neutral sugar compositions within the range of the control populations, but inspection of the spectra indicates that two of these have altered ester content and a further four vary in protein content although we have not assayed for these directly (data not shown). FTIR spectra are also sensitive to conformational changes in polymers (McCann *et al.*, 1997) and we have not assayed for changes in molecular architecture as distinct from composition. For example, a mutation affecting hydrogen bonding in the wall would be detected by FTIR but not necessarily by sugar analysis.

Discussion

We have demonstrated that FTIR spectroscopy is a remarkably efficient means of screening for cell-wall mutants. Large mutagenised populations of plants can be scored in a relatively short period of time, and a limited population of potential mutants can then be identified for subsequent cytological, biochemical and biophysical analysis. For example, our selected flax mutants now require testing for heritability of the altered sugar trait, as well as for other possible wall defects, such as uronic acid content, pectin organization, cellulose crystallinity, etc. Particular cell-wall features may impinge on quality traits in some plant breeding programs or in plant-derived food products, and there is a clear application in the screening of transgenic populations.

The PC loadings derived are more or less specific to the carbohydrate fingerprint region of the spectrum but, unfortunately, we cannot assign specific changes at any frequency to a specific sugar or polysaccharide. Polysaccharides interact with each other, have absorbing frequencies in common, and adopt characteristic conformations to which infrared spectra are sensitive. The loadings simply reflect any correlated changes in the spectra. As more FTIR spectral information about polymer conformation becomes available, we may be able to correlate spectral features with structural or biophysical properties of cell walls in instances where assignments for specific peaks are difficult to make. Despite this shortcoming, the utility of FTIR spectroscopy as a primary screen for alterations in structure and architecture is clear. For example, the *mur1* mutant is the most extensively characterised of the neutral-sugar mutants. The phenotype is a deficiency in the de novo synthesis of fucose (Bonin *et al.*, 1997), which accounts for only 0.5% of sugars in the wall (Reiter *et al.*, 1993). Our studies using PCA indicate that the fucose deficiency must result in more global changes in wall architecture. Furthermore, peaks diagnostic of pectin are well-resolved in the spectrum making it possible to screen for a variety of pectin mutants (McCann *et al.*, 1997). Even using a curtailed spectral region to minimise spectral variability from cytoplasmic protein, the 1420 cm^{-1} stretch from pectin carboxylate is a suitable diagnostic peak.

Arabidopsis has previously been shown to have a cell wall typical of most higher plants (Zablackis *et al.*, 1995), making it an appropriate model for understanding the genetic basis of wall structure and architecture. While several cell wall mutants, such as the initial *Arabidopsis* collection (Reiter *et al.*, 1997), are now beginning to be characterised, these form a far from complete collection. Furthermore, mutants in *Arabidopsis* will be of limited use in understanding the special walls of grasses (Carpita, 1996), or the developmental regulation involved in building a tree (Whetten and Sederoff, 1995). Hence, the search for cell wall mutants must be extended to crop species and other plants, as well as to finding additional *Arabidopsis* mutants.

Experimental procedures

Plant material

Twenty-three mutant lines of *Arabidopsis thaliana* (ecotype Columbia), representing 11 different loci were selected either for the absence of a particular cell-wall sugar, substantially lower amounts of one sugar, or alterations in the amounts of several sugars (Reiter *et al.*, 1997). Some of these lines have been re-selected after up to six backcrosses to wild-type. Plants were either grown in a greenhouse maintained at 23°C with a 16 h photoperiod supplied with supplemental high-pressure Na-lamps or in growth chambers maintained at 23°C and constant light. Leaves were harvested from each of the plants at the onset of flowering. A set of wild-type, *mur1* and *mur4* plants were placed in darkness at 23°C for 48 h to reduce or eliminate starch in the leaves before harvest.

Seeds of flax (*Linum usitatissimum* L. cv. Novotorzhskii) were mutagenized with 0.05, 0.1, or 0.2% ethylmethane sulfonate and planted in a garden plot in Indiana or field plots in Torzhak, Russia. The M_2 population of seeds was planted in a greenhouse maintained at 16–18°C with a 16 h photoperiod supplied by high-pressure Na vapor lamps. Individual plants were tagged, and several leaves from each plant were collected at the onset of the fast-growth stage when the plants were about 30 cm high.

The excised leaves of both *Arabidopsis* and flax were plunged into ethanol and heated to 85°C for 20 min to extract chlorophyll, sugars and other small molecules. Two additional extractions were made with 80% ethanol (v/v) at 85°C for 20 min each until the leaves were white (*Arabidopsis*) to pale tan (flax) in colour. The leaves were washed with water, suspended in a small volume of water, frozen and lyophilized.

FTIR spectroscopy

The lyophilized leaves were placed on a barium fluoride window supported on the stage of a UMA500 microscope accessory of a Bio-Rad FTS175c FTIR spectrometer equipped with a liquid nitrogen-cooled mercury cadmium telluride detector. An area of leaf (100×100 μm) away from the mid-vein was selected for spectral collection. Sixty-four interferograms were collected in transmission mode with 8 cm^{-1} resolution and co-added to improve the signal-to-noise ratio for each leaf sample. Three spectra were

collected from different areas of each *Arabidopsis* leaf and then averaged and baseline-corrected. The triplicate-averaged spectrum was then used in the multivariate analysis. For the flax screen, a single spectrum was collected in the same way from each of three leaves from each plant.

Data analysis

PCA and LDA analyses of area-normalised spectra in the region 1800–800 cm^{-1} were carried out using WIN-DISCRIM software (E.K. Kemsley, Institute of Food Research, Norwich, UK).

Quantitation of cell-wall neutral sugars

Portions of the flax leaves used in the FTIR analysis of the potential mutants selected by PCA were hydrolyzed with 2 M TFA containing 1 μmol of myo-inositol for 90 min at 120°C. The TFA was evaporated under a stream of nitrogen and the sugars were converted to alditol acetates (Carpita and Shea, 1989). The alditol acetates were separated by gas-liquid chromatography on a 0.25 mm × 30 m vitreous silica capillary column of SP-2330 (Supelco, Bellefonte, PA, USA). Temperature was programmed from 170°C to 240°C at 5°C min^{-1} with a 6 min hold at the upper temperature. The neutral sugar composition was verified by electron-impact mass spectrometry.

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