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Screening of *Arabidopsis thaliana* stems for variation in cell wall polysaccharides

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Abstract

A high-throughput method is described by which *Arabidopsis thaliana* stems can be screened for variation in cell wall composition after hydrolysis with Driselase or trifluoroacetic acid (TFA). Driselase, a mixture of fungal enzymes, hydrolyses cellulose (to glucose) and all the major matrix polysaccharides (to monosaccharides and/or characteristic disaccharides); TFA hydrolyses the matrix polysaccharides, but not cellulose, to monosaccharides. Two different wild-type ecotypes, Columbia and Wassilewskija, showed only minor differences in wall carbohydrate composition. A small number of T-DNA-tagged populations that were screened contained individuals in which the proportion of cellulose, xyloglucan or xylan differed quantitatively from the wild-type. Differences from the wild-type were also observed in the susceptibility of the hemicelluloses to hydrolysis by Driselase, probably reflecting differences in wall architecture.

Keywords: *Arabidopsis thaliana*; Cruciferae; Cell walls; Polysaccharides; Xyloglucan; Xylan; Cellulose; Pectins; Driselase; Screening; Chromatography

1. Introduction

An ability to manipulate plant cell walls genetically is valuable both in fundamental studies of growth and development and in commercial endeavours to improve the physical properties of plant products, including food and paper. *Arabidopsis thaliana* is an excellent species in which to isolate and characterize mutants with altered cell wall composition. The primary walls of its leaves are closely comparable to those of other dicots, apart from an unusually high proportion of cold-water-soluble pectic polysaccharides (Zablackis et al., 1995), a feature characteristic of a minority of dicot species (Kooiman, 1969). *A. thaliana* leaf primary walls contain homogalacturonan, rhamnogalacturonan-I (RG-I), RG-II, a xylan (glucuronoarabinoxylan), xyloglucan and cellulose (Zablackis et al., 1995). The secondary walls of *A. thaliana* xylem have not been thoroughly characterized chemically.

Relatively few mutants with wall defects have been isolated (Dolezal and Cobbett, 1991; Cobbett et al., 1992; Reiter et al., 1993, 1997; Potikha and Delmer, 1995; Xu et al., 1995; Turner and Somerville, 1997; Turner and Hall, 2000). Reiter et al. (1993, 1997) identified 11 mutants with altered wall monosaccharide residue composition from a chemically mutagenised population of *A. thaliana*. These mutants had abnormal quantities of Fuc, Ara, Rha, Xyl or Man residues in the wall polymers. For example, a weak-stemmed dwarf mutant, *mur1*, was deficient in GDP-L-Fuc owing to a lack of an isoform of GDP-D-Man 4,6-dehydratase; the L-Fuc residues of polysaccharides were partially replaced in the mutant by L-Gal (Reiter et al., 1993; Zablackis et al., 1996; Bonin et al., 1997).

Screens for mutants altered in wall polysaccharide chemistry have so far examined the monosaccharide profile obtained after acid hydrolysis. A consequence of this strategy is that a mutant defective in xylan but with compensating levels of xyloglucan might not readily

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show up because the total Xyl content would be unaffected. To overcome this difficulty, we now describe a method in which cell walls are hydrolysed with either acid or Driselase (Fry, 2000), the latter yielding products that readily distinguish xylans from xyloglucans. Acid- and Driselase-hydrolysis also yield distinguishable products from several other major wall polysaccharides (Table 1).

We have tested this methodology by screening insertionally mutagenised populations of *A. thaliana* for new putative mutants with wall abnormalities. The *A. thaliana* genome includes a surprisingly large number of genes that appear to encode enzymes of cell wall metabolism (*Arabidopsis* Genome Initiative, 2000). Random mutagenesis, therefore, has a disproportionately high probability of causing a dysfunction in wall biochemistry. To increase further the probability of our detecting plants with altered wall properties, we screened populations containing 'form' mutants, i.e. those reported to exhibit (minor) morphological defects. Since the cell wall is the key determinant of plant shape, many 'form' mutants are likely to have their biochemical basis in an altered cell wall composition or architecture.

Table 1
The probable polymeric sources from which identified hydrolysis-products were generated

Hydrolysis product	Major polymeric sources	Minor polymeric sources
Characteristic products of acid-hydrolysis		
Xyl	xylans, xyloglucans	RG-I, xylogalacturonan
Glc[b]	xyloglucan, starch	glucomannan, callose
GlcA	xylans, RG-II	
Characteristic products of Driselase-hydrolysis		
Glc[c]	cellulose	xyloglucan
Xylobiose (X_2)	xylans	
Isoprimeverose (IP)	xyloglucan	
Xyl	xylans	RG-I
GlcA	RG-II?	
Products where no clear difference is expected between acid- and Driselase-hydrolysis		
GalA[a]	homogalacturonan, RG-I	RG-II, xylogalacturonan
Gal	RG-I, xyloglucan	RG-II, glycoproteins
Ara	RG-I, xylans	RG-II, glycoproteins
Rha	RG-I	RG-II
Man	glucomannan	glycoproteins
Fuc	xyloglucan	RGs

[a] Galacturonic acid glycosyl bonds are relatively resistant to the acid hydrolysis conditions used so the yield of galacturonic acid in the hydrolysate was unlikely to reflect its true level in the AIR.

[b] Cellulose is not hydrolysed efficiently by TFA (Selvendran and Ryden, 1990).

[c] Driselase does not efficiently break down starch to the monosaccharide.

2. Results

2.1. Screening of A. thaliana populations

Flowering stems from the T4 generation (i.e. fourth generation after transformation) of mutagenised populations of *A. thaliana* were screened for altered profiles of cell wall components. Screening of the acid- and Driselase-hydrolysis products was in two stages: (i) initial screen by paper chromatography (PC) or TLC; (ii) repeat PC or TLC to test the reproducibility of the data of (i). The products detected in the acid and Driselase hydrolysates, and the polymers from which they would have arisen, are listed in Table 1.

Within each Feldmann population, 90% of the T4 individuals were expected to be kanamycin-resistant and to carry a mutant allele of the *A. thaliana* gene into which the insertion had occurred (70% homozygous, 20% heterozygous). Thus, although each line is a genetically non-uniform population, a majority of the individuals within it are expected to carry the same mutation. If this mutation influences wall chemistry, a pooled sample of the flowering stems from several randomly chosen individuals is likely to have a wall composition measurably different from the wild-type.

2.2. Initial screen

The aim of the initial screen was to pick out populations containing potential wall mutants quickly. The concentration of each hydrolysis product was scored visually. After acid hydrolysis of alcohol-insoluble residues (AIRs), 44 of 105 screened populations appeared to deviate from the wild-type (Table 2). The most common deviations involved Xyl, Gal or uronic acids (Table 3); 70% of the deviations were decreases. Among each of the three sources of mutagenised populations, ~40% of the populations tested deviated from the wild-type.

After Driselase hydrolysis, 47 of 110 screened populations appeared to deviate from the wild-type (Table 2). The most common deviations involved xylobiose [X_2; β-D-Xyl*p*-(1→4)-D-Xyl] or Gal (Table 3); 92% of the deviations were decreases. Of the three sources of potential mutants, those that had been T-DNA-tagged by seed transformation gave the highest proportion of populations (~60%) that differed from the wild-type. Subsequent work was therefore focused on this source.

2.3. Repeat-screen

Nine (from the acid hydrolysis screen) and 18 (from the Driselase screen) of the apparently deviant populations selected in the initial screen were repeat-screened. AIRs from numerous plants of each population were pooled, and 2–3 replicate sub-samples were hydrolysed as before. In the acid screen, five of the nine chosen populations again deviated from the wild-type (Table 4). Three (B14, B51 and B52) were low in Xyl. In the

Table 2
Frequency of hits in the initial screen of AIRs from tagged populations of *A. thaliana* (pooled individuals from T4 generation). AIR samples from flowering stems were hydrolysed by acid (TFA) or an enzyme mixture (Driselase) and the products analysed by PC and TLC. Deviation from wild-type ecotype Columbia was judged visually

Source of mutant populations[a]	No. of populations screened	% of populations differing from wild-type	No. of populations screened	% of populations differing from wild-type
	TFA screen		Driselase screen	
T-DNA-tagged, organ[a]	14	43	25	20
T-DNA-tagged, seed[b]	61	41	68	62
Ds-tagged[c]	30	43	17	0
Total	105	42	110	43

[a] T-DNA-tagged form mutants from organ transformation (Koncz et al., 1992).
[b] T-DNA-tagged form mutants from seed transformation (Feldmann and Marks 1987; Feldmann et al. 1989).
[c] Ds-tagged lines (Bancroft and Dean 1993).

Driselase screen, 9 of the 18 chosen populations again deviated from the wild-type (Table 4). Seven were low in X_2: in two of these, isoprimeverose [IP; α-D-Xyl*p*-(1→6)-D-Glc] was also decreased, whereas in one IP was increased.

2.4. HPLC analysis

For quantification, hydrolysis products from individual stems were assayed by HPLC method 1 [acid (Fig. 1) and Driselase products (Fig. 2)] and method 2 [Driselase products only (Fig. 3)]. Data for the two wild-type ecotypes (Table 5) and for the putative mutants (Figs. 4 and 5) are presented.

2.5. Identified carbohydrate hydrolysis products of wild-type (Wassilewskija)

2.5.1. TFA hydrolysis

The major components of the TFA hydrolysate of ecotype Wassilewskija (Xyl, GalA, Glc, Gal) together made up 78 mol% of the total identified products (Table 5).

2.5.2. Driselase hydrolysis

The major components of the Driselase digest of ecotype Wassilewskija (Glc, GalA) together accounted for 63 mol% of the identified products (Table 5). Yields of IP and (X_2 + Xyl) indicate xyloglucan and xylan respectively. Thus the ratio of xyloglucan-associated Xyl residues to xylan-associated Xyl residues was 1:2.63. The primary walls of *A. thaliana* leaves have a xyloglucan:xylan ratio of ~5:1 (Zablackis et al., 1995). However, there will be a greater proportion of xylan in bolting stems owing to the abundance of xylem.

2.5.3. Comparison between acid- and Driselase-digestibility

Acid and Driselase solubilised 65 and 56% of the AIR, respectively (Table 5). The proportion solubilised and the profile of identifiable products will be determined by several factors: non-carbohydrate components (e.g. lignin and cutin) will be a more effective barrier to enzymes than to H^+; crystallinity (of cellulose) will be more of a barrier to 2 M acid than to enzymes; the presence of rare glycosidic linkages (e.g. α-GlcA in xylans and aceric acid in RG-II) and non-carbohydrate side-chains e.g. acetyl groups will be a more effective barrier to enzymes than to H^+; the chemical resistance of uro-

Table 3
Nature of hits in the initial screen of AIR samples from tagged populations of *A. thaliana* (pooled individuals from T4 generation). Other details as in Table 2; 105 populations were screened by TFA hydrolysis, 110 by Driselase hydrolysis

Hydrolytic agent	Hydrolysis products[a] obtained in a yield different from the wild-type	No. of populations altered in yield of the products specified
TFA	UAs	5
	Gal	8
	Glc	2
	Ara	4
	Xyl	16
	UAs, Ara	1
	UAs, Xyl	2
	Gal, Xyl	2
	Glc, Xyl	1
	Ara, Xyl	1
	UAs, Gal, Xyl	1
	UAs, Gal, Xyl, Ara	1
Driselase	G_2	2
	IP	1
	X_2	17
	Gal	1
	G_2, X_2	2
	X_2, Gal	15
	X_2, Glc	1
	UAs, X_2, Glc	2
	G_2, X_2, Gal	3
	X_2, Gal, Glc	2
	G_2, IP, X_2, Gal	1

[a] G_2 = cellobiose; UAs = uronic acids.

Table 4
Results of the repeat-screens of AIRs from tagged populations of *A. thaliana* (pooled individuals from T4 generation). Other details were as in Table 2

Line[a]	NASC accession number	Hydrolytic agent	Deviation[b] from wild-type yield of						
			Uronic acids	Gal	Ara	Xyl	G_2[c]	IP	X_2
A21	N4123	TFA	↓		↓				
B12	N2855	TFA							
B14	**N2858**	TFA				↓			
B16	**N2860**	TFA	↑		↑				
B19	N2863	TFA							
B27	N2871	TFA							
B39	N2883	TFA							
B51	N2895	TFA				↓			
B52	N2896	TFA				↓			
A33	N4279	Driselase							
B05	**N2848**	Driselase						↓	
B11	N2854	Driselase							
B17	**N2861**	Driselase						↓	↓
B24	**N2868**	Driselase					↓		↓
B25	N2869	Driselase							
B27	**N2871**	Driselase		↓					↓
B28	N2872	Driselase					↓		↓
B29	N2873	Driselase							
B34	N2878	Driselase						↑	↓
B40	N2884	Driselase							
B44	N2888	Driselase							
B47	N2891	Driselase							
B52	N2896	Driselase						↓	↓
B54	N2898	Driselase		↓					↓
B55	N2899	Driselase							
B58	**N2902**	Driselase		↓					
B64	N2908	Driselase							

[a] Lines with prefix 'A' = T-DNA-tagged form mutants from organ transformation (Koncz, 1992); lines with prefix 'B' = T-DNA-tagged form mutants from seed transformation (Feldmann and Marks 1987; Feldmann et al. 1989). Lines shown in **bold type** (B5, B14, B16, B17, B24, B27 and B58) were subsequently analysed by HPLC.

[b] Symbols used: ↑ = greater yield than wild-type; ↓ = lesser yield than wild-type; blank = no appreciable difference from wild-type.

[c] G_2 = cellobiose.

nosyl glycosidic bonds will be more of a barrier to acid than to enzymes; and the degradation of liberated uronic acids will be greater in acid than in enzyme solutions. These considerations explain the differences between Driselase- and acid-generated products: Driselase gives more Glc because it can hydrolyse cellulose; other neutral monosaccharides are generated in higher yield by acid, presumably because lignin partially protects polysaccharides against Driselase.

The identified soluble products of the two hydrolytic agents made up approximately the same proportion of the AIR (17–18% of the AIR; Table 5). About 39–47% of the initial AIR was solubilised but not identified: this material would include non-carbohydrate components of the AIR (e.g. proteins), carbohydrates that were solubilised but not completely hydrolysed to mono- or disaccharides (e.g. RG-II and starch, by Driselase), and degradation-products formed by the action of hot acid on released monosaccharides.

The difference between the total glucose (Glc + IP = 358 μmol/g) released by Driselase and that released by acid (121 μmol/g) enables a rough estimate of the Driselase-susceptible cellulose content (~237 μmol cellulosic Glc per g AIR).

Assuming that all the xylan and xyloglucan present in the AIR was hydrolysed by acid, the difference between the yield of Xyl after acid hydrolysis and the summed yield of the three relevant products (IP + X_2 + Xyl) after Driselase digestion indicates that ~66% of the total Xyl was Driselase-resistant in ecotype Wassilewskija.

2.6. Comparison of wild-type Wassilewskija with wild-type Columbia

Columbia AIR showed a slightly lower digestibility than Wassilewskija in the two screens, but the difference was not significant (Table 5). The two ecotypes were almost identical in total yield of identified carbohydrate products.

On acid hydrolysis, Columbia yielded a significantly higher proportion of Glc and lower proportion of Man than Wassilewskija (Table 5), but the differences were small. In the Driselase screen, Columbia gave significantly lower yields of Fuc, Ara, Xyl and Man, and a significantly higher yield of Glc, than Wassilewskija (Table 5), but again the differences were small. The percentage of total Xyl that was Driselase-resistant was only slightly lower (61%) in Columbia than in Wassilewskija (66%). The ratio of xyloglucan-associated Xyl residues to xylan-associated Xyl residues was 1 : 2.63 in the Wassilewskija wild-type and 1 : 2.32 in Columbia wild-type. Driselase-digestible cellulose constituted roughly 3.84% and 4.63% of the total AIR in Wassilewskija and Columbia, respectively.

These results show that the differences between Wassilewskija and Columbia are minor. This lends support to a genetic basis for the deviations found in the Feldmann lines, described below.

2.7. Comparison of wild-type (Wassilewskija) with T-DNA-tagged populations

Seven of the populations which came through a repeat-screen (B5, B14, B16, B17, B24, B27 and B58) were investigated quantitatively by HPLC (Figs. 4 and 5; Table 6). These populations had all been T-DNA-tagged by seed transformation (Feldmann and Marks, 1987; Feldmann et al., 1989) and each population was genetically heterogeneous [Nottingham Arabidopsis Stock Centre (NASC) Seed List, 1994]. Such a population cannot be treated statistically as a group. Therefore, the HPLC data on individual plants were compared with those of the wild-type group using

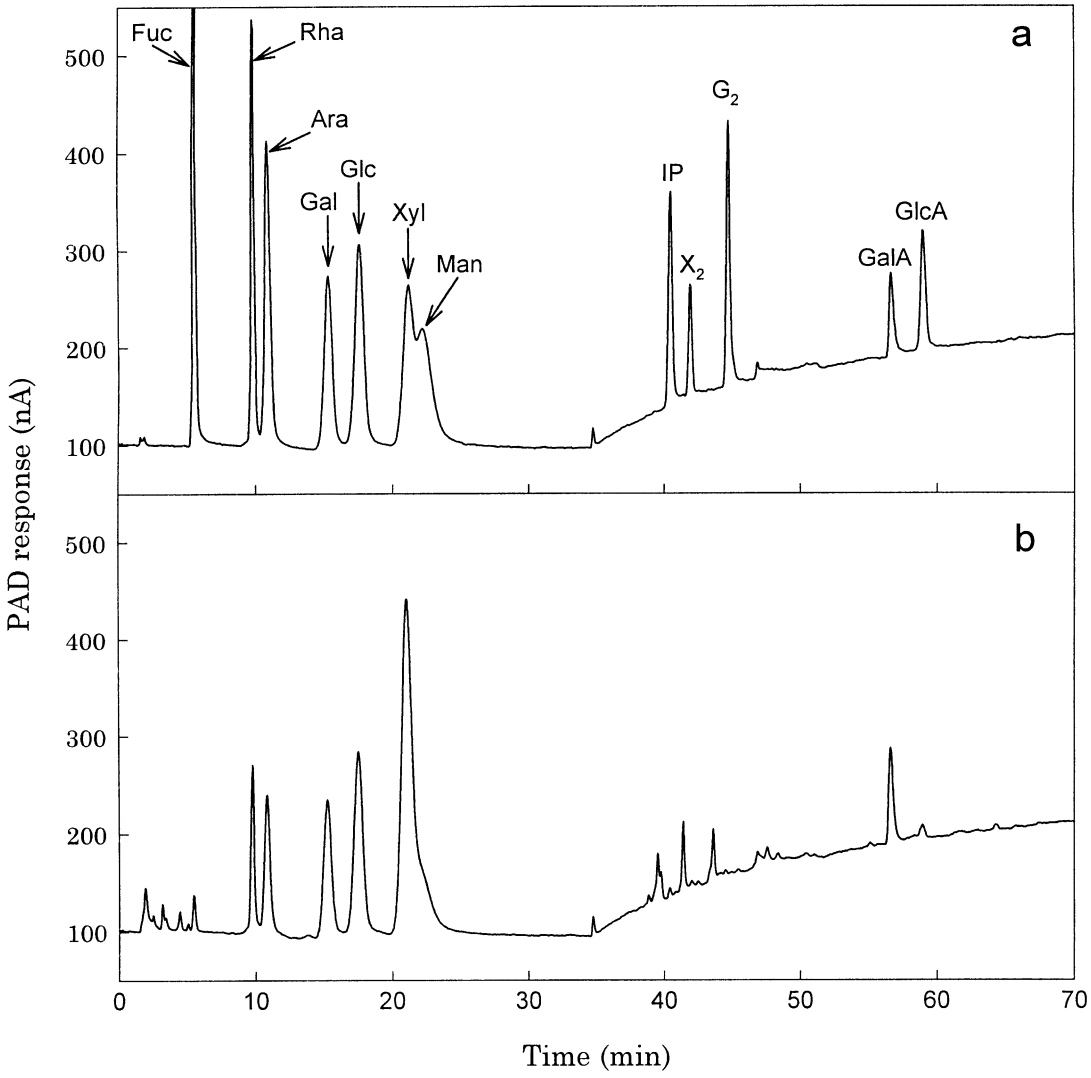

Fig. 1. TFA hydrolysis products of AIR of flowering stems from *A. thaliana* ecotype Wassilewskija. HPLC method 1. (a) Marker mixture, (b) AIR hydrolysis products.

Student's t-distribution. For a difference between Wassilewskija wild-type and a Feldmann line to be due to the presence of the T-DNA tag in the Feldmann line (T4 generation), the percentage of the population with the mutant phenotype should be 90% (if the proposed mutation is dominant) or 70% (if recessive).

2.7.1. Population B24

Three B24 individuals had significantly decreased Driselase digestibility, one increased (Fig. 5). However, these effects did not correlate with any qualitative difference in the profile of digestion products. In the Driselase–HPLC analysis there was a significantly decreased proportion of X_2 in five of the seven B24 plants (Fig. 5), confirming the phenotype previously observed in the repeat-screen (Table 4). The same five individuals all exhibited an increase in Driselase-generated Glc (significant in four of the five). Since the acid hydrolysis screen did not show altered proportions of Glc (Fig. 4), the results indicate that certain B24 plants had a higher content of enzymically digestible cellulose. The Glc:X_2 ratio in the five low-X_2 plants was considerably higher (range = 18–29) than in wild-type Wassilewskija (mean ratio = 7.3; $n = 6$), suggesting that the presence of the T-DNA increased the cellulose:xylan ratio. Similarly, the IP:X_2 ratio was higher in the five low-X_2 plants (range 1.9–2.3) than in the wild-type (mean ratio = 0.97; $n = 6$). Four of the five low-X_2 plants also had increased proportions of Rha.

In the acid hydrolysis screen (Fig. 4), three plants gave high Ara, Gal and Fuc and low Xyl. The Gal:Xyl ratio in these individuals was higher (range 0.63–2.0) than in wild-type (mean ratio = 0.26; $n = 6$). Three plants had low GalA but one had increased GalA. The low Xyl in the acid hydrolysis screen suggests that the low X_2 proportion in the Driselase screen was due to a low xylan content rather than to a high resistance to enzymic digestion.

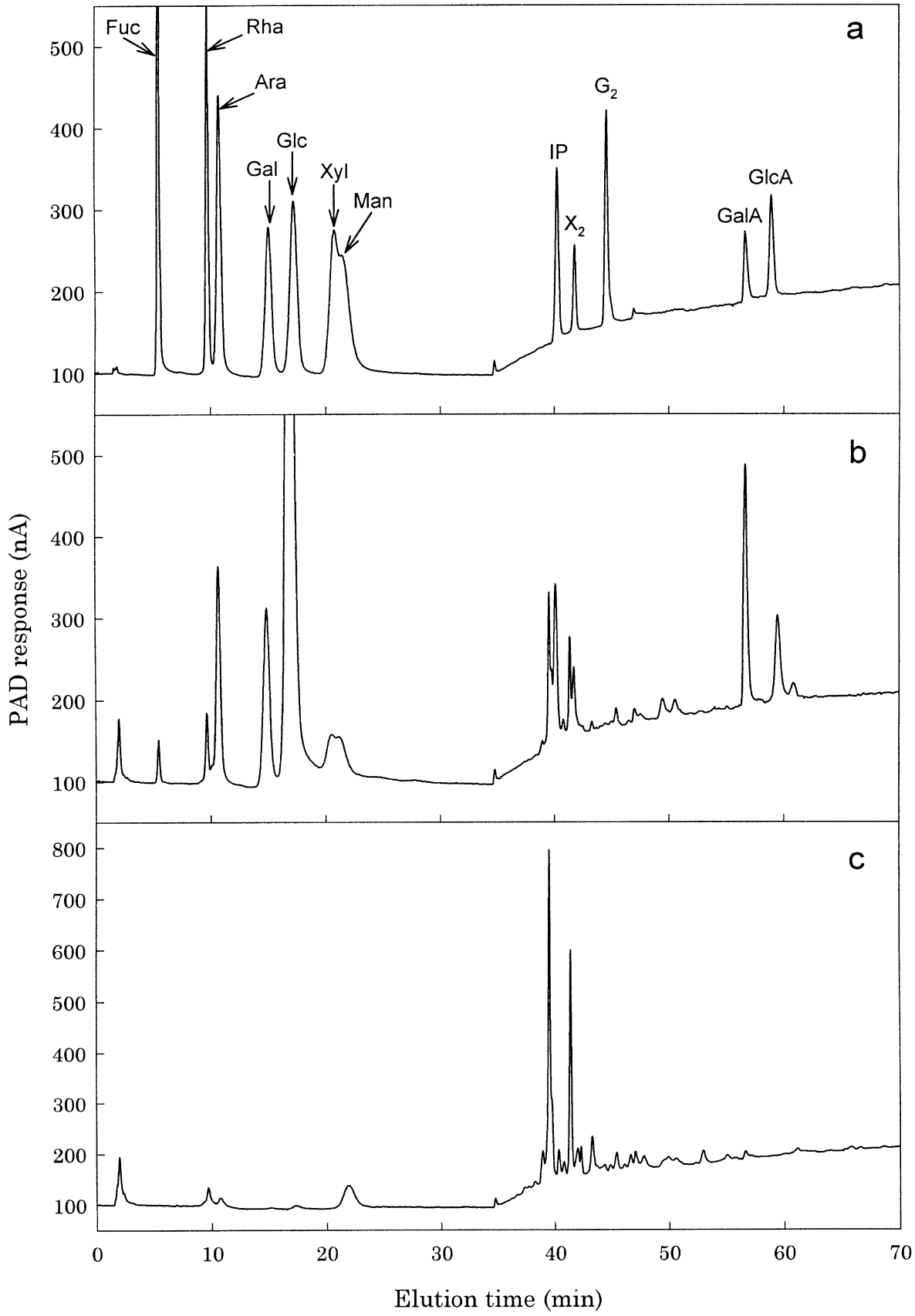

Fig. 2. Driselase hydrolysis products of AIR of flowering stem of *A. thaliana* ecotype Wassilewskija. HPLC method 1. (a) Marker mixture, (b) AIR hydrolysis products, (c) Driselase-only control [injected at 10× concentration of (b)].

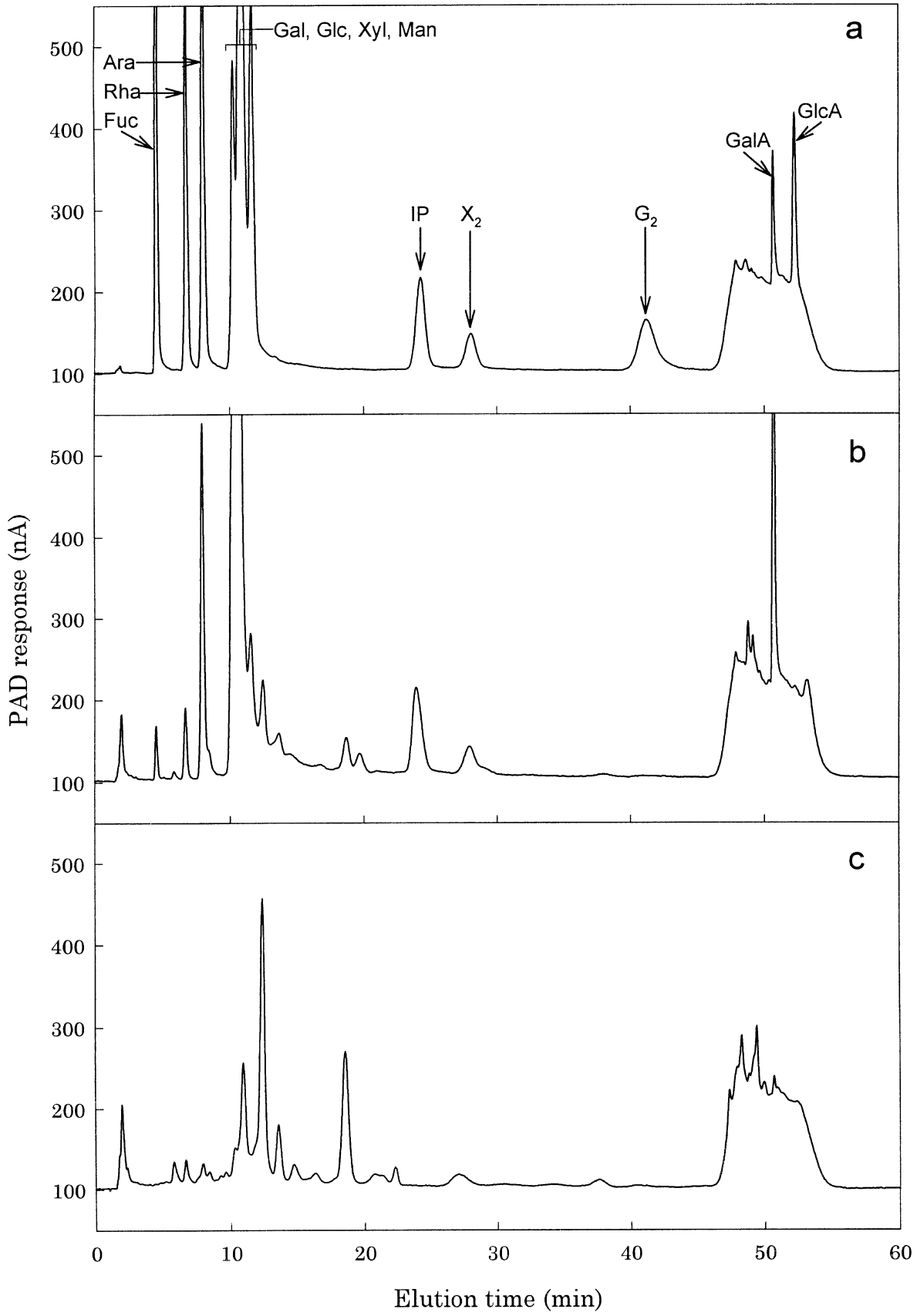

Fig. 3. Driselase digestion products of the AIR of the flowering stem of *A. thaliana* ecotype Wassilewskija. HPLC method 2. (a) Marker mixture, (b) AIR hydrolysis products, (c) Driselase-only control [injected at 10× concentration of (b)].

2.7.2. Population B27

In four of the five B27 individuals put through the Driselase–HPLC analysis, there was a significantly lower proportion of X_2 and Gal than in the wild-type (Fig. 5), confirming the phenotype previously observed in the repeat-screen (Table 4). The same four plants also had a significantly elevated proportion of Driselase-generated Glc and a significantly decreased proportion of GlcA;

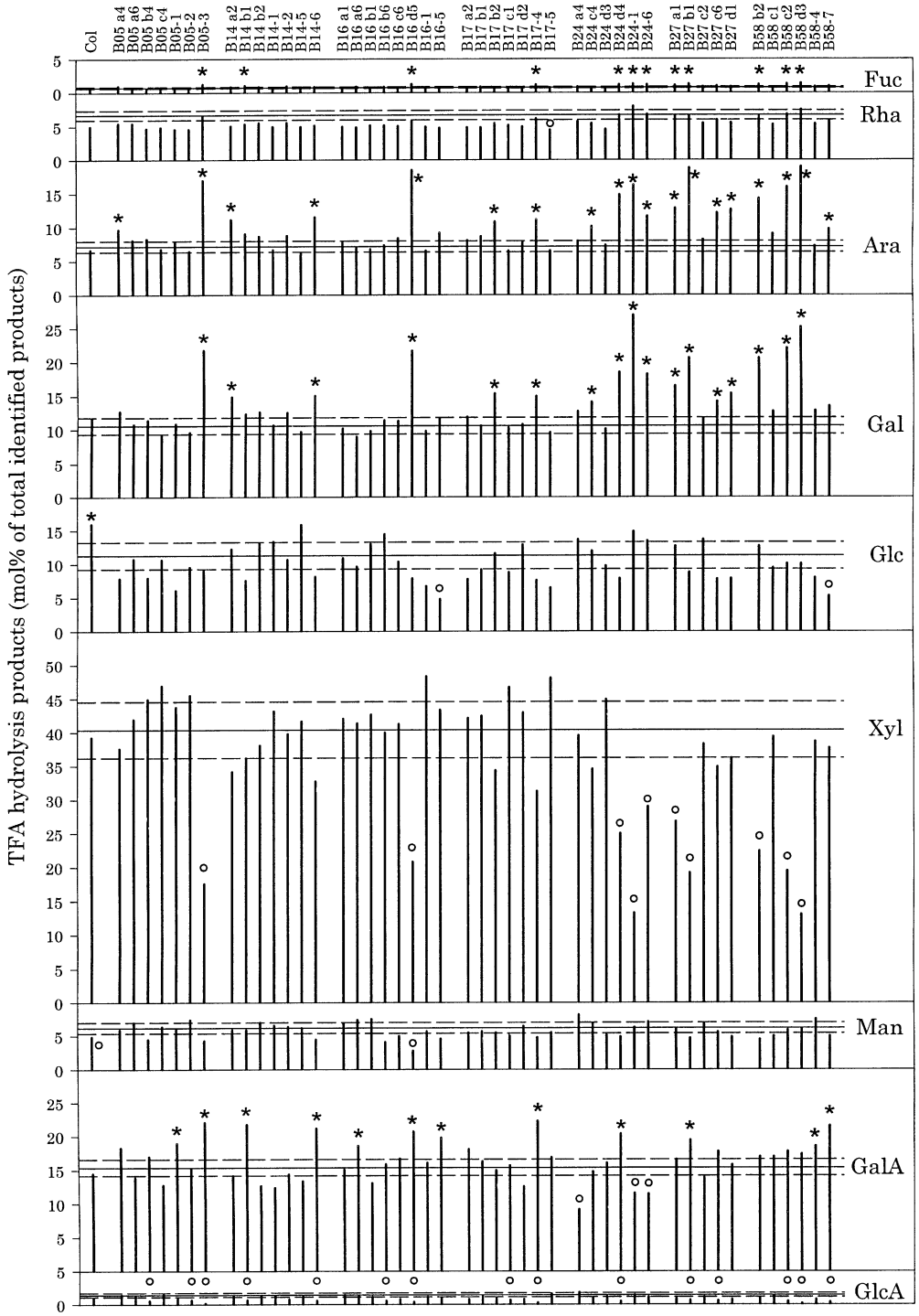

Fig. 4. Quantitative analysis of products obtained by acid hydrolysis of AIR from flowering stems of populations B5, B14, B16, B17, B24, B27 and B58 (= NASC accession numbers N2848, N2858, N2860, N2861, N2868, N2871, N2902, respectively). The hydrolysates were analysed by HPLC. Each set of vertically aligned bars on the histogram gives data for an individual plant, except 'Col' (= Columbia ecotype wild-type, which gives the mean of 6 individuals). Horizontal lines represent the means (———)±standard deviation (- - - -) ($n=6$) of the Wassilewskija ecotype wild-type. Individuals that differed significantly ($P<0.05$) from the Wassilewskija wild-type population are indicated (⋆, ○).

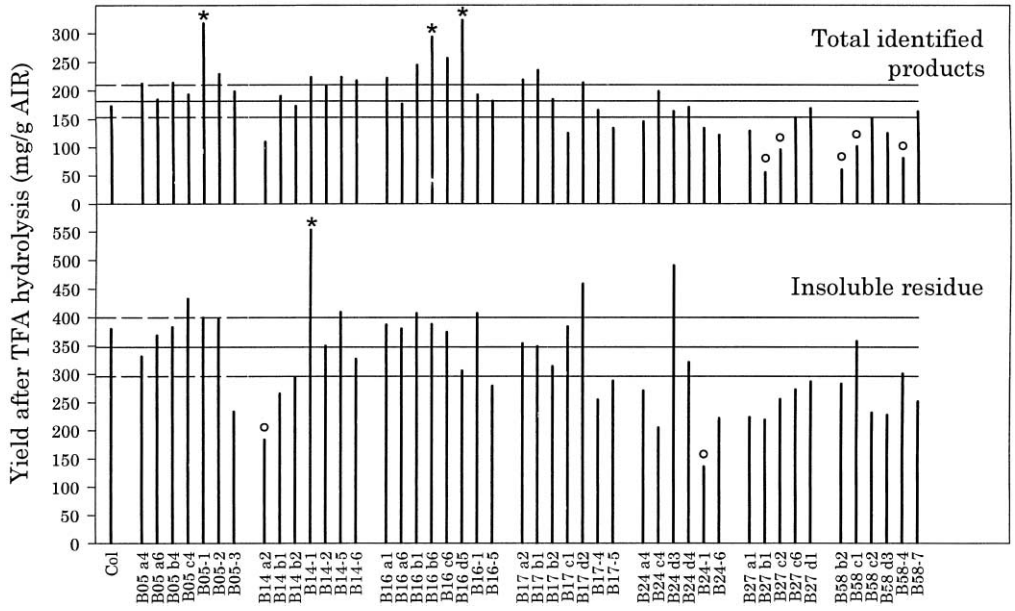

Fig. 4 (*continued*).

Table 5
Quantification by HPLC of hydrolysis products from the AIR of two wild-type ecotypes of *A. thaliana*

Hydrolysis product	Yield of specified product (mean±S.D.)[c]			
	mol% of total identified products		µmol/g AIR	
	Wassilewskija	Columbia	Wassilewskija	Columbia
After acid hydrolysis				
Fuc	0.8±0.1	0.8±0.2	8.9±1.1	8.1±1.6
Rha	6.7±0.7	5.0±0.3	72.6±8.6	49.2±2.3
Ara	7.2±0.8	6.7±0.8	77.8±11.5	72.7±9.5
Gal	10.6±1.2	11.7±1.1	114±14	124±9
Glc	11.3±2.0	16.0±3.4[a]	121±20	175±42
Xyl	40.4±4.2	39.3±3.5	444±103	434±47
Man	6.2±0.8	4.9±0.4[a]	68.3±17.2	58.3±7.0
GalA	15.4±1.2	14.5±1.5	167±24	164±13
GlcA	1.4±0.3	1.0±0.2	11.5±4.9	10.7±1.9
			mg/g AIR	
Total identified products	(100)	(100)	182±28	173±14
Residue[b]	–	–	348±52	380±14
After Driselase hydrolysis			µmol/g AIR	
Fuc	0.9±0.1	0.7±0.1[a]	6.9±0.6	5.9±0.8
Rha	1.7±0.2	1.7±0.2	12.8±1.2	14.8±1.5
Ara	8.0±1.0	6.9±1.0[a]	60.4±7.5	61.1±12.9
Gal	9.6±1.0	9.4±0.5	73.0±9.1	82.4±8.0
Glc	41.4±2.0	47.1±2.4[a]	316±49	415±45
Xyl	3.2±0.5	2.5±0.4[a]	24.3±5.1	21.7±4.7
Man	2.5±0.6	1.5±0.1[a]	18.7±3.4	13.6±1.7
IP	5.6±0.4	5.2±0.6	42.1±4.1	46.3±7.8
X_2	5.7±0.6	4.8±1.5	43.2±6.8	42.8±16.7
GalA	21.2±1.7	20.0±3.1	162±32	176±35
GlcA	0.3±0.1	0.2±0.1	2.2±0.5	2.1±1.0
			mg/g AIR	
Total identified products	(100)	(100)	168±59	169±19
Residue[b]	–	–	444±40	459±38

[a] Significantly different from Wassilewskija ($P<0.05$).
[b] Insoluble after hydrolysis.
[c] For acid hydrolysis, $n=6$; for Driselase hydrolysis, $n=6$ (Wassilewskija) or $n=10$ (Columbia).

the proportion of Fuc and Ara was significantly low in three of them. The Glc:X_2 ratio of the four variant plants was in the range 23–32 (cf. wild-type = 7.3; $n = 6$); the fifth individual was normal (ratio = 6.1). Similarly, the IP:X_2 ratio was higher (2.1–2.7) for the four variant plants than for the wild-type (ratio = 0.97). This pattern resembles that exhibited by many of the B24 individuals.

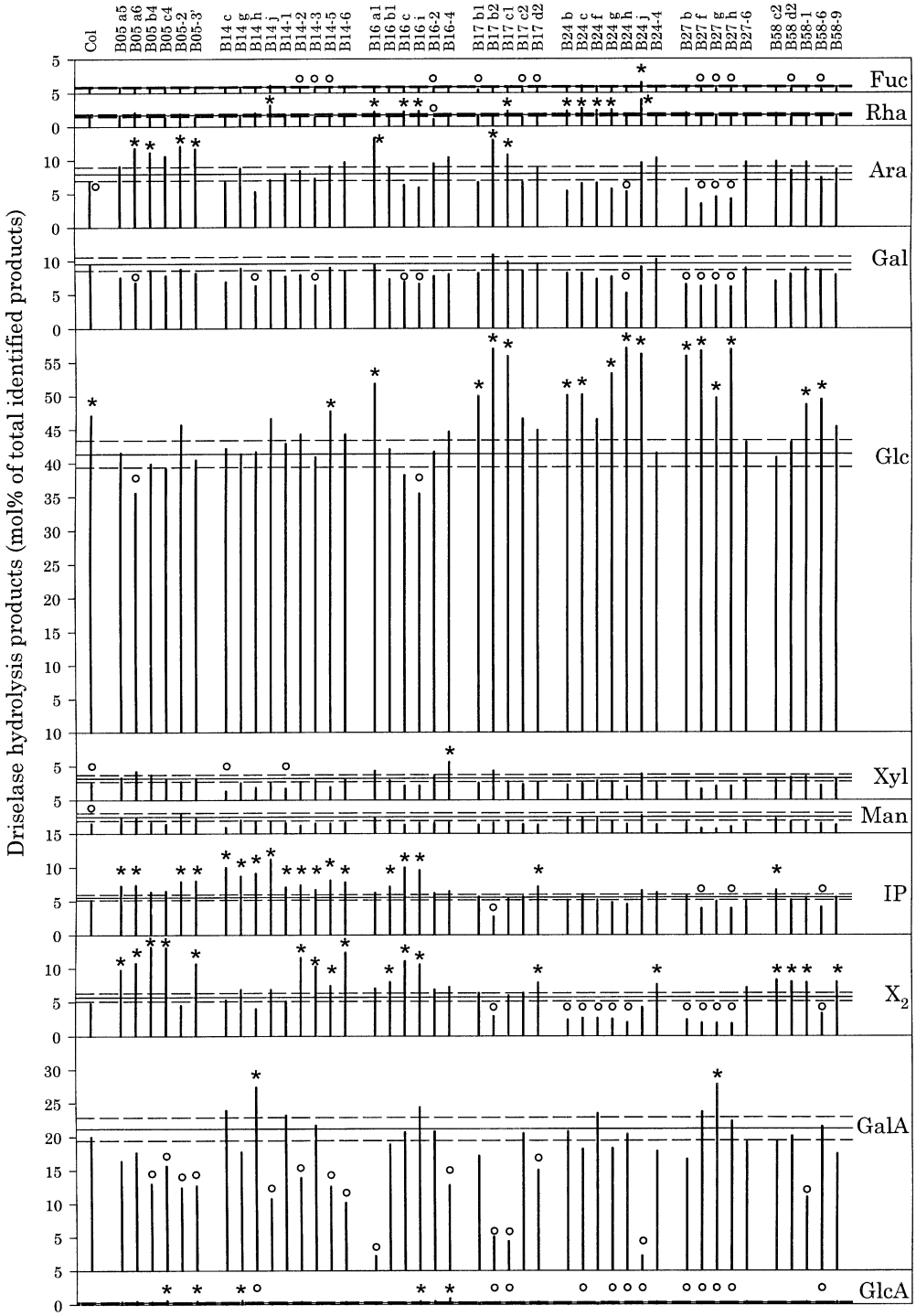

Fig. 5. Quantitative analysis of products obtained by Driselase digestion of AIR from flowering stems of populations B5, B14, B16, B17, B24, B27 and B58. Other details as in Fig. 4, except that the 'Col' data are means of 10 individuals.

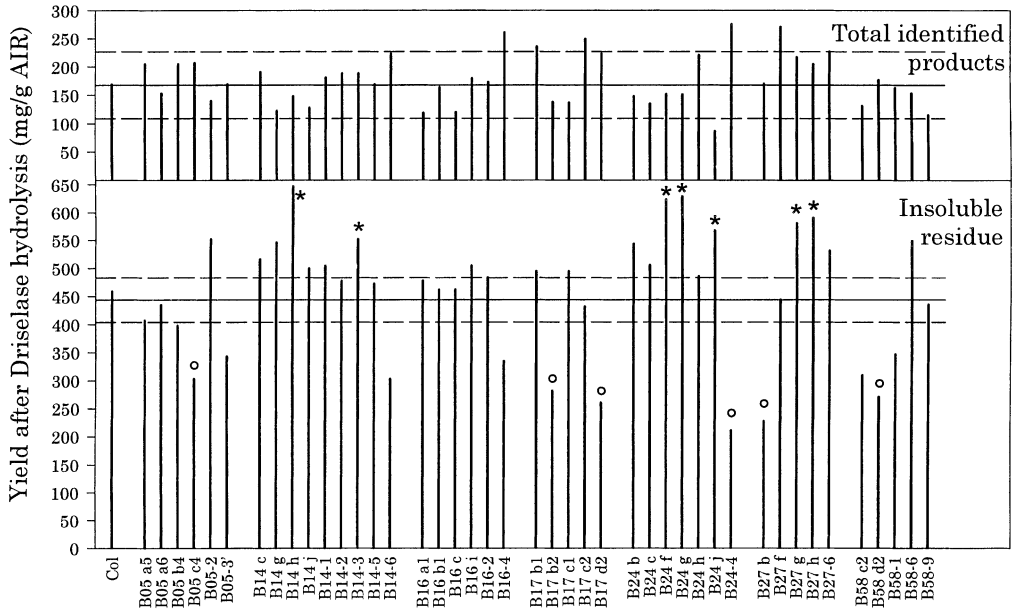

Fig. 5 (*continued*).

Table 6
Summary of the main recurrent deviations seen in six of the populations analysed by HPLC and their deduced biochemical basis

Line	Recurrent phenotypes[a] seen in		Possible biochemical basis of phenotype
	Driselase hydrolysate	Acid hydrolysate	
B24	$-X_2$, −GlcA, +Glc	−Xyl, •Glc, +Ara, +Gal	Increased digestible cellulose[b]; decreased absolute xylan
B27	$-X_2$, −GlcA, +Glc	−Xyl, •Glc, +Ara, +Gal	Increased digestible cellulose[b]; decreased absolute xylan
B5	$+X_2$, +IP, +Ara	•Xyl	Increased enzymic digestibility of hemicelluloses
B14	+IP, ($+X_2$), •Fuc	•Xyl, •Fuc, •Glc	Increased enzymic digestibility of xyloglucan backbone
B17	+Glc, •IP	•Glc	Increased digestible cellulose[b]
B58	$+X_2$	+Ara, +Gal, +Fuc, −Xyl, −GlcA	Decreased absolute xylan with decreased glucuronidation

[a] Symbols used: +, −, • = increased, decreased and normal proportion relative to wild-type.
[b] The data do not allow us to tell whether there was an increased absolute cellulose content or a normal cellulose content that was unusually susceptible to enzymic hydrolysis.

Acid hydrolysis showed normal non-cellulosic Glc in all individuals tested (Fig. 4), so the Driselase data suggest that the T-DNA insert had caused an increase in the proportion of (digestible) cellulose. In two out of five plants, the proportion of Xyl was diminished and these two plants also had relatively high levels of Gal and Ara; thus their Gal:Xyl and Ara:Xyl ratios were 2.4 to 5.4 times those of the wild-type.

2.7.3. Population B5

The Driselase–HPLC analysis (Fig. 5) did not corroborate the phenotype (low IP) previously seen in the repeat-screen of population B5 (Table 4). However, five of the six individuals tested gave unusually high proportions of X_2 (on average 2.5× that of the wild-type). Some individuals also gave somewhat higher proportions of IP (mean 1.3× wild-type) and Ara (mean 1.4× wild-type). This suggests that the presence of the T-DNA in the B5 population caused increased levels of (digestible) xylans and xyloglucan. The Glc:X_2 ratio (range 3.0–4.2 in the five affected plants) was lower than in the wild-type (mean = 7.3). The increase in proportion of X_2, IP and Ara was balanced by small decreases in several other products, especially GalA.

Of the seven individuals tested by acid hydrolysis, six gave relatively normal HPLC profiles (Fig. 4). Since the proportion of Xyl in the acid hydrolysate was approximately normal, the elevated proportions of X_2 and IP in the Driselase digests were probably due to increased digestibility, rather than quantity, of xylans and xyloglucans.

2.7.4. Population B14

The acid–HPLC analysis did not corroborate the phenotype (low Xyl) previously seen in the repeat-screen of population B14 (Table 4). However, all nine individuals tested in the Driselase–HPLC analysis (Fig. 5) gave unusually high proportions of IP (mean 1.7× that of the

wild-type). The proportion of X_2 was often also elevated. This suggests that the presence of the T-DNA in the B14 population caused increased levels of (digestible) hemicelluloses, especially xyloglucan. The increase in IP was balanced by small decreases in several other products—often GalA, Gal and Xyl. Acid hydrolysis indicated normal proportions of total Xyl (Fig. 4); thus the T-DNA insert in the B14 population appeared to lead to increased digestibility (rather than quantity) of the xyloglucan. There was no corresponding increase in Driselase-susceptible Fuc, suggesting that the Fuc residues of xyloglucan were always accessible to Driselase but that in the wild-type some of the backbone was shielded from the enzymes by groups (possibly *O*-acetyl groups) that were absent from the B14 plants.

2.7.5. Population B16

Of eight B16 individuals tested in the acid–HPLC analysis (Fig. 4), only one showed with statistical significance the phenotype previously observed (high Ara and high uronic acids; Table 4). This individual also showed numerous other significant differences from the wild-type. Two other individuals showed high GalA but not high Ara. The Driselase–HPLC analysis showed no recurring pattern of abnormalities (Fig. 5).

2.7.6. Population B17

Only one of the five B17 individuals reproduced the phenotype (low IP and low X_2) previously noted in the repeat-screen (Table 4). However, in three of the five B17 individuals subjected to the Driselase–HPLC analysis, the proportion of Glc was significantly higher than in the wild-type (Fig. 5). The high Glc was associated with decreases in several other digestion-products (different in different individuals), including IP, X_2 and GalA. None of the three high-Glc individuals had an abnormally high proportion of IP, indicating that the additional Glc arose from cellulose rather than xyloglucan. The acid hydrolysis data showed no recurring pattern of abnormalities (Fig. 4), supporting the conclusion that the mutation in certain B17 individuals affected cellulose (which is resistant to hydrolysis by 2 M TFA) rather than the matrix polysaccharides.

2.7.7. Population B58

The Driselase–HPLC analysis (Fig. 5) did not corroborate the repeat-screen (Table 4) in indicating a low proportion of Gal. However, the acid–HPLC analysis did reveal a recurrent phenotype (high Ara, Gal and Fuc; low Xyl and GlcA), which was exhibited by three of the six B58 individuals tested (Fig. 4). In the affected individuals, the proportions of Ara, Gal and Fuc were on average 2.3-, 2.1- and 1.6-fold higher, respectively, than in the wild-type, whereas the proportions of Xyl and GlcA were on average 0.45× and 0.38×, respectively, those of the wild-type. In the Driselase digests, there was a higher than normal proportion of X_2 in four out of five individuals tested; this was not associated with any other consistent trend. It is thought that after Driselase hydrolysis the GlcA and MeGlcA residues of xylans are mainly present in large, acidic oligosaccharides, which would not have been identified in the present analysis. A high proportion of X_2 in the Driselase digest, combined with a low proportion of total Xyl and GlcA in the acid hydrolysate, could indicate that there was a diminished xylan content but that the xylan that was present had a low degree of substitution with GlcA residues and was thus efficiently hydrolysed by Driselase to yield X_2.

3. Discussion

Mutagenised *A. thaliana* populations (T4 generation) were screened for alterations in detectable wall polysaccharides. The high-throughput screens developed here using PC and TLC enabled large numbers of lines to be tested easily. Individuals from seven populations of potential interest were also subjected to quantitative analysis by HPLC.

Our screens detect variants both with altered concentrations of specific wall components and with altered wall architecture (Table 6). For example, the proportion of X_2 (a xylan product) in the Driselase digests of affected individuals was elevated in populations B5 and B58 and diminished in B24 and B27. However, a high or low X_2 yield does prove abnormal xylan synthase activity; the dysfunction could equally be in the deposition of wall components that protect xylan from Driselase digestion. In fact, the high-X_2 individuals did not have an elevated proportion of Xyl in the acid hydrolysate, indicating that in B5 and B58 the abnormality was an increased susceptibility to Driselase digestion.

Changes in wall composition or architecture could be due to mutations either in genes directly related to cell wall metabolism or in genes involved in developmental regulation. Mutations of either type would be valuable both for basic studies of growth and development and for commercially orientated efforts to improve plant products. The screens presented here will facilitate the detection of such mutants.

4. Experimental

4.1. A. thaliana populations

Mutagenised populations of *A. thaliana*, supplied by NASC, were (in each case T4 generations): T-DNA tagged 'form-mutants' from organ transformation of ecotype Columbia (Koncz, 1992) and seed transformation of ecotype Wassilewskija (Feldmann and Marks,

1987; Feldmann et al., 1989) and Ds-tagged lines of ecotype Landsberg erecta (Bancroft and Dean, 1993). Wild-type ecotype Wassilewskija was also from NASC (accession no. N1601) and wild-type ecotype Columbia was from the collection at Advanced Technologies (Cambridge) Ltd.

The Feldmann lines had been produced by seed-transformation of Wassilewskija ecotype with *Agrobacterium tumefaciens* strain C58C1rif, containing Ti plasmid 3850:1003 (Feldmann and Marks, 1987), during which the T-DNA region of the plasmid is inserted into the plant's nuclear genome (Feldmann and Marks, 1987; Feldmann et al., 1989). This plasmid carries a neomycin phosphotransferase gene (NPT II), which confers kanamycin-resistance. Within each line, the T2 and T3 generations were both selected for kanamycin-resistance; therefore 90% of the T4 plants are expected to be kanamycin-resistant and to carry a mutant allele of the *A. thaliana* gene into which the insertion had occurred (70% homozygous, 20% heterozygous).

4.2. Cultivation of plants

Seeds were scattered on autoclaved compost (Fisons F2) soaked in nutrient soln. Pots were covered with a propagator lid to increase humidity, and placed in a growth room [12-h day (4 000 lumen/m^2); constant 22 °C]. Plants were watered with nutrient soln as required.

4.3. Alcohol-insoluble residue

The substrate for the screens was the AIR of the flowering stem, prepared by an adaptation of the method of Fry (2000) to remove low-M_r sugars and other metabolites, while retaining macromolecules (proteins, RNA and starch as well as wall polymers). Once the first siliquae had begun to form, the stems were collected, stored frozen in 50-mg aliquots, thawed into 70% EtOH (1 ml per 50 mg) and heated at 60 °C for 6–7 h, until the tissue appeared free of chlorophyll. The AIR was then washed in fresh 70% EtOH and dried in vacuo.

4.4. Marker sugars

Authentic sugars were from Sigma Chemical Co., Poole, Dorset, UK except IP and X_2, which were prepared by Driselase digestion of tamarind xyloglucan and birchwood xylan, respectively, and purified by preparative PC in EtOAc/pyridine/H_2O (8:2:1 by vol.).

4.5. Screening methods

4.5.1. Hydrolysis by acid

AIR (10 mg) was heated in 1 ml of 2 M TFA at 120 °C for 1 h. The residue was H_2O-washed, dried in vacuo and weighed. The hydrolysate and washings were dried in vacuo, re-dissolved in 1 ml H_2O and analysed by TLC and HPLC. For TLC, 6 μl was run on a cellulose plate (Merck) in BuOH/HOAc/H_2O (3:1:1) for 9 h, followed by EtOAc/pyridine/H_2O (10:4:3) for 4 h in the same dimension (Fry, 2000). Staining was with aniline hydrogen-phthalate (Fry, 2000).

4.5.2. Hydrolysis by Driselase

Driselase (Sigma) was partially purified (Fry, 2000). Driselase is a mixture of exo-hydrolases including α-D-galactopyranosidase, β-D-galactopyranosidase, β-D-glucopyranosidase, α-D-mannopyranosidase, β-D-mannopyranosidase, α-L-arabinofuranosidase, β-D-xylopyranosidase, α-L-fucopyranosidase, α-D-galacturonidase and cellulose-cellobiohydrolase, and endo-hydrolases including cellulase [endo-β-(1→4)-D-glucanase], endo-β-D-galactanase, endo-α-L-arabinanase, pectinase [endo-α-(1→4)-D-galacturonanase], endo-β-(1→4)-D-mannanase and endo-β-(1→4)-D-xylanase. The β-glucosidases include an enzyme that releases the disaccharide, IP, from the non-reducing end of xyloglucan and its oligosaccharides; there is no detectable α-D-xylosidase, so IP is an end-product (Fry, 2000). Driselase releases free GalA from homogalacturonan (whether or not methyl-esterified) and from RG-I.

AIR (10 mg) was incubated with 250 μl 0.5% Driselase in pyridine–acetic acid–H_2O (1:1:98, pH 4.7, containing 0.5% chlorobutanol) at 37 °C for 48 h. The residue was washed in H_2O, dried and weighed. Solution (30 μl) was mixed with 15 μl 30% formic acid and subjected to PC by the descending method on Whatman No. 1 paper (46 × 57 cm) in EtOAc–pyridine–H_2O (8:2:1) for 70 h. The chromatogram was stained with aniline hydrogen-phthalate (Fry, 2000).

4.6. HPLC protocols

The Driselase digest was mixed with 5 vols EtOH, left 16 h at −20 °C, then bench-centrifuged to pellet protein and undigested polysaccharides. The soln was dried in vacuo and resuspended in a vol. of H_2O equal to the original vol of Driselase soln. All samples were filtered through a 0.2-μm Gelman Supor Acrodisc filter prior to analysis on a Dionex HPLC with a CarboPac PA1 column. The eluent flow-rate was 1 ml/min, NaOH (0.5 M, 0.6 ml/min) was added post-column, and sugars were quantified with a pulsed amperometric detector (with gold electrode).

In method 1, for monosaccharides, the programme of NaOH concns was: 0–5 min, 10 mM; 5–30 min, 0 mM; 30–70 min, 0→800 mM (linear gradient); 70–75 min, 800 mM; 75–76 min, 800→10 mM; 76–85 min, 10 mM. In method 2, for disaccharides, the programme of NaOH concns was: 0–42 min, 30 mM; 42–43 min, 30→600 mM (linear); 43–48 min, 600 mM; 48–49 min, 600→30 mM; 49–61 min, 30 mM.

Two dilutions of each sample were analysed. Samples were run on the HPLC as batches of up to 40, with a range of calibration standards (0.02–0.10 mg/ml) at the beginning of the batch. Standards of 0.05 mg/ml were run between groups of samples to check that there was no drift in retention times or sensitivity. For Driselase digests, an enzyme-only control was also run; Driselase autolysis products (mainly Man) have been subtracted from the data presented.

4.7. Statistical analysis

HPLC data were analysed statistically. Analysis of variance (anova; Rohlf and Sokal, 1981; Sokal and Rohlf, 1995) was used to assess differences in product concentration between groups of replicate samples from wild-type Wassilewskija and Columbia (which are genetically homogenous populations).

As the plants within any given T4-generation population were not genetically identical, they could not be pooled to obtain a mean. Therefore, we used Student's t-distribution (Rohlf and Sokal, 1981; Sokal and Rohlf, 1995) to compare HPLC data from each individual transformant with the mean data from the relevant group of replicate wild-types.

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