Rapid Structural Phenotyping of Plant Cell Wall Mutants by Enzymatic Oligosaccharide Fingerprinting[1]

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Various biochemical, chemical, and microspectroscopic methods have been developed throughout the years for the screening and identification of mutants with altered cell wall structure. However, these procedures fail to provide the insight into structural aspects of the cell wall polymers. In this paper, we present various methods for rapidly screening Arabidopsis cell wall mutants. The enzymatic fingerprinting procedures using high-performance anion-exchange-pulsed-amperometric detection liquid chromatography, fluorophore-assisted carbohydrate electrophoresis, and matrix-assisted laser-desorption ionization time of flight (MALDI-TOF) mass spectrometry (MS) were exemplified by the structural analysis of the hemicellulose xyloglucan. All three techniques are able to identify structural alterations of wall xyloglucans in *mur1*, *mur2*, and *mur3*, which in comparison with the wild type have side chain defects in their xyloglucan structure. The quickest analysis was provided by MALDI-TOF MS. Although MALDI-TOF MS per se is not quantitative, it is possible to reproducibly obtain relative abundance information of the various oligosaccharides present in the extract. The lack of absolute quantitation by MALDI-TOF MS was compensated for with a xyloglucan-specific endoglucanase and simple colorimetric assay. In view of the potential for mass screening using MALDI-TOF MS, a PERL-based program was developed to process the spectra obtained from MALDI-TOF MS automatically. Outliers can be identified very rapidly according to a set of defined parameters based on data collected from the wild-type plants. The methods presented here can easily be adopted for the analysis of other wall polysaccharides. MALDI-TOF MS offers a powerful tool to screen and identify cell wall mutants rapidly and efficiently and, more importantly, is able to give initial insights into the structural composition and/or modification that occurs in these mutants.

The cell wall of plants is an extracellular matrix with both structural and growth-regulating functions. In dicots, the primary wall in growing cells consists of a network of cellulose microfibrils and xyloglucan cross-links embedded in a matrix consisting of a complex mixture of pectic polysaccharides and proteins. The rigidity and strength of the cell wall is related to the integrity of this cellulose/hemicellulose network (Pauly et al., 1999a). Furthermore, during cell growth, wall expansion has been found to be dependent on the enzymatic modification of the hemicellulosic component (Talbot and Ray, 1992; Pauly et al., 2001b). However, complementary information of the structure, organization, and metabolism of this network is still necessary to fully understand the biological process leading to plant cell elongation and its regulation.

Progress has been made in the isolation and characterization of cell wall polymers and on their dynamic changes occurring during cell division, expansion, and differentiation (Carpita and Gibeaut, 1993). However, very little is known about the biosynthesis, in muro assembly, and turnover of cell wall polymers on a molecular level, although numerous putative genes involved in these processes have been identified (e.g. Henrissat et al., 2001; Reiter and Vanzin, 2001). The knowledge of the functions of these genes will greatly enhance our understanding of the involvement of wall polysaccharides during plant growth and development. This will give us an opportunity to alter the wall polymers in a defined manner and thus enable us to relate the structure of the various wall polysaccharides to their functions. The selection of mutant plants with altered cell wall composition is a particularly useful approach that can potentially provide new opportunities to study the functions of cell wall polysaccharides and to identify the gene(s) encoding enzymes involved in the biosynthesis of nucleotide sugars or cell wall polysaccharide.

Selection of cell wall mutants can be labor intensive because of the complexity of the wall. Cellulose mutants have been selected on the basis of developmental phenotypes (Baskin et al., 1992; Turner and Som-

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erville, 1997; Arioli et al., 1998; Fagard et al., 2000). As an alternative, spectroscopic methods, such as birefringence (Potikha and Delmer, 1995) and Fourier transform infrared microspectroscopy (Chen et al., 1998), have also been developed for the selection of cell wall mutants. The latter is particularly efficient in the screening of mutants exhibiting defects with regard to their cellulose and non-cellulosic polymers (G. Mouille and H. Höfte, unpublished data). An alternative method for the screening of cell wall mutants was facilitated by Reiter and co-workers (1993, 1997) on the basis of the quantification of neutral cell wall monosaccharides using gas chromatography. A series of Arabidopsis *mur* mutants were selected based on the contents of their neutral sugars using this strategy. However, this method did not give a detailed characterization of the structural wall defect, failing e.g. to indicate which particular wall polysaccharide was affected. The characterization of a wall polysaccharide alteration usually involves its purification using differential extraction procedures and additional structural characterization using glycosidic linkage compositions by methylation analysis or NMR spectrometry (Zablackis et al., 1996; Vanzin et al., 2002). These procedures are unfortunately labor intensive, and they require large amounts of material and are therefore not suitable for mutant screens.

Here, we describe the use of various biochemical enzymatic fingerprinting methods that allow rapid and detailed structural analysis of wall polysaccharides. These methods are based on the analysis of enzymatically generated wall oligosaccharides by liquid chromatography, electrophoresis, or matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis. As a proof of concept, we focused on the analysis of xyloglucans, the most abundant hemicellulose present in the primary cell walls of dicots and non-graminaceous monocots to demonstrate the feasibility of the techniques.

RESULTS

Enzymatic Fingerprinting of Xyloglucans

A method for fast enzymatic fingerprinting analysis of Arabidopsis xyloglucans was assessed by treating cell wall material with xyloglucan-degrading enzymes, endoglucanase or xyloglucan-specific endoglucanase (XEG), followed by characterization of the resulting solubilized xyloglucan fragments. These enzymes cleave the xyloglucan backbone after the non-substituted Glc residues and release heptasaccharide XXXG to decasaccharide XLFG fragments, as well as the shorter XXG and GXXG fragments, according to the nomenclature reported by Fry et al. (1993; see also Table I). To minimize the number of experimental steps, plant material from Arabidopsis was treated with alcohol, and the resulting alcohol-insoluble material was directly subjected to hydrolysis with xyloglucan-degrading enzymes without any prior solubilization of the hemicellulose polymers. Under these conditions, the enzyme-susceptible xyloglucan domain is hydrolyzed (Pauly et al., 1999a). The resulting fragments, recovered in the enzyme buffer, were then analyzed by liquid chromatography (high-performance anion-exchange pulsed-amperometric detection [HPAE-PAD]), by fluorophore-assisted carbohydrate electrophoresis (FACE), or by MALDI-TOF MS without any further purification step.

HPAE-PAD Analysis of Endoglucanase-Generated Xyloglucan Fragments

The analytical procedures and protocols were validated by comparing results obtained from seedlings of wild-type (WT) Arabidopsis and *mur* mutants using the commercially available endoglucanase. *mur* mutants were selected on the basis of an alteration of the cell wall composition (Reiter et al., 1997). Among those mutants, three have been characterized as affected in the biosynthesis of xyloglucan. In *mur1*, the inactivation of the GDP-Man-4,6-dehydratase results

Table I. *Nominal masses, composition, putative structures, and significance of ions generated by MALDI-TOF MS of the various mur mutants*

Nominal Mass[a]	Composition[b]	Putative Structure[c]	mur 1[d]	*mur 2*	*mur 3*	*mur 4*	*mur 5*	*mur 6*	*mur 7*	*mur 9*	*mur 10*	*mur 11*
791.233	Hex_3Pen_2	XXG				+				+		
953.286	Hex_4Pen_2	GXXG						+			+	+
1085.328	Hex_4Pen_3	XXXG		+	+	+			+	+		
1247.381	Hex_5Pen_3	XXLG/XLXG	+	+						+		
1289.391	$Hex_5Pen_3OAc_1$	XXLG/XLXG + 1OAc	+	+	+			+				
1393.439	$Hex_5Pen_3Dox_1$	XXFG	+	+	+					+		
1435.449	$Hex_5Pen_3Dox_1OAc_1$	XXFG + 1OAc	+	+	+			+	+			
1555.491	$Hex_6Pen_3Dox_1$	XLFG	+	+	+			+	+	+	+	+
1597.502	$Hex_6Pen_3Dox_1OAc_1$	XLFG + 1OAc	+	+	+						+	

[a]Nominal mass of xyloglucan oligosaccharides generated by XEG digestion of Arabidopsis leaf cell wall material $[M + Na^+]^+$. [b]Hex, Hexose; Pen, pentose; Dox, deoxyhexose; OAc, *O*-acetyl substituent. Nos. in subscript indicate no. of substituent. [c]Based on xyloglucan oligosaccharides structures found in Pauly et al. (2001b). [d]Significance of relative ion areas by *t* test (nWT = 69, n*mur* = 5). +, Significantly different compared with WT with the probability $P = 0.01$; other ion areas were not significantly different.

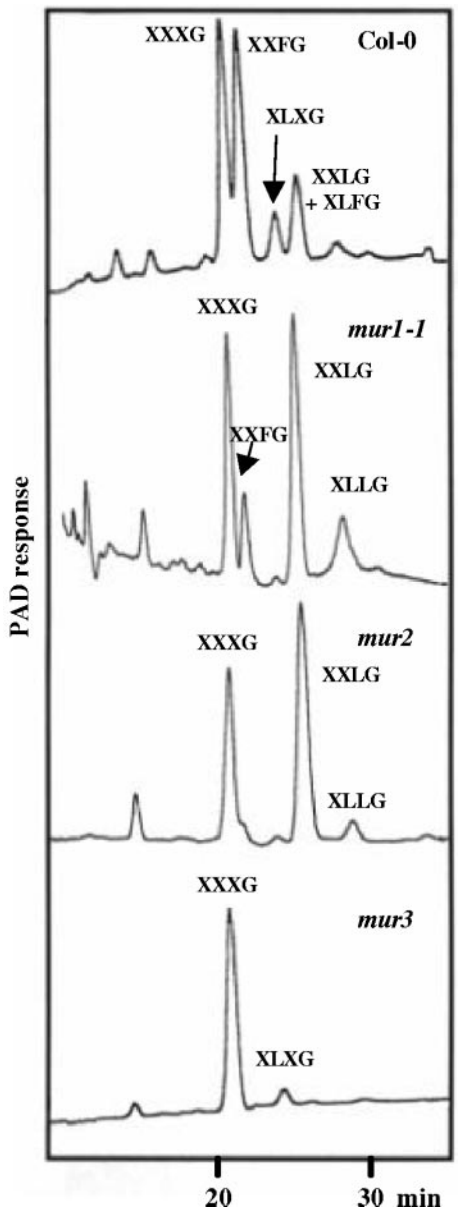


Figure 1. HPAE-PAD profiles of endoglucanase-generated xyloglucan fragments from seedlings of WT and *mur* Arabidopsis mutants. XXXG to XLFG refers to xyloglucan fragments according to the nomenclature reported in Fry et al. (1993).

in a strong decrease of L-Fuc (<5%; Reiter et al., 1993), and its replacement by L-Gal in the xyloglucan (Zablackis et al., 1996) and *N*-linked glycans (Rayon et al., 1999. *Mur2* (Vanzin et al., 2002) and *mur3* are altered in the xyloglucan fucosyl and galactosyltransferase, respectively. These mutations lead to an altered xyloglucan structure.

The pools of endoglucanase-generated xyloglucan fragments from *mur1* to *mur8* mutants were analyzed by HPAE-PAD chromatography (Fig. 1). Small changes in the substitution pattern of the xyloglucan oligomers result in considerable changes in their retention time in HPAE-PAD (Vincken et al., 1996). As a consequence, the alterations of xyloglucans in mutants give rise to HPAE-PAD profiles that strongly differ from the WT reference profile. This can be illustrated through the HPAE-PAD analysis of *mur1-1*, *mur2*, and *mur3*. In these mutants, the decrease of the Fuc content and/or the inactivation of the xyloglucan galactosyl and fucosyltransferase lead to the generation of a restricted number of xyloglucan fragments (Fig. 1). In contrast, other *mur4* to *mur8* mutants did not show any significant modification of the profiles of the pool of xyloglucan fragments (data not shown).

HPAE-PAD chromatography of endoglucanase-generated xyloglucan fragments was found to be highly reproducible. Quantification of the major oligosaccharides peaks from multiple repeats carried out on WT seedlings show that the SD in the peak area hardly ever exceed 1% (Table II). Furthermore, HPAE-PAD chromatography allows the separation of closely related oligosaccharides such as positional isomers. For instance, XLXG and XXLG are easily separated, which allows in *mur1-1*, *mur2*, and *mur3* the unambiguous identification of xyloglucan fragments that accumulate in these cell wall mutants. Consistent with published data (Reiter et al., 1993; Vanzin et al., 2002), the modification of the Fuc content in *mur1-1* and the inactivation of the fucosyltransferase in *mur2* result in the decrease or the absence of fucosylated fragments and in the increase of XXLG isomer. In contrast, only the XLXG isomer is detected in *mur3*, confirming that the mutation affects the galactosyltransferase involved in the transfer of the Gal residue onto the third Glc unit.

FACE

FACE has been described for oligosaccharides analysis (Jackson, 1990; Stack and Sullivan, 1992; Bardor et

Table II. *Comparison of relative abundance of xyloglucan oligosaccharides obtained from Arabidopsis seedlings by HPAEC-PAD and by MALDI-TOF MS*

Mutant	Xyloglucan Fragments	Relative Abundance (Percentage) as Determined by	
		HPAE-PAD[a]	MALDI-TOF MS[b]
mur1–1	XXXG	32 ± 1[c]	32 ± 3
	XXLG/XLXG	42 ± 1	47 ± 1
	XXFG	11 ± 1	11 ± 1
	XLLG/XXJG	15 ± 1	10 ± 2
mur2	XXXG	38 ± 1	38 ± 4
	XXLG/XLXG	55 ± 2	54 ± 5
	XLLG	7 ± 1	9 ± 1
mur3	XXXG	94 ± 3	86 ± 5
	XXLG/XLXG	6 ± 1	13 ± 3

[a]HPAEC-PAD signals (Fig. 1) representing the xyloglucan fragments were integrated. [b]Ion signals obtained by MALDI-TOF MS (Table I and Fig. 3) of the different xyloglucan fragments were integrated. The areas of the ion signals of *O*-acetylated compounds were added to their respective non-acetylated species. [c]Data represent the mean and SD of three independent experiments.

al., 2000), in which reducing oligosaccharides released were labeled by reductive amination with the negatively charged fluorophore 8-amino-naphtalene-1,3,6-trisulfonic acid (ANTS). The resulting fluorescent derivatives are separated according to their hydrodynamic size with high resolution by PAGE. Xyloglucan fragments, released from WT Arabidopsis and from *mur1* to *mur8* seedlings by endoglucanase treatment, were coupled to ANTS and analyzed by FACE (Fig. 2A). In the WT preparation, the four main bands could be assigned to XXXG, XXLG/XLXG, XXFG, and XLFG. Quantification of the major ANTS bands from four individual WT preparations show that the band intensities are highly reproducible (Fig. 2B).

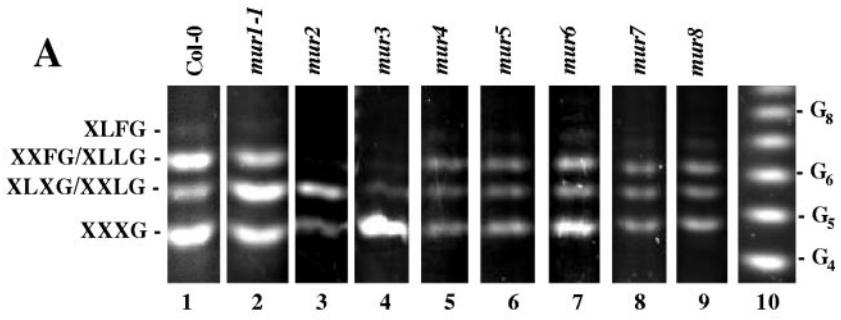
As depicted in Figure 2A, the FACE profiles of ANTS-xyloglucan fragments prepared from *mur1* to *mur8* can be visualized on a single gel. By comparison with the WT profile, no differences were observed in the electrophoretic profiles of *mur4* to *mur8* (Fig. 2A). In contrast and consistent with HPAE-PAD profiles, the alterations of the side chain biosynthesis in *mur2* and *mur3* result in strongly different electrophoretic patterns because of the absence of fucosylated ANTS-oligosaccharides. Compared with the WT profile, the FACE profile of *mur1-1* only shows a slight difference because ANTS derivatives of XLLG and XXFG comigrate on the gel. However, as described in Figure 2B, the quantification of the ANTS bands clearly allows the detection of xyloglucan alteration in this mutant.

MALDI-TOF MS Analysis

The pool of xyloglucan fragments, generated by endoglucanase treatment of ethanol-insoluble material from Arabidopsis seedlings, were analyzed by MALDI-TOF MS. The main ions detected in the MALDI-TOF MS of endoglucanase-generated fragments from WT seedlings were assigned to $(M+Na)^+$ adducts of XXG, GXXG, XXXG, XXLG/XLXG, XXFG, and XLFG on the basis of their M_r and literature data (Zablackis et al., 1995; Pauly et al., 2001a; Fig. 3; Table I). Furthermore, ions having 42 additional mass units were assigned to fragments containing *O*-acetyl substituents on the Gal residues (Kiefer et al., 1989; Pauly et al., 2001a). As observed for the HPAE-PAD analysis of *mur* mutants, only *mur1*, *mur2*, and *mur3* gave MALDI-TOF MS that strongly differed from the WT (Fig. 3). As expected, MALDI-TOF MS of xyloglucan fragments from *mur2* and *mur3* mutants are consistent with the inactivation of glycosyltransferases involved in the biosynthesis of the side chains, which leads to truncated xyloglucans. Interestingly, MALDI-TOF MS also allowed the fast comparison of different alleles. For illustration, two different alleles of *mur1* were analyzed: a strong allele, *mur1-1*, and one leaky allele, *mur1-7*. In both mutants, fucosylated fragments were detected. However, compared with the leaky *mur1-7* allele, *mur1-1* is characterized by a strong decrease of ions assigned to the fucosylated oligosaccharides and the increase of L-Gal-containing or XLLG fragments.

Quantitative Aspects of MALDI-TOF MS Analysis

Of all of the methods presented here, MALDI-TOF MS allows the quickest qualitative analysis of the xyloglucan structure because spectra can be obtained in less than 1 min. However, MALDI-TOF MS is not quantitative because of ion suppression and different ionization properties of different compounds. This disadvantage of MALDI-TOF MS is demonstrated when spectra were collected from endoglucanase extracts of five, 10, or 30 seedlings. The spectra were virtually the same (data not shown) despite the different amounts of wall material. Nevertheless, the ion area of a particular ion signal can be integrated allowing the establishment of a relative abundance profile of xyloglucan oligosaccharides present in a digest (Table II). The percentage of the various xyloglucan oligosaccharide ion signals is in good agreement with the percentage of the xyloglucan oligosaccharides determined by HPAE-PAD (Table II), a



B

	XXXG	XLXG/ XXLG	XXFG/ XLLG	XLFG
Col-0	32.6 ± 0.8	20.8 ± 0.7	32.8 ± 0.8	13.8 ± 0.5
mur1-1	33.1	37.9	29.1	

Figure 2. A, FACE profile of endoglucanase-generated xyloglucan fragments from seedlings of WT and *mur* Arabidopsis mutants. Lanes 1 through 9, ANTS-xyloglucan fragments from WT Col-0 and *mur1-mur8*. Lane 10, ANTS-Glc oligomers, prepared by partial hydrolysis of dextran and coupling to ANTS. XXG to XLFG, Xyloglucan fragments (for nomenclature, see Fry et al., 1993) according to their relative electrophoretic migration and based on migration of standard oligosaccharides. For confirmation, the four bands were analyzed by MS analysis as previously described (Lemoine et al., 2000). B, Quantification of ANTS-derivatives of isolated xyloglucan fragments isolated from WT and *mur1-1* seedlings. Values represent percentage of total quantified bands.

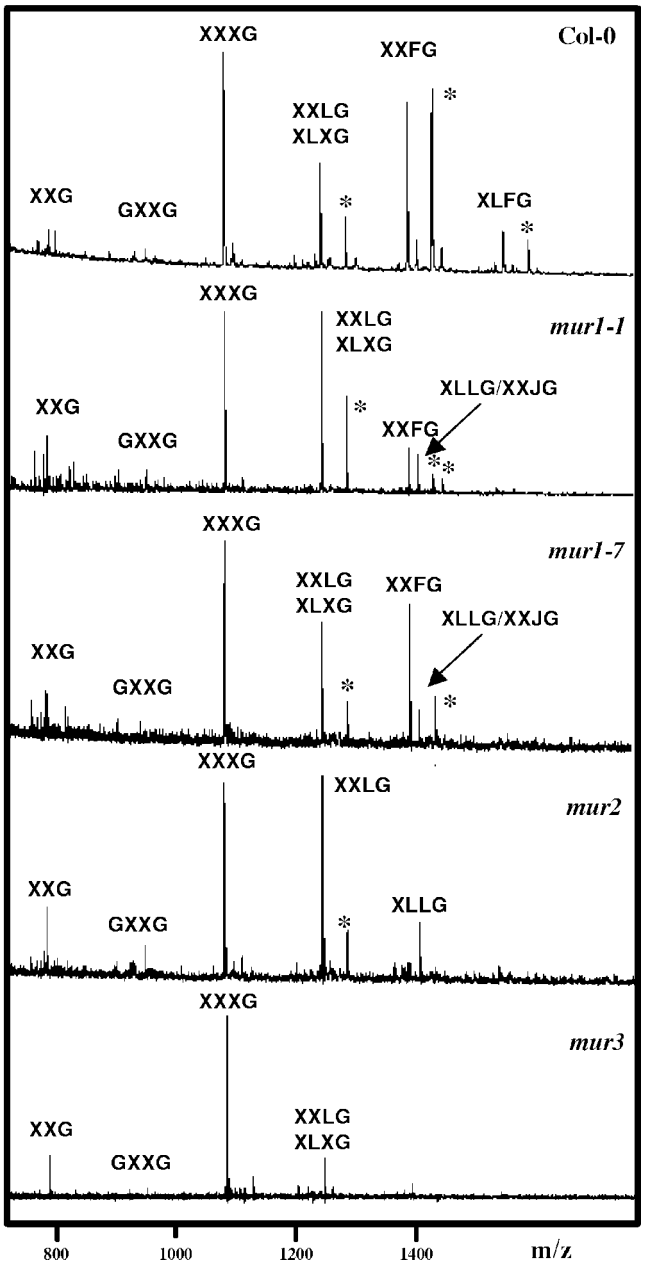


Figure 3. MALDI-TOF mass spectra of endoglucanase-generated xyloglucan fragments from seedlings of WT and *mur1-mur3* Arabidopsis mutants. XXG to XLFG, Xyloglucan fragments according to the nomenclature reported by Fry et al. (1993). The asterisk indicates the respective acetylated xyloglucan fragments.

method that allows not only the relative but also an absolute quantitation of the released material (Vincken et al., 1996) as shown for several of the *mur* mutants. This is probably attributable to the fact that the oligosaccharides are chemically related compounds. These results demonstrate that a MALDI-TOF profile gives a good indication of the relative quantitative distribution of the xyloglucan oligosaccharides present in the digest. The quantitation of the ion signals is most importantly reproducible, albeit with a larger SD than with the HPAE method, allowing a clear distinction between mutants.

The issue of absolute amounts of released xyloglucan oligosaccharides can be addressed by replacing the endoglucanase, which in addition to xyloglucan oligosaccharides also solubilizes some cello-oligosaccharides (Pauly et al., 1999b), with an XEG. The amount of solubilized material (only xyloglucan oligosaccharides) can then easily be quantified using a conventional colorimetric assay. The XEG/MALDI-TOF procedure was carried out with the WT (Col-0) seedling material, resulting in MALDI-TOF mass spectra that could be tested for reproducibility by integration of the area under the major ion signals (see also structures in Table I) and calculating their relative abundance (Fig. 4A). Again, the data clearly demonstrate the reproducibility of such MALDI-TOF oligosaccharides fingerprints. In addition, the amount of enzyme accessible xyloglucans present in the wall material could be reproducibly established (Fig. 4B). The amount of released xyloglucan oligosaccharides was independent of the extraction procedure used (boiling versus non-boiling; data not shown).

Another factor important in the analysis of mutant plants is that an apparent difference in wall structure might be related to the developmental stage rather than an actual alteration in wall structure. Using the XEG/MALDI-TOF procedure described above leaves

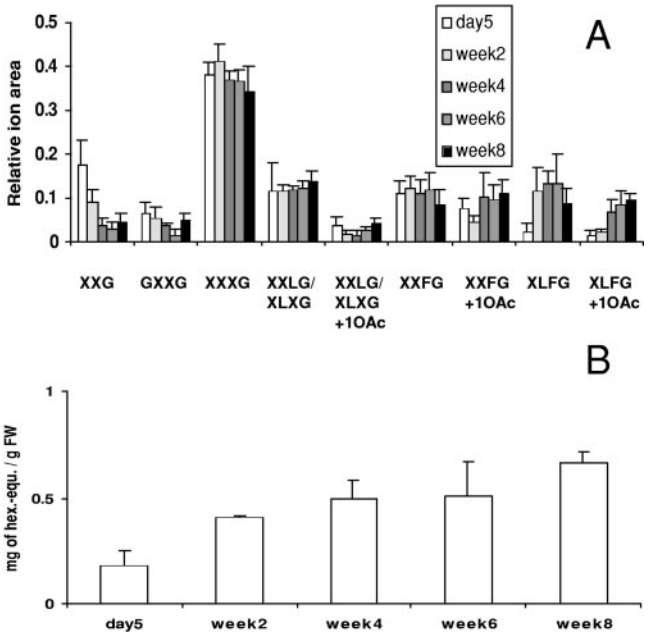


Figure 4. Analysis of xyloglucan oligosaccharides released by a XEG from Arabidopsis WT Col-0 seedlings (d 5) and leaf material at different developmental stages (weeks 2–8). A, Relative ion areas of selected ions representing xyloglucan oligosaccharides according to Table I obtained by MALDI-TOF MS. The data represent 10 individual plants. B, Amount of solubilized xyloglucan oligosaccharides using the anthrone assay expressed in hexose (Glc) equivalence per gram fresh weight.

from 2- to 8-week-old Arabidopsis plants were analyzed and compared with the seedling material (Fig. 4, A and B). The data indicates that there are very few differences in the xyloglucan oligosaccharides composition during the development of the plant, just a slight increase in *O*-acetylated xyloglucan oligosaccharides species. On the other hand, there is a substantial decrease in the proportion of the smaller oligosaccharides, such as XXG, whereas larger oligosaccharides, particularly XLFG (also *O*-acetylated) increase in relative abundance when the leaf material is compared with seedlings. These changes might be attributable to age, but it is more likely that they might be attributable to the tissue composition of the plant material analyzed. Further analysis of the xyloglucan oligosaccharides profiles of various tissues would be necessary to answer this question. There is also a continuous increase in the amount of xyloglucan that can be solubilized using the enzyme. However, the few differences found in the oligosaccharides profile suggest that under the conditions and time frame used, a difference in xyloglucan structure/accessibility of a potential mutant might be attributable to the differences in cell wall structure.

Bioinformatic Processing of the MALDI-TOF Spectra

The developed XEG fingerprinting procedure was applied to the *mur* mutants to establish relative quantitative differences. Using the colorimetric assay, the quantities of xyloglucan oligosaccharides released from WT and the *mur* mutants with the XEG showed large differences (Fig. 5B). *mur7*, *mur10*, and *mur11* showed reduced amounts of solubilized xyloglucans in comparison with WT. However, more xyloglucans were enzymatically released from *mur1-1*, *mur3*, *mur4*, *mur5*, *mur6*, whereas *mur2* and *mur9* showed comparable results as in the WT. These results demonstrate that the amounts of xyloglucans that are enzyme accessible can vary in different wall mutants.

MALDI-TOF fingerprints facilitating the XEG were performed on single leaves from sixty-five 4-week-old WT plants and four to five plants of each *mur* mutant. As expected, the fingerprint of *mur1-1*, *mur2*, and *mur3* can easily and reproducibly be distinguished from the fingerprint of WT plants (Fig. 5A). Although the fingerprint of the other *mur* mutants looks at a first glance similar to that of WT plants, statistical analysis (Student's *t* test) reveals that the relative abundance of some of the ions representing

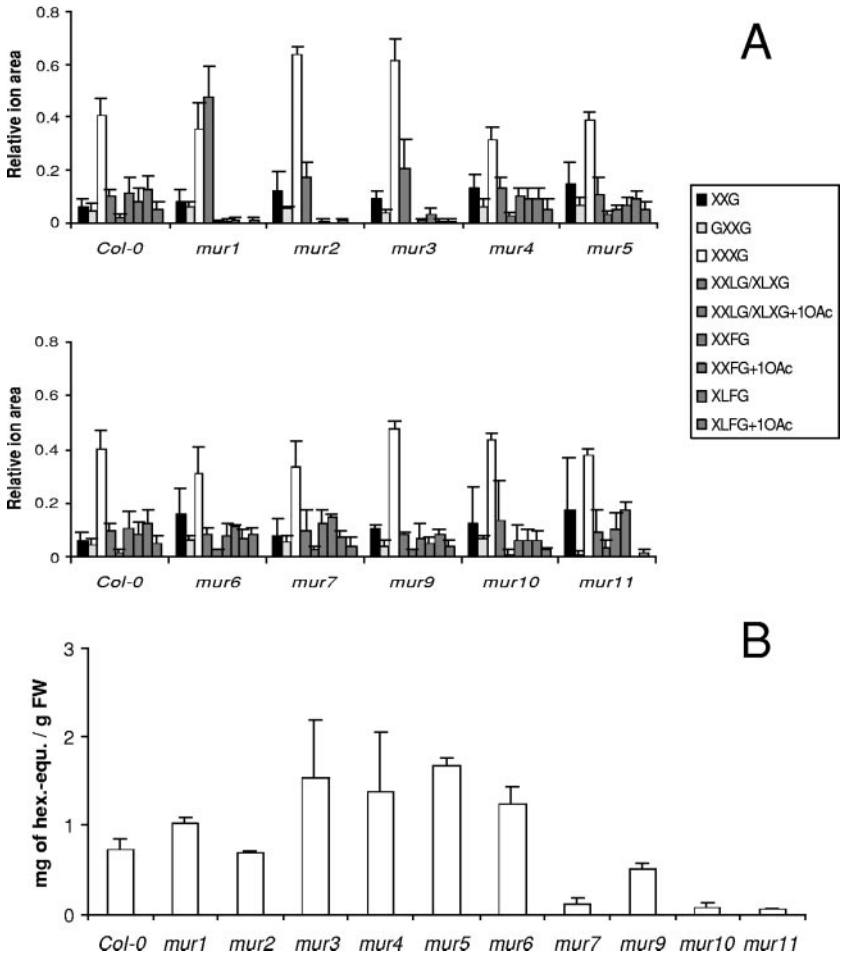


Figure 5. Analysis of xyloglucan oligosaccharides released by a XEG from WT Col-0 and selected *mur* mutants. A, Relative ion areas of selected ions representing xyloglucan oligosaccharides according to Table I obtained by MALDI-TOF MS. The data represents 65 individual WT plants and five individual plants of each *mur* mutant. B, Amount of solubilized xyloglucan oligosaccharides using the anthrone assay expressed in hexose (Glc) equivalence per gram fresh weight.

specific xyloglucan oligosaccharide structures is significantly different in all of the other *mur* mutants (Table I), albeit in not as many different ions as in the analysis of *mur1*, *-2*, and *-3*.

Should this method be used for a high throughput mutant screen, it is necessary to process the mass spectra obtained from solubilized xyloglucans from numerous plants. Furthermore, it is desirable to assess a single plant sample to reduce the labor involved in the screening process. Using the software provided by the MALDI-TOF MS company (Data Explorer, Applied Biosystems, Langen, Germany), it is possible to automatically obtain spectra of up to 192 samples that can be spotted on a single target plate. These spectra are then processed by an in-house developed PERL-based program (http://www.mpimp-golm.mpg.de/pauly under "MS-based tools"), that allows the evaluation of the acquired spectra in a fast and comfortable way. The program imports all of the acquired spectra in one run and compares each of them ($n = 1$) with the WT data set ($n = 65$; Fig. 6). As a selection criterion, a difference from the standard mean of the WT set by at least three SDs was chosen to reduce the number of potential false positives (1% in case of a normal distribution) and to only identify the mutants with the most pronounced differences. The program flags these spectra as outliers as well as spectra where at least one peak falls under the absolute value of 0.005 (Fig. 6, indicated by an asterisks). Should more than one such deviation occur, the probabilities for such occurrences are multiplied to make up for what we call the "outlier value" (Fig. 6), even though the individual peaks are not independent of each other. The graphical enhanced outlier value facilitates the quick examination of the spectra

Evaluated Data		
Outlier?	Filename	@Peaks?
100***	murs/mur 1.pkt	XXLG 13.99 SD; XXLG+AC *; XXFG *; XLFG *;
7***	murs/mur 2.pkt	XXXG 3.43 SD; XXLG+AC *; XXFG+AC *; XLFG+AC*;
15**	murs/mur 3.pkt	XXXG 3.05 SD; XXLG 4.03 SD; XXLG+AC *; XLFG *;
	murs/mur 4.pkt	
	murs/mur 5.pkt	
	murs/mur 6.pkt	
	murs/mur 7.pkt	
	murs/mur 9.pkt	
	murs/mur10.pkt	
*	murs/mur11.pkt	XLFG *;

Figure 6. Identification of outliers using a PERL-based program. The graph represents the graphical output of this program, taken into account the quantitation of the spectra as presented in Figure 5. The outlier value is calculated as described in the text and graphically enhanced (bold or inverted letters) to flag the most pronounced outliers. The putative structures of ions that fulfill the outlier criteria (see text) and their -fold difference in SD are explicitly shown. An asterisk denotes putative structures of ions that fall under the detection threshold (see text for details). The number of the asterisks ions are also accumulated in the column "Outlier?".

to point out mutants that have a substantial alteration in xyloglucan structure. In addition, the program points out specifically which ions, i.e. their putative structures, fulfill the abovementioned criteria (Fig. 6). One disadvantage of the current program is that only the masses of preset ions are taken into account, i.e. the ions occurring in the WT xyloglucan oligosaccharide sample. Should a mutant contain novel structures that have different masses than those preset ions, then they will not be taken into account. This is the case, for example, in the *mur1-1* xyloglucan, where novel ions for XXJG and XLJG also occur. However, the occurrence of these novel structures apparently changes the ratio of other examined ions, and hence the mutant was unambiguously identified (see Figs. 3–5).

The MALDI-TOF spectra obtained from the *mur* mutants were subjected to the analysis by the outlier program (Fig. 6), and as expected, *mur1* showed the highest outlier value, followed by *mur3*, and then by *mur2*. Interestingly, *mur11* was also identified as a potential outlier. It therefore seems that the xyloglucan structure in the latter mutant is altered, indicating that this mutant is a very good candidate for further more detailed structural analysis of the xyloglucan.

DISCUSSION

Enzymatic fingerprinting of xyloglucans seems to be a fast and efficient method to identify mutants altered in the xyloglucan structure, biosynthesis, and metabolism. In contrast to the previously described biochemical, chemical, and microspectroscopy methods, much more structural information is obtained, leading to a clearer picture of the alterations of a particular wall structure. Treatment of cell wall material prepared from Arabidopsis seedlings or leaves with endoglucanase or XEG releases xyloglucan fragments that can be characterized by chromatographic, electrophoretic, or spectroscopic analysis. The enzymes used here only hydrolyze about one-third of the xyloglucan present in Arabidopsis leaves (Pauly et al., 1999a). However, structural analysis of the remaining xyloglucan in the wall was also found to be very similar in the mutants demonstrating that the enzymatic method is sufficient to identify changes in overall xyloglucan structure (Pauly et al., 1999a, 2001a, 2001b). The pools of xyloglucan fragments were analyzed by HPAE-PAD, FACE, and MALDI-TOF MS. All analytical techniques were found to be able to easily characterize the defects in the side chain biosynthesis in *mur1* to *mur3* mutants by comparison with the WT data. Among these techniques, FACE has recently been described as a powerful tool for plant polysaccharide analysis (Goubet et al., 2002). Electrophoretic mobilities of ANTS-labeled oligosaccharides, relative to the standard ladder, are highly reproducible, allowing a fast and easy com-

parison of gels (Bardor et al., 2000). Applied to the analysis of xyloglucan fragments, this allows a fast identification of xyloglucan mutants by comparing the profiles and FACE band intensities with those of WT preparations. This method is a well-adapted technique for fast and inexpensive comparison of polysaccharide digests. However, the low resolution of this technique does not permit separation of closely related oligosaccharides such as XXFG and XLLG. In this study, the MALDI-TOF MS fingerprinting protocol was found to be sensitive enough for an accurate fingerprinting analysis on the low amount of material available from seedlings. For instance, we were able to record reproducible MALDI-TOF MS spectra on xyloglucan fragment pools released from one Arabidopsis seedling. However, the use of leaf material allows the analysis to be carried out on less diverse cell types. As illustration, the *mur1* xyloglucan structural phenotype is much more marked in leaves than in seedlings, because the mutation mainly affected the Fuc content in aerial parts of the plants. In addition to *mur* mutants, some Arabidopsis mutants affected in the biosynthesis of cellulose, i.e. *procuste* (*prc*) altered in cellulose synthases (Fagard et al., 2000) and *korrigan* (*kor*) altered in an membrane-bound endoglucanase (Nicol et al., 1998), were also subjected to the enzymatic fingerprinting. No differences were observed in both the HPAE-PAD profiles and the MALDI-TOF MS spectra of the xyloglucan fragments by comparison with the corresponding Wassilewskija WT fingerprint. This indicates that the decrease of the cellulose content in *prc* and *kor* does not qualitatively alter the structure of the xyloglucan associated in the cellulose/hemicellulose network (data not shown).

Analysis of the pools of xyloglucan fragments by MALDI-TOF MS allows a fast and reproducible identification of enzymatic fingerprints of xyloglucans. It has been demonstrated that the MALDI-TOF fingerprint reflects well the proportions of the different oligosaccharides present in the mixture. However, in contrast to HPAE-PAD, MALDI-TOF MS does not discriminate between structural oligosaccharide isomers such as XXLG and XLXG, unless a more time-consuming fragmentation analysis of the oligosaccharides by post-source decay analysis is employed (Yamagaki et al., 1998). Despite this limitation, one major advantage of the enzymatic fingerprinting by MALDI-TOF MS is the possibility to easily detect the native xyloglucan fragments including those bearing alkali-labile substituents such as *O*-acetyl substituents. The function of cell wall polysaccharide *O*-acetylation is not known. It was postulated that the presence of *O*-acetyl substituents may affect the rate to which an xyloglucan binds to cellulose (Pauly et al., 1999a). In contrast to HPAE-PAD and FACE in which analytical conditions resulted in the deacetylation of oligosaccharides, *O*-acetylated xyloglucan fragments could be visualized by MALDI-TOF MS. As a consequence, MS analysis of endoglucanase-susceptible xyloglucans may provide the opportunities to identify plants affected in the xyloglucan-specific acetyltransferases and to study the functions of such polysaccharide modifications.

MALDI-TOF spectra are generated in less than 1 min. Taken together with the potential of generating automatically spectra of up to 192 samples per MS run and the subsequent automated statistical analysis, this method allows the screening of a vast number of plants rapidly identified by their structural alterations in wall polysaccharides. Thus, this method appears to be a powerful tool that allows the identification of putative mutants in an automated and high-throughput manner. Applied to *mur* mutants, the automated analysis was found to be able to select not only strongly affected xyloglucan *mur1* to *mur3* mutants harboring side chain alterations, but also less affected mutants such as *mur11*, allowing the detection of new candidates for the analysis of the xyloglucan biosynthesis in plants.

The use of specific enzymes, such as XEG, allows even the absolute quantitation of solubilized material using e.g. conventional assays. This data reflects the enzyme accessibility of polysaccharides in the wall. Strong differences in the amounts of solubilized xyloglucan were observed between *mur* leaf material regardless of the extraction method used (methanol versus ethanol, boiling versus non-boiling). These differences cannot be related to a phenotype because *mur3* to *mur6*, showing a higher accessibility to xyloglucan-degrading enzymes, as well as *mur7* and *mur11*, showing a lower accessibility, do not exhibit any visible phenotypes (Reiter et al., 1997). In the same way, similar xyloglucan fingerprints were obtained from *mur4* to *mur6* and *mur11* plants, which demonstrates that the different enzyme accessibility of the xyloglucan in these mutants compared with the WT plants is not related to modifications in the xyloglucan structure. However, one exception that we cannot exclude is the possibility that substitutions of the xyloglucan backbone, such as arabinosylation (Kiefer et al., 1991), may lead to a partially XEG-resistant xyloglucan and thus to a decrease of the amount of released material. Nevertheless, our data indicate that changes in the xyloglucan accessibility in *mur* mutants is likely attributable to changes in the topology of other polymers and thus overall wall architecture. As a consequence, quantitation of enzyme extractable material from cell wall appears to be an alternative and simple protocol for detection of cell wall mutants.

The success of the study of cell walls with altered composition arising from either environmental adaptation or genetic modification is related to the development of biochemical methods well adapted to fast biochemical screens. Enzymatic fingerprinting of cell wall polysaccharides particularly by MALDI-TOF MS appears to be the most promising method allow-

ing a fast overview of a polymer on basis of the structure of fragments released by a specific enzyme. Once this initial information has been obtained it is worthwhile to employ more time-consuming methodologies to describe the structural differences in detail. This strategy, described herein on xyloglucan, can easily be adopted for the analysis of other cell wall components such as pectins or arabinoxylans potentially allowing rapid elucidation of the entire polysaccharidome, i.e. all classes of polysaccharides present in the wall, of the walls of mutants and other transgenic plants.

MATERIALS AND METHODS

Endoglucanase was purchased from Megazyme International Ireland (Bray, County Wicklow, Ireland; endo-β(1,4)-glucanase, EC 3.2.1.4, catalog no. E-CELTR). XEG was provided by Novozymes (Bagsvaerd, Denmark), and purified according to Pauly et al. (1999a). Seeds of the *mur* mutants were obtained from the Arabidopsis Biological Resource Center at the Ohio State University (Columbus). *mur1*(*1-1* and *1-7*) and *mur2* to *mur11* were in a Col-0 background (Reiter et al., 1997). *prc* (Fagard et al., 2000) and *kor* (Nicol et al., 1998) cellulose mutants were in a Wassilewskija background.

Plant Material and Growth Conditions

Seedlings were grown for 4 d in highly standardized conditions in the dark to minimize environmental influences. Seeds were sterilized and allowed to germinate as described by Santoni et al. (1994) without Suc in a dark growth chamber at 21°C. Seeds were imbibed for 48 h at 4°C and exposed to white light (200 μmol m^{-2} s^{-1}) for 2 h before transfer to final dark growth conditions, and plates were wrapped in three layers of aluminum foil. Days of growth were counted after transfer of the culture plates from 4°C to the growth chamber. Arabidopsis plants (Col-0, *mur1–mur11*) were grown on soil with 120 μmol m^{-2} s^{-1} at 20°C, 16-h light/8-h dark period with 60% humidity. Single leaves were harvested after 4 weeks unless otherwise indicated.

Preparation of Xyloglucan Fragments

Endoglucanase

Two hundred seedlings were combined and heated at 70°C for 15 min in 70% (v/v) ethanol to inactivate enzymes. The tissues were then ground in a potter homogenizer, and the homogenate was washed two times with hot 70% (v/v) ethanol and one time with water. The remaining pellet was then freeze-dried. Xyloglucan fragments were generated by treating 500 μg of the ethanol-insoluble material with 5 units of endoglucanase in 500 μL of 10 mM sodium acetate buffer, pH 5, for 18 h at 37°C.

XEG

Leaf material (15–50 mg fresh weight) or the seedling material was harvested and transferred to an Eppendorf tube (2-mL volume) before freezing in liquid nitrogen. The tissue was then homogenized using a Retschmill machine (model MM200, Retsch, Haan, Germany) at 25 Hz for 1 min. The ground leaf tissue was suspended in 100% methanol, vortexed, and pelleted by centrifugation at 10,000*g* for 10 min. The methanol was decanted, and this methanol washing procedure was then repeated. The remaining pellet was dried under vacuum for 5 min and then washed with distilled water once. The remaining wall residue was treated with 1 unit of XEG (1 unit releases 1 μmol of reducing xyloglucan oligosaccharide per hour) in 200 μL of 50 mM ammonium formate, pH 5, for 16 h at 37°C. The solubilized xyloglucan oligosaccharides present in the supernatant after centrifugation (1,000*g*, 5 min) were then passed through a mini centrifuge filter (0.45 μm, Nalge Nunc International, Essen, Germany) filled with 100 μL of Q-Sepharose material (high-performance material, Amersham Biosciences AB, Uppsala). The solublized xyloglucan oligosaccharides were quantified according to Dische (1962) using a Glc standard curve.

HPAE-PAD Chromatography

HPAE-PAD chromatography of endoglucanase-generated xyloglucan fragments was achieved on a DX 500 system (Dionex, Sunnyvale, CA) equipped with a GP 50 gradient pump and a CarboPac PA1 column. Oligosaccharides were separated using a gradient from 100 mM NaOH (solvent A) to 1 M NaOAc in 100 mM NaOH (solvent B) at 1 mL min^{-1} in the following conditions: 0 min, 100% A; 5 min, 95% A; and 30 min, 92% A. Assignments of peaks to xyloglucan fragments were carried out according to published data (Vincken et al., 1996) and by comparison of their retention times with XXXG, XLXG, XXLG, and XLLG fragments (nomenclature according to Fry et al. [1993]) prepared by digestion of xyloglucan from tamarind with endoglucanase.

Fluorophore Labeling and Electrophoretic Separation of Oligosaccharides

Freeze-dried xyloglucan fragments were labeled by adding 15 μL of a stock solution of 8-aminonaphtalene-1,3,6 trisulfonic acid ANTS (0.15 M ANTS in acetic acid:water 3:17, v/v) and 15 μL of a freshly made solution of 1.0 M sodium cyanoborohydride in dimethyl sulfoxide and incubating at 37°C for 16 h. The reaction mixture was dried under vacuum for 4 h in a Speed-Vac (Savant Instruments, Holbrook, NY) at 45°C and dissolved in 100 μL of glycerol:water (1:4, v/v). Two to 5 μL of the solution of ANTS-labeled oligosaccharides was loaded on homemade polyacrylamide gels and was subjected to PAGE according to the method of Laemmli, but without SDS. The gel size was 80 mm high, 100 mm wide, and 0.75 mm deep. Ten milliliters of the resolving gel solution was obtained by mixing 5.0 mL of a stock solution containing 60% (w/v) acrylamide and 1.6% (w/v) *N*,*N*′-methylenebisacrylamide, 2.5 mL of stock gel buffer (1.5 M Tris-HCl buffer, pH 8.5), 2.5 mL of water, 50 μL of 10% (w/v) fresh made ammonium persulfate solution, and 10 μL of TEMED. The stacking gel was made by mixing 0.63 mL of stock acrylamide solution, 2.5 mL of gel buffer, 100 μL of 10% (w/v) ammonium persulfate solution, 10 μL of TEMED, and water to a final volume of 10 mL. The sample was loaded on the gel and separated by gel electrophoresis at room temperature for 2 h at 15 mA of constant current using a Tris-Gly buffer (25 mM Tris-HCl and 192 mM Gly, pH 8.5). Electrophoresis was performed until the unreacted ANTS exited from the gel. The separation was visualized on a standard UV light box (TFX-35M transilluminator, Bioblock Scientific, Illkirch, France). The gels were imaged using an imager system (Vilber Lourmat, Marne-la-Vallée, France), a fluorescence-imaging device equipped with an array CCD camera. The image was displayed on a computer screen, and the quantification was determined with Bio-capt and Bio-1D Software (Vilber Lourmat). Assignments of bands to non-fucosylated xyloglucan fragments were carried out by comparison of the electrophoretic mobilities with ANTS derivatives of XXXG, XLXG/XXLG, and XLLG fragments prepared by digestion of xyloglucan from tamarind with endoglucanase (Lemoine et al., 2000).

MS Analysis

MALDI-TOF MS of the xyloglucan oligosaccharides solubilized by endoglucanase was recorded on a Tof spec E MALDI-TOF mass spectrometer (Micromass, Manchester, UK). Xyloglucan oligosaccharides solubilized with XEG were analyzed by a Voyager DE-Pro MALDI-TOF instrument (Applied Biosystems) using an acceleration voltage of 20,000 V with a delay time of 350 ns. Mass spectra were obtained in the reflectron mode using 2,5-dihydroxybenzoic acid (10 mg mL^{-1}) as matrix mixed with the solubilized sugars 1:1 (v/v).

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