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ARTICLE

MALDI-TOF MS and CE-LIF Fingerprinting of Plant Cell Wall Polysaccharide Digests as a Screening Tool for *Arabidopsis* Cell Wall Mutants

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Cell wall materials derived from leaves and hypocotyls of *Arabidopsis* mutant and wild type plants have been incubated with a mixture of pure and well-defined pectinases, hemicellulases, and cellulases. The resulting oligosaccharides have been subjected to MALDI-TOF MS and CE-LIF analysis. MALDI-TOF MS analysis provided a fast overview of all oligosaccharides released, whereas CE-LIF-measurements enabled separation and characterization of many oligosaccharides under investigation. Both methods have been validated with leaf material of known mutant *Arabidopsis* plants and were shown to be able to discriminate mutant from wild type plants. Downscaling of the MALDI-TOF MS and CE-LIF approaches toward the hypocotyl level was established, and the performance of MALDI-TOF MS and CE-LIF was shown in the successful recognition of the *Arabidopsis* mutant *gaut13* as an interesting candidate for further analysis.

KEYWORDS: *Arabidopsis thaliana*; MALDI-TOF MS; CE-LIF; cell wall; oligosaccharides; screening

INTRODUCTION

Primary plant cell walls of dicotyledonous plants are mainly composed of the polysaccharides cellulose, hemicelluloses (mainly xyloglucan and (glucurono)-arabinoxylans), and pectin (homogalacturonan, rhamnogalacturonan I and II, xylogalacturonan). Knowledge of their biosynthesis is rather incomplete, especially concerning the pectin structures. The elucidation of the biosynthesis of cell wall polysaccharides is nowadays mainly based on comparable analysis of wild type and mutant plants of *Arabidopsis thaliana* (*1*−*3*). As its genome is known completely and many mutants can be obtained relatively easy, the dicotyledonous plant *A. thaliana* has broadly been accepted as a model plant in plant sciences. The cell wall polysaccharides of *A. thaliana* have been described quite intensively (*4*−*6*) and are comparable to the ones identified in other dicotyledonous plants (e.g., apple). For further elucidation of the cell wall polysaccharide biosynthesis, the identification of more (new) *Arabidopsis* mutant plants, which differ specifically in the cell wall polysaccharide structure, is necessary.

Sugar composition analysis after extraction of the cell wall material has frequently been used as a screening tool for *Arabidopsis* cell wall mutants (*7*). However, due to complete hydrolysis of the polymer, crucial information of the origin and linkage of the various sugars is lost. Fourier transform infrared (FTIR) microspectroscopy is a fast and nondisruptive method, which has successfully been shown to select cell wall mutant *Arabidopsis* hypocotyls (*8*). However, no detailed information about the nature of the polysaccharide altered is provided. Other screening techniques are mostly based on enzymatic degradation of a specific polysaccharide followed by analysis of the oligosaccharides released. Such an approach by using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis after enzymatic degradation has been shown to be efficient in selecting *Arabidopsis* mutants, which differ in xyloglucan (XG) oligosaccharides released by xyloglucan specific endoglucanase (XEG; *9*). Recently, the same approach has been described for homogalacturonan (HG) oligosaccharides released by endopolygalacturonase (PG) and pectin methyl esterase (PME; *25*). The degradation of all enzyme-accessible polysaccharide structures in the cell wall by using a mixture of various cell wall degrading enzymes prior to MALDI-TOF MS analysis to recognize changes in cell wall polysaccharides as well as cell wall architecture has, however, not yet been investigated. In this study we present a multipolysaccharide screening method by using a mixture of 10 pure and well-defined cell wall degrading enzymes followed by MALDI-TOF MS analysis of the oligosaccharides released to enable the detection of *Arabidopsis* mutants with alterations in any polysaccharide of the whole cell wall. As MALDI-TOF MS detection is based on ionization of the molecule, which is highly dependent on the nature (especially charge) of the molecule, capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) has been evaluated as a complementary medium-throughput quantitative screening method. CE-LIF has been described recently to be a suitable method to analyze complex oligosaccharides, enabling the separation and mole-based detection of various cell wall oligosaccharides within 15 min (*10*−*12*). This makes it an outstanding method as a medium-throughput quantitative screening method.

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Table 1. Overview of the Analyses Performed for Various Leaf and Hypocotyl Materials

			leaf material		hypocotyl material		
		background	MALDI-TOF MS	CE-LIF	sugar composition	MALDI-TOF MS	CE-LIF
Col0	wild-type		x	x	x	x	x
qrt	wild-type		x	x			
WS	wild-type		x	x			
mur3	XG-altered	*Col0*	x	x			
xgd1	XGA-deficient	*qrt*	x	x			
qua1	HG-deficient	*WS*	x	x			
irx14 (SALK 038212)	xylan-deficient	*Col0*			x	x	x
qua1 (SALK 102380)	HG-deficient	*Col0*			x	x	x
At4g21060 (SALK 033674)	not known	*Col0*			x	x	x
gaut13 (SALK 122602)	not known	*Col0*			x	x	x

MATERIALS AND METHODS

Plant Material. Wild type and mutant leaves (6−12 weeks old) and hypocotyls (4 days old, dark grown) of *A. thaliana* have been kindly provided by partners of the European framework 6 project "WallNet" (Herman Höfte, INRA, Versailles, France; Markus Pauly, University of Berkeley, Berkeley, CA; Henrik Scheller, JBEI, Berkeley, CA). All materials have been delivered freeze-dried. An overview of all samples and the various analyses performed are listed in **Table 1**.

Preparation of Alcohol Insoluble Solids (AIS) of Leaf Material. About 10 g of freeze-dried sample was milled and subsequently extracted with 70% (v/v) aqueous ethanol at 50 °C as described in Hilz et al. (*13*). The extraction was repeated twice, and the residue (AIS) was dried after solvent exchange with acetone at ambient temperature.

Preparation of AIS of Hypocotyls. Twenty hypocotyls (4 days old, dark grown) were weighed into a reaction vial (1 mL; Alltech Biotechnology, Lexington, KY), and the tissue was destroyed by ball milling in liquid nitrogen (4 min, 3 × 1 mm stainless steel balls, Retsch Mill M200, Retsch, Haan, Germany). Subsequently, the material was extracted three times with 70% (v/v) aqueous ethanol at 70 °C (0.75 mL, 2 min ball-milling) followed by centrifugation (1 min, 1500*g*, ambient temperature). The residue (AIS) was dried by solvent exchange using acetone at ambient temperature. The whole residue was used for further enzyme treatments.

Enzyme Digestion of Leaf and Hypocotyl Materials. Suspensions of 5 mg/mL and 0.02−0.05 mg/100 μL AIS derived from leaf material and 20 hypocotyls, respectively, were treated with an enzyme cocktail of 10 different enzymes. As buffers, 50 and 10 mM sodium acetate buffers (pH 5.0) for leaves and hypocotyls, respectively, were used. The enzyme cocktail contained α-(1,4)-polygalacturonase (endo-PG, *Kluyveromyces fragilis marxianus*; *14*), pectin methyl esterase (PME, *Aspergillus niger*), rhamnogalacturonan hydrolase (RGH, *Aspergillus aculeatus*; *15*), xylogalacturonan hydrolase (XGH, *A. niger*; *16*), β-(1,4)-galactanase (*A. niger*; *17*), α-(1,5)-arabinanase (*A. aculeatus*), β-(1,4)-xylanase (*Aspergillus awamori*; *18*), xyloglucan specific endoglucanase (XEG, *A. aculeatus*; *19*), endoglucanase I and cellobiohydrolase (*Trichoderma viride*; *20*). Enzymes were added in an overdose to ensure that the enzyme reactions reach their end point within 6 h, and the samples were incubated for 22 h at 37 °C. The enzymes were inactivated by boiling the digests for 5 min. Subsequently, the digests were centrifuged (5 min, 1500*g*, ambient temperature), and the supernatants were used for MALDI-TOF MS analysis and CE-LIF labeling.

MALDI-TOF MS Analysis. The supernatants (10 and 15 μL for leaves and hypocotyls, respectively) of the enzyme digests were desalted with AG 50W-X8 resin (Bio-Rad Laboratories, Hercules, CA). For leaves, one spatula spoon of AG 50W-X8 resin was added to 100 μL of each enzyme digest and desalting was allowed for ∼30 min (*21*). For hypocotyls, the desalting step has been standardized by always incubating 15 μL of sample with 10 mg of AG 50W-X8 resin for 60 min. Each desalted sample solution (1 μL) was mixed on a MALDI plate (Bruker Daltonics, Bremen, Germany) with 1 μL of matrix solution of 12 mg/mL 2,5-dihydroxybenzoic acid (Bruker) in 30% (v/v) acetonitrile and dried under a stream of air (*22*). MALDI-TOF MS analysis was performed by using an Ultraflex workstation (Bruker Daltonics) equipped with a nitrogen laser of 337 nm and operated in positive mode. After a delayed extraction time of 350 ns, the ions were accelerated to a kinetic energy of 22000 V. The ions were detected using the reflector mode. The lowest laser power required to obtain good spectra was used. The mass spectrometer was calibrated with a mixture of maltodextrins (AVEBE, Foxhol, The Netherlands; average degree of polymerization = 5; mass range m/z 500−2000). Data were processed using flexAnalysis version 2.2 (Bruker Daltonics).

Oligosaccharide Separation by CE-LIF. To 75 and 30 μL of the nondesalted enzyme digests were added 5 and 2 nmol of maltose as internal standard for leaf and hypocotyl material, respectively. After this solution had been dried (SpeedVak concentrator; Savant ISS110, Thermo Scientific, Waltham, MA), the oligomers were labeled with 9-aminopyrene-1,4,6-trisulfonate (APTS) using the ProteomeLab Carbohydrate Labeling and Analysis Kit (Beckman Coulter, Fullerton, CA). This solution (4 μL) was filled to 50 μL with Millipore water and subsequently diluted with Millipore water, 20 times for the leaf enzyme digests and 10 times for the hypocotyl enzyme digests. The labeled oligosaccharides were separated on a polyvinyl alcohol (NCHO) coated capillary (50 μm i.d. × 50.2 cm, detection window after 40 cm; Beckman Coulter) using a ProteomLab PA 800 capillary electrophoresis system, equipped with a laser-induced fluorescence detector (excitation, 488 nm; emission, 520 nm; Beckman Coulter). Samples were loaded hydrodynamically (7 s at 0.5 psi, representing approximately 25 nL sample solution) on the capillary. Separation was carried out in reversed polarity at 30 kV with 25 mM acetate buffer (pH 2.98) containing 0.4% polyethylene oxide and 0.3% formic acid. The capillary was kept at 25 °C.

Statistical Analysis of MALDI-TOF MS Data. From each wild type and mutant leaf material AIS was extracted, and subsequently each AIS was incubated with the enzyme cocktail in triplicate. Every digest was subjected twice to MALDI-TOF MS, and for every spot two times 200 shots were taken to exclude interferences due to different crystallization. In total, 12 MALDI-TOF mass spectra per sample were transferred to a statistical program (Grams, Thermo Scientific). Prior to principal component analysis (PCA), the MALDI-TOF mass spectra (m/z 500−2000) were exported from the flexAnalysis 2.2 software and were preprocessed. For each data point (in total about 66000 points) the absolute logarithm was calculated to receive a normal distribution of the data set. Afterward, a normalization of the data set on the peak XXXG (m/z 1085; xyloglucan-heptamer; *23*) was performed.

Enzyme digests of hypocotyls were obtained from three biological replicates (each 20 hypocotyls). The digests were subjected to MALDI-TOF MS (2 spots each, at least 2 times 200 shots), and the obtained spectra were subjected to PCA as has been described for leaf material.

Sugar Composition Analysis of Hypocotyl Material. Ten hypocotyls (4 days old, dark grown) were weighed into a reaction vial (1 mL; Alltech Biotechnology, Lexington, KY). AIS from the hypocotyls was obtained as described earlier. The obtained AIS was incubated with 0.75 mL of 2 M trifluoroacetic acid for 1 h at 121 °C (*1*), followed by evaporation at ambient temperature. The material obtained was redissolved twice with methanol and subsequently dissolved in 0.25 mL of Millipore water. The solution was analyzed with HPAEC-PAD for its sugar composition (*24*).

RESULTS AND DISCUSSION

MALDI-TOF MS of Enzyme Digests of Wild Type *Arabidopsis* Plants (*Col0*). A mixture of 10 pure and well-defined enzymes was used to degrade all enzyme-accessible polysaccharide structures

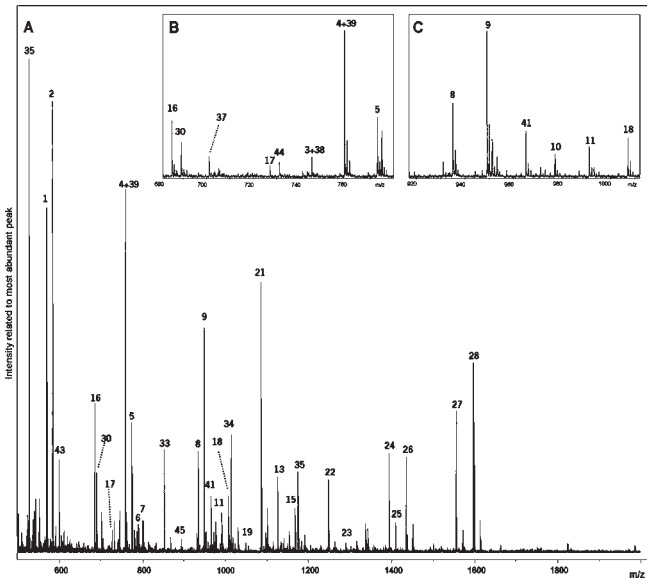

Figure 1. MALDI-TOF mass spectrum of *Col0* leaf material after digestion with the enzyme cocktail: (**A**) *m*/*z* 500−2000; (**B**) zoom of *m*/*z* 680−780; (**C**) zoom of *m*/*z* 920−1010. Given numbers correspond to oligosaccharides as described in **Table 2**.

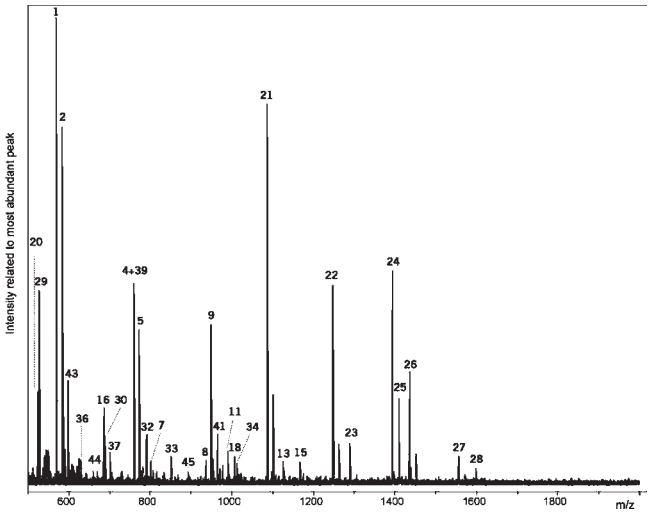

Figure 2. MALDI-TOF mass spectrum of *Col0* hypocotyls after digestion with the enzyme cocktail. Given numbers correspond to oligosaccharides as described in **Table 2**.

of the cell wall of leaves and hypocotyls derived from *A. thaliana* prior to MALDI-TOF MS analysis. By using such a multi-enzyme-assisted MALDI-TOF MS approach, a fingerprint of all characteristic oligosaccharides released from various enzyme-accessible cell wall polysaccharides becomes visible in a single mass spectrum. This is exemplified in **Figure 1** for wild type leaves (*Columbia-0* (*Col0*)) and in **Figure 2** for 4-day-old dark-grown wild type hypocotyls (*Col0*). In **Table 2** a summary of *m*/*z* values, which could be annotated to various oligosaccharides, is given. Although some peaks are doubly annotated, for example, *m*/*z* 569 can be explained by either a galacturonic acid trimer or a pentose tetramer (**Table 2**, peaks 1 and 36), 80% of the peaks present in the mass spectra are annotated unambiguously.

The most abundant HG oligomers released are $GalA_3$, $GalA_3Me$, $GalA_4Me$, and $GalA_5Me_2$ (**Figures 1** and **2**, peaks 1, 2, 4, and 9, respectively). Furthermore, some smaller peaks corresponding to HG oligosaccharides are visible in the MALDI-TOF mass spectra (**Figures 1** and **2**, peaks 7, 10, 11, and 15). These *m*/*z* values can be annotate either as multiple methyl-esterified

Table 2. *m*/*z* Ratios of Possible Degradation Products after Digestion with the Enzyme Cocktail[a]

peak	*m*/*z*	annotation $[M + Na]^+$
	Oligomers Released from Homogalacturonan by Endo-PG and PME	
1	569	$GalA_3$
2	583	$GalA_3Me$
3	745	$GalA_4$
4	759	$GalA_4Me$
5	773	$GalA_4Me_2$
6	787	$GalA_4Me_3$ and/or $GalA_4Ac$
7	801	$GalA_4MeAc$
8	935	$GalA_5Me$
9	949	$GalA_5Me_2$
10	977	$GalA_5Me_4$ and/or $GalA_5MeAc$
11	991	$GalA_5Me_2Ac$
12	1097	$GalA_6$
13	1125	$GalA_6Me_2$
14	1153	$GalA_6Me_4$ and/or $GalA_6MeAc$
15	1167	$GalA_6Me_5$ and/or $GalA_6Me_2Ac$
	Oligomers Released from Rhamnogalacturonan by RGH	
16	685	$(Rha\text{-}GalA)_2$
17	727	$(Rha\text{-}GalA)_2Ac$
18	1007	$(Rha\text{-}GalA)_3$
19	1049	$(Rha\text{-}GalA)_3Ac$
	Oligomers Released from Xylogalacturonan by XGH	
20	525	$GalA_2Xyl$
	Oligomers Released from Xyloglucan by XEG	
21	1085	XXXG
22	1247	X[XL]G
23	1289	X[XL]G + Ac
24	1393	XXFG
25	1409	XLLG or XXFG (potassium adduct)
26	1435	XXFG + Ac
27	1555	XLFG
28	1597	XLFG + Ac
	Oligomers Released from β-(1,4)-Galactan by β-(1,4)-Galactanase	
29	527	Gal_3
30	629	Gal_2Ara_2
31	689	Gal_4
32	791	Gal_3Ara_2
33	851	Gal_5
34	1013	Gal_6
35	1175	Gal_7
	Oligomers Released from β-(1,4)-Xylan by β-(1,4)-Xylanase and α-(1,5)-Arabinan by α-(1,5)-Arabinanase	
36	569	Ara_4 and/or Xyl_4 and/or mixtures
37	701	Ara_5 and/or Xyl_5 and/or mixtures
38	745	Xyl_4GlcA
39	759	$Xyl_4GlcAMe$ and/or $Xyl_3RhaGalA$
40	833	Ara_6 and/or Xyl_6 and/or mixtures
41	965	Ara_7 and/or Xyl_7 and/or mixtures
42	1097	Ara_8 and/or Xyl_8 and/or mixtures
	Mixed Oligosaccharides (Pentoses and Hexoses)	
43	599	P_3H_1
44	731	P_4H_1
45	893	P_4H_2
46	1055	P_4H_3

[a] GalA, galacturonic acid; GlcA, glucuronic acid; Rha, rhamnose; H, hexose; P, pentose; Ara, arabinose; Xyl, xylose; Ac, acetyl group; Me, methyl ester; xyloglucan nomenclature according to Fry et al. (*23*).

HG oligosaccharides or as acetylated HG oligosaccharides. In contrast to Obel and co-workers (*25*), we prefer to interpret the annotation of these peaks as oligosaccharides having one acetyl group attached (**Table 2**) rather than as multiple methyl-esterified oligosaccharides (e.g., $GalA_4AcMe$ rather than $GalA_4Me_4$), because the endo-PG used is hindered in its action by methyl esters (*14*). Comparison of the mass spectra of leaves and hypocotyls reveals that there are clear changes within the HG oligosaccharides released (**Figures 1** and **2**). Especially the relative abundances of the low methyl-esterified oligosaccharides (peaks 2, 4, 8, and 9; normalized to $GalA_3$ (peak 1)) are higher in leaves compared to hypocotyls. The most abundant RG-I oligosaccharides released are $(Rha\text{-}GalA)_2$ and $(Rha\text{-}GalA)_3$ (**Figures 1** and **2**, peaks 16 and 18). Traces of acetylated RG-I oligosaccharides (**Figures 1** and **2**, peaks 17 and 19) could be detected as well. In agreement with Zandleven and co-workers no peaks corresponding to xylogalacturonan (XGA) oligosaccharides could be identified in *Col0* leaf digest, because XGH is not active on the XGA present in the AIS of *Arabidopsis* leaves (*4*). In contrast to the *Col0* leaf digest, in *Col0* hypocotyls the XGA oligosaccharide $GalA_2Xyl$ (**Figure 2**, peak 20) could be annotated, indicating a substantial difference of the XGA present in hypocotyls compared to XGA in leaves. Enzymes degrading rhamnogalacturonan II (RG-II), representing 8% of the cell wall material of *Col0* leaves (*5*, *26*), are not yet available. To degrade the side chains of RG-I, α-(1,5)-arabinanase and β-(1,4)-galactanase have been added to the enzyme mixture. β-(1,4)-Xylanase has been included to enable the identification of possible xylan mutants (*27*). In **Figures 1** and **2** several *m*/*z* values, which can be annotated to either arabinan-, (arabino)-galactan or (arabino)-xylan oligosaccharides, have been identified, for example, *m*/*z* 569, 629, 701, and 791 (**Figures 1** and **2**, peaks 36, 30, 37, and 32, respectively). Due to the addition of XEG to the enzyme mixture, the XG oligosaccharides released could be recognized by MALDI-TOF MS (**Figures 1** and **2**, peaks 21−28) as described earlier (*9*). The relative abundances of the individual XG oligosaccharides released from *Arabidopsis* leaves differ compared to data from the literature (*9*, *29*), which can most likely be explained by different extraction techniques as well as by the use of an enzyme cocktail within this research. It can be hypothesized that the use of pectinases and cellulases in combination with XEG liberates XG moieties, which are tightly bound to cellulose and/or pectin (*28*). By comparison of the relative abundance of the XG oligosaccharides in *Col0* hypocotyl digests to the ones in *Col0* leaf digests, clear differences can be observed. Apparently, the XLFG oligosaccharide (peak 27) is substantially less abundant, whereas the X[XL]G and XXFG oligosaccharides (peaks 22 and 24, respectively) are more abundant in *Col0* hypocotyl digests (**Figures 1** and **2**). Additionally, the level of acetylated XG oligosaccharides (XXFG and XLFG, peaks 26 and 28, respectively) appears to be lower in hypocotyls than in leaf tissue.

All enzyme-accessible polysaccharides are represented by oligosaccharides in a single MALDI-TOF mass spectrum as demonstrated for *Col0* leaves and hypocotyls, respectively. Hence, this MALDI-TOF MS method after digestion of *Arabidopsis* tissue with a mixture of 10 pure and well-defined enzymes gives a good fingerprint of the complete enzyme-accessible *Arabidopsis* cell wall, which qualifies this method to be used for screening of *Arabidopsis* plants or even other dicotyledonous plants.

PCA of Mass Spectra of Known Mutant *Arabidopsis* Leaf Digests for Method Validation. The enzyme cocktail was subjected to AIS of leaves from various *Arabidopsis* wild type and known mutant plants, namely, *mur3*, *qua1*, and *xgd1*, and their corresponding wild type plants as given in **Table 1**. The mutant plant *mur3* is described to have an altered XG structure (*29*), whereas *qua1* and *xgd1* have altered pectic structures (*qua1*, HG deficient, *2*, *30*; *xgd1*, XGA deficient, *31*). As all of the mutant plants were grown in different wild type backgrounds (used within the EU project "WallNet"), the different wild type plants were included in the analysis resulting in the following pairs: *Col0*−*mur3*, *quartet* (*qrt*)−*xgd1*, and *Wassilewskija* (*WS*)−*qua1*. All MALDI-TOF mass spectra were subjected to PCA, resulting in a total variance of 65% (PC1 and PC2). This value is acceptable when taking into account that the whole MALDI-TOF mass spectra (with 66000 data points each) were used to perform PCA. The results of the PCA performed are presented in **Figure 3A** by plotting PC1 against PC2. In the lower left quadrant of the graph all of the wild type plants cluster (*Col0* (○), *qrt* (□), and *WS* (+)), whereas the mutants under investigation cluster either in the upper right quadrant (*mur3* (◆) and *qua1* (△)) or in the lower right quadrant (*xgd1* (∗)). In **Figure 3B**−**D** we highlighted the mutant−wild type pairs *Col0*−*mur3*, *qrt*−*xgd1*, and *WS*−*qua1*, respectively. It can be seen that PCA results in the distinction of all mutant *Arabidopsis* plants from their corresponding wild type plants. Even the three different wild type plants (*Col0*, *qrt*, and *WS*) clustered mainly in three distinct clouds (**Figure 3A**), although a complete distinction could not be achieved. Due to this partial distinction between different wild type plants, analysis of the corresponding wild type plant in combination with the mutant plant is always required in the screening for *Arabidopsis* mutants.

PCA of Mass Spectra of Known Mutant *Arabidopsis* Hypocotyl Digests for Method Validation. Much research in plant sciences is performed with *Arabidopsis* hypocotyls because they can be obtained quickly and strongly defected mutants may not even reach the leaf state. Thus, after the successful distinction of all (known) mutant leaf materials as described above, the multi-enzyme-assisted MALDI-TOF MS screening method has been downscaled to hypocotyls. The use of 20 4-day-old dark-grown hypocotyls for each enzyme digest and the analysis of three independent grown biological replicates ensure a good representation of every mutant plant (*25*). Due to much less material available when working with hypocotyls, we observed that the MALDI-TOF MS measurements became much more sensitive to external influences such as salts and matrix effects. Especially the ratio between the XG and HG oligosaccharides appeared to be very sensitive toward small differences in sample preparation. Therefore, some general requirements had to be defined for the data processing of the mass spectra obtained. First, mass spectra were excluded from further data processing when peaks, which were annotated as the potassium adduct of a component, account for >40% of the sodium adduct as this is a sign of incorrect desalting. Second, the data processing excludes data points below *m*/*z* 568 as peaks originating from the DHB matrix (*m*/*z* < 550) were not reproducible and disturbed the PCA substantially. Third, all mass spectra, which were subjected to PCA, had to be analyzed parallel as interday differences were visible in PCA, even though a standardized desalting step within the sample preparation procedure was introduced to minimize this effect. The PCAs, which were performed on different days with the same hypocotyl material, showed independently the same distinction between the mutant and corresponding wild type hypocotyls. Therefore, the use of this modified MALDI-TOF MS method has been explored to be used as screening method for *Arabidopsis* hypocotyls. Four mutant hypocotyls have been chosen for analysis: *gaut 13* (*32*), the β-(1,4)-xylan-deficient *irx14* (*27*), the HG-deficient *qua1* (*2*), and the mutant line SALK 033674, which lacks the gene At4g21060 (**Table 1**). Whereas the chemical composition of the mutants *irx14* and *qua1* has been described earlier (at least for leaf material), no or hardly any cell wall analysis has been performed for *gaut13* and the mutant line

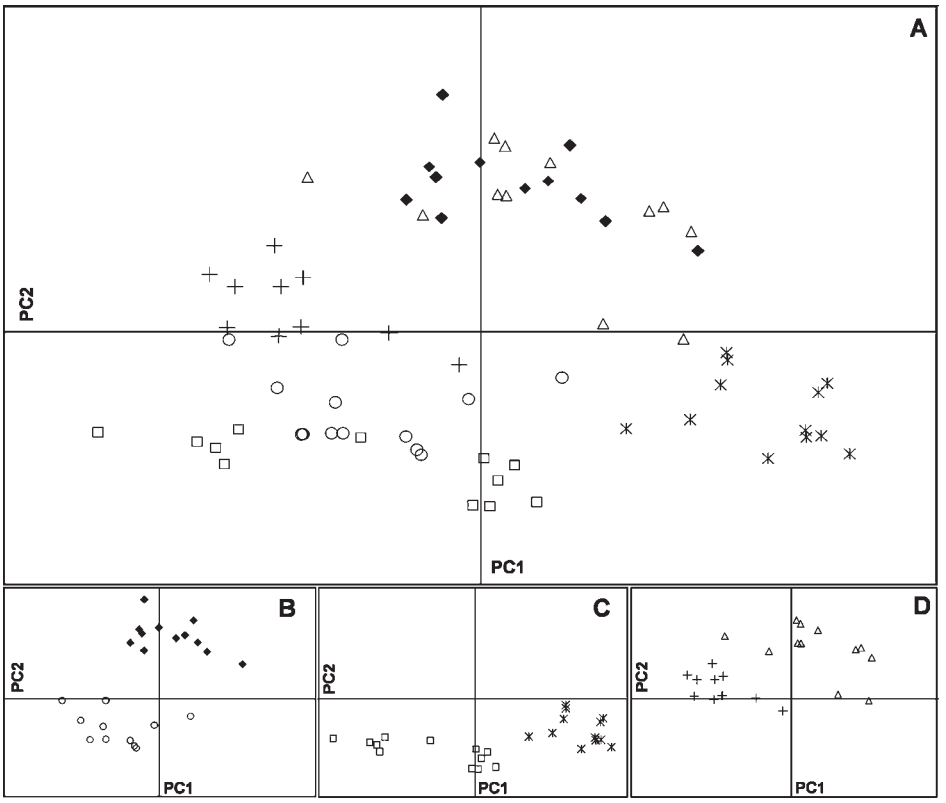

Figure 3. Principal component analysis of MALDI-TOF mass spectra of different wild type and mutant leaf materials after digestion with the enzyme cocktail: (**A**) all data points; (**B**) *Col0*−*mur3*; (**C**) *qrt*−*xgd1*; (**D**) *WS*−*qua1*; (○) *Col0*; (◆) *mur3*; (□) *qrt*; (∗) *xgd1*; (+) *WS*; (△) *qua1*.

Table 3. Sugar Composition of Hypocotyls of Different Mutant Lines after Hydrolysis with TFA Analyzed with HPAEC-PAD[a]

	mol %								
	Fuc	Ara	Rha	Gal	Glc	Xyl	Man	GalA	GlcA
Col0	2	16	5	19	13	15	2	26	1
gaut13(SALK_122602)	2	14	5	18	9	15	3	32	1
At4g21060 (SALK_033674)	2	15	5	17	13	16	3	29	1
irx14(SALK_038212)	2	16	6	18	11	12	2	32	1
qua1(SALK_102380)	2	16	4	18	23	14	2	21	1

[a] *Col0*, $n = 7$; SALK 102380 (*qua1*), $n = 4$; SALK 122602 (*gaut13*), $n = 3$; SALK 038212 (*irx14*), $n = 3$; and SALK 033674, $n = 3$.

SALK 033674 (At4g21060). In **Table 3** the results of the sugar composition analysis (mol %) of the four mutant hypocotyls is presented. The sugar composition analysis of *qua1* (hypocotyls from the SALK line 102380; *Col0* background) and *irx14* (SALK 038212; *Col0* background) is in good agreement with experiments performed earlier (significant decrease of xylose and galacturonic acid, respectively; *2*, *27*). The sugar composition of the mutant line SALK 033674 (At4g21060) shows a significant decrease of galactose without a significant change of any other monosaccharide (**Table 3**). The *gaut13* hypocotyls (SALK line 122602; *Col0* background) showed a significant increase in galacturonic acid, which was recently also found for *gaut13* inflorescences and stems (*33*). This is in good agreement with the literature predicting *gaut13* to be involved in pectic biosynthesis (*32*). In **Figure 4** the PCA of the mass spectra of the mutant and wild type hypocotyl enzyme digests has been plotted, and it can be seen that *irx14* (■), *gaut13* (△), and At4g21060 (SALK 033674 (∗)) are clearly separated from the corresponding wild type hypocotyls (*Col0* (◇)).

The *qua1* (▲) mutant clusters with the wild type *Col0*, which is in disagreement with FTIR studies, in which the *qua1* mutant hypocotyls were distinguished from the wild type hypocotyls (*8*, *34*). A possible explanation for this deviant behavior could be that the *enzyme-accessible* part of HG is not (yet) affected in 4-day-old *qua1* hypocotyls.

Detailed Analysis of the Oligosaccharides Released after Enzyme Treatment To Identify Possibly Altered Polysaccharide(s). After successful distinction of the wild type and mutant *Arabidopsis* leaves and hypocotyls by PCA, the mass spectra obtained by the MALDI-TOF MS screening method as well as CE-LIF analysis (see below) were evaluated to enable the identification of the altered structure(s) within the mutant tissue.

Interpretation of the Mass Spectra of the Wild Type and Known Mutant Leaf Digests. In **Figure 5** typical mass spectra of the wild type and mutant leaf materials analyzed are given. In the mass spectrum of *mur3* the peaks of (acetylated) XXFG and XLFG (**Figure 5B**, peaks 24 and 26−28, respectively) are absent, whereas the intensity of the peaks XXXG and X[XL]G (peaks 21−22) is increased compared to the wild type plant (*Col0*; **Figure 5A**). Both observations are in good agreement with the literature describing *mur3* to lack the fuco-galactosyl structures (*29*). Obviously, MALDI-TOF MS is not suitable to distinguish XLXG and XXLG, because these structures have the same m/z values (1247). Thus, the increase of XLXG and decrease of XXLG described before (*29*) cannot be observed by MALDI-TOF MS. Besides the XG alteration, the rest of the oligosaccharides released, including HG, RG-I, and (arabino)-galactan oligosaccharides, remain the same (**Figure 5A**,**B**). The *xgd1* mutant has been grown in *qrt* background, which is a *Col0* plant differing in a gene encoding a pectin methyl esterase (*35*). By comparison of the mass spectra of the two different wild type plants *Col0* and *qrt* (**Figure 5A**,**C**), it can be seen that the signal intensities of the methyl-esterified HG oligosaccharides (**Figure 5A**−**C**, peaks 2 and 5) are increased when normalized to $GalA_3$ (peak 1), which is in good agreement with earlier data (*35*). The differences between the mass spectra of the mutant *xgd1* and its wild type *qrt* are

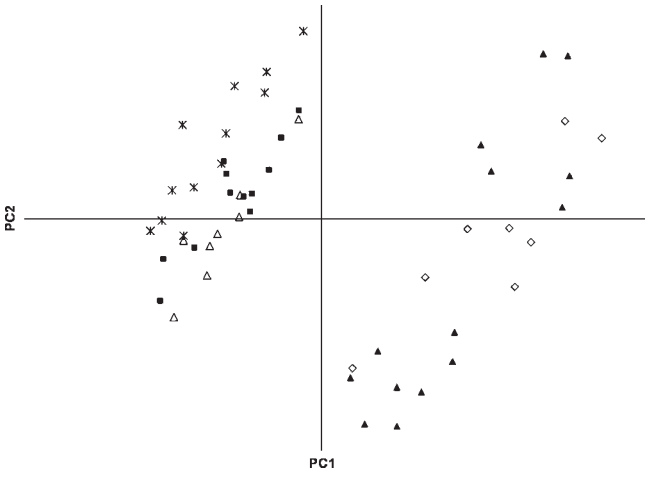

Figure 4. Principal component analysis of MALDI-TOF mass spectra of wild type (*Col0*) and mutant *Arabidopsis* hypocotyls after digestion with the enzyme cocktail: (◇) *Col0*; (■) *irx14*; (∗) galactose-deficient mutant (At4g21060); (△) *gaut13*; (▲) *qua1*.

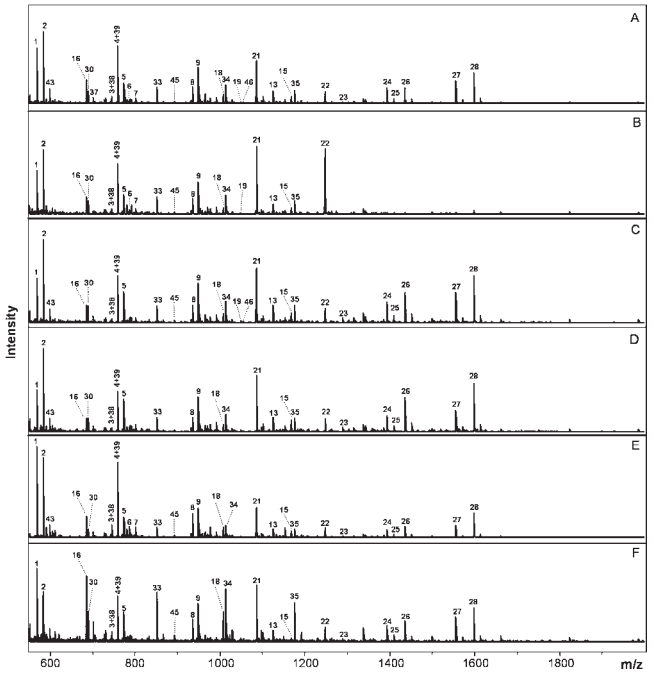

Figure 5. MALDI-TOF mass spectra of different wild type and mutant *Arabidopsis* leaf materials after AIS extraction and digestion with the enzyme cocktail: (**A**) *Col0*; (**B**) *mur3*; (**C**) *qrt*; (**D**) *xgd1*; (**E**) *WS*; (**F**) *qua1*. Annotation of oligosaccharides based on *m*/*z* values; given numbers correspond to oligosaccharides as described in **Table 2**.

minimal. These results were expected as *xgd1* is deficient in XGA and the enzyme XGH is not active on AIS of *Arabidopsis* leaf material (*4*). Nevertheless, the performed PCA of the MALDI-TOF mass spectra could distinguish between *xgd1* and *qrt* (**Figure 3**), most likely on the basis of the different levels of acetylation of the XG oligosaccharides (**Figure 5C**,**D**; ratio of peak 26 to 24 and peak 28 to 26). In the mass spectrum of *qua1* (**Figure 5F**) a clear decrease in the relative abundance of the HG oligosaccharides released can be observed compared to the corresponding wild type leaf material (**Figure 5E**,**F**, $GalA_3$−$GalA_5$, with and without methyl esters (peaks 2, 4, 5, 8, 9; ratios of these peaks toward peak 1)), which is in good agreement with the earlier described HG deficiency (*2*). The RG-I oligosaccharides (including side chains thereof) and XG oligosaccharides seem to remain unaffected.

Thus, this multienzyme-assisted MALDI-TOF MS high-throughput screening method is also capable of giving directions for further detailed analysis by pointing to specific altered polysaccharides, but, obviously, it must be kept in mind that MS is not capable of distinguishing between two different components with the same *m*/*z* values, as has been shown for XXLG and XLXG in the *mur3* mutant. Furthermore, the ionization in MALDI-TOF MS analysis depends on the charge of the oligosaccharides. Thus, drawing conclusions by comparing the relative abundances of different groups of oligosaccharides such as neutral and acidic oligosaccharides with each other might not result in correct data. Therefore, another sensitive method able to quantify the oligosaccharides released might be suitable for complementary analysis. Recently, CE-LIF analysis has been shown to enable the separation and mole-based quantification of complex oligosaccharide structures in a short time requiring only very low amounts of sample (*10*−*12*). This indicates that CE-LIF enables the measurement of leaf and hypocotyl digests, even though only very low amounts of sample might be present. Therefore, CE-LIF analysis has been evaluated as medium-throughput screening method for *Arabidopsis* leaves and hypocotyls to provide complementary data, using the same enzyme digests, which were used for MALDI-TOF MS measurements. As we start with the same enzyme digest, the extra time for CE-LIF analysis is reduced to the labeling of the oligosaccharides present after enzyme incubation (∼100 samples/day) and the analysis time itself (15−20 min/sample).

CE-LIF Analysis of Wild Type and Known Mutant Leaf Material. In **Figure 6** are given the electropherograms of the various enzyme digests of the leaf materials. Even though complete annotation of all oligosaccharides released has not been achieved, many of the main degradation products could be identified using the literature (*11*, *36*). Although not further described in this paper, a first exploration of CE-(LIF)-MS (*12*) seems to be extremely useful for further improvement of the analysis of *Arabidopsis* digests. Due to the unexpected α-amylase side activity in the enzyme cocktail (e.g., the presence of maltotriose), the added maltose could be used as a mobility marker but proved to be an unreliable internal standard.

For *Col0* leaf material it was calculated that about 20−25% of the cell wall polysaccharides were released as oligosaccharides (degree of polymerization (DP) of 2−12). The main peak in all enzyme digests is cellobiose (4.70 min; about 30% of the identified oligosaccharides for *Col0* leaves), which results from cellulose degradation by endoglucanase I and cellobiohydrolase. HG oligosaccharides elute earlier than neutral oligosaccharides as has been indicated in **Figure 6** ($GalA_{1-3}$, 4.30, 4.48, and 4.86 min, respectively). For *Col0* leaves about 10% of the oligosaccharides released can be accounted for by $GalA_{1-3}$. RG-I and XG oligosaccharides elute significantly later (5.62 min $(Rha\text{-}GalA)_2$ and 6.4−7.6 min (XXXG, XXLG+XXLG, XXFG+XXFG, XLFG+XLFG; acetylation indicated by underlined letter), respectively) with nonacetylated and monoacetylated XG oligosaccharides coeluting within CE-LIF analysis (*36*). Whereas MALDI-TOF MS analysis of *Arabidopsis* leaf materials (**Figure 5A**) indicated a relatively high amount of HG oligosaccharides compared to XG oligosaccharides, CE-LIF (**Figure 6A**) analysis reveals that MALDI-TOF MS analysis is overestimating the amount of HG oligosaccharides released, especially when the mole-based detection is taken into account.

CE-LIF analysis of *mur3* leaves (**Figure 6B**) shows clearly the absence of XXFG and XLFG, which confirms the MALDI-TOF MS results. Furthermore, although not visible in MALDI-TOF

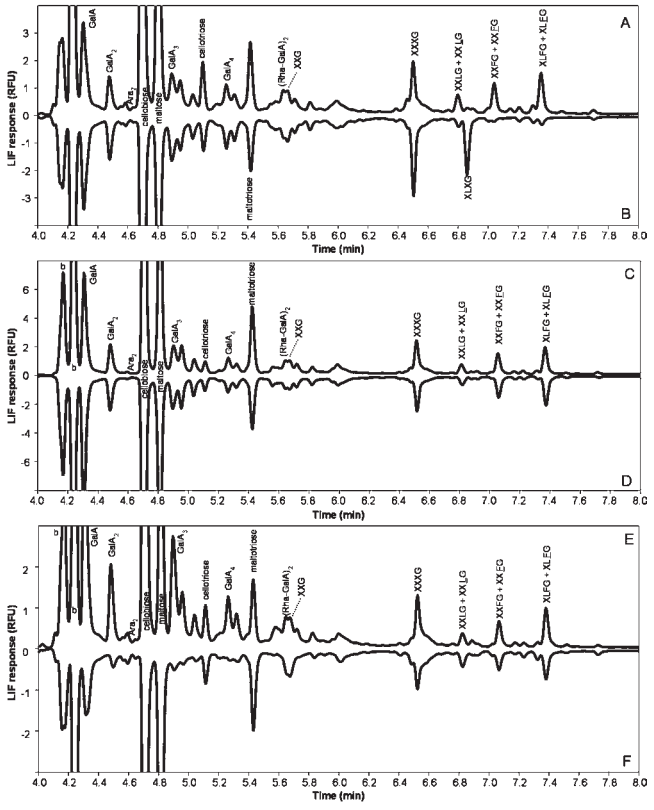

Figure 6. CE-LIF electropherograms of different wild type and mutant *Arabidopsis* leaf materials after AIS extraction and digestion with the enzyme cocktail: (**A**) *Col0*; (**B**) *mur3*; (**C**) *qrt*; (**D**) *xgd1*; (**E**) *WS*; (**F**) *qua1*. Acetylation is indicated by underlined letters.

MS analysis, an 18-fold increase of XLXG and a 14-fold decrease of XXLG can be observed in the electropherogram of *mur3*. No variation in any other oligosaccharide was detected, as predicted by MALDI-TOF MS analysis. CE-LIF analysis of *qrt* and *xgd1* leaves did not show any significant differences between both enzyme digests (**Figure 6C**,**D**), besides an increase in galacturonic acid (GalA) in *xgd1* leaves, which cannot be explained yet. The differences between *WS* and *qua1* leaves are obvious in the CE-LIF electropherograms (**Figure 6E**,**F**), and especially the amount of oligosaccharides released from the enzyme-accessible HG is reduced substantially ($GalA_{1-4}$, −69, −86, −87, and −85%, respectively). The RG-I and XG oligosaccharides are much less affected, which is in good agreement with the MS results (**Figures 5E**,**F**). In addition, the amount of cellobiose is decreased (−37%, data not shown), indicating that the *qua1* mutation affects not only the pectin moiety but as well cellulose or even the whole cell wall architecture.

MALDI-TOF MS and CE-LIF Analysis of *Col0* and *gaut13* Hypocotyl Material. To exemplify the potential of MALDI-TOF MS and CE-LIF analysis performed for further identification of deviating structures within mutant *Arabidopsis* hypocotyls, the MALDI-TOF mass spectra and CE-LIF electropherograms of *gaut13* and its corresponding wild type *Col0*, as presented in **Figures 7** and **8**, are discussed. Both CE-LIF and MALDI-TOF MS analysis revealed that the XG oligosaccharides released by enzyme treatment are similar in *gaut13* and *Col0* hypocotyls as the ratios of these XG oligosaccharides to each other remain constant (**Figures 7** and **8**). Furthermore, MALDI-TOF MS analysis revealed that in *gaut13* hypocotyls fewer galacturonic acid oligomers of higher DP (DP 4−5) are released compared to $GalA_3$ (peaks 4 and 9 compared to peak 1; **Figure 7**), which was

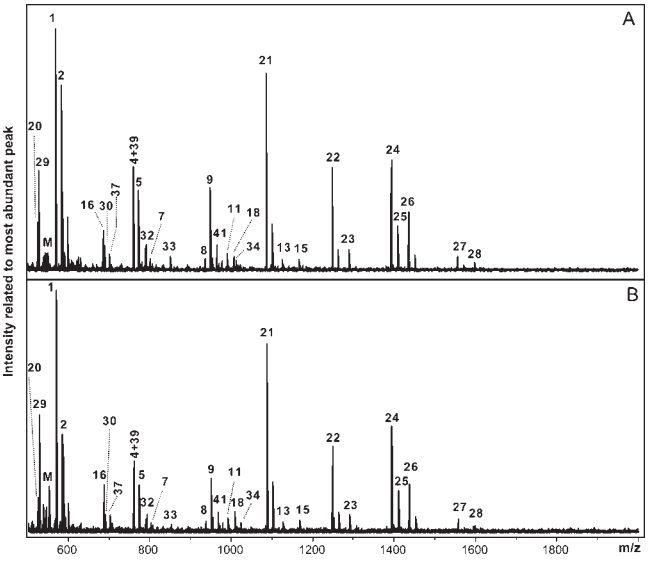

Figure 7. MALDI-TOF mass spectra of (**A**) *Col0* and (**B**)*gaut13* hypocotyls after AIS extraction and digestion with the enzyme cocktail. Annotation of oligosaccharides based on *m*/*z* values; given numbers correspond to oligosaccharides as described in **Table 2**.

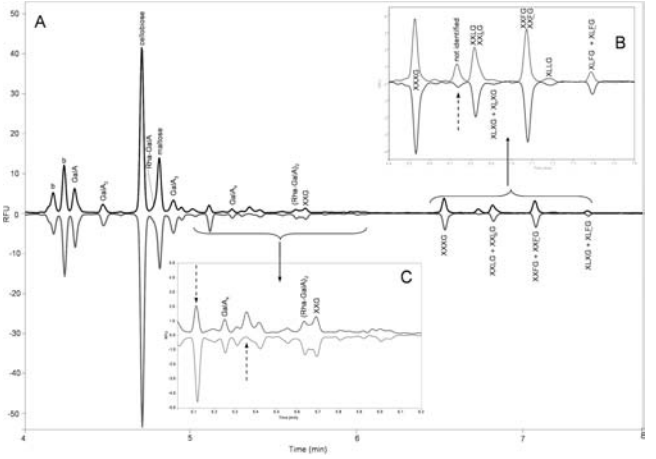

Figure 8. CE-LIF electropherograms of *Col0* and *gaut13* hypocotyls after AIS extraction and digestion with the enzyme cocktail: *Col0* (upper line), *gaut13* (lower line). Acetylation is indicated by underlined letters.

confirmed by CE-LIF analysis (**Figure 8A**,**B**). Opposite to the $GalA_4$, $GalA_{1-3}$ are slightly increased in *gaut13* enzyme digest as can be observed in the CE-LIF electropherograms (**Figure 8A**,**B**). In addition, fewer methyl-esterified HG oligosaccharides are released from *gaut13* hypocotyls compared to *Col0* hypocotyls as can be seen in MALDI-TOF-MS (peaks 2 and 5 compared to peak 1; **Figure 7**). In total, these results could indicate either a different degree of methyl esterification of the HG present and/or another distribution of these methyl esters for the *gaut13* hypocotyls. Cellobiose (4.70 min) and cellotriose (5.12 min) are more abundant in *gaut13* hypocotyls compared to *Col0* hypocotyls, whereas unknown peaks at 5.35 and 6.75 min are not (or much less) present in *gaut13* (**Figure 8**).

In conclusion, this research demonstrated the potential of a multienzyme-assisted MALDI-TOF MS screening method for the recognition of altered cell wall polysaccharide structures. In addition, CE-LIF analysis has been shown to be a suitable method for the detailed analysis of cell wall polysaccharide derived oligosaccharides, whereas also quantitative data on

oligosaccharide levels could be obtained. Even though only a very low amount of, for example, hypocotyls was presented, CE-LIF showed to be much less affected by matrix or salt effects. The accuracy of such quantification will increase when an internal standard other than maltose is used.

The combination of techniques was used to screen for *Arabidopsis* mutants, and it is anticipated that this approach could also be used to analyze (changed) cell wall polysaccharide structures in other (dicotyledonous) plant materials.

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