Development of a new high-throughput method to determine the composition of ten monosaccharides including 4-*O*-methyl glucuronic acid from plant cell walls using ultra-performance liquid chromatography

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Abstract Plant cell walls are an important dietary source for livestock, and could be an enormous resource for production of next-generation bioethanol and more valuable materials. Because polysaccharides are major components of plant cell walls, analysis of their composition is important. In this report, we established a high-throughput method to determine the composition of ten monosaccharides from plant cell walls simultaneously using ultra-performance liquid chromatography with *p*-aminobenzoic ethyl ester-labeling technology. Complete separation of a mixture of internal standards, 2-deoxy-glucose and 3-*O*-methyl glucose, and ten monosaccharides, consisting of seven neutral and three acidic sugars including 4-*O*-methyl-*D*-glucuronic acid, which are frequently found in plant cell wall polysaccharides, can be obtained within 7 min using this system. Relative standard deviations of retention time and peak area value are lower than 1%. Linearity for broader dynamic ranges (0.02–2000 mg l⁻¹), faster analysis and higher sensitivity than other traditional methods, including one that employs widely used high-performance anion exchange chromatography, are achieved. We evaluated this new method by analyzing the composition of cell walls from three model plants (*Arabidopsis thaliana*, rice and hybrid aspen) and confirmed that the obtained results for most monosaccharides are consistent with those in previous studies. These data suggest that our newly developed system could greatly contribute to the study of plant cell walls, especially research requiring high-throughput analysis.

Key words: 4-*O*-Methyl glucuronic acid, monosaccharide composition, plant cell wall, UPLC.

Plant cell wall polysaccharides are essential compounds for plant bodies, and are an important dietary source for livestock. In addition, it has recently been considered that plant cell walls could be the most abundant terrestrial organic resource for production of next-generation bioethanol and more valuable materials. Plant cell walls contain three major classes of polysaccharides, namely cellulose, hemicellulose and pectin. The composition of polysaccharides varies in each plant species and also in each tissue (Bauer et al. 2006). These polysaccharides are polymers of neutral monosaccharides such as *D*-glucose (Glc), *D*-xylose (Xyl), *L*-fucose (Fuc), *L*-arabinose (Ara), *D*-mannose (Man), *D*-galactose (Gal), *L*-rhamnose (Rha) and/or acidic monosaccharides such as *D*-glucuronic acid (GlcA), *D*-galacturonic acid (GalA), and 4-*O*-methyl-*D*-

glucuronic acid (4-m-GlcA). Neutral monosaccharides are components of cellulose, and the backbones and/or side chains of hemicelluloses, while acidic monosaccharides are components of the pectin backbone or side chains of heteroxylan (Albersheim et al. 2010). Recently, not only cellulose (Somerville 2003) and the backbone of hemicelluloses (Brown et al. 2009; Jensen et al. 2014), but also the modification of GalA residues in pectin of primary cell wall (Hongo et al. 2012) and GlcA/4-m-GlcA side chains of heteroxylan of secondary cell wall (Lee et al. 2012) have been demonstrated to be essential for normal plant growth. Regarding saccharification of plant biomass, the arabinofuranose side chains promote hydrolysis of heteroxylan by some xylanases (Shallom and Shoham 2003). In addition, the

Abbreviations: ABEE, *p*-aminobenzoic ethyl ester; AIR, alcohol insoluble residue; Ara, *L*-arabinose; Fuc, *L*-fucose; GC, gas liquid chromatography; GC-MS, gas liquid chromatography-mass spectrometry; 2-d-Glc, 2-deoxy-glucose; Gal, *D*-galactose; GalA, *D*-galacturonic acid; HPAEC, high-performance anion exchange chromatography; HPLC, high-performance liquid chromatography; Glc, *D*-glucose; GlcA, *D*-glucuronic acid; Man, *D*-mannose; 3-m-Glc, 3-*O*-methyl glucose; 4-m-GlcA, 4-*O*-methyl-*D*-glucuronic acid; PA, peak area; PAD, pulsed amperometric detection; RSD, relative standard deviation; TFA, trifluoroacetic acid; UPLC, ultra-performance liquid chromatography; Rha, *L*-rhamnose; Xyl, *D*-xylose.

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reduction of the degree of 4-*O*-methylation to GlcA on the glucuronoxylan of *Arabidopsis* mutants was found to increase the release of Xyl during hydrothermal pretreatment of plant cell wall (Urbanowicz et al. 2012). These reports suggest that the profiling of neutral and acidic monosaccharides from total plant cell walls is important for characterizing plant cell wall in the context of growth properties and/or for achieving improved conversion efficiency of the cell wall to ethanol.

To this end, chromatographic analysis is the main technique; gas liquid chromatography (GC) and GC-mass spectrometry (GC-MS) analyses are widely employed to analyze acid hydrolysates of polysaccharides and provide accurate and reproducible data of the composition of neutral and acidic monosaccharides in plant cell walls (Albersheim et al. 1967; Blakeney et al. 1983; Doco et al. 2001; Hoebler et al. 1989). However, this method based on the GC system requires time-consuming derivatization of monosaccharides to alditol acetates or trimethylsilyl ethers (McNeil et al. 1982; Pettolino 2012). On the other hand, high-performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) had been developed as a method to improve the sensitivity of monosaccharide detection without derivatization and is widely used for the plant cell wall analysis (De Ruiter et al. 1992). A recent study improved the HPAEC-PAD method to quantify two acidic monosaccharides in addition to seven neutral monosaccharides taking only 60 min of analytic time with high resolution (Nagel et al. 2014). However this advanced method has different linearity range for some monosaccharides, such as that for GalA ($1.7\text{--}128\mu\text{mol l}^{-1}$) is different from those of other sugars ($0.28\text{--}30.3\mu\text{mol l}^{-1}$, Nagel et al. 2014). In addition, Chong et al. (2013) has reported that the PAD response of 4-m-GlcA is one-third from that of GlcA. These differences necessitate several injections at different concentration levels for the comprehensive quantification of monosaccharides.

A method for high-performance liquid chromatographic (HPLC) analysis of monosaccharides with pre-column derivatization to produce a fluorophore is also commonly employed (Takemoto et al. 1985), which involves *p*-aminobenzoic ethyl ester (ABEE) derivatization of carbohydrates (Matsuura and Imaoka 1988). In the ABEE-labeling method, monosaccharides are derivatized for 1 h and excess reagents are easily removed by chloroform (Yasuno et al. 1999). In addition, the labeling efficiency of ABEE is mostly comparable for a broad range of neutral and acidic saccharide species and ABEE-labeled monosaccharides are efficiently separated in HPLC systems using borate buffer as an eluent, resulting in excellent resolution and wide linearity of 1–1000 pmol at a chromatographic injection for each monosaccharide (Yasuno et al. 1999). This

suggests that the ABEE-labeling procedure is one of the most suitable methods for simultaneous determination of neutral and acidic monosaccharides from plant cell walls. However, the total run time of ABEE-labeled monosaccharide separation with HPLC is ca. 75 min for each sample analysis and therefore longer than the HPAEC-PAD method (Nagel et al. 2014). Recently, Kumagai et al. (2012) applied the ultra performance liquid chromatography (UPLC) system, which is a relatively new technique giving new possibilities in liquid chromatography by shortening run time, improving peak resolution, and sensitivity (Swartz 2005), to analyze the composition of trifluoroacetic acid (TFA)-hydrolyzable polysaccharides from Hinoki cypress (*Chamaecyparis obtusa*) slurry. However, chromatographic conditions such as the running time, peak resolutions, and the linearity ranges of each monosaccharide, which are important to evaluate the accuracy of their conditions, remained to be validated. In addition, the amount of 4-m-GlcA in their material was not intended to be analyzed even though Hinoki xylan is enriched with 4-m-GlcA (Ishii et al. 2010). The internal standard which is used for the calibration and estimation of the injection volume and the loss of monosaccharide during the acid hydrolysis (McNeil et al. 1982; Pettolino et al. 2012), also remained to be established (Kumagai et al. 2012). Therefore further development of the chromatographic conditions for UPLC with ABEE-labeling system is still needed for the rapid, accurate and comprehensive quantification of the monosaccharide composition of the plant cell wall including 4-m-GlcA using internal standard.

In this study, we established a system to rapidly analyze major 10 plant cell wall monosaccharides by combining UPLC with the ABEE-labeling technique. We also demonstrated that 2-deoxy-glucose (2-d-Glc) and 3-*O*-methyl glucose (3-m-Glc) can be used as the internal standard in this system. A total run time is shortened to 6.5 min, sensitivity is increased to 2–5 fmol per injection and the linearity range is broadened to $0.02\text{--}2000\mu\text{g ml}^{-1}$. We applied this simultaneous monosaccharide determination method to the cell wall samples of inflorescence stem of *Arabidopsis thaliana*, culm of rice, and poplar wood for the method evaluation. We verified that this method allows us a rapid and sensitive determination of 10 cell-wall-composing sugars including 4-m-GlcA in actual biomass samples in reasonable ranges, indicating that this new high-throughput chromatographic method could be an innovative tool for studying plant cell wall composition.

Materials and methods

Chemicals

The standard monosaccharides, Gal, Man, Ara, Xyl, Fuc, and Rha were purchased from Kanto Chemical Inc. (Japan), GlcA,

GalA, and 2-D-Glc were obtained from Sigma-Aldrich Inc. (USA), and Glc and 3-m-Glc was sourced from Wako Pure Chemical Industries, Ltd. (Japan). An ABEE-labeling kit was purchased from J-Oil mills Inc. (Japan). 4-m-GlcA purified from the sap of a lac tree (Kuroyama et al. 2001; Nakamura et al. 1984) was kindly gifted from Prof. Y. Tsumuraya (Saitama University, Japan). Synthetic 4-m-GlcA is also commercially available from Toronto Research Chemicals Inc. (www.trc-canada.com, Cat. #: M308350).

Plant materials

A. thaliana ecotype Columbia-0 were grown in soil at 22°C under a 16 h day (60–70 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)/8 h night cycle after 3 weeks of germination on 1/2 Murashige and skoog medium. Regenerated rice plants (*Oryza sativa* cv. Nippon-bare) from seed callus (Hiei et al. 1994) were grown in soil for 8 months at 28°C under a 13 h day (130–150 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)/11 h night cycle in a phytotron. Rooted cuttings of hybrid aspen (*Populus tremula* × *Populus tremuloides*) were grown in the greenhouse at ambient temperature under natural light.

Preparation of cell wall residue

The inflorescence stem of 2-month-old *A. thaliana*, the culms of 8-month-old *O. sativa*, was cut into 1 cm-segments and fixed with methanol in a 50 ml-Pyrex glass tube with a screw cap (Asahi Glass Inc., Japan). The fixed samples were treated with methanol twice at 80°C for 10 min, twice with acetone at 70°C for 5 min, and twice with methanol/chloroform (1:1, v/v) at 70°C for 5 min and then dried at 65°C for 18 h. The dried tissue segments were powdered with a stainless steel bead (6 mm, Biomedical Science, Japan) and three zirconia beads (3 mm, Nikkato Corp., Japan) using a Shake Master NEO (Biomedical Science Inc., Japan). In case of poplar, xylem tissues were collected from 1–2 years old twigs and then ground into fine powder. The starch in resulting powder of *A. thaliana*, *O. sativa*, and poplar was degraded with α -amylase solution and de-starched residue was prepared as previously described (Sakamoto and Mitsuda 2014). The de-starched residue was designated as alcohol-insoluble residue (AIR).

Sulfuric acid hydrolysis of the cell wall

The AIR was hydrolyzed by the two-step hydrolysis method based on NREL protocol (Sluiter et al. 2008) with slight modification as previously described (Sakamoto and Mitsuda 2014). As a recovery standard, we prepared the authentic monosaccharide-standards including 7 neutral monosaccharides and 3 acidic monosaccharides (20 μg each in a tube, see Figure 1) in a 2 ml-Eppendorf safe-lock microtube and added 1.45 ml of 4% sulfuric acid. After addition of 10 μl of 3-m-Glc solution (1 mg ml⁻¹) as an internal standard, the resulting suspension was autoclaved at 121°C for 1 h and cooled at room temperature. The hydrolysate was neutralized and adjusted to around pH 5.0 with calcium carbonate powder. The supernatant of neutralized hydrolysate was used for ABEE-labeling.

Figure 1. UPLC chromatogram of monosaccharides labeled with ABEE. A monosaccharide mixture (20 $\mu\text{g ml}^{-1}$ of each, Standard) and ultrapure water (Blank) was reacted with ABEE reagent and 2 μl of reacted solution containing 0.89 ng of ABEE-labeled monosaccharides was injected for chromatographic analysis. Peak assignment: 1, GlcA; 2, GalA; 3, Gal; 4, Man; 5, Glc; 6, 4-m-GlcA; 7, Ara; 8, Xyl; 9, Fuc; 10, Rha; 11, 2-d-Glc; 12, 3-m-Glc.

Preparation of ABEE-labeled monosaccharides

The acidic and neutral monosaccharides were labeled with an ABEE labeling kit (J-Oil mills Inc., Japan) based on the method of Yasuno et al. (1999). Twenty-microliter of ABEE reagent, which was prepared as described in Yasuno et al. (1999), was added to 5 μl of the neutralized hydrolysates in a 1.5 ml-microtube. The mixture was heated at 80°C for 1 h and then cooled to room temperature. Two hundred-microliter of distilled water and 200 μl of chloroform were added. After vigorous vortexing, the mixture was centrifuged at 20,000 × *g* for 1 min at 25°C, and then the upper aqueous phase was collected. This aqueous phase containing ABEE-labeled monosaccharides was filtrated with syringe filter unit (Millex-LG 0.2 μm pore, Millipore Inc., USA) and the resulting solution was analyzed with UPLC system.

UPLC conditions

The chromatographic separation and detection of monosaccharides labeled with ABEE was performed using an ACQUITY UPLC H-Class system (Waters Inc., USA) equipped with ACQUITY UPLC BEH C18 column (100 mm × 2.0 mm, id, 1.7 μm particle size, Waters Inc.) and fluorescence detector (ACQUITY UPLC FLR Detector, Waters Inc.). The eluent A was 200 mM potassium borate buffer (pH 8.9). The eluent B was 100% acetonitrile. The column was equilibrated with the mixture of 97% (v/v) eluent A and 3% (v/v) eluent B at a flow rate of 0.7 ml min⁻¹. The elution was started with a linear gradient of eluent B from 3 to 21% at a flow rate of 0.7 ml min⁻¹ for 4.0 min. The elution program including washing and regeneration of the column was shown in gradient profile 4 in Supplementary Table S1. The temperature of column and auto-sampler was maintained at 50°C and 10°C respectively. The ABEE-labeled monosaccharides were detected with 305 nm of emission and 360 nm of excitation using fluorescence detector. The injection volume of all samples was 2 μl .

Table 1. Comparison of assay conditions between a previous study^{*1} and this study.

	Kumagai et al. (2012)	This study
Eluent method	Isocratic	One step gradient
Detector	UV	Fluorescence
Column length (mm)	150	100
Initiate buffer composition	A ^{*2} containing 10% B ^{*3}	Mixture of 97% A and 3% B
Flow rate (ml/min)	0.3	0.7
Column temperature (°C)	30	50

^{*1} Kumagai et al. (2012), ^{*2} A, 200 mM potassium borate (pH 8.9), ^{*3} B, 100% acetonitrile.

Determination of peak resolution ($R_{P1, P2}$)

The resolution ($R_{P1, P2}$; Equation 1) between two adjacent peaks ($P1$ and $P2$) was deduced from the retention times of the two analytes (RT_{P1} , RT_{P2}) and the baseline widths (W_{P1} and W_{P2}) resulting from the tangents of the inflection points of each peak (Nagel et al. 2014).

$$R_{P1, P2} = 2(RT_{P2} - RT_{P1}) / (W_{P2} + W_{P1}) \quad (1)$$

Results and discussion

Assay condition to separate twelve monosaccharides by UPLC was established

Based on established methodologies for the ABEE labeling of monosaccharides (Kwon and Kim 1993; Yasuno et al. 1997, 1999), Kumagai et al. (2012) determined the monosaccharide composition of slurry of *Chamaecyparis obtusa* (Hinoki cypress) wood after the method transfer from HPLC to UPLC. However, (1) the established column performance such as specificity and stability remained to be validated. (2) 4-m-GlcA, which is present in major side chains of heteroxylan in secondary cell wall of eudicot, was not intended to be analyzed and (3) the internal standard correction was not applied. Because of these reasons, evaluation of the method transfer from HPLC to UPLC is still needed in addition to establishment of the condition to separate 4-m-GlcA, two other acidic monosaccharides, seven neutral monosaccharides and two modified monosaccharides (2-d-Glc or 3-m-Glc) which are not usually detected in the plant cell wall (as the potential internal standards). Using the same chromatographic conditions as those reported previously except for employing a shorter column in this study to shorten the total run time, all compounds were eluted within 10 min of injection in their isocratic elution with 10% acetonitrile (Supplementary Figure S1, isocratic 1). It was found that Glc co-eluted with 4-m-GlcA at 5.02 min under these conditions, although the separation of other monosaccharides was mostly acceptable (Supplementary Figure S1, isocratic 1). In addition, the total run time including wash and regeneration of the column was 25 min. Although this time frame is better than those of GC or HPLC methods, further shortening is desirable. Therefore, we needed to develop the different

chromatographic conditions to separate all of the major monosaccharides from the plant cell wall and to shorten run time. Through many trial-and-error procedures, as described in detail in Supplementary Text, we finally established appropriate assay conditions that allowed rapid separation of all the monosaccharides (Figure 1). Under these conditions, the elution-program, the mobile phase, flow rate and column temperature were entirely changed from those of the reported method by Kumagai et al. (2012) (Table 1). Total analysis time was shortened to 6.5 min, including column washing and re-equilibration (Gradient profile 4 in Supplementary Table S1), and 4-m-GlcA was able to be detected simultaneously with the other monosaccharides and two internal standard candidates.

The established method has improved linearity and sensitivity

The reproducibility of the developed method was assessed by carrying out 12 independent injections of a mixture of twelve authentic monosaccharides. As listed in Tables 2 and 3, the relative standard deviation (RSD) values of retention time (RT) and peak area (PA) were less than 1% for all examined monosaccharides. These data indicate that the established system has a sufficient robustness for repeated analyses of chromatographic injection.

Supplementary Table S2 shows that the peak resolution values between each pair of two neighboring monosaccharides in the chromatograph except for “4-m-GlcA/Ara (0.80 ± 0.01)” and “Fuc/Rha (0.82 ± 0.01)” were between 0.99 ± 0.01 and 2.25 ± 0.05 , when $0.89 \text{ ng}/2 \mu\text{l}$ of sample was injected. This indicates that the separations of all tested monosaccharides were acceptable. Reduction of the injected sample amount from $0.89 \text{ ng}/2 \mu\text{l}$ to $0.05 \text{ ng}/2 \mu\text{l}$ markedly increased the peak resolution values for five pairs of monosaccharides without affecting those of the other four pairs of monosaccharides (Supplementary Table S3).

We also investigated the relationship between peak area and the concentration of monosaccharide labeled with ABEE to estimate the linearity range for the determination of monosaccharide concentration. As shown in Figure 2, the linearity of GlcA and 4-m-GlcA, for example, in our established method ranged from

Table 2. Reproducibility of retention time of ABEE-labeled monosaccharides using the established separation conditions shown in gradient profile 4 of Table S1.

Monosaccharide*	Retention time (min)
	AVE $\pm$ SD (RSD %, $n=12$)
GlcA	2.04 $\pm$ 0.004 (0.18)
GalA	2.21 $\pm$ 0.003 (0.16)
Gal	2.55 $\pm$ 0.004 (0.14)
Man	2.69 $\pm$ 0.004 (0.14)
Glc	2.77 $\pm$ 0.004 (0.15)
4-m-GlcA	2.83 $\pm$ 0.004 (0.15)
Ara	2.89 $\pm$ 0.004 (0.13)
Xyl	3.04 $\pm$ 0.004 (0.13)
Fuc	3.28 $\pm$ 0.004 (0.12)
Rha	3.34 $\pm$ 0.004 (0.11)
2-d-Glc	3.49 $\pm$ 0.004 (0.10)
3-m-Glc	3.82 $\pm$ 0.003 (0.09)

* ABEE-labeled monosaccharide mixture (0.89 ng of each monosaccharide per chromatographic injection) was injected.

Table 3. Detected area value of various monosaccharides using the established separation conditions shown in gradient profile 4 of Table S1.

Monosaccharide*	Peak area value ($\mu\text{V}\cdot\text{s}^{-1} \times 10^4$)
	AVE $\pm$ SD (RSD %, $n=12$)
GlcA	83 $\pm$ 0.61 (0.74)
GalA	99 $\pm$ 0.45 (0.45)
Gal	113 $\pm$ 0.32 (0.28)
Man	135 $\pm$ 0.39 (0.29)
Glc	142 $\pm$ 0.48 (0.34)
4-m-GlcA	75 $\pm$ 0.66 (0.88)
Ara	121 $\pm$ 1.08 (0.89)
Xyl	207 $\pm$ 0.76 (0.37)
Fuc	117 $\pm$ 0.43 (0.37)
Rha	58 $\pm$ 0.33 (0.54)
2-d-Glc	140 $\pm$ 0.56 (0.40)
3-m-Glc	181 $\pm$ 0.53 (0.29)

* ABEE-labeled monosaccharide mixture (0.89 ng/ $2\mu\text{l}$ of each monosaccharide) was injected.

0.02 to 2000 $\mu\text{g ml}^{-1}$ (approximately 0.004–400 pmol per chromatographic injection) with a correlation coefficient r^2 value of 0.99 (Supplementary Table S4). This is much better than the previous report in which the detection linearity of detected monosaccharides was 1–1000 pmol per chromatographic injection in HPLC (Yasuno et al. 1999). Our results indicate that the reestablished UPLC method shows markedly improved linearity range (up to 100 fold) and sensitivity (up to 250 fold) for the determination of all examined monosaccharides compared with those of HPLC.

Application of the established method to three different types of plant cell walls produced reasonable results

To evaluate the analytical procedure with the UPLC system in actual plant cell wall samples, we quantified the monosaccharide contents in sulfuric acid-hydrolysate

Figure 2. Relationship between area value and monosaccharide concentration of GlcA and 4-m-GlcA. Each concentration (0.02–2000 mg/l) of ABEE-labeled monosaccharide was prepared by dilution. The peak area and concentration of 4-m-GlcA (filled circles) and GlcA (open circles) are plotted.

of three types of plant cell walls; namely, inflorescence stem of *A. thaliana*, culm of rice, and woody tissues of poplar stem. According to the recovery rate of sulfuric acid hydrolysis described in Supplementary text, 3-m-Glc is resistant to sulfuric acid hydrolysis, while 2-d-Glc is intensely degraded (Supplementary Table S5). Therefore 3-m-Glc was used for internal standard in sulfuric acid hydrolysate in this application.

As shown in Figure 3A, the chromatogram of cell wall hydrolysate revealed the 2 major peaks (peak No. 5 and 8) and 9 minor but significant peaks (peak No. 1 to 4, 6 to 10, and 12 as an internal standard, See Figure 3A–D), whose elution times were identical to those of monosaccharide standards, in all tested plant tissues. These two major monosaccharides were Glc and Xyl, respectively and other minor monosaccharides are three uronic acids (GalA, GlcA, and 4-m-GlcA) and five neutral sugars (Ara, Fuc, Gal, Man and Rha). The chromatogram also revealed that some unknown peaks eluted between GlcA and GalA around the elution time of 2.2 min (Figure 3A, B). These unknown peaks might be products from incomplete hydrolysis with sulfuric acid, such as substance with aldobiuronic acid structures presumably from arabinogalactan protein (Yates et al. 1996) or glucuronoxylans, which are relatively resistant to acid hydrolysis (Chong et al. 2013; Vignon and Gey 1998).

We calculated the monosaccharide contents in the cell wall of tested plants with the recovery rate (Supplementary Table S6) and compared our data with the previous reports in same or similar plant species (Figure 4). Most monosaccharide contents quantified in this study were in the range of previous studies, except for Rha in *A. thaliana* and GalA in poplar species (Figure 4). Rha content in *A. thaliana* cell wall in this study ($20.5 \pm 1.5 \mu\text{g mg}^{-1}$ AIR) was ca. 1.5 to 3 times

Figure 3. UPLC chromatogram of plant cell wall hydrolyzed by sulfuric acid. Monosaccharide profiles of cell walls from three plants *A. thaliana*, rice culm, and poplar stem are shown. The hydrolysates of cell wall samples were neutralized using calcium carbonate and then the supernatant was labeled with ABEE. Labeled monosaccharide in hydrolysate (2 μ l) was injected for chromatographic analysis. Peak assignments are shown in Figure 1. A: Total chromatogram of cell wall acid hydrolysates from three plants. B to D: Expanded chromatograms from (A) to show minor but significantly detected peaks of monosaccharides.

higher than the previous studies, but relatively close to the data (14.4 and 17.3 μ g mg⁻¹ AIR) determined by the combination of the two step-hydrolysis with sulfuric acids and the quantification with GC (Lee et al. 2011b, 2012), while the data determined with HPAEC-PAD system (9.6 and 10.6 μ g mg⁻¹ AIR, Eudes et al. 2012; Iwase et al. 2009) and the data determined by the combination of one-step hydrolysis and GC (6.72 μ g mg⁻¹ AIR, Persson et al. 2007) were low. Therefore higher content of Rha in *A. thaliana* in this study might be caused by the difference of peak resolution of quantification apparatus (HPAEC or GC) and hydrolysis condition between our study (two-step) and the previous studies.

In case of 4-m-GlcA in rice and Fuc in poplar, few report showed the quantification data though we detected significant peaks in the all tested plant species (peak No. 6 in Figure 3C, No. 9 in Figure 3D, Supplementary Table S6). Leplé et al. (2007) used Fuc as an internal standard for HPAEC analysis of the monosaccharide composition in poplar cell wall, indicating that the Fuc in poplar wood sample is below

Figure 4. Comparison of monosaccharide compositions of cell walls from stem/culm in *Arabidopsis*, poplar, and rice between this study and previous studies. Contents of three major and seven minor monosaccharides of cell walls were determined for *Arabidopsis* inflorescence stems (A, D), poplar woody stems (B, E), and rice culms (C, F). The positions of monosaccharides are arranged in order of higher amounts determined in this study. The filled diamonds indicate the monosaccharide contents determined in this study ($n=4$ for *Arabidopsis* and rice, $n=2$ for poplar). Gray boxplots represent a range of values in previous studies; seven studies of *Arabidopsis*, six studies of poplar species, and six studies of rice, respectively (See supplementary Table S9 in detail). Horizontal bar in the gray box indicates the median of the reported values. Upper and lower hinges of gray box indicate 75%- and 25%-ranges of reported values, respectively. The upper and lower extreme bars of box plot indicate maximum (upper) and minimum (lower) of reported values, respectively.

the detection limit of HPAEC-PAD system. Hence, our improvement of the sensitivity may contribute to more detailed quantification of the monosaccharides in poplar wood sample.

Our analysis of monosaccharide composition of plant cell walls clearly revealed the difference between *A. thaliana*, poplar, and rice as reviewed previously (Albersheim et al. 2010; Carpita and Gibeaut 1993). The contents of Glc, Xyl and GalA were higher than those of other monosaccharides in *Arabidopsis* (Figure 4, Supplementary Table S6), which is consistent with the previous report that cellulose, xyloglucan, xylan and polygalacturonic acid are major cell wall components in eudicot plants (Zabackis et al. 1995). The contents of Glc, Xyl, and 4-m-GlcA were higher than those of other monosaccharides in poplar cell wall, which is also consistent with the previous review in which cellulose

Table 4. Comparison of chromatographic analyses used to determine monosaccharide composition.

Procedure	Derivatization (h)	Analysis (min/run)	Linearity (mg l ⁻¹)	Reference
UPLC-ABEE	1	6.5	0.02–2000	This study
HPLC-ABEE	1	75	4.5–4500* ¹	Yasuno et al. (1999)
HPEAC-PAD	None	60	0.05–5.5* ² 3.3–24.9* ³	Nagel et al. (2014)
GC-FID	3	30	0.1–150* ⁴	Blakeney et al. (1983)
	3	30	0.3–3.0* ⁵	Jones and Albersheim (1972)
GC-MS	17	65	0.6–1500 µg* ⁶ 4.4–475 µg* ⁷	Guadalupe et al. (2012)

*¹ Concentration was calculated from the linearity range shown as 1–1000 pmol. *² Neutral monosaccharide and GlcA, *³ GalA, *⁴ Neutral monosaccharides from Jones and Albersheim (1972), *⁵ GlcA and GalA, *⁶ Monosaccharides except GlcA, *⁷ GlcA.

and 4-*O*-methyl-glucurono xylan are described as major components of dicotyledonous woody plants (Sannigrahi et al. 2010). In case of rice cell wall, contents of Glc, Xyl and Ara were relatively high, while the content of GalA was lowest among three tested species, which well describes the characteristics of *Poaceae* cell wall; namely, cellulose, mixed linkage-glucan, and arabinoxylan are major components but polygalacturonic acid is minor (Smith and Harris, 1999).

In terms of GlcA methylation, we found that 95%, 77%, and 30% of GlcA were methylated in poplar, *Arabidopsis*, and rice cell walls, respectively (calculated from Supplementary Table S6). According to previous studies, almost all and 60% of GlcA were described to be methylated in poplar (Lee et al. 2011a, 2012) and *Arabidopsis* (Lee et al. 2012; Zhong et al. 2005), respectively, and therefore our data is consistent with these previous studies. By contrast in case of rice, it is difficult to evaluate this value because few report succeeded to quantify the amount of 4-m-GlcA in rice as mentioned above.

Conclusion

The UPLC system established here greatly improves the throughput and quantification range of seven neutral and three acidic monosaccharides including 4-m-GlcA from plant cell walls in addition to 3-m-Glc as an internal standard. As listed in Table 4, we were able to shorten the total time for the chromatographic analysis of monosaccharides to 6.5 min and improved both the linearity range and detection limit, with a 100-fold increase of the linearity range and 200–500-fold increase of the detection limit compared to those of the HPLC-ABEE method. Furthermore, the developed method gives mostly comparable data for the monosaccharide compositions of plant cell walls with those of previous studies using GC or HPAEC-PAD systems. We believe this new UPLC system will greatly contribute to the study of plant cell walls and assist development of production of bioethanol and other valuable materials.

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