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Analysis of plant nucleotide sugars by hydrophilic interaction liquid chromatography and tandem mass spectrometry

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ABSTRACT

Understanding the intricate metabolic processes involved in plant cell wall biosynthesis is limited by difficulties in performing sensitive quantification of many involved compounds. Hydrophilic interaction liquid chromatography is a useful technique for the analysis of hydrophilic metabolites from complex biological extracts and forms the basis of this method to quantify plant cell wall precursors. A zwitterionic silica-based stationary phase has been used to separate hydrophilic nucleotide sugars involved in cell wall biosynthesis from milligram amounts of leaf tissue. A tandem mass spectrometry operating in selected reaction monitoring mode was used to quantify nucleotide sugars. This method was highly repeatable and quantified 12 nucleotide sugars at low femtomole quantities, with linear responses up to four orders of magnitude to several 100 pmol. The method was also successfully applied to the analysis of purified leaf extracts from two model plant species with variations in their cell wall sugar compositions and indicated significant differences in the levels of 6 out of 12 nucleotide sugars. The plant nucleotide sugar extraction procedure was demonstrated to have good recovery rates with minimal matrix effects. The approach results in a significant improvement in sensitivity when applied to plant samples over currently employed techniques.

Plant cell walls have been the focus of recent efforts to convert biomass into liquid transportation fuels with the intention of providing a sustainable alternative to fossil fuels [1]. The majority of nucleotide sugar substrates of plant cell wall polymers are synthesized through a series of nucleotide sugar interconverting enzymes in the cytosol and Golgi apparatus from UDP-α-D-glucose or GDP-α-D-mannose [2]. Cell wall UDP-sugar precursors include UDP-α-D-glucose (UDP-Glc), -α-D-galactose (UDP-Gal), -α-D-glucuronate (UDP-GlcA), -α-D-galacturonate (UDP-GalA), -α-D-xylose (UDP-Xyl), -α-D-apiose (UDP-Api), -β-L-arabinose (UDP-Ara) and -β-L-rhamnose (UDP-Rha) [3]. Cell wall GDP-sugar precursors include GDP-α-D-glucose (GDP-Glc), -α-D-mannose (GDP-Man), -β-L-galactose (GDP-Gal), and -β-L-fucose (GDP-Fuc) [3]. Other nucleotide sugar precursors include CMP-D-ketodeoxyoctonate (CMP-Kdo), which is an activated acid sugar that is incorporated into pectic rhamnogalacturonan II fractions of primary cell walls of higher plants [4]. There are also numerous minor nucleotide sugars that also exist in plants, although evidence for their incorporation into plant cell walls is rare or unknown [5]. Glycosyltransferases utilize these activated nucleotide sugar donors as substrates to produce the major plant cell wall polysaccharides: cellulose, and matrix polysaccharides (e.g., hemicellulose and pectin) [6].

Reverse genetic studies of enzymes involved in cell wall biosynthesis using the model dicot plant *Arabidopsis thaliana* have shown that nucleotide sugars involved in cell wall biosynthesis are essential for normal plant growth and development. For example, the *mur1* mutant is a nonfunctional cytosolic GDP-D-mannose 4′,6′-dehydratase catalyzing the first step in converting GDP-Man into GDP-Fuc. In the pectic component rhamnogalacturonan-II of Arabidopsis *mur1* mutants, GDP-Fuc is substituted with GDP-Gal, preventing the formation of borate-dependent dimers and *mur1* plants display dwarf phenotypes [7]. In addition, the *mur4* mutant is a Golgi-targeted UDP-xylose 4′-epimerase partially defective in the last step of UDP-Ara synthesis. Analyses of

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leaf samples from Arabidopsis *mur4* plants show a 50% decrease in L-arabinose in its cell wall compared with leaves from wild-type plants [8,9]. Finally, knockout mutants of two nucleotide sugar mutases (*RGP1* and *RGP2*) that interconvert UDP-L-arabinopyranose (UDP-Arap) to UDP-L-arabinofuranose (UDP-Araf) have markedly lower total L-arabinose content (12 to 31%) compared with wild-type plants [10]. Down regulation of their expression levels was detrimental to the development of affected plants, along with virtually no L-arabinose in their cell walls [10].

Measuring metabolic changes within the network of cell wall biosynthetic reactions is difficult because of the high number of metabolites involved. Metabolic analysis of plant cell wall biosynthesis requires a highly sensitive and robust method for detecting changes in levels of precursors such as nucleotide sugars. Recently a number of quantitative methods based on porous graphitic carbon (PGC)[1] [11], anion-exchange [12,13], and ion-pair reversed-phase chromatography [14] coupled to mass spectrometry (MS) have been developed. Several of these approaches have been employed to directly measure nucleotide sugars from plant material, namely *Arabidopsis thaliana* cell cultures and rosette leaves [11,12].

An emerging chromatographic method has gained interest for its capacity to separate polar metabolites from complex biological mixtures. Hydrophilic interaction liquid chromatography (HILIC) consists of a polar chromatographic surface with the starting mobile phase containing low aqueous content in low-polarity solvent (i.e., acetonitrile) [15,16]. Polar compounds are retained in the water-rich hydrophilic stationary phase away from the solvent mobile phase. They are eluted in order of increasing polarity under higher aqueous conditions in the mobile phase [16]. Low overall aqueous content (typically 5 to 40%) and the limited amount of salts required in the mobile phase solutions allow HILIC to be highly compatible with electrospray ionization (ESI) mass spectrometry. A recent metabolomic study applied zwitterionic silica (ZIC)-based stationary phase in HILIC mode to separate and quantify over 200 hydrophilic intracellular metabolites, including several nucleotide sugars from extracts of β-lactam antibiotic fermentation broths, demonstrating the utility of the separation approach [17].

Here, we present a highly sensitive and robust LC–MS/MS method using ZIC-HILIC coupled with a triple quadrupole operating in multiple reaction monitoring mode to compare nucleotide sugar levels from leaves of two plants with different cell wall compositions: the model dicot plant species, *Arabidopsis thaliana,* and the model monocot plant species rice (*Oryza sativa*).

Materials and methods

Nucleotide sugar standards and reagents

All chemicals were analytical grade or higher and were used as received without any further purification. Nucleotide sugar standards were obtained from the following sources: UDP-α-D-xylose (*p*), UDP-β-L-arabinose (*p*), UDP-α-D-galacturonic acid (*p*) (Carbosource Services, Complex Carbohydrate Research Center, Athens, GA); UDP-α-D-glucuronic acid (*p*), UDP-α-D-glucose (*p*), UDP-α-D-galactose (*p*), UDP-*N*-acetyl-α-D-glucosamine, UDP-*N*-acetyl-α-D-galactosamine, GDP-α-D-mannose (*p*), GDP-β-L-fucose, GDP-α-D-glucose (*p*) (Sigma–Aldrich, St. Louis, MO); UDP-β-L-arabinose (*f*) (Peptides International, Louisville, KY).

[1] *Abbreviations used:* HILIC, hydrophilic interaction liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; PGC, porous graphitic carbon; ZIC, zwitterionic silica.

Plant growth and sample harvest

Arabidopsis thaliana (Col-0) plants were grown with a 16 h photoperiod at 22 °C with 90 μmol m^{-2} s^{-1} illumination intensity during the day period. Arabidopsis rosettes from 4-week-old plants (three separate individuals) were sampled simultaneously in the middle of the light period. These were immediately frozen in liquid nitrogen and stored at −80 °C until used for metabolic extraction. Rice (*Oryza sativa*, cultivar Nipponbare) plants were grown in chambers under the following conditions: 12 h daylight, 470 μmol m^{-2} s^{-1} illumination intensity, 80% relative humidity, 26 °C for 1 h at the beginning and end of the cycle, and 28 °C for the remaining 10 h; 12 h dark, 80% relative humidity, 26 °C. Leaf material from three individual plants (4–5 weeks old) was sampled in the middle of the day period. These were immediately frozen in liquid nitrogen and stored at −80 °C until used for metabolic extraction.

Monosaccharide composition analysis of extracted cell wall material

Leaf material from three individual plants of either rice (4–5 weeks old) or Arabidopsis (4 weeks old) was harvested during the day period and dried in an oven at 40 °C for 3 days. Dried material was ground with a bead beater (Retsch, GmbH, Germany) to a fine powder at 30 Hz for 1 to 2 min. Preparation and hydrolysis of alcohol-insoluble residues from ground material were separately prepared from three independent biological replicates for both Arabidopsis and rice as previously outlined [18]. Monosaccharide composition was measured by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Dionex, Sunnvale CA) using a CarboPac PA20 column as previously outlined [18].

Nucleotide sugar extraction from plant material

After grinding the frozen leaf material to a fine powder using a bead beater (Retsch GmbH, Germany), nucleotide sugars were extracted from 10 mg fresh weight (FW) as previously described [19]. The freeze-dried extract was dissolved in 1.2 mL of 10 mM ammonium bicarbonate before using an ENVI-Carb SPE column (Sigma–Aldrich, St. Louis, MO) using a previously established purification protocol for bacterial samples [20]. Purified extracts were dried in a CentriVap Vacuum Concentrator System (Labconco, Kansas City, MO) and immediately stored at −80 °C.

Hydrophilic interaction liquid chromatography (LC–MS/MS)

Metabolite extracts were initially reconstituted in 10 μL of 10 mM ammonium acetate (pH 7) and then diluted 1:10 with a solution of 94% acetonitrile and 10 mM ammonium acetate (pH 7) to produce ~85% acetonitrile. Thus, FW equivalents of 300 to 700 μg of extracts (10 μL) in ~85% acetonitrile, 10 mM ammonium acetate (pH 7) were used for analysis by LC–MS/MS. Liquid chromatography was performed on an 1100 series capillary HPLC system (Agilent Technologies, Santa Clara, CA) with a 20 μL flow sensor, a 40 μL sample loop, and appropriate capillaries for the flow rate used. During chromatographic runs, the injection volume was 10 μL, plate cooler temperature was set to 10 °C, and column compartment was 50 °C. Nucleotide sugars were separated with a ZIC-HILIC stationary phase column (150 mm × 1 mm, 3.5 μm, 200 Å) and a ZIC-HILIC guard column cartridge (5 mm × 1 mm, 5 μm, 200 Å) (Merck SeQuant, Umeå Sweden). The flow rate was 20 μL/min with the mix rate set at 400 μL/min and coupled directly to the mass spectrometer for analysis. The mobile phase was 10 mM ammonium acetate (pH 7), in (A) 90% acetonitrile and (B) H_2O. At the start of the run, (A) was set at 85% for 2 min. Gradient