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Metabolic Isotope Labeling of Polysaccharides with Isotopic Glucose for Quantitative Glycomics in Cell Culture

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The attachment of sugars to the side chains of proteins via glycosylation is a common post-translational modification that plays significant roles in a wide range of biological processes in living organism.[1,2] The majority of extracellular matrix and the membranous proteins are highly glycosylated by N-linked glycans as well as other types of glycans.[3] A change in glycome profiles and the expression levels of proteins responsible for glycan complexity has been reported in a variety of disease states and during the course of developmental processes.[4–6]

In order to understand the biological relevance of glycans, many mass spectrometry (MS)-based methods have been developed for the relative quantitation of glycan. The simplest strategy is a label-free method in which the quantification is relied on the prevalence of each glycan signal in order to calculate the proportion of an individual glycan to the total glycans in the sample.[7] Several *in vitro* chemical-based isotopic labeling methods have also been developed to introduce signature tags at the reducing end of N-linked glycans.[8,9] Another version of relative isotopic labeling of glycans utilizes heavy vs. light iodomethane ($^{13}CH_3$ vs. $^{12}CDH_2$) in a standard permethylation workflow for the incorporation of isobaric structures.[10–12] Also, it is possible to incorporate ^{18}O-atoms at the reducing end of N-glycans during deglycosylation of enzyme glycosidase in order to achieve the purpose of quantification.[13,14]

In contrast to the aforementioned *in vivo* labeling methods, a metabolic labeling method, isotopic detection of aminosugars with glutamine (IDAWG), has been reported by Wells and coworkers for comparative glycomic studies using the murine embryonic stem cells.[15,16] In this strategy, amide-^{15}N glutamine is used as sole donor source of nitrogen for aminosugars in the production of nucleotide sugars through the hexosamine biosynthetic pathway.

Herein, we describe a novel *in vivo* labeling method using either light or heavy glucose (1-$^{13}C_1$) to tag glycans from rice (*Oryza sativa*) culture. As shown in Figure 1(a), isotopic glucose enters the glucose metabolic pathway, which in turn produces nucleotide sugar precursors that are subsequently used for all glycan biosynthesis. We named this method *M*etabolic *I*sotope *L*abeling of *P*olysaccharides with *I*sotopic *G*lucose (MILPIG) and provide evidence for the quantitative power of MILPIG on N-linked glycans after 2 weeks on labeling (Figure 1(b)). In addition, we foresee that other glycoconjugates such as O-linked glycans, glycolipids, and extracellular matrix polysaccharides should be susceptible to MILPIG labeling.

We validated the application of this approach by MS-based analysis of N-linked glycans released from proteins of rice cultures grown in both light and heavy glucose. Figure 2 shows the full mass spectra of N-linked glycans prepared by mixing equal amounts of normal and heavy labeled samples. The isotopic pairs of N-linked glycans on the full MS demonstrate a robust incorporation of 1-$^{13}C_1$ glucose into N-linked glycans of rice plants. Comparison of the isotopic pairs reveals an increase in glycan ions for samples that were previously grown in 1-$^{13}C_1$ glucose containing media and the increment is correlated with the number of sugars on the glycans. For example, the paucimannosidic N-glycan with core β1,2-xylose and α1,3 fucose residues (Man3XylFucGlcNAc2), 7 sugar-containing glycan, measured at *m/z* 1505.472 as $[M+Na]^+$ with normal glucose and *m/z* 1512.572 as $[M+Na]^+$ with heavy glucose, shows a 7 mass unit difference. Table 1 provides the quantification of the 10 most abundant N-linked glycans by their ratio of peak areas. The selected glycans are nearly free of spectral inferences and we obtained on average a ratio of 1.05:1 (theoretically 1:1) with $^{13}C_1/^{12}C_1$ glucose.

In conclusion, MILPIG labeling strategy relies on the influx of isotopic glucose through the hexosamine biosynthesis pathway where the formation of isotopic uridine

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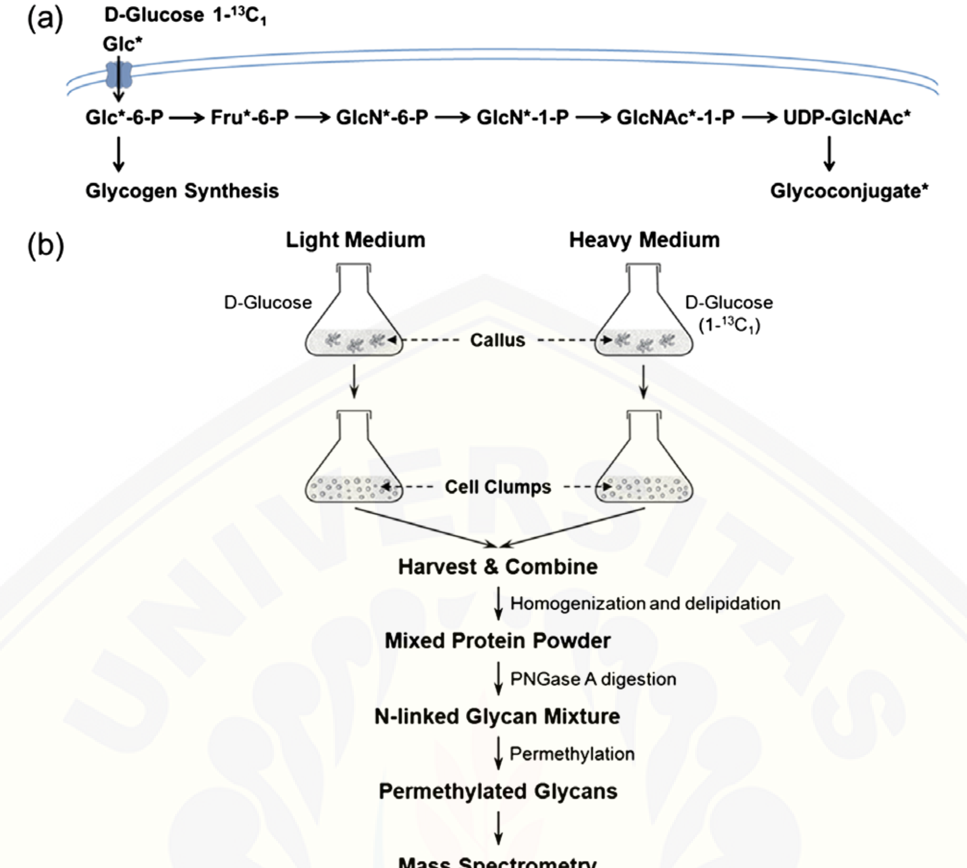

Figure 1. The glucose metabolic pathway and MILPIG labeling strategy. (a) A schematic of the glucose metabolic pathway that illustrates the formation of UDP-GlcNAc. (b) Schematic of the MILPIG *in vivo* labeling method in plant cell culture and MS for relative quantification of glycans.

diphosphate N-acetylglucosamin (UDP-GlcNAc) is subsequently used for the incorporation of isotopic tags onto all glycoconjugates. Using N-glycans in rice cells as a model, we demonstrate that the replacement of normal media supplements with $1\text{-}^{13}C_1$ glucose allows for practically complete labeling of glycans within little more than a 2-week labeling experimental phase for relative quantitation.

Experimental

Rice Cell Culture. Oc cells, a rice cell line derived from the roots of *O. sativa* seedlings[17] (kindly provided by Dr. Syōno), was maintained in liquid N6 media (pH 5.8) supplemented with 1 mg/L 2,4-D-(2,4-dichlorophenoxyace tic acid) and 3% sucrose.[18] Five milliliters of the suspension culture were transferred into 25 mL of fresh Murashige and Skoog medium in a 100 mL Erlenmeyer flask every week. Suspension cells were cultured on a gyratory shaker with a setting of 80 rpm at 25°C in the dark. For stable isotope labeling experiments, sucrose was replaced by 3% glucose either as the $[1\text{-}^{13}C_1]$-labeled isotope isomer (99%; Cambridge Isotope Laboratories, Tewksbury, MA) or as a natural glucose. The cells were allowed to grow utilizing the labeled feed or a natural glucose as a control for 2 weeks.

N-Linked Glycan Extraction and Permethylation. Protein samples were harvested from 2-week-old Oc cells. Plant tissues (3 g) were ground in liquid nitrogen, resuspended in 10 mL of a 50 mM Hepes (pH 7.5) buffer containing 20 mM sodium metabisulfite, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.1% (w/v) sodium dodecyl sulfate (SDS), and 1.7% polyvinylpolypyrrolidone, and then insoluble material was eliminated by centrifugation (15 min at 13 000 × *g*) at 4°C. Protein samples were processed by acetone precipitation three times at 4°C for 3 h to remove contaminants. Release of N-glycans from protein was performed as previously described.[6,19] Equivalent amounts (200 μg) of protein samples were mixed and digested with 20 μg of trypsin in reaction buffer (40 mM NH_4HCO_3) at 37°C overnight. The tryptic peptides were desalted by a reverse phase column (Sep-Pak C18, Restek) and dried by lyophilization. The dried samples were resuspended in 50 μL of 50 mM sodium citrate buffer (pH 5.0). The resuspended glycopeptide sample in sodium citrate buffer was incubated with 0.2–0.5 mUnits of peptide-N-glycosidase A (Calbiochem) for 18 h at 37°C. The released N-linked glycans were purified from peptides with a reverse

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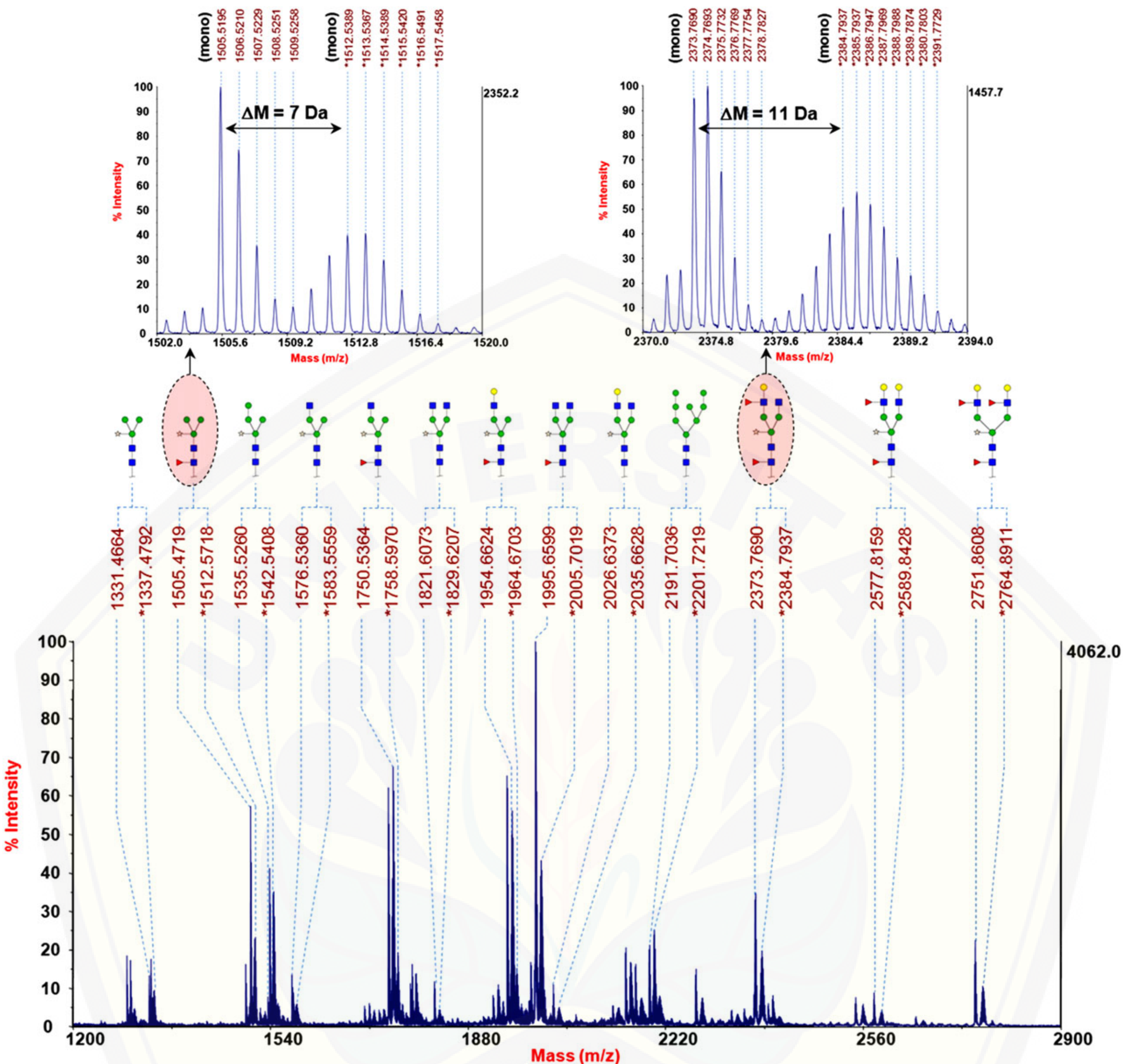

Figure 2. Full mass spectra of N-linked glycans from normal and heavy labeled rice cells. A mixture of N-linked glycans from a 1:1 mixture of heavy/light labeled rice cells was analyzed on MALDI-MS. Magnified spectra (top panel) show the expected mass shift for the number of sugar residues and the underincorporation in the heavy spectra, which allowed us to calculate the labeling efficiency in an average 86.5% incorporation of 1-$^{13}C_1$ into N-linked glycans.

phase column (Sep-Pak C18, Restek) using 5% acetic acid elution buffer. The samples were dried and permethylated. Briefly, each of the dried N-glycan mixtures was resuspended in 200 μL anhydrous dimethyl sulfoxide (DMSO) and 250 μL of fresh dehydrated NaOH/DMSO reagent (mixture of 50 mg NaOH in 2 mL of anhydrous DMSO). After sonication and vortexing under nitrogen gas, 100 μL of iodomethane (CH_3I) was added and the mixtures were vortexed vigorously for 5 min. 2 mL of distilled water was added to the samples and the excess iodomethane was removed by bubbling with a nitrogen stream. The permethylated N-glycan mixtures were extracted with dichloromethane and dried under nitrogen gas. The permethylated glycans were further cleaned using a reverse phase column (Sep-Pak C18, Restek), dried by lyophilization, and analyzed by matrix-assisted laser-desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS).

Analysis of N-Linked Glycans by Mass Spectrometry. MALDI-TOF MS of permethylated glycans was performed as previously described.[6,19] The analysis was performed in reflector positive ion mode using 2,5-dihydroxybenzoic acid (20 mg/mL solution in 50% methanol) as a matrix. The spectrum was obtained by using an AB SCIEX TOF/TOF 5800 MALDI-MS.

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Table 1. Quantitative analysis of representative N-linked glycans from rice cells.

No.	Structure	Number of sugars	Measured $[M+Na]^+$ (mono) Normal ($^{12}C_1$)	Measured $[M+Na]^+$ (mono) Heavy (1-$^{13}C_1$)	ΔM	Ratio of areas[a] ($^{13}C_1/^{12}C_1$)
1		7	1505.472	1512.572	7.100	0.85 ± 0.08
2		7	1535.526	1542.541	7.015	1.17 ± 0.01
3		7	1576.536	1583.556	7.020	1.28 ± 0.13
4		8	1821.607	1829.621	8.013	0.84 ± 0.02
5		9	1995.660	2005.702	10.042	0.76 ± 0.01
6		9	2026.637	2035.663	9.025	1.26 ± 0.14
7		10	2191.704	2201.722	10.018	0.94 ± 0.01
8		11	2373.769	2384.794	11.025	1.22 ± 0.01
9		12	2577.816	2589.843	12.027	1.17 ± 0.05
10		13	2751.861	2764.891	13.030	1.00 ± 0.02

[a] Values represent the mean ± standard deviation (SD) of three replicates.

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