A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

CHEMBIOCHEM

SYNTHETIC BIOLOGY & BIO-NANOTECHNOLOGY

Accepted Article

Title: Expanding the Scope of Metabolic Glycan Labeling in Arabidopsis Thaliana

Authors: Yuntao Zhu and Xing Chen

To be cited as: *ChemBioChem* 10.1002/cbic.201700069

Link to VoR: http://dx.doi.org/10.1002/cbic.201700069

A Journal of

ChemPubSoc Europe

WILEY-VCH

www.chembiochem.org

10.1002/cbic.201700069

WILEY-VCH

Expanding the Scope of Metabolic Glycan Labeling in *Arabidopsis Thaliana*

Yuntao Zhu, and Xing Chen*

Dedicated to the life and work of Professor Werner Reutter

Abstract: Metabolic glycan labeling (MGL) has gained wide utility and has become a useful tool for probing glycosylation in living systems. For the past three decades, the development and application of MGL have been mostly focused on animal glycosylation. Recently, exploiting MGL for studying plant glycosylation has started to gain interest. Here, we describe a systematic evaluation of MGL for fluorescence imaging of root glycans in *Arabidopsis thaliana*. A collection of nineteen monosaccharide analogs containing a bioorthogonal group (i.e., an azide, alkyne, or cyclopropene) were synthesized and evaluated for metabolic incorporation into root glycans. Among these unnatural sugars, fourteen, including three new compounds, were evaluated in plants for the first time. Our results showed that five unnatural sugars could be used to metabolically label root glycans efficiently, and enabled fluorescence imaging via bioorthogonal conjugation with fluorophores. Furthermore, we optimized the experimental procedures for MGL in *Arabidopsis*. Finally, distinct distribution patterns of newly synthesized glycans were observed along the root developmental zones, indicating regulated biosynthesis of glycans during root development. We envision that MGL will find broad applications in plant glycobiology.

Introduction

All cells in all organisms synthesize glycans, one of the four classes of biomacromolecules that mediate a variety of physiological and pathological processes.[1] Methods and tools for visualizing glycans in vivo are vital for studying glycobiology, but have been challenging to develop. Seminal works by the Reutter group and the Bertozzi group in 1990s opened up the development of the metabolic glycan labeling (MGL) strategy,[2-3] which has emerged as a powerful tool for probing glycosylation in living systems.[4-8] MGL exploits the substrate promiscuity of the enzymes in the glycan biosynthetic pathways to metabolically incorporate monosaccharide analogs or unnatural sugars containing a bioorthogonal group (e.g., an azide or alkyne) into cellular glycans. Subsequent bioorthogonal reactions (e.g., click chemistry) covalently conjugate the unnatural sugars with desired functionalities, such as imaging probes and enrichment tags. For the past three decades, MGL has been widely developed and utilized in various animals including mice,[9-10] rats,[11] zebrafish,[12] *Caenorhabditis elegans*,[13] and *Drosophila* melanogaster.[14] However, applying MGL for studying plant glycobiology has received much less attention.

Plant glycans play important roles in regulating plant development and provide biomass for generating renewable energy.[15] Fluorescent labeling and imaging of glycans in plants have been enabled by using carbohydrate-recognizing lectins or antibodies,[16-17] and small molecule dyes binding specific polysaccharides.[18] Inspired by the success of MGL in probing animal glycosylation, it is envisioned that extending MGL to the plant system would expand the toolkit for probing plant glycosylation. In particular, MGL can potentially be used to study the dynamics of plant glycosylation in vivo, in a way similar to probing glycosylation dynamics in zebrafish and rats.[11-12]

Recently, several research groups have successfully demonstrated MGL in *Arabidopsis thaliana*, the most widely used model organism in plant biology. The Somerville group first showed that an alkynyl fucose analog, Ac_4FucAl, could be used to metabolically label the cell wall polysaccharide pectin in the root tissue.[19] Subsequently, our group and several other groups reported metabolic labeling and fluorescent imaging of *Arabidopsis* root glycans by using several azido or alkynyl analogs of *N*-acetylglucosamine (GlcNAc), 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo), glucose (Glc), *N*-acetylgalactosamine (GalNAc), and L-arabinose (Arab).[20-23] These works have demonstrated the applicability of MGL in probing plant glycobiology. The distinctive differences between plant and animal glycosylation require re-evaluation of unnatural sugars in plants, and development of new sugar reporters for plant glycans. Herein, we report a systematic evaluation of a collection of unnatural sugars, which include several newly synthesized and ones that have been reported, for metabolic glycan labeling in *Arabidopsis*. The procedures for metabolic labeling and click reactions were optimized for fluorescence imaging of glycans in *Arabidopsis* roots. The metabolic fates of unnatural sugars and distribution of the metabolically labeled glycans in the developmental zones of roots were also investigated.

Results and Discussion

Design and synthesis of unnatural sugars

[a] Y. Zhu, Prof. X. Chen
College of Chemistry and Molecular Engineering
Peking Univeristy, Beijing, 100871, China
E-mail: xingchen@pku.edu.cn

Prof. X. Chen
Peking-Tsinghua Center for Life Sciences,
Synthetic and Functional Biomolecules Center, and
Key Laboratory of Bioorganic Chemistry and Molecular Engineering of Ministry of Education
Peking Univeristy, Beijing, 100871, China

Supporting information for this article is given via a link at the end of the document.

Plants synthesize a wealthy repertoire of glycans, some of which are unique in plants (Figure 1A).[1] While animal glycans are made of nine kinds of monosaccharides, more than a dozen of monosaccharides are needed for plants.[1, 24] Plant N-linked glycans have the conserved pentasaccharide core structure, but several unique glycosidic linkages are used for extending the core.[25] Intracellular protein O-GlcNAcylation occurs in both animals and plants.[26] In addition, structural cell wall proteins are modified with unique O-glycans, arabinose or galactose attached to hydroxyproline residues.[27] The cell wall also contain an abundant pool of high-molecular-weight polysaccharides, which mainly consist of: i) cellulose, a linear polymer of β1,4-linked glucose which protects plant cells from the osmotic pressure; ii) hemicelluloses, branched polysaccharides with β1,4-linked backbones and xylose-containing side chains that crosslink cellulose fibrils; and iii) pectins, uronic acid-containing polymers forming the acidic jelly including homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan-I (RG-I), and rhamnogalacturonan-II (RG-II).[28-29] The *Arabidopsis* roots can absorb unnatural sugars supplied in the media. If the unnatural sugars can enter the glycan biosynthetic pathways and tolerated by the underlying enzymes, the corresponding glycans are metabolically incorporated with the chemical reporters for subsequent bioorthogonal conjugation with imaging probes (Figure 1B).

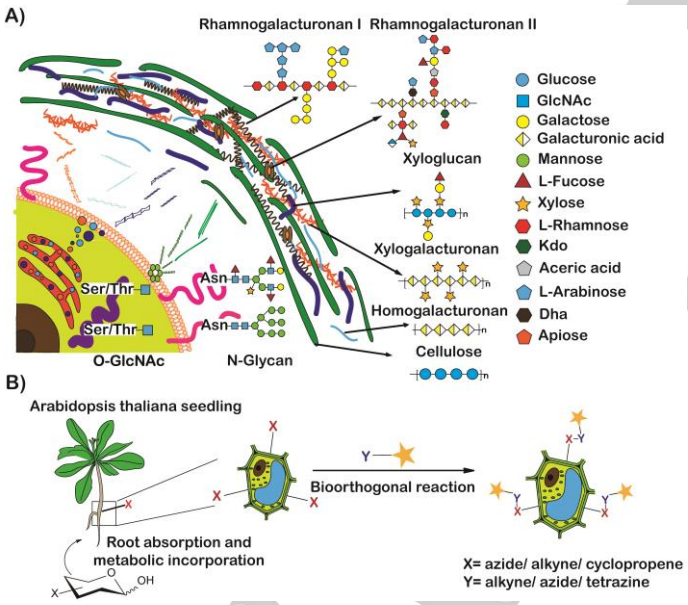
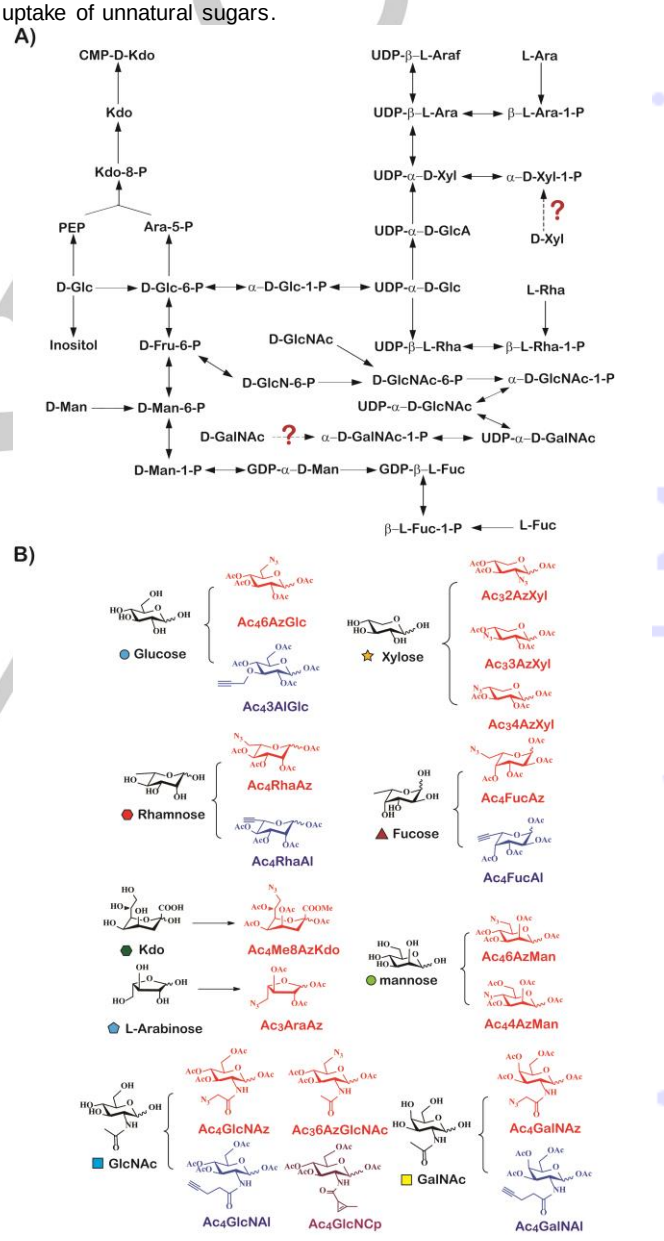


Figure 1. Metabolic glycan labeling enables visualization of root glycans in *Arabidopsis*. A) Schematic of various glycans synthesized in plant cells. B) *Arabidopsis* seedlings were cultured with monosaccharide chemical reporters (i.e., unnatural sugars containing a bioorthogonal group). The unnatural sugars were taken up via root absorption, and metabolically incorporated into cellular glycans through the underlying glycan biosynthetic machinery. Subsequent bioorthogonal labeling of the seedlings with fluorophores conjugated with a complementary bioorthogonal group enables fluorescence imaging of root glycans.

We first surveyed the known biosynthetic and interconversion pathways of nucleotide monosaccharides in plants (Scheme 1A).[24] For designing monosaccharide chemical reporters for different plant glycans, we chose to target monosaccharides that can either be used directly as biosynthetic precursors or enter a salvage pathway. We avoided using phosphorylated monosaccharides due to their troublesome chemical synthesis, and low cellular uptake due to the negative charge. Aiming to label different plant glycans including pectin, cellulose, hemicellulose, and glycoproteins, we designed and synthesized a panel of monosaccharide analogs including several ones that have been previously evaluated in *Arabidopsis* (Scheme 1B). The hydroxyl groups were acetylated and the carboxylic group was methyl esterified to facilitate the cellular uptake of unnatural sugars.

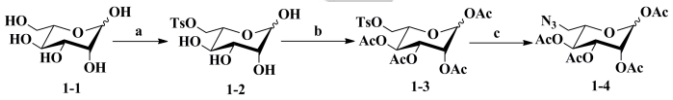


Scheme 1. A) Biosynthetic and interconversion pathways of several important monosaccharides in *Arabidopsis*. The question marks indicate that the existence of the salvage pathways for GalNAc and Xyl is speculated. B) Structures of unnatural sugars used in this work.

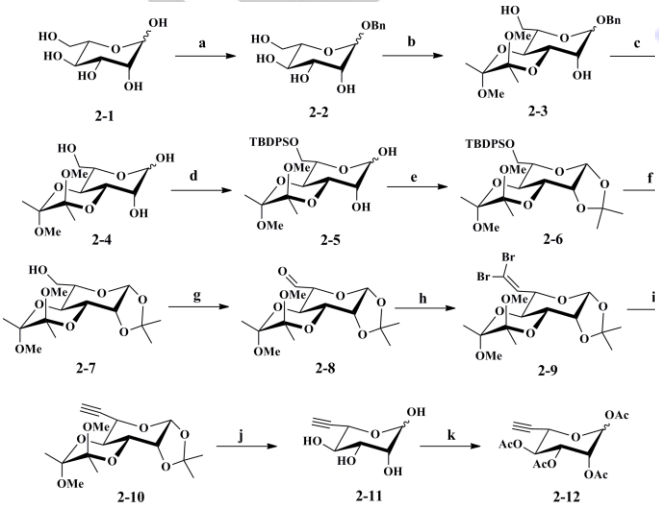
Targeting cellulose or/and hemicellulose, we synthesized peracetylated 6-azido-6-deoxy-D-glucose ($Ac_4$6AzGlc) and peracetylated 3-*O*-propargyl-D-glucose ($Ac_4$3AlGlc) using the previously reported synthetic procedures.[30-31] Three azido analogs of xylose, peracetylated 2-azido-2-deoxy-D-xylose ($Ac_3$2AzXyl), peracetylated 3-azido-3-deoxy-D-xylose ($Ac_3$3AzXyl), and peracetylated 4-azido-4-deoxy-D-xylose ($Ac_3$4AzXyl),[32] were synthesized as reported and used as potential reporters for hemicellulose, N-linked glycans, or/and pectins.

Among the pectin components, fucose, rhamnose, Kdo and L-arabinose are also possible parent monosaccharides. We synthesized peracetylated alkynyl fucose (Ac_4FucAl) and peracetylated 6-azidofucose (Ac_4FucAz) using the reported synthetic procedures.[33-34] The metabolic incorporation of Ac_4FucAl and Ac_4FucAz into cell wall glycans has been recently demonstrated.[19, 23] To synthesize the azido analog of rhamnose, peracetylated 6-azidorhamnose (Ac_4RhaAz, **1-4**), L-mannose was reacted with 4-toluenesulfonyl chloride. The resulting 6-*O*-tosyl-L-mannose was globally acetylated and reacted with sodium azide to give the product in a yield of 30% (Scheme 2). To synthesize the alkynyl rhamnose (**2-12**), we oxidized the 6-hydroxyl group of a selectively protected L-mannose (**2-7**, Scheme 3), followed by dibromomethylation of the aldehyde group and elimination to obtain the alkynyl compound (**2-10**). After deprotection and full acetylation, the final product was obtained in an overall yield of 1.4% (Scheme 3). A different synthetic route for Ac_4RhaAl was recently reported.[35] For the synthesis of Ac_4Me8AzKdo (**3-8**), we synthesized 8AzKdo (**3-7**) as reported,[36] followed by methyl esterification of the carboxylate and acetylation of the hydroxyl groups to give the final product in a yield of 3.8% (Scheme 4). It was recently shown that 8AzKdo could be metabolically incorporated into root glycans in *Arabidopsis*.[21] The synthesis of Ac_3AraAz (**4-4**) started by reacting L-arabinose with 4-toluenesulfonyl chloride, followed by full acetylation and substitution reaction using sodium azide to give the product in a yield of 18% (Scheme 5). A different synthetic route for Ac_3AraAz was recently reported.[23]

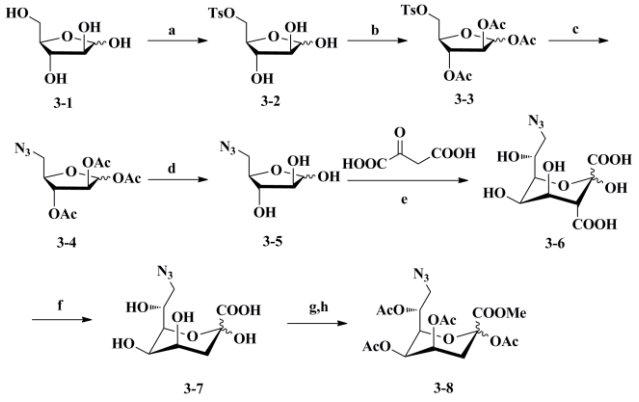
To label glycoproteins, we synthesized peracetylated *N*-azidoacetylglucosamine (Ac_4GlcNAz),[37] peracetylated *N*-4-pentynoylgalactosamine (Ac_4GlcNAl),[38] peracetylated 6-azido-6-deoxy-*N*-acetylglucosamine ($Ac_3$6AzGlcNAc),[39] peracetylated 6-azido-6-deoxy-mannose ($Ac_4$6AzMan),[40] peracetylated 4-azido-4-deoxy-mannose ($Ac_4$4AzMan),[40] peracetylated *N*-azidoacetylgalactosamine (Ac_4GalNAz),[41] and peracetylated *N*-4-pentynoylgalactosamine (Ac_4GalNAl)[38] as previously reported. Ac_4GlcNAz and Ac_4GalNAz have been recently shown to label glycoproteins in *Arabidopsis*.[20, 23, 42] In addition, we designed and synthesized a GlcNAc analog containing a cyclopropene, peracetylated *N*-2-methylcycloprop-2-enoylglucosamine (Ac_4GlcNCp), which can react with tetrazine-containing probes via inverse electron demand Diels-Alder (invDA) reaction.[43] The synthesis of Ac_4GlcNCp (**5-4**) was achieved by synthesizing of 2-methylcycloprop-2-enecarboxylic acid (**5-3**), followed by coupling with tetra-*O*-acetylglucosamine to give the product with an overall yield of 12% (Scheme 6). A slightly different cyclopropene-containing GlcNAc analog, peracetylated (2-methylcycloprop-2-enoyl) methoxycarbonylglucosamine (Ac_4GlcNCyoc) was recently shown to be metabolically incorporated into *Arabidopsis* root glycans and labeled by invDA reaction.[23] It should be noted that the one unnatural sugar might be metabolically incorporated in multiple types of glycans, which will be discussed further below.



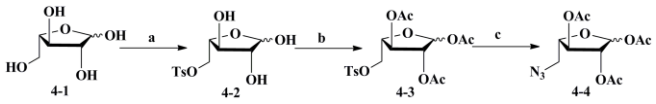
Scheme 2. Synthesis of Ac_4RhaAz. a) TsCl, pyridine, 0 °C-r.t., 12 h, 78%; b) Ac_2O, pyridine, 0 °C-r.t., 12 h, 84%; c) NaN_3, DMF, 65 °C, 16 h, 46%.



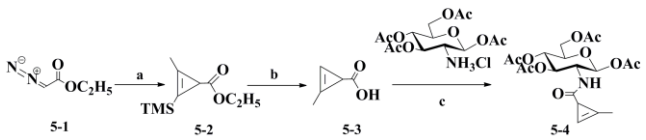
Scheme 3. Synthesis of Ac_4RhaAl. a) $PhCH_2OH$, $HCl_{(g)}$, 50 °C, overnight, 98% b) $BF_3.Et_2O$, $HC(OMe)_3$, 2,3-butanedione, MeOH, r.t., 17 h, 86%; c) $Pd(OH)_2/C$ MeOH, 18 h, 30 °C, 79%; d) TBDPSCl, imidazole, DMF, 0 °C-r.t., 12 h, 70%; e Acetone, $Me_2C(MeO)_2$, CSA, r.t., 16 h, 73%; f) *N*-Bu_4NF, THF, r.t., 6 h, 77%; g 4Å-Molecular Sieve, PCC, DCM, r.t., 3 h, 56%; h) PPh_3, CBr_4, DCM, 0 °C-r.t., 5 h, 43%; i) *n*-BuLi, THF, -78°C, 4 h; j) TFA:H_2O=9:1, 0 °C-r.t., 2 h; k) Ac_2O, pyridine, 0 °C-r.t., 12 h, 21% over three steps.



Scheme 4. Synthesis of Ac_4Me8AzKdo. a) TsCl, pyridine, 0 °C-r.t., 12 h; b) Ac_2O, pyridine, 0 °C-r.t., 12 h; c) NaN_3, DMF, 65 °C, 16 h, 27% over three steps; d) MeONa, MeOH, r.t., 30 min; e) H_2O, pH=11, r.t., 2 h; f) 80 °C, 30 min, 32% over three steps; g) Acetyl chloride, MeOH, 0 °C-r.t., 3 h; h) Ac_2O, pyridine, 0 °C-r.t., 12 h, 44% over two steps.



Scheme 5. Synthesis of Ac_3AraAz. a) TsCl, pyridine, 0 °C-r.t., 12 h; b) Ac_2O, pyridine, 0 °C-r.t., 12 h; c) NaN_3, DMF, 65 °C, 16 h, 18% over three steps.



Scheme 6. Synthesis of $Ac_4GlcNCp$. a) $Rh_2(OAc)_4$, 1-(Trimethylsilyl)-1-propyne, DCM, 0 °C, 2 h, 50%; b) KOH, DCM, MeOH, H_2O, 4°C, 24 h; c) EDCI, DMAP, TEA, DCM, r.t., 12 h, 25% over two steps.

Evaluation of azido and alkynyl unnatural sugars for metabolic incorporation in *Arabidopsis* roots

The *Arabidopsis* Col-0 seeds were planted in Murashige and Skoog (MS) solid media, and vernalized in dark at 4 °C for 3 d. The resulting seedlings were cultured under long-day conditions at 22 °C for 4 d, transferred to a liquid culture medium containing the azido or alkynyl monosaccharides, and cultured for 2 or 3 d. The incubation time was chosen for specific unnatural sugars so that high labeling could be achieved. The azido sugar- or alkynyl sugar-treated seedlings were then reacted with alkyne-Alexa Fluor 488 or azide-Alexa Fluor 488, respectively, via copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC, also termed click chemistry),[44-45] followed by fluorescence imaging of root tissues using confocal fluorescence microscopy (Figure 2). Robust fluorescence signal was observed for seedlings treated with Ac_4FucAl, Ac_4FucAz, $Ac_4Me8AzKdo$, $Ac_4GlcNAz$, and $Ac_4GalNAz$. Weak fluorescent labeling was observed in Ac_4RhaAl-treated samples. The rest of the unnatural sugars did not show significant cell surface or cell wall labeling in *Arabidopsis* roots.

Glucose is the major component of plant cellulose and hemicellulose. Our results showed that 6AzGlc with the 6-hydroxyl group replaced by an azide or 3AlGlc with a 3-*O*-propargyl group could not be metabolically incorporated into *Arabidopsis* glycans (Figure 2, top row). Interestingly, another alkynyl glucose, peracetylated 6-deoxy-D-gluco-6-ynopyranose, was recently reported to be specifically incorporated into root hair tips in *Arabidopsis*, and arrest root hair and root growth.[22] This unnatural monosaccharide, however, may also be regarded an alkynyl analog of xylose, 5-ethynylxylose; its metabolic fate in *Arabidopsis* has not been completely clear. These results indicate that the biosynthesis of cellulose and hemicellulose does not tolerate well the modifications on glucose. Xylose is a major component of the hemicellulose xyloglucan. It is also present in the pectin XGA and N-linked glycans. Although the xylose salvage pathway does not exist in animals, some evidence indirectly indicated that plants may have a salvage pathway for xylose.[46] Our results showing that azidoxylose with the 2-, 3-, or 4-hydoxyl group substituted with an azide could not be metabolically incorporated can be explained either by intolerance of the chemical modifications on xylose or by inexistence of the xylose salvage pathway (Figure 2, top row).

Rhamnose is a stereoisomer of fucose and both of them are major components of plant cell wall pectins. Although Ac_4FucAl and Ac_4FucAz showed robust metabolic incorporation and fluorescence labeling, in consistent with previous reports,[19, 23] Ac_4RhaAz and Ac_4RhaAl with similar substitutions only exhibited very weak labeling, indicating that the enzymes processing rhamnose are not as promiscuous as the ones for fucose (Figure 2, middle row). $Ac_4Me8AzKdo$ showed robust fluorescence labeling (Figure 2, middle row). Because Kdo only exists in the pectin RG-II, $Ac_4Me8AzKdo$ is presumably a RG-II-specific chemical reporter. In this work, we observed that Ac_3AraAz did not result in significant incorporation (Figure 2, middle row). However, the same compound was recently shown to be able to label *Arabidopsis* root glycans.[23] This discrepancy might be attributed to different experimental conditions for plant culture and metabolic labeling. In addition, we used peracetylated *N*-azidoacetylmannosamine ($Ac_4ManNAz$),[47] a designated precursor for sialic acid biosynthesis in animals that is not metabolized by plants, as a negative control. No fluorescence labeling was observed in $Ac_4ManNAz$-treated *Arabidopsis* roots (Figure 2, middle row), ruling out the possibility of non-specific physical absorption of unnatural sugars to the roots.

For metabolic labeling of glycoproteins in *Arabidopsis*, $Ac_4GlcNAz$ and $Ac_4GalNAz$ showed robust fluorescence labeling (Figure 2, bottom row), in agreement with the previous results.[20, 23] $Ac_4GlcNAz$ was metabolically incorporated into N-linked glycans and O-GlcNAc through the GlcNAc salvage pathway. Since *Arabidopsis* does not synthesize GalNAc-containing glycans, GalNAc was probably converted to UDP-GlcNAc. However, the interconversion pathway remains incompletely known.[48-49] The alkynyl analogs of GlcNAc and GalNAc did not show significant metabolic labeling. Moreover, the azido analogs of mannose did not appear to be incorporated into N-linked glycans, which contain multiple mannose residues (Figure 2, bottom row).

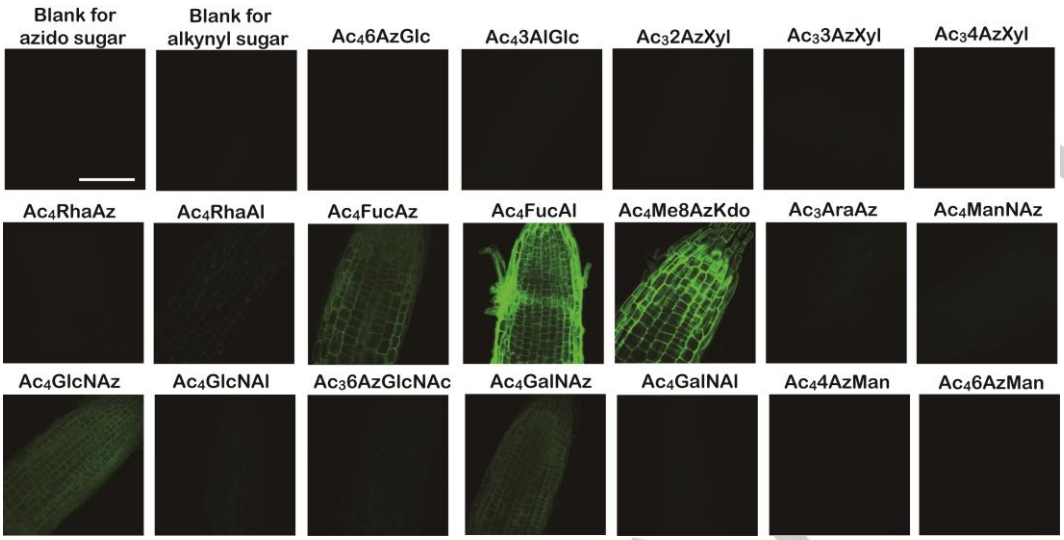


Figure 2. Evaluation of azido and alkynyl unnatural sugars for metabolic incorporation in *Arabidopsis* roots. The 4-d-old *Arabidopsis* seedlings were incubated with DMSO for 2 d (negative control for azido monosaccharides), DMSO for 2 d (negative control for alkynyl monosaccharides), 200 μM $Ac_4$6AzGlc for 2 d, 200 μM $Ac_4$3AlGlc for 2 d, 100 μM $Ac_3$2AzXyl for 2 d, 100 μM $Ac_3$3AzXyl for 2 d, 100 μM $Ac_3$4AzXyl for 2 d, 10 μM Ac_4FucAl for 4 h, 100 μM Ac_4FucAz for 1 d, 1 mM Ac_4RhaAl for 3 d, 1 mM Ac_4RhaAz for 3 d, 200 μM Ac_4Me8AzKdo for 2 d, 200 μM Ac_3AraAz for 3 d, 200 μM Ac_4GalNAz for 2 d, 200 μM Ac_4GalNAl for 2 d, 200 μM Ac_4GlcNAz for 2 d, 200 μM Ac_4GlcNAl for 2 d, 200 μM $Ac_3$6AzGlcNAc for 2 d, 200 μM $Ac_4$4AzMan for 2 d, 200 μM $Ac_4$6AzMan for 2 d, or 200 μM Ac_4ManNAz for 2 d. The azido monosaccharide-treated seedlings were reacted with alkyne-Alexa Fluor 488 and the alkynyl monosaccharide-treated seedlings were reacted with azide-Alexa Fluor 488 via CuAAC, followed by analysis with confocal fluorescence microscopy. The images were collected with a 63× oil-immersion objective lens on a confocal microscope. Scale bar, 100 μm.

Optimization of the metabolic labeling procedures

We investigated the concentration and time dependence of the metabolic incorporation of unnatural sugars in *Arabidopsis*, by using Ac_4FucAz and Ac_4Me8AzKdo as two representatives (Figure 3A and B). The seedlings were treated with Ac_4FucAz at varied concentrations for 1 d, followed by click labeling and fluorescence imaging. Fluorescence signal was observable at the concentration of 25 μM and saturated at 100 μM. Incubation time-dependent fluorescence imaging showed that labeling was distinguishable from the background starting at 6 h and saturated at 24 h (Figure 3A). For Ac_4Me8AzKdo, the metabolism is slightly less efficient and slower, with the obvious signal at concentration of 50 μM and incubation time of 1 d, and saturated signal at the concentration of 200 μM and incubation time of 3 d (Figure 3B).

Global protection of the hydroxyl groups of unnatural sugars with acetylation has been widely used to increase the labeling efficiency in mammalian cells by improving cellular uptake.[4] The peracetylated unnatural sugars can passively diffuse through the plasma membrane more efficiently. It should be noted that the uptake of some unprotected sugars may involve active cellular internalization pathways.[50] We therefore evaluated whether peracetylation can improve labeling efficiency of monosaccharide analogs in *Arabidopsis* (Figure 3C). Lipophilic protection increased the labeling efficiency of both 8AzKdo and GalNAz. The extent of improvement is more significant for GalNAz. In particular, Ac_4Me8AzKdo showed minimal improvement when the incubation time is longer than 1 d. It is worth noting that a recent study showed better labeling for GlcNAz than Ac_4GlcNAz in *Arabidopsis*.[23]

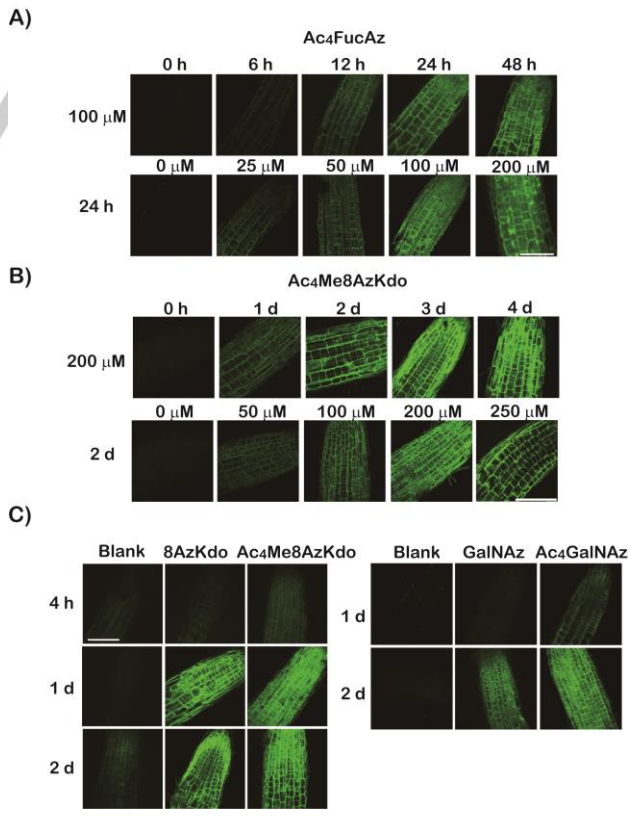


Figure 3. Optimization of the experimental conditions for metabolic labeling with unnatural sugars . A) The dose- and time-dependence of Ac_4FucAz incorporation. Seedlings were treated with Ac_4FucAz at varying concentrations for 24 h or with 100 μM Ac_4FucAz for varying durations of time, followed by labeling with alkyne-Alexa Fluor 488 and confocal fluorescence imaging. Scale bar, 100 μm. B) The dose- and time-dependence of $Ac_4Me8AzKdo$ incorporation. Seedlings were treated with $Ac_4Me8AzKdo$ at varying concentrations for 48 h or with 200 μM $Ac_4Me8AzKdo$ for varying durations of time, followed by labeling with alkyne-Alexa Fluor 488 and confocal fluorescence imaging. Scale bar, 100 μm. C) The seedlings were treated with 200 μM $Ac_4GalNAz$, 200 μM GalNAz, DMSO or 200 μM $Ac_4Me8AzKdo$, 200 μM 8AzKdo, DMSO for varying durations of time followed by click-labeling with alkyne-Alexa Fluor 488. Scale bar, 100 μm.

We used the root length assay[19-20, 23] to evaluate the biocompatibility of the azido and alkynyl sugars that can be metabolized by *Arabidopsis*. The seedlings were grown on solid MS media for 2 d, transferred to liquid MS media containing the unnatural sugars, and cultured for another 5 d, followed by measurement of the root length. Except that $Ac_4GalNAz$ showed moderate inhibition on root growth, the other five unnatural sugars did not cause significant toxic effect (Figure 4).

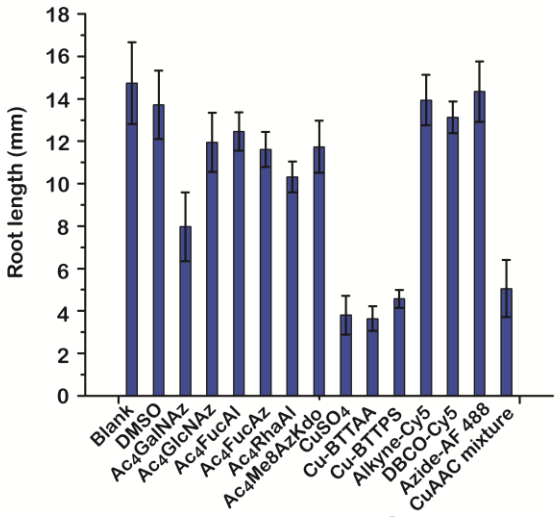


Figure 4. Toxicity analysis of unnatural sugars and labeling reagents in *Arabidopsis*. The seedlings were allowed to grow on 0.7% solid MS for 2 d, and then transferred to liquid MS (blank), MS only containing 0.2% DMSO or MS containing 0.2% DMSO and one of the following reagents: 200 μM $Ac_4GalNAz$, 200 μM $Ac_4GlcNAz$, 10 μM Ac_4FucAl, 100 μM Ac_4FucAz, 1 mM Ac_4RhaAl, 200 μM $Ac_4Me8AzKdo$, 1 mM $CuSO_4$, 1 mM $CuSO_4$ + 2 mM BTTAA, 1 mM $CuSO_4$ + 2 mM BTTPS, 1 μM dyes, 1 μM azide-Alexa Fluor 488 + 1 mM $CuSO_4$ + 1 mM ascorbic acid (CuAAC mixture). The seedlings were cultured for another 5 d, followed by measurement of the root length. The error bars represent the S.D. from 20 seedlings.

Optimization of the click labeling procedures

MGL using azido or alkynyl sugars hinges on click chemistry. We therefore performed several experiments to optimize the click-labeling procedures for *Arabidopsis* labeling. For CuAAC, increasing the concentration of $CuSO_4$ up to 1 mM improved the labeling efficiency (Figure 5A). The Cu(I) ligands BTTAA and BTTPS have been shown to accelerate CuAAC in mammalian cells.[51] We therefore evaluated them in plant labeling and the results showed that they did not improve the labeling efficiency in *Arabidopsis* (Figure 5B and S1). This is probably because the negatively charged plant cell walls repelled the negatively charged ligands.

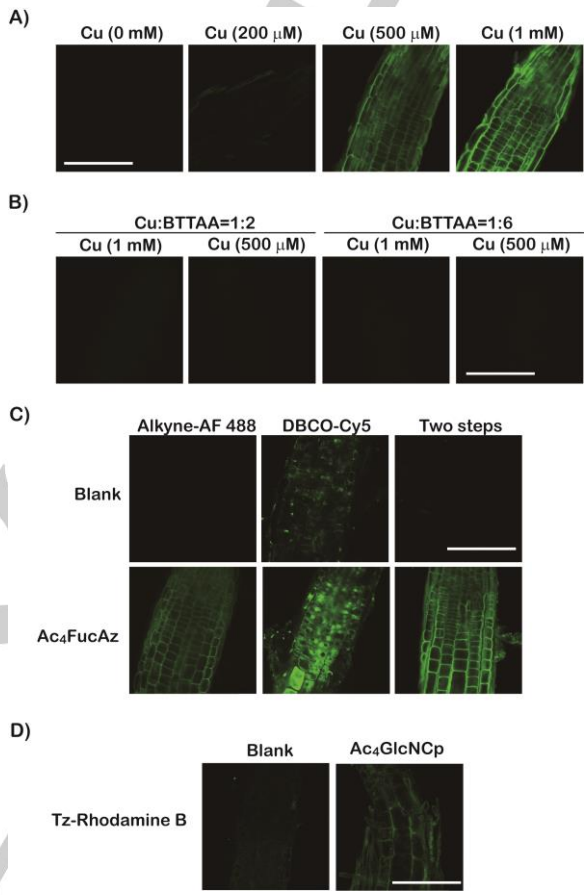


Figure 5. Optimization of the click labeling procedures. A) Labeling efficiency test under the concentration gradient of copper ion. The seedlings were incubated with 10 μM Ac_4FucAl for 4 h followed by click-labeled with azide-Alexa Fluor 488 under a designated concentrations of $CuSO_4$. Scale bar, 100 μm. B) Labeling efficiency test under the present of BTTAA ligands. The seedlings were incubated with 10 μM Ac_4FucAl for 4 h followed by click-labeled with azide-Alexa Fluor 488 under a designated concentrations of $CuSO_4$ and BTTAA. Scale bar, 100 μm. C) Labeling strategy study, the seedlings were incubated with DMSO or 100 μM Ac_4FucAz for 24 h. Left group the seedlings were labeled with alkyne-Alexa Fluor 488 by using copper-catalyzed reaction. Middle group, the seedlings were labeled with DBCO-Cy5 by using copper-free strain- promoted reaction. Right group, the seedlings were firstly labeled with alkyne-biotin, followed by BSA-blocked then treated with streptavidin-AF 647. Scale bar, 100 μm. D) Metabolic labeling using tetrazine-containing probes via the inverse electron demand Diels-Alder reaction. The seedlings were incubated with DMSO or 200 μM $Ac_4GlcNCp$ for 2 d then reacted with tetrazine-Rhodamine B. Scale bar, 100 μm.

Although most unnatural sugars caused minimal toxicity in *Arabidopsis*, copper induced significant inhibition of root growth (Figure 4 and S2). We therefore evaluated the strain-promoted azide-alkyne cycloaddition (i.e., copper-free click chemistry) for *Arabidopsis* labeling. In agreement with a recently published study,[23] reaction of Ac_4FucAz-treated seedlings with aza-

dibenzocyclooctyne (DBCO-Cy5) resulted in robust labeling; however, a higher background signal and more intracellular signal were observed in DBCO-Cy5-labeled samples, which might be due to non-specific absorption of the lipophilic DBCO-Cy5 (Figure 5C). We also compared a two-step protocol in CuAAC labeling. The Ac_4FucAz-treated seedlings were first reacted with alkyne-biotin and then stained with streptavidin-Alexa Fluor 647. This two-step protocol resulted in a slightly improved labeling efficiency and signal-to-noise ratio.

In addition, we tested the metabolic labeling using Ac_4GlcNCp, which can react with tetrazine-containing probes via the invDA reaction, another bioorthogonal reaction without the need of copper catalyst. The Ac_4GlcNCp-treated seedlings were reacted with tetrazine-Rhodamine B and confocal fluorescence microscopy showed a moderate fluorescence signal in the root tissues, indicating that Ac_4GlcNCp is metabolized in *Arabidopsis* with a relatively lower efficiency (Figure 5D). Ac_4GlcNCyoc was recently shown to result in higher fluorescence labeling, which might be owing to higher metabolism efficiency or faster reaction kinetics for the invDA reaction.[23]

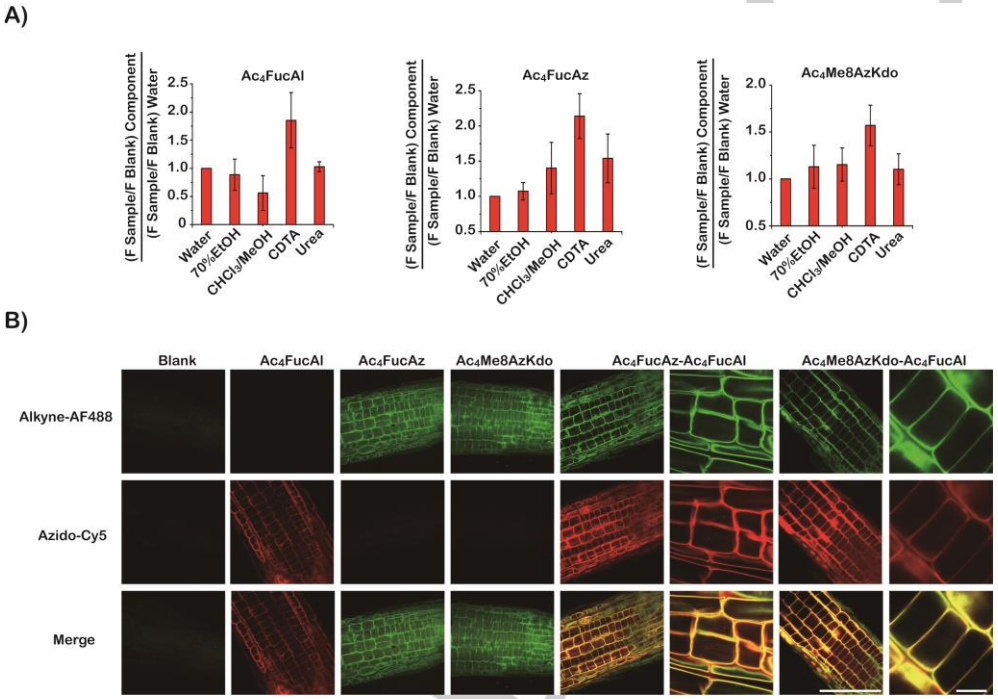


Figure 6. Metabolic fates of Ac_4FucAz and Ac_4Me8AzKdo. A) Glycan extraction analysis. The seedlings treated with 10 μM Ac_4FucAl for 4 h, 100 μM Ac_4FucAz for 1 d, 200 μM Ac_4Me8AzKdo for 2 d or DMSO for 2 d were harvested and click-labeled with the Alexa Fluor 488 dyes. After washed with fresh MS medium, the seedlings were grinded into powder and extract with water, 70% (vol/vol) EtOH in water, 50% (vol/vol) chloroform in methanol, 50 mM CDTA and 8 M urea for 24 hours. The Alexa Fluor 488 fluorescence of supernatant from each extraction was measured and the ratio of probe-treated to DMSO-treated fluorescence intensity was calculated and normalized. B) Colocalization of FucAz/ 8AzKdo with FucAl. *Arabidopsis* seedlings were incubated with DMSO, 10 μM Ac_4FucAl, 100 μM Ac_4FucAz, 200 μM Ac_4Me8AzKdo for 48 h in the single labeling groups, or incubated with 100 μM Ac_4FucAz + 10 μM Ac_4FucAl or 200 μM Ac_4Me8AzKdo + 10 μM Ac_4FucAl for 48 h in the double labeling groups, followed by sequentially reacted with azide-Cy5 and alkyne-Alexa Fluor 488. Scale bars, 100 μm (zoom out panel) and 10 μm (zoom in panel).

Metabolic fates of Ac_4FucAz and Ac_4-8-AzKdoMe

The metabolic fate of Ac_4FucAl was previously determined by detection of the presence of FucAl in extraction fractions of various cell wall components.[19] The FucAl was mainly detected in pectin RG-I. We performed the same assay to determine the metabolic fate for Ac_4FucAz and Ac_4Me8AzKdo (Figure 6A). The water and ethanol fractions mainly contained small molecules including the non-specifically adsorbed dyes. The chloroform and methanol fraction contained lipids and some proteins. CDTA removed Ca^{2+}, so that the Ca^{2+}-crosslinked pectin components containing galacturonan were dissolved in this fraction. As expected, Ac_4FucAz exhibited a similar extraction pattern to that of Ac_4FucAl, indicating that they have a similar metabolic fate. AzKdo was also mainly present in the CDTA fraction, confirming that AzKdo was incorporated into pectins. Since Kdo has only been found in pectin RG-II, it is reasonable to conclude that 8AzKdo is specially incorporated into RG-II in *Arabidopsis*.

We further performed double labeling experiments to confirm their metabolic fates (Figure 6B). The seedlings were simultaneously treated with two monosaccharides, Ac_4FucAl together with Ac_4FucAz or Ac_4FucAl together with Ac_4Me8AzKdo. After sequential click reactions with alkyne-Alexa Fluor 488 and azido-Cy5, the seedlings were subjected to imaging by two-color confocal fluorescence microscopy. The fluorescence signals of Alexa Fluor 488 and Cy5 were nicely colocalized, supporting that Ac_4FucAz and Ac_4Me8AzKdo mainly label cell wall pectin components.

Distribution patterns of Ac_4FucNAl and Ac_4Me8AzKdo in the developmental zones of growing roots

Arabidopsis roots are divided into three developmental zones—meristematic zone, elongation zone, and differentiation zone—according to three distinct developmental stages that new cells produced from stems cells undergo.[52] We previously showed that newly synthesized N-linked glycans containing the metabolically incorporated GlcNAz was distributed distinctly in these zones, with significantly lower fluorescence labeling in the elongation zone.[20] We therefore visualized the distribution patterns of the metabolically incorporated Ac_4FucAl and Ac_4Me8AzKdo in the developmental zones by confocal fluorescence microscopy (Figure 7 and S3). In comparison to Ac_4GlcNAz, Ac_4FucAl-treated seedlings showed an evenly distributed fluorescence signal along the three developmental zones. Ac_4Me8AzKdo-treated seedlings exhibited a similar distribution pattern to Ac_4GlcNAz-treated seedlings. In the elongation zone, cell division stops, while the cells expand rapidly in volume by increasing their length.[53] At the same time, cell wall glycans were vigorously synthesized and distributed. Our results suggest that the synthesis of different kinds of glycans may be differentially regulated in distinct developmental stages. Of note, we currently cannot completely rule out the possibility that the cells in different developmental zones have varied metabolic incorporation efficiency for unnatural sugars.

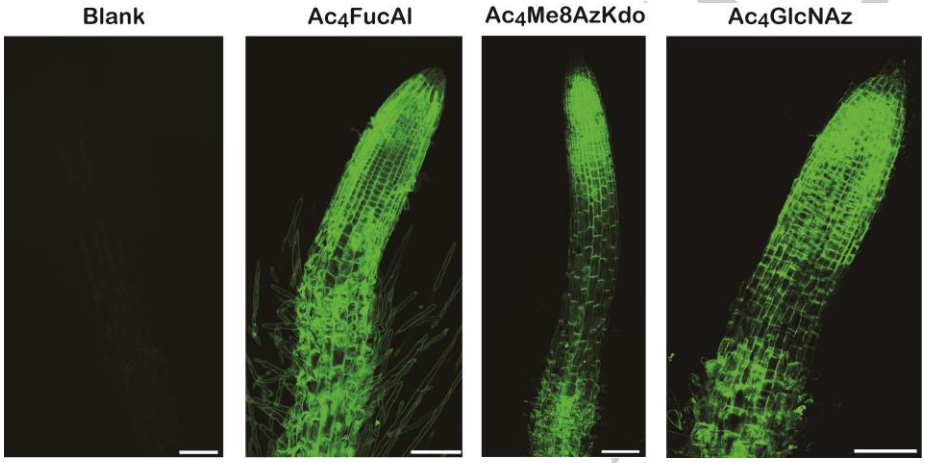


Figure 7. Distribution of newly synthesized glycans in the developmental zones of growing roots labeled by Ac_4Me8AzKdo, Ac_4FucAl or Ac_4GlcNAz. The seedlings were treated with 10 μM Ac_4FucAl, 200 μM Ac_4GlcNAz, 200 μM Ac_4Me8AzKdo or DMSO for 48 h, followed by click-labeling. Confocal fluorescence images of a longitudinal section were collected through the root with a 63× oil-immersion objective lens. Each mosaic was then constructed from 12 titles (10 titles for Ac_4Me8AzKdo) (each 246 μm × 246 μm). Scale bar, 100 μm.

Conclusion

In summary, we have synthesized a collection of 19 unnatural sugar reporters, including three new compounds (Ac_4Me8AzKdo, Ac_4RhaAz and Ac_4GlcNCp) and 14 that have not previously been evaluated for MGL in *Arabidopsis*. The systematic evaluation and comparison of the metabolic incorporation of those monosaccharide reporters into root glycans of *Arabidopsis* provides a guideline for selecting appropriate unnatural sugars for probing glycosylation in plants. The conditions for bioorthogonal labeling have also been evaluated and optimized in the context of fluorescence imaging of *Arabidopsis* roots. Furthermore, our results reveal that the biosynthesis of glycans in roots may be developmentally regulated, and this regulation may be glycan type-dependent. Expanding MGL in *Arabidopsis* adds a toolkit for studying glycosylation in plants.

Experimental Section

Synthesis of the new compounds: Compounds and reagents used in this paper and details describing the synthesis of the new compounds can be found in **Supporting Information**.

Plant materials and growth conditions: *Arabidopsis thaliana* Col-0 seeds were sterilized in 75% (vol/vol) ethanol aqueous solution for 8 min, in pure ethanol for 2 min, and dried under the reduced pressure for 30 min at 25 °C. The sterilized seeds were deposited on solid MS [4.4 g/L Murashige and Skoog salts (Caisson), 1% (wt/vol) sucrose, 0.7 % (wt/vol) agar, pH = 5.72] plates and incubated at 4 °C in the dark for 3 d. The plates were then moved to a greenhouse under long-day conditions (16 h light/8 h dark cycles) and incubated at 22 °C for 4 d.

Metabolic labeling of seedlings with unnatural sugars and click reaction: The 4-d-old seedlings in the plates were transferred to 1.8 mL liquid MS medium containing 0.2% DMSO for 2 d as the negative control, or 0.2% DMSO with unnatural sugars (200 μM $Ac_4$6AzGlc for 2 d, 200 μM $Ac_4$3AlGlc for 2 d, 100 μM $Ac_3$2AzXyl for 2 d, 100 μM $Ac_3$3AzXyl for 2 d, 100 μM $Ac_3$4AzXyl for 2 d, 10 μM Ac_4FucAl for 4 h, 100 μM Ac_4FucAz for 1 d, 1 mM Ac_4RhaAl for 3 d, 1 mM Ac_4RhaAz for 3 d, 200 μM Ac_4Me8AzKdo for 2 d, 200 μM Ac_3AraAz for 3 d, 200 μM Ac_4GalNAz for 2 d, 200 μM Ac_4GalNAl for 2 d, 200 μM

$Ac_4GlcNAz$ for 2 d, 200 μM $Ac_4GlcNAl$ for 2 d, 200 μM $Ac_3$6AzGlcNAc for 2 d, 200 μM $Ac_4GlcNCp$ for 2 d, 200 μM $Ac_4$4AzMan for 2 d, 200 μM $Ac_4$6AzMan for 2 d, 200 μM $Ac_4ManNAz$ for 2 d) and incubated at long day condition under 22 °C. Before labeling reactions, the seedlings were washed with fresh liquid MS medium for three times. For copper-catalyzed reaction, the seedlings were incubated with liquid MS medium containing 1 μM alkyne-Alexa Fluor 488 (Invitrogen, Cat. # A10267) for azido probes or 1 μM azide-Alexa Fluor 488 (Invitrogen, Cat. # A10266) for alkynyl probes, 1 mM $CuSO_4$, and 1 mM ascorbic acid at 25 °C in the dark for 1 h, followed by washing with liquid MS medium for three times. For copper-free strain- promoted reaction, the seedlings were incubated with liquid MS medium containing 1 μM DBCO-Cy5 (Click Chemistry Tools, Cat. # A130-5) at 25 °C in the dark for 1 h, followed by washing with liquid MS medium for 12 h. For two-steps labeling, the seedlings were incubated with alkyne-biotin (Click Chemistry Tools, Cat. # TA105-1000), 1 mM $CuSO_4$, and 1 mM ascorbic acid at 25 °C for 1 h, followed by washing with liquid MS medium for three times. Then the seedlings were blocked with 1% BSA in MS medium for 1 h followed by three washes, 5 μg/mL streptavidin-AF 647 (Biolegend, Cat. # 405237) in MS was used as a secondary tag to incubate the seedlings for an hour followed by MS washing overnight. For tetrazine-containing probes used in inverse electron demand Diels-Alder reaction, the seedlings were incubated with liquid MS medium containing 1 μM tetrazine-Rhodamine B at 25 °C in the dark for 1 h, followed by washing with liquid MS medium for three times.

For the labeling efficiency test under the concentration gradient of copper ion, the seedlings were treated with 10 μM Ac_4FucAl for 4 h followed by washing with liquid MS medium for three times. Then the seedlings were incubated with liquid MS medium containing 1 μM azide-Alexa Fluor 488, a designated concentrations $CuSO_4$ and BTTAA, 1 mM ascorbic acid at 25 °C in the dark for 1 h, followed by washing with liquid MS medium for three times.

For lipophilic protection study experiment, the seedlings were treated with 200 μM $Ac_4GalNAz$, 200 μM GalNAz, DMSO or 200 μM Ac_4Me8AzKdo, 200 μM 8AzKdo, DMSO for varying durations of time followed by click-labeling with alkyne-Alexa Fluor 488.

For the dose- and time-dependence experiments for Ac_4FucAz, the seedlings were treated with Ac_4FucAz at varying concentrations for 24 h or with 100 μM Ac_4FucAz for varying durations of time, followed by click-labeling with alkyne-Alexa Fluor 488. For the dose- and time-dependence experiments for Ac_4Me8AzKdo, the seedlings were treated with Ac_4Me8AzKdo at varying concentrations for 48 h or with 200 μM Ac_4Me8AzKdo for varying durations of time, followed by labeling with alkyne-Alexa Fluor 488.

For double labeling experiments, the seedlings were incubated with DMSO, 10 μM Ac_4FucAl, 100 μM Ac_4FucAz, 200 μM Ac_4Me8AzKdo for 48 h in the single labeling groups, or incubated with 100 μM Ac_4FucAz + 10 μM Ac_4FucAl or 200 μM Ac_4Me8AzKdo + 10 μM Ac_4FucAl for 48 h in the double labeling groups, followed by sequentially reacted with azide-Cy5 (Click Chemistry Tools, Cat. # AZ118-25) and alkyne-Alexa Fluor 488.

Fluorescence microscopy and image analysis: Confocal microscopy of labeled seedlings was performed on a Leica TCS SP8X scanning confocal microscope equipped with a white light laser (WLL, wavelength range from 470 nm to 670 nm). Alkyne-Alexa Fluor 488 and azide-Alexa Fluor 488 were excited at 488 nm by using the WLL and collected with a 510–535 nm band-pass filter. DBCO-Cy5, azide-Cy5, alkyne-Cy5 (Click Chemistry Tools, Cat. # TA116-25) and Streptavidin- AF 647 was excited at 632 nm and collected with a 660–685 nm band-pass filter, tetrazine-Rhodamine B was excited at 540 nm and collected with a 570-590 nm band-pass filter. The mosaic was then constructed from 12 titles (10 titles for Ac_4Me8AzKdo) (each 246 μm × 246 μm). The experiments were repeated three times (n ≥ 20 seedlings were selected) and imaged using identical exposure settings.

Toxicity analysis of unnatural sugars and labeling reagents: The seedlings were grown on 0.7% solid MS for 2 d, and then transferred to liquid MS (blank), MS containing only 0.2 % DMSO or MS containing 0.2% DMSO and one of the following reagents: 200 μM $Ac_4GalNAz$, 200 μM $Ac_4GlcNAz$, 10 μM Ac_4FucAl, 100 μM Ac_4FucAz, 1 mM Ac_4RhaAl, 200 μM Ac_4Me8AzKdo, 1 mM $CuSO_4$, 1 mM $CuSO_4$ + 2 mM BTTAA, 1 mM $CuSO_4$ + 2 mM BTTPS, 1 μM dyes, 1 μM azide-Alexa Fluor 488 + 1 mM $CuSO_4$ + 1 mM ascorbic acid (CuAAC mixture). The seedlings were cultured for another 5 d, followed by measurement of the root length. The error bars represent the S.D. from 20 seedlings.

Glycan extraction analysis: The seedlings treated with 10 μM Ac_4FucAl for 4 h, 100 μM Ac_4FucAz for 1 d, 200 μM Ac_4Me8AzKdo for 2 d or DMSO for 2 d were harvested and click-labeled with the Alexa Fluor 488 dyes. The seedlings were washed with fresh MS medium then grinded into powder in liquid nitrogen. The powder from about one hundred seedlings was extracted in 1.8 mL water under vigorous rocking for an hour in the dark. The mash was centrifuged under 13000 g for 5 min and the supernatant was collected for analysis. Then the residue was sequentially treated in the same way with 70% (vol/vol) EtOH twice for an hour and 1:1 (vol/vol) chloroform/methanol once for an hour. The obtained alcohol insoluble residue (AIR) was further extracted with 50 mM CDTA (trans-1, 2-diaminocyclohexane-*N, N, N', N'*-tetraacetic acid) in 50 mM Hepes-NaOH buffer (pH=7.5) once for 24 hour and 8 M urea in 50 mM Hepes-NaOH buffer (pH=7.5) once for 24 hour. The Alexa Fluor 488 fluorescence of supernatant from each extraction was measured, and the ratio of probe-treated to DMSO-treated fluorescence intensity was calculated and normalized by using the water group. The experiments were repeated three times.

Acknowledgements

We thank Prof. H. Guo and Y. Zhu for the assistance on plant culture and growth experiments. This work is supported by the

National Natural Science Foundation of China (No.21425204, No. 21521003 and No. 21672013) and the National Key Research and Development Projects (No. 2016YFA0501500).

Keywords: metabolic glycan labeling • *Arabidopsis thaliana* • cell wall polysaccharide • plant glycobiology • click chemistry

[1] A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, G. W. Hart, M. E. Etzler, in *Essentials of Glycobiology*, Cold Spring Harbor Laboratory Press, **2008**.

[2] H. Kayser, R. Zeitler, C. Kannicht, D. Grunow, R. Nuck, W. Reutter, *J. Biol. Chem.* **1992**, *267*, 16934-16938.

[3] L. K. Mahal, K. J. Yarema, C. R. Bertozzi, *Science* **1997**, *276*, 1125-1128.

[4] B. Cheng, R. Xie, L. Dong, X. Chen, *Chembiochem* **2016**, *17*, 11-27.

[5] S. T. Laughlin, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 12-17.

[6] S. H. Rouhanifard, L. U. Nordstrom, T. Zheng, P. Wu, *Chem. Soc. Rev.* **2013**, *42*, 4284-4296.

[7] T. J. Sminia, H. Zuilhof, T. Wennekes, *Carbohydr. Res.* **2016**, *435*, 121-141.

[8] P. R. Wratil, R. Horstkorte, W. Reutter, *Angew. Chem. Int. Ed.* **2016**, *55*, 9482-9512.

[9] J. A. Prescher, D. H. Dube, C. R. Bertozzi, *Nature* **2004**, *430*, 873-877.

[10] R. Xie, L. Dong, R. Huang, S. Hong, R. Lei, X. Chen, *Angew. Chem. Int. Ed.* **2014**, *53*, 14082-14086.

[11] J. Rong, J. Han, L. Dong, Y. Tan, H. Yang, L. Feng, Q. W. Wang, R. Meng, J. Zhao, S. Q. Wang, X. Chen, *J. Am. Chem. Soc.* **2014**, *136*, 17468-17476.

[12] S. T. Laughlin, J. M. Baskin, S. L. Amacher, C. R. Bertozzi, *Science* **2008**, *320*, 664-667.

[13] S. T. Laughlin, C. R. Bertozzi, *ACS Chem. Biol.* **2009**, *4*, 1068-1072.

[14] T. W. Liu, M. Myschyshyn, D. A. Sinclair, S. Cecioni, K. Beja, B. M. Honda, R. D. Morin, D. J. Vocadlo, *Nat. Chem. Biol.* **2017**, *13*, 161-167.

[15] M. E. Himmel, S. Y. Ding, D. K. Johnson, W. S. Adney, M. R. Nimlos, J. W. Brady, T. D. Foust, *Science* **2007**, *315*, 804-807.

[16] S. Pattathil, U. Avci, D. Baldwin, A. G. Swennes, J. A. Mcgill, Z. Popper, T. Bootten, A. Albert, R. H. Davis, C. Chennareddy, *Plant Physiol.* **2010**, *153*, 514-525.

[17] W. G. T. Willats, C. G. Steele-King, L. Mccartney, C. Orfila, S. E. Marcus, J. P. Knox, *Plant Physiol. Biochem.* **2000**, *38*, 27-36.

[18] Anderson, T. Charles, Carroll, Andrew, Akhmetova, Laila, Somerville, Chris, *Plant Physiol.* **2010**, *152*, 787-796.

[19] C. T. Anderson, I. S. Wallace, C. R. Somerville, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 1329-1334.

[20] Y. Zhu, J. Wu, X. Chen, *Angew. Chem. Int. Ed.* **2016**, *55*, 9301-9305.

[21] M. Dumont, A. Lehner, B. Vauzeilles, J. Malassis, A. Marchant, K. Smyth, B. Linclau, A. Baron, J. Mas Pons, C. T. Anderson, D. Schapman, L. Galas, J. C. Mollet, P. Lerouge, *Plant J.* **2016**, *85*, 437-447.

[22] D. D. Mcclosky, B. Wang, G. Chen, C. T. Anderson, *Phytochemistry* **2016**, *123*, 16-24.

[23] J. Hoogenboom, N. Berghuis, D. Cramer, R. Geurts, H. Zuilhof, T. Wennekes, *BMC Plant Biol.* **2016**, *16*, 220.

[24] M. Bar-Peled, M. A. O'Neill, *Annu. Rev. Plant Biol.* **2011**, *62*, 127-155.

[25] P. Lerouge, M. Cabanes-Macheteau, C. Rayon, A. C. Fischette-Laine, V. Gomord, L. Faye, *Plant Mol. Biol.* **1998**, *38*, 31-48.

[26] N. E. Olszewski, C. M. West, S. O. Sassi, L. M. Hartweck, *Biochim Biophys. Acta* **2010**, *1800*, 49-56.

[27] D. T. A. Lamport, *Nature* **1967**, *216*, 1322-1324.

[28] D. J. Cosgrove, *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 850-861.

[29] C. Somerville, S. Bauer, G. Brininstool, M. Facette, T. Hamann, J. Milne, E. Osborne, A. Paredez, S. Persson, T. Raab, *Science* **2004**, *306*, 2206-2211.

[30] Z. Györgydeàk, L. Szilàgyi, *Eur. J. Org. Chem.* **1987**, *1987*, 235-241.

[31] A. Singhamahapatra, L. Sahoo, S. Kundu, D. Loganathan, *Trends Carbohydr. Res.* **2013**, *5*, 39-43.

[32] B. J. Beahm, K. W. Dehnert, N. L. Derr, J. Kuhn, J. K. Eberhart, D. Spillmann, S. L. Amacher, C. R. Bertozzi, *Angew. Chem. Int. Ed.* **2014**, *53*, 3347-3352.

[33] D. Rabuka, S. C. Hubbard, S. T. Laughlin, S. P. Argade, C. R. Bertozzi, *J. Am. Chem. Soc.* **2006**, *128*, 12078-12079.

[34] M. Sawa, T. L. Hsu, T. Itoh, M. Sugiyama, S. R. Hanson, P. K. Vogt, C. H. Wong, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 12371-12376.

[35] B. Wang, D. D. Mcclosky, C. T. Anderson, G. Chen, *Carbohydr. Res.* **2016**, *433*, 54-62.

[36] A. Dumont, A. Malleron, M. Awwad, S. Dukan, B. Vauzeilles, *Angew. Chem. Int. Ed.* **2012**, *51*, 3143-3146.

[37] D. J. Vocadlo, H. C. Hang, E. J. Kim, J. A. Hanover, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 9116-9121.

[38] B. W. Zaro, Y. Y. Yang, H. C. Hang, M. R. Pratt, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 8146-8151.

[39] K. N. Chuh, B. W. Zaro, F. Piller, V. Piller, M. R. Pratt, *J. Am. Chem. Soc.* **2014**, *136*, 12283-12295.

[40] S. Marchesan, D. Macmillan, *Chem. Commun.* **2008**, 4321-4323.

[41] H. C. Hang, C. Yu, D. L. Kato, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 14846-14851.

[42] R. Zentella, J. Hu, W. P. Hsieh, P. A. Matsumoto, A. Dawdy, B. Barnhill, H. Oldenhof, L. M. Hartweck, S. Maitra, S. G. Thomas, S. Cockrell, M. Boyce, J. Shabanowitz, D. F. Hunt, N. E. Olszewski, T P. Sun, *Genes Dev.* **2016**, *30*, 164-176.

[43] D. M. Patterson, L. A. Nazarova, B. Xie, D. N. Kamber, J. A. Prescher, *J. Am. Chem. Soc.* **2012**, *134*, 18638-18643.

[44] V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem. Int. Ed.* **2002**, *41*, 2596-2599.

[45] C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057-3064.

[46] C. Geserick, R. Tenhaken, *Plant J.* **2013**, *74*, 239-247.

[47] E. Saxon, C. R. Bertozzi, *Science* **2000**, *287*, 2007-2010.

[48] M. Nozaki, M. Sugiyama, J. Duan, H. Uematsu, T. Genda, Y. Sato, *Plant Cell* **2012**, *24*, 3366-3379.

[49] K. Furo, M. Nozaki, H. Murashige, Y. Sato, *FEBS Lett.* **2015**, *589*, 3258-3262.

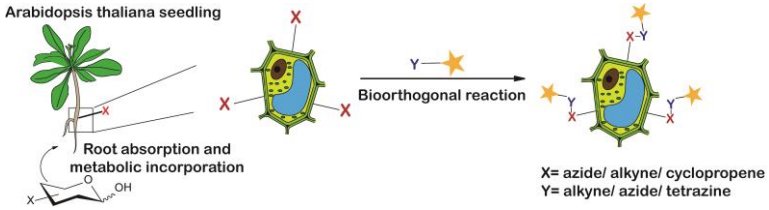
[50] P. A. Gilormini, C. Lion, D. Vicogne, T. Levade, S. Potelle, C. Mariller, Y. Guerardel, C. Biot, F. Foulquier, *Chem. Commun.* **2016** *52*, 2318-2321.

[51] C. Besanceney-Webler, H. Jiang, T. Zheng, L. Feng, D. Soriano del Amo, W. Wang, L. M. Klivansky, F. L. Marlow, Y. Liu, P. Wu, *Angew. Chem. Int. Ed.* **2011**, *50*, 8051-8056.

[52] J. J. Petricka, C. M. Winter, P. N. Benfey, *Annu. Rev. Plant Biol.* **2012**, *63*, 563-590.

[53] J. P. Verbelen, C. T. De, J. Le, K. Vissenberg, F. Baluska, *Plant Signal Behav.* **2006**, *1*, 296-304.

FULL PAPER



Metabolic Glycan Labeling (MGL) in plants: A collection of 19 unnatural sugars containing a bioorthogonal group (i.e., an azide, alkyne, or cyclopropene) were synthesized and evaluated of MGL for fluorescence imaging of root glycans in *Arabidopsis thaliana*.

*Y. Zhu, X. Chen**

Page No. – Page No.

Expanding the Scope of Metabolic Glycan Labeling in *Arabidopsis Thaliana*