TECHNICAL ADVANCE

Plant cell wall imaging by metabolic click-mediated labelling of rhamnogalacturonan II using azido 3-deoxy-ß-manno-oct-2-ulosonic acid

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SUMMARY

In plants, 3-deoxy-ß-manno-oct-2-ulosonic acid (Kdo) is a monosaccharide that is only found in the cell wall pectin, rhamnogalacturonan-II (RG-II). Incubation of 4-day-old light-grown Arabidopsis seedlings or tobacco BY-2 cells with 8-azido 8-deoxy Kdo (Kdo-N3) followed by coupling to an alkyne-containing fluorescent probe resulted in the specific in muro labelling of RG-II through a copper-catalysed azide–alkyne cycloaddition reaction. CMP-Kdo synthetase inhibition and competition assays showing that Kdo and D-Ara, a precursor of Kdo, but not L-Ara, inhibit incorporation of Kdo-N3 demonstrated that incorporation of Kdo-N3 occurs in RG-II through the endogenous biosynthetic machinery of the cell. Co-localisation of Kdo-N3 labelling with the cellulose-binding dye calcofluor white demonstrated that RG-II exists throughout the primary cell wall. Additionally, after incubating plants with Kdo-N3 and an alkynated derivative of L-fucose that incorporates into rhamnogalacturonan I, co-localised fluorescence was observed in the cell wall in the elongation zone of the root. Finally, pulse labelling experiments demonstrated that metabolic click-mediated labelling with Kdo-N3 provides an efficient method to study the synthesis and redistribution of RG-II during root growth.

Keywords: rhamnogalacturonan-II, click chemistry, Kdo-N3, cell wall, Arabidopsis, BY-2 cells, technical advance.

INTRODUCTION

Expression of target proteins fused to green fluorescent protein tags is routinely used in cell biology to study protein localisation in living cells. However, this efficient strategy cannot be extended to glycans and lipids. The use of bio-orthogonal chemical reporters offers an alternative for in vivo labelling of biomolecules without the requirement for genetic manipulation. In this method, a chemical reporter is incorporated into the target biomolecule using the endogenous biosynthetic machinery of the cell. This reporter can then be covalently coupled in a highly selective and efficient reaction at room temperature to an exogenously delivered probe. The copper-catalysed azide–alkyne
cycloaddition, a type of so-called ‘click’ chemistry, is the most widely used click-mediated labelling method in biology (Rostovtsev et al., 2002; Tornøe et al., 2002). An azide chemical reporter is usually selected for the step of incorporation into biomolecules because of its small size, metabolic stability and the lack of reactivity with naturally occurring biomolecules. Labelling is then performed by copper-catalysed linkage to reporter molecules carrying terminal alkynes. The study of cell-surface glycans has benefited greatly from the emergence of click chemistry for use in cell biology. These experiments have mostly been performed in vertebrates and bacteria by metabolic glycan labelling of cells, using a modified monosaccharide containing a bio-orthogonal chemical reporter. Chemical ligation to a fluorescent probe then enables the visualisation of glycoconjugates that accumulate at the cell surface (Chang et al., 2009; Dehnert et al., 2011; Dumont et al., 2012; Mas Pons et al., 2014).

Although click chemistry has been widely used in animal and bacterial biology, this approach has received limited attention in plant biology to date. Click chemistry was recently used to label cells undergoing DNA synthesis while following a Golgi protein marker fused to a fluorescent protein (Bourge et al., 2015). Several recent studies have reported the imaging of plant cell wall components using a click chemistry approach and two groups have recently reported the imaging of plant cell wall lignification using different azido- and alkynyl-tagged monolignol analogues (Bukowski et al., 2014; Tobimatsu et al., 2014; Pandey et al., 2015). In addition, an alkynyl derivative of L-fucose (Fuc-Al) has been used for cell wall labelling in Arabidopsis thaliana (Anderson et al., 2012). In this study, the authors demonstrated that seedlings fed with the peracetylated alkynyl derivative of L-fucose incorporated this L-Fuc analogue into cell wall pectic rhamnogalacturonan-I (RG-I) but not into xyloglucan, the main fucose-containing cell wall polysaccharide. Chemical ligation to a fluorescent azide-containing probe enabled labelling of primary cell walls of the roots of Arabidopsis seedlings. This fucose-alkyne-based labelling strategy was also used to investigate the secretion of RG-I in mutants lacking the kinesin FRAGILE FIBER 1 (FRA1) that is defective in the secretion of RG-I in mutants lacking the kinesin (Zhu et al., 2015).

Pectins are composed of α-1,4-linked homogalacturonan (HG) and RG-I, which consists of arabinan and galactan chains linked to a [→2]-α-L-Rhap(1→4)-α-D-GalpA(1→) repeating disaccharide backbone. A third pectic molecule, rhamnogalacturonan II (RG-II), represents only about 2–4% of the primary cell wall in plants and is found in the primary walls of angiosperms, gymnosperms, lycophytes and pteridophytes (O’Neill et al., 2004; Bar-Peled et al., 2012). RG-II is composed of a HG backbone that is substituted with four oligosaccharide side chains (Figure 1a) (O’Neill et al., 2004). At least 12 different glycosyl residues are present in RG-II, including several rare sugars such as aceric acid, apoiose, 3-deoxy-α-lyxo-hept-2-ulosic acid and 3-deoxy-α-manno-oct-2ulosonic acid (Kdo) (O’Neill et al., 2004). Despite its complex structure, RG-II is evolutionarily conserved in the plant kingdom and is present in the primary cell wall predominantly in the form of a dimer that is cross-linked by a borate di-ester bond between two apiosyl residues (Kobayashi et al., 1996; O’Neill et al., 1996). The demonstration that RG-II exists in primary walls as a dimer was a major advance in the understanding of the structure-function relationship of this cell wall polysaccharide. Dimer formation results in the covalent cross-linking of two HG chains involved in the in planta formation of a pectic network. This network contributes to the mechanical properties of the primary wall and is required for normal plant growth and development. Boron deficiency or defects in RG-II biosynthesis result in altered plant growth and changes in cell wall architecture, demonstrating the crucial function of boron-mediated RG-II cross-linking in generating the covalently cross-linked pectic network (Fleischer et al., 1998, 1999; O’Neill et al., 2001; Voxeur et al., 2011; Dumont et al., 2014). Nevertheless, the precise function of RG-II remains unclear to date. Moreover, despite its importance in primary cell wall synthesis and elongation, little is known about RG-II biosynthesis and in planta localisation and turnover. Carbohydrate-binding proteins, such as lectins and antibodies, have been demonstrated to be highly valuable tools for the investigation of the complexity and diversity of RG-I, HG and hemicelluloses at the cell and tissue levels (Lee et al., 2011). With the exception of rabbit polyclonal and Fab antibodies reported by Matoh et al. (1998) and Williams et al. (1996), respectively, most of the efforts to obtain specific antibodies against RG-II for immunocytochemistry studies have been unsuccessful. As a consequence, appropriate tools for studying the synthesis and distribution of RG-II within the plant cell are lacking.

As well as being a constituent of RG-II in plants, Kdo is also present in the inner core of the lipopolysaccharide (LPS) of Gram-negative bacteria. As in bacteria, in plants this sugar is synthesised in the cytosol by the isomerisation of D-ribulose-5-P (D-Rul-5-P) into D-arabinose-5-P (D-Ara-5-P), followed by its coupling to phosphoenol pyruvate (PEP) to yield Kdo-8-phosphate (Kdo-8-P) (Matsuura et al., 2003; Delmas et al., 2008) (Figure 1b). After dephosphorylation of Kdo-8-P and coupling to CMP by a CMP-Kdo synthetase (Royo et al., 2000), the activated CMP-Kdo sugar nucleotide is transported into the Golgi apparatus and transferred to the RG-II backbone, probably by sialyl-like transferases (Deng et al., 2010; Dumont et al., 2014). 8-Azido 8-deoxy Kdo (Kdo-N3; Figure 1c) was recently used to detect living bacteria through the labelling of LPS (Dumont et al., 2012). This study demonstrated that Kdo-N3 was efficiently utilised by the bacterial glycosylation
machinery. Moreover, modification of the C8 position of Kdo with an azido group prevents reverse metabolism by Kdo-8-P phosphatase and incorporation of the azido moiety into other cell metabolites, which might result in a loss of labelling specificity.

In this paper we report the metabolic labelling of plant cell walls using Kdo-N3 (Figure 1c). We show that the azido Kdo analogue can be metabolically integrated into the pectin-rich cell walls of 4-day-old light-grown Arabidopsis seedlings and Nicotiana tabacum L. cv Bright Yellow-2 (tobacco BY-2) cells and can then be efficiently coupled to an alkyne-containing fluorescent probe via a copper-catalysed azide–alkyne cycloaddition, allowing for specific metabolic labelling of RG-II.

RESULTS

Metabolic click-mediated labelling of plant tissues was investigated with the 8-azido Kdo analogue because this monosaccharide is specific for RG-II and the Kdo C-8 hydroxy group is not involved in linkage to the rhamnose residue in the Kdo-containing side chain of RG-II (Figure 1a). Four-day-old light-grown Arabidopsis seedlings were incubated for 16 h in liquid Murashige and Skoog (MS) medium containing from 0 to 50 μM Kdo-N3. Seedlings were then transferred for 1 h to a solution of Alexa Fluor® 488-alkyne, a membrane-impermeable probe, in copper-catalysing conditions before observation of fluorescence in cell walls by confocal microscopy. As shown in Figure 2(a–g), seedlings treated with Kdo-N3 exhibited a strong labelling of their root cell walls. The highest labelling intensity was obtained with 50 μM Kdo-N3 (Figure 2d–g). X-Z projections of z-series showed that the labelling is restricted to the root surface (Figure 2f), indicating that the fluorescent probe did not diffuse within the root tissues. The labelling occurs throughout all the primary root epidermal cell walls in the division and elongation zones of seedling roots, as well as in root hairs (Figure 2b) and the developing root hair bulges (Figure 2e). Labelling experiments were also carried out on suspension cultured tobacco BY-2 cells. As illustrated in

Figure 1. Schematic representation of RG-II and biosynthetic pathway of CMP-Kdo. (a) Structure of rhamnogalacturonan II (RG-II) with the location of 3-deoxy-D-manno-oct-2ulosonic acid (Kdo) indicated in a dashed box. (b) Biosynthetic pathway of CMP-Kdo. (c) Structure of the 8-azido 8-deoxy Kdo (Kdo-N3).
Figure 2. Confocal microscope images of roots of 4-day-old light-grown Arabidopsis thaliana seedlings and tobacco BY-2 cells. (a)–(e) Seedlings were incubated for 16 h in MS medium containing from 0 to 50 μM 8-azido 8-deoxy Kdo (Kdo-N3; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid) and treated with Alexa Fluor® 488 alkyne in copper-catalysed conditions. In the upper part of the elongation zone (e), the emerging sites of root hairs are strongly labelled. Scale bars = 100 μm (a, b, c) and 50 μm (d, e). (f) X-Z projections of z-series of epidermal cells showing that the labelling is restricted to the root surface. Scale bars = 50 μm. (g) Overall view of a Kdo-N3-labelled root. Scale bar = 100 μm. (h), (i) Tobacco BY-2 cells were collected 3 days after subculture and incubated in liquid MS medium containing 0 μM (h) or 100 μM Kdo-N3 (i) for 16 h and then labelled with 0.1 μM Alexa 488-alkyne for 1 h in copper-catalysed conditions. Scale bars = 20 μm. Inserts correspond to the transmitted light images.

Figure 2(h,i), strong labelling of the cell walls of tobacco cells was observed.

Control experiments were performed to demonstrate the specificity of the metabolic click-mediated labelling of the cell wall. No labelling of cell walls was observed in seedlings treated with Kdo-N3 or the fluorescent probe alone (Figure S1 in Supporting Information). To investigate whether cell viability is required for incorporation of Kdo-N3, seedlings were treated with the Kdo analogue before or after fixation with 4% paraformaldehyde and then...
labelled with Alexa Fluor® 488-alkyne. Seedlings treated with Kdo-N3, then fixed before coupling to the fluorophore tag, exhibited strong cell wall labelling. In contrast, no fluorescence of the root tissues was observed in seedlings fixed prior to labelling, thus demonstrating that the incorporation of Kdo-N3 requires living tissue (Figure 3a,b). Click-mediated labelling of Arabidopsis seedlings was then performed in the presence of 2β-deoxy Kdo, an inhibitor of cytosolic CMP-Kdo synthetase (Figure 1b) (Smyth et al., 2013). In these conditions, a strong decrease in cell wall labelling was observed compared with the control (Figure 4a). This observation supports the hypothesis that incorporation of Kdo-N3 in the cell wall occurs through the endogenous Kdo biosynthetic machinery. Together, these data indicate that the fluorescence of click-labelled cell walls in roots of Arabidopsis seedlings results from the incorporation of Kdo-N3 into RG-II, the sole Kdo-containing cell wall polysaccharide. This probably occurs through the endogenous metabolism of living plant cells and not from non-specific adsorption of Kdo-N3 to the cell wall.

D-Ara-5-P is the metabolic precursor of Kdo in plants (Figure 1b). In the presence of D-Ara, metabolic click-labeling of Arabidopsis seedlings exposed to Kdo-N3 was strongly inhibited, whereas no inhibition was observed with L-Ara, the isomer found in several cell wall polysaccharides (Figure 4b). Inhibition of labelling was also observed in seedlings co-incubated with Kdo (Figure 4b). This competitive inhibition observed with D-Ara and Kdo further confirmed that incorporation of Kdo-N3 occurred through the Kdo biosynthesis pathway. Root elongation was not affected in these experiments (Figure 4c), indicating that inhibition of Kdo-N3 incorporation was not due to the toxicity of D-Ara or Kdo.

To investigate the co-localisation of the observed Kdo-N3 labelling with cellulose in primary cell walls, seedlings were treated with Kdo-N3 labelled with Alexa Fluor® 488-alkyne and stained with the glucan-binding calcofluor white, a fluorescent dye that labels cellulose (Figure 5). Dual-colour confocal imaging shows that newly synthesised RG-II and cellulose co-localise throughout the cell walls of the root at the limit of resolution of this technique. Interestingly, the pattern of the fluorescence of Kdo-N3 and calcofluor is different in radial and longitudinal walls, with a more intense fluorescence of Kdo-N3 in longitudinal walls (Figure 5a) and a more intense signal of calcofluor in radial walls (Figure 5b). This may indicate that pectins are synthesised in a greater quantity in the longitudinal growing walls compared with the radial, non-growing walls.

Double labelling was tested using Fuc-Al and Kdo-N3 to take advantage of the fact that these two sugar analogues carry different bio-orthogonal chemical reporters. Fuc-Al has been used for imaging of the cell wall RG-I in A. thaliana (Anderson et al., 2012). Four-day-old light-grown Arabidopsis seedlings were incubated for 16 h in MS medium containing 50 μM of Kdo-N3 and 50 μM of peracetylated Fuc-Al. Coupling to Alexa Fluor® 488-alkyne (Figure 6a) and Alexa Fluor® 594-azide (Figure 6b) fluorescent probes enabled double labelling of the cell wall (Figure 6c). Co-localisation of the labelling (Figure 6e) using the two monosaccharide analogues shows that newly synthesised RG-I and RG-II are deposited with similar distributions in root cell walls.

Pulse labelling experiments (Figure 7) were performed by incubating Arabidopsis seedlings with Kdo-N3, washing out free analogue, and then incubating in analogue-free medium for 22 h to investigate RG-II synthesis and redistribution in muro. After 4 h of incubation with Kdo-N3, the pattern of incorporation was homogeneous in the walls of cells in the elongation zone (Figure 7a). After 22 h, only the apical/basal borders of the elongated cells showed a strong labelling in the elongation zone (Figure 7c). In contrast, the labelling of the longitudinal/lateral cell walls was weak and diffuse, revealing a dilution of Kdo-N3-containing RG-II during the elongation of the cells. Cells in the differentiation zone are not expected to greatly expand or add large amounts of new RG-II to their walls. This is reflected by a lack of labelling in the differentiation zone after the 4 h incorporation (Figure 7b). In contrast, the cells in the

Figure 3. Control experiments for 8-azido 8-deoxy Kdo (Kdo-N3; Kdo, 3-deoxy-o-manno-oct-2-ulosonic acid) incorporation.

Chemical fixation of seedlings using 4% paraformaldehyde after (a) or before (b) Kdo-N3 incorporation and labelling. Seedling viability is required for Kdo-N3 incorporation but not for the click chemistry reaction. Inserts correspond to the transmitted light images. Scale bars = 100 μm.

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played homogeneous labelling (Figure 7d). These results suggest that when root cells become differentiated, RG-II synthesis is reduced.

DISCUSSION

Several lines of evidence indicate that incubation of Arabidopsis seedlings or tobacco BY-2 cells with Kdo-N$_3$, followed by coupling to an alkyne-containing fluorescent probe in the presence of Cu(I), results in the specific in muro labelling of RG-II. Firstly, Kdo is only found in RG-II. Secondly, click-mediated labelling of cell walls is abolished in presence of 2β-deoxy Kdo, an inhibitor of the cytosolic CMP-Kdo synthetase that is able to activate free Kdo into its nucleotide sugar in the cytosol (Misaki et al., 2009; Smyth et al., 2013). Thirdly, incorporation of Kdo-N$_3$ only occurs in living tissues and can be inhibited by addition of Kdo and D-Ara. D-Ara is only involved in plant cell wall metabolism as a precursor of Kdo in the RG-II biosynthetic pathway (Figure 1b). In contrast, its L-isomer is widely distributed in arabinan, arabinoxylan and proteoglycan cell wall polymers (Carpita and Gibeaut, 1993). The inhibition of labelling with Kdo-N$_3$ upon feeding seedlings with D-Ara suggests that D-Ara is likely to enter cellular metabolism via the salvage pathway where it can compete with D-Ara-5-P for cytosolic Kdo synthesis (Figure 1b). Plant Kdo-8-P synthetases have been reported to be specific for D-Ara-5-P, which arises from the cytosolic isomerisation of D-ribulose-5-P (Figure 1b). Activity of these synthetases was studied by complementation assays of bacterial strains impaired in endogenous Kdo-8-P synthetase activity (Brabetz et al., 2000; Delmas et al., 2003; Matsuura et al., 2003) or using D-Ara-5-P as a substrate in enzyme biossays (Delmas et al., 2003). Inhibition of Kdo-N$_3$ incorporation with D-Ara suggests that phosphorylation of D-Ara is not required for its coupling to PEP by cytosolic Kdo-8-P synthetases (Figure 1b).

Click-mediated metabolic labelling using Kdo-N$_3$ resulted in efficient imaging of RG-II in the Arabidopsis root primary cell wall, thus providing an alternative to the use of antibodies for studying RG-II synthesis and distribution. Labelling is observed in cells located in the division and elongation zones where pectin synthesis occurs. Immunolocalisation studies using antibodies raised against RG-II have shown that labelling was stronger in the vicinity of the plasma membrane. However, cell walls treated with alkali to promote de-esterification, showed homogeneous labelling of the entire cell wall. This suggests that the apparent anisotropic distribution of RG-II in primary cell walls resulted from an increasing gradient in RG-II esterification from the inner to the outer faces of the cell wall since the antibodies used were raised against de-esterified RG-II (Williams et al., 1996; Matoh et al., 1998). In our study, co-localisation of labelled Kdo-N$_3$ with the cellulose-binding dye calcofluor white confirmed that RG-II exists throughout Arabidopsis primary cell walls.

**Figure 4.** Inhibition of 8-azido 8-deoxy Kdo (Kdo-N$_3$; Kdo, 3-deoxy-o-manno-oct-2-ulosonic acid) incorporation.

(a) Quantification of root-tip fluorescence in 4-day-old seedlings grown on MS medium supplemented with or without 150 μM 2β-deoxy-Kdo. Seedlings were then transferred in liquid MS medium containing 50 μM Kdo-N$_3$ with or without 150 μM 2β-deoxy-Kdo. Data are expressed as SEM (n > 5 seedlings from two experiments). Similar letters indicate that no significant difference was observed whereas different letters indicate a highly significant difference between the conditions (Mann-Whitney test, P < 0.01).

(b) Quantification of the root-tip fluorescence in seedlings treated for 16 h with 50 μM Kdo-N$_3$ with or without 25 mM Kdo, L-arabinose (L-Ara) or D-Ara. Error bars represent the SEM (n = 10–12 seedlings for each experiment). Data are the mean of at least two experiments. Similar letters indicate that no significant difference was observed whereas different letters indicate a highly significant difference between the conditions (one-way ANOVA test, P < 0.05).

(c) Root length of 4-day-old seedlings grown in liquid MS alone or with 25 mM Kdo, D-Ara or L-Ara for 16 h. Error bars represent the SEM (n = 12 seedlings). No significant difference was observed for the root elongation (Mann-Whitney test, P > 0.05).
tion of tobacco BY-2 cells with Kdo-N₃ also resulted in an efficient labelling of the primary cell wall. It should be mentioned that labelling assays of BY-2 cells using Fuc-Al were unsuccessful (Figure S2), and as a consequence Kdo-N₃ is the unique probe for cell wall click labelling experiments in this widely used plant cell model.

Imaging of RG-I in the cell walls of roots of Arabidopsis seedlings was previously reported using the alkyne derivative of L-fucose Fuc-Al as a probe (Anderson et al., 2012). In our study, double labelling was tested using Fuc-Al and Kdo-N₃, taking advantage of the fact that these two sugar analogues carry different bio-orthogonal chemical reporters. Co-localisation of the labelling using the two monosaccharide analogues suggests that newly synthesised RG-I and RG-II molecules are deposited with a similar spatial distribution in root cell walls. Pulse labelling experiments were used to track the fate of RG-II in the cell wall during elongation. After 22 h, our results indicated that expansion of lateral cell walls during elongation led to the dilution of RG-II signal due to wall expansion and the delivery of newly synthesised, unlabelled cell wall polymers.

Although the click labelling strategy appears to be a good alternative to the use of antibodies for studying cell wall polysaccharides, this strategy has some limitations. Several monosaccharides are present in multiple polymer types, and click-mediated labelling using analogues of these monosaccharides carrying an azide or alkyne bio-orthogonal chemical reporter may not be restricted to a single cell wall polysaccharide. In this respect, Kdo-N₃ is appropriate for pectin studies since Kdo is only found in RG-II. Furthermore, Kdo-N₃ is efficiently incorporated into both root tissues and suspension-cultured cells. However, cell wall imaging is restricted to the root epidermis of tissues that are able to efficiently incorporate exogenous sugar analogues (Figure 2f) and/or because of a lack of diffusion of the fluorophore in the internal tissues. In addition, the high cytotoxicity of copper ions required for the azide-alkyne cycloaddition limits its use in living cells – thus pulse labelling experiments have to be performed in order to investigate the dynamics of labelling during root growth (Anderson et al., 2012). To circumvent this limitation, copper-free click reactions have recently been developed using a ring-strained cyclooctyne moiety. This approach allows labelling to be performed in living organisms with minimal physiological perturbation (Chang et al., 2009; Laughlin and Bertozzi, 2009; Boyce and Bertozzi, 2011; Dehnert et al., 2011). However, considering the large size of cyclooctyne motifs, this biocompatible click chemistry strategy is likely to require the metabolic incorporation of azido sugars. With regard to plant cell wall imaging using fucose analogues, it was reported that plant cells were able to incorporate the alkyne derivative of L-fucose but not the azido analogue (Anderson et al., 2012). In this respect, the Kdo-N₃ analogue is thus far the only azido sugar reported to be assimilated by plant cells. Given that the azido sugar is suitable for in vivo labelling using cyclooctyne-containing fluorescent probes, this study opens new avenues for following the real-time trafficking of pectins within the cell and their delivery to the apoplast.

**EXPERIMENTAL PROCEDURES**

**Sugar analogues**

Kdo-N₃ was synthesised according to Dumont et al. (2012). 2β-Deoxy Kdo was synthesised according to a published procedure.
seeds were surface sterilised in 50% (v/v) ethanol for 5 min, in

Four-day-old Arabidopsis seedlings were incubated in liquid MS medium supplemented with 50 μM Fuc-Al and 50 μM Kdo-N₃ for 16 h at 22°C. Seedlings were then washed three times with liquid MS and transferred in 1 ml of liquid MS containing 0.1 μM Alexa Fluor® 488-alkyne (Thermofisher, http://www.fisher.co.uk/), 1 mM CuSO₄ and 1 mM ascorbic acid. The solution was incubated for 1 h at room temperature (22°C) in the dark. Seedlings were then washed once in liquid MS supplemented with 0.01% (v/v) Tween 20 for 5 min, three more times in liquid MS and finally with distilled water. Control experiments were carried out by omitting either the Kdo-N₃ or the fluorescent probe in the click labelling assays. Inhibition of CMP-Kdo synthetase was carried out by adding 150 μM 2-Deoxy Kdo to the solid MS medium, then complementing the liquid MS with the same concentration of inhibitor as during the Kdo-N₃ incorporation step (Smyth et al., 2013). Inhibition assays were carried out by adding 25 mM D-Ara, L-Ara or Kdo to the liquid medium. Root lengths of 4-day-old seedlings were measured using ImageJ software (Abramoff et al., 2004).

For labelling of tobacco BY-2 cells, Kdo-N₃ was diluted to 200 μM in 0.5 ml of liquid MS medium. The 0.5 ml of a 3-day-old suspension of BY-2 cells collected 3 days after subculture in logarithmic growth was added. Cells were then incubated for 16 h. Cells were rinsed three times with liquid MS medium by centrifugation (2 min, 700 g). Click reactions were performed by incubating BY-2 cells in 500 μl of labelling solution containing 0.1 μM Alexa Fluor® 488-alkyne, 1 mM CuSO₄ and 1 mM ascorbic acid in liquid MS for 1 h, in the dark with gentle shaking. Cells were then rinsed once with 500 μl liquid MS, then incubated for 5 min in liquid MS supplemented with Tween 20 (0.01%, v/v), rinsed three more times with liquid MS for 5 min each and finally with water.

Double staining using Kdo-N₃ and calcofluor

Four-day-old seedlings treated with Kdo-N₃ and labelled with Alexa 488-alkyne were then incubated with 1 mg L⁻¹ calcofluor white M2R (Sigma, http://www.sigmaaldrich.com/) for 30 min in the dark. After careful washing with distilled water, the roots were observed.

Double staining using Fuc-Al and Kdo-N₃

A 1 mM solution of peracetylated 6-alkyne L-fucose (Fuc-Al; Thermofisher, http://www.fisher.co.uk/) was prepared by dissolution in DMSO and then dilution to the desired concentration in MS medium. Four-day-old light-grown seedlings were transferred from MS medium plates to 1 ml of liquid MS containing 50 μM of Fuc-Al and 50 μM Kdo-N₃ for 16 h at 22°C. Seedlings were washed and then incubated in 1 ml of labelling solution (liquid MS containing 0.1 μM Alexa Fluor® 594-azide (Thermofisher, http://www.fisher.co.uk/), 1 mM CuSO₄, 1 mM ascorbic acid) for 1 h at room temperature.

Plant culture and growth conditions. Arabidopsis Col-0 seeds were surface sterilised in 50% (v/v) ethanol for 5 min, in
After one washing step with liquid MS, seedlings were then labelled with 1 ml of 0.1 \text{M} Alexa Fluor® 488-alkyne. Seedlings were washed as described for single labelling and imaged.

Staining of BY-2 cells using Fuc-Al

Tobacco BY-2 cells in the exponential growth phase were incubated, with shaking, for 24 h in medium containing 1 \text{mM} fucose-alkyne peracetate (‘FucAl’, Life Technologies). The cultures were then rinsed over a cell strainer with 10 ml liquid BY-2 media [4.3 g L\(^{-1}\) Murashige and Skoog salts (Caisson Laboratories MSP0501, http://www.caissonlabs.com/), 100 mg L\(^{-1}\) inositol, 1 mg L\(^{-1}\) thiamine, 0.2 mg L\(^{-1}\) 2,4-D, 255 mg L\(^{-1}\) KH\(_2\)PO\(_4\) and 30 g L\(^{-1}\) sucrose] lacking FucAl, then resuspended in click reaction mixture consisting of 1 mM CuSO\(_4\), 1 mM ascorbic acid and 1 \text{µM} Alexa488-alkyne (Thermofisher, http://www.fisher.co.uk/) for 1 h, with rocking. The reaction was halted by rinsing away the reaction mixture as before. Cells were imaged with a Zeiss Axio Observer spinning disc confocal microscope with a 488-nm excitation laser and a 20 \times 0.8 NA objective. Fluorescence images are presented as maximum \(z\)-projections, \(z\)-distance between slices: 1 \text{µm}.

Pulse labelling experiment

For pulse experiments, 4-day-old Arabidopsis seedlings were incubated in 200 \text{µM} Kdo-N\(_3\) in liquid MS for 4 h, washed three times with liquid MS and then transferred to solid MS lacking Kdo-N\(_3\). Seedlings were labelled as previously described after 0 and 22 h, washed and imaged.

Microscopy

The roots of labelled seedlings and the BY-2 cells were observed with the Leica TCS SP2 AOBS confocal microscope (488-nm excitation laser; 495-550 nm emission detection for Alexa 488 and 594 nm excitation laser; 610–670 nm emission detection for Alexa 594). For the double staining experiment, images were acquired in the sequential scan mode. Images were acquired with identical exposure settings. The fluorescence intensity of the root was calculated with ImageJ by thresholding (pixel intensity >16) to select the root boundary and measuring the mean of pixel intensity inside the area defined by the threshold. The ImageJ co-localisation Colormap plugin was used to determine the correlation index (\(I_{\text{cor}}\)), which is the fraction of positively correlated pixels between two fluorescent populations. The plug-in is based on the method originally described by Jaskolski et al. (2005).

For the double staining experiment, epidermal root cells were observed with the Leica TCS SP8 CFS confocal microscope (488-nm excitation laser, 500–550 nm emission detection for Alexa 488 and the 405 nm excitation laser, 420–470 nm emission detection for calciofluor). Images were acquired in sequential scanning mode. After acquisition, a deconvolution software, Huygens Professional (http://www.svi.nl) was used to deblur images.

Statistical analysis

The data were analysed statistically using GraphPad Software (http://www.graphpad.com/). Differences were considered statistically significant when \(P \leq 0.05\).

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Control experiments for Kdo-\(\text{N}_{2}\) labelling.

**Figure S2.** Tobacco BY-2 cells do not incorporate appreciable amounts of furose-alkyne into their cell walls.

**Methods S1.** Experimental procedure for the synthesis of 2\(\text{j}\)-deoxy Kdo.

**REFERENCES**


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