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1 **Dynamic imaging of cell wall polysaccharides by metabolic click-mediated labelling**
2 **of pectins in living elongating cells.**

3

4 Marc Ropitiaux¹, Quentin Hays¹, Aurélie Baron², Laura Fourmois², Isabelle Boulogne¹,
5 Boris Vauzeilles², Patrice Lerouge¹, Jean-Claude Mollet¹, Arnaud Lehner^{1,*}

6

7 ¹ Normandie Université, UNIROUEN, Laboratoire Glyco-MEV UR 4358, Structure
8 Fédérative de Recherche Normandie Végétal FED 4277, Innovation Chimie Carnot,
9 76000 Rouen, France

10 ² Université Paris-Saclay, CNRS, Institut de Chimie des Substances Naturelles, UPR
11 2301, 91198, Gif-sur-Yvette, France

12

13

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15

16 **Key words**

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19

20 ***Correspondence** : Arnaud Lehner

21 arnaud.lehner@univ-rouen.fr

22 Normandie Université, UNIROUEN, Laboratoire Glyco-MEV UR 4358, Structure
23 Fédérative de Recherche Normandie Végétal FED 4277, Innovation Chimie Carnot,
24 76000 Rouen, France.

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27 **Summary**

28 Investigation of protein tracking in living plant cells has become a routine experiment
29 with the emergence of reporter genes encoding fluorescent tags. Unfortunately, this
30 imaging strategy is not applicable to glycans because their synthesis is not directly
31 encoded by the genome. Indeed, complex glycans result from sequential additions and/or
32 removals of monosaccharides by the glycosyltransferases and glycosidases of the cell's
33 biosynthetic machinery. To date, the imaging of cell wall polymers mainly relies on the
34 use of antibodies or dyes that exhibit variable specificities. However, as
35 immunolocalisation typically requires sample fixation, it does not provide access to the
36 dynamics of living cells. The development of click chemistry in plant cell wall biology
37 offers an alternative for live-cell labelling. It consists of the incorporation of a
38 carbohydrate containing a bio-orthogonal chemical reporter into the target polysaccharide
39 using the endogenous biosynthetic machinery of the cell. Once synthesized and deposited
40 in the cell wall, the polysaccharide containing the analogue monosaccharide is covalently
41 coupled to an exogenous fluorescent probe. Here, we developed a metabolic-click
42 labelling approach which allows the imaging of cell wall polysaccharides in living and
43 elongating cells without affecting cell viability. The protocol was established using the
44 pollen tube, a useful model to follow cell wall dynamics due to its fast and tip-polarized
45 growth, but was also successfully tested on Arabidopsis root cells and root hairs. This
46 method offers the possibility of imaging metabolically incorporated sugars of viable and
47 elongating cells, allowing the study of the long-term dynamics of labelled extracellular
48 polysaccharides.

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55 **Introduction**

56 Expression of proteins of interest fused to fluorescent protein tags is routinely used in cell
57 biology to investigate protein localization and trafficking in living cells. However, this
58 efficient strategy cannot be applied to glycans and lipids. In contrast, the use of bio-
59 orthogonal chemical reporters now offers an alternative for *in vivo* labelling of such
60 biomolecules. In this method, a chemical reporter is incorporated into the target
61 biomolecule using the endogenous biosynthetic machinery of the cell. This reporter is
62 then covalently coupled in a highly selective and efficient reaction to an exogenously
63 delivered probe. The azide–alkyne cycloaddition is the most widely used click-mediated
64 labelling method in biology (Rostovtsev *et al.*, 2002). The azide and alkyne chemical
65 reporters can be either linked to the biomolecule or to the probe and the chemical coupling
66 is catalyzed by copper (I) ions in biological conditions.

67 The study of cell-surface glycans in vertebrates and bacteria has greatly benefited from
68 the emergence of click chemistry. These experiments have mostly been performed by
69 metabolic glycan labelling by feeding cells with monosaccharides containing a bio-
70 orthogonal chemical reporter (Prescher *et al.*, 2004). In contrast, click chemistry has
71 received limited attention in plant biology to date. An alkyne derivative of L-fucose has
72 been used for cell wall labelling in *Arabidopsis thaliana* (Anderson *et al.*, 2012). In this
73 pioneering study, the authors demonstrated that seedlings fed with the per-acetylated
74 alkyne derivative of L-fucose incorporated this analogue into the cell wall, most likely in
75 pectic rhamnogalacturonan-I (RG-I). Chemical ligation to a fluorescent azide-containing
76 probe enabled efficient labelling of primary cell walls of the roots of *Arabidopsis*
77 seedlings. This methodology was then successfully performed on *Arabidopsis* roots and
78 tobacco cell-suspension cultures using 8-azido-3-deoxy-D-manno-oct-2-ulosonic acid, an
79 azido derivative of Kdo (Kdo-N₃), a monosaccharide that is found in the cell wall in pectic
80 rhamnogalacturonan-II (Dumont *et al.*, 2014; Dumont *et al.*, 2016). More recently,
81 imaging of lignin in plants was performed using click-compatible derivatives of
82 monolignols that were incorporated in lignin in the apoplast (Bukowski *et al.*, 2014; Lion
83 *et al.*, 2017) and various clickable monosaccharide analogs have been developed for the
84 glycan labeling of plant cell walls (Hoogenboom *et al.*, 2016; Wang *et al.*, 2016; Zhu and
85 Chen, 2017).

86 Although the feeding of plant cells with appropriate azide- or alkyne-containing sugars
87 and their coupling to fluorophore tags allows for efficient imaging of the cell wall, the
88 concentration of copper (I) ions required for the click coupling is lethal for plant cells.
89 Thus, click chemistry labelling only results in a snapshot of cell wall polysaccharides
90 without any access to their *in vivo* dynamics. In order to bypass the copper toxicity,
91 alternative “copper-free” methods, such as the Strain-Promoted Azide-Alkyne
92 Cycloaddition (SPAAC) have been developed and were shown to induce minimal
93 physiological perturbations in living animal and bacterial cells (Prescher *et al.*, 2004)
94 (Figure 1). Indeed, the mild azide–alkyne cycloaddition involving a strained alkyne
95 functionality occurs in biological conditions without altering cell physiology. Recent
96 works by Hoogenboom *et al.* (2016) and Zhu and Chen (2017) confirmed the possibility
97 to label the cell wall with SPAAC after metabolic incorporation of different azido sugars.
98 However, these works did not investigate the dynamic monitoring of the cell wall
99 polymers after labelling with the fluorescent probes. As a consequence, we developed a
100 protocol for the metabolic labelling of cell wall polymers in the pollen tube, a living and
101 rapidly elongating cell. This method is based on the metabolic incorporation of azido
102 sugars and their subsequent observation after the SPAAC reaction with a fluorophore on
103 a living and elongating cell. This methodology allows the monitoring of the long-term
104 dynamics of labelled extracellular polysaccharides without affecting cell elongation.

105

106 **Results and discussion**

107 **Procedure for labelling cell wall pectic polysaccharides**

108 Development of the copper-free click-chemistry protocol for live imaging was performed
109 on pollen tubes. Pollen tubes are a suitable model to follow cell wall dynamics due to
110 being fast-growing, tip-polarized expanding cells. This single fast-growing cell elongates
111 from 500 to 1000 μm per hour for most angiosperm species (Williams, 2012) and can be
112 easily monitored under a confocal microscope. Moreover, pollen tubes do not display
113 autofluorescence (Dardelle *et al.*, 2010) and can be monitored continuously during
114 elongation for up to 4 to 6 hours after germination. The pollen tube cell wall is mainly
115 composed of pectins and other matrix components secreted *via* Golgi-mediated vesicles

116 at the apical end of the cell, allowing for tip growth (Mollet *et al.*, 2013; Dehors *et al.*,
117 2019). For the dynamic labelling of pectins, pollen tubes were incubated with Kdo-N₃
118 (Dumont *et al.*, 2012), a monosaccharide derivatives that is incorporated into pectins
119 through the endogenous biosynthetic machinery (Hoogenboom *et al.*, 2016; Dumont *et*
120 *al.*, 2016). In addition, labelling of polysaccharides (pectin and xyloglucan) and
121 glycoproteins containing fucose (Fuc) was monitored after incubation with 6-azido L-
122 fucose (Fuc-N₃). Then, coupling to the fluorescent probe was achieved by using strained
123 cyclooctyne probes (SPAAC) (Figure 1) that do not require toxic copper ions for
124 activation (Zhu and Chen, 2017). Indeed, the strained alkyne function reacts with an azide
125 group in biological conditions without any requirement of Cu⁺ ions for coupling. This
126 allows the pollen tube to remain viable after coupling to the fluorescent tag that occurs
127 spontaneously in the culture medium.

128 The procedure is detailed in Figure 2 and consists of incubating germinating pollen grains
129 and/or growing pollen tubes with modified sugars (Figure 2, step ❶). To do so, freshly
130 harvested pollen grains were incubated in liquid germination medium for 1 h. The
131 medium contained populations of germinated pollen grains with growing pollen tubes,
132 plus ungerminated and germinating pollen grains. Germinated and germinating pollen
133 grains were centrifuged and rinsed before being incubated in the dark in culture medium
134 supplemented with 50 μM peracetylated 6-azido L-fucose (Fuc-N₃) or 200 μM Kdo-N₃
135 (Figure 2, step ❷). The metabolic incorporation and the subsequent synthesis and
136 deposition of the clickable polymers in the cell wall occurred during a 2 h period while
137 the clickable sugar was available for incorporation into newly synthesized cell wall
138 material (Figure 2, step ❷). During this step ❷, the population of ungerminated pollen
139 grains started germinating, producing a growing tube incorporating the clickable sugar
140 from the first instant of growth. During the same time (Step ❷), already germinated pollen
141 tubes started to incorporate clickable sugars in newly synthesized cell wall. At the end of
142 the incorporation step, pollen tubes were washed three times with BK-PEG by gentle
143 centrifugation (2 min, 700 g) in order to keep them alive. Pollen tubes were then
144 transferred into a germination medium supplemented with 1 μM of the clickable
145 fluorescent reporter (Alexa Fluor 488 sDIBO). Incubation with the fluorescent reporter
146 lasted for 1 h (Figure 2, step ❸). Samples were washed once in BK-PEG supplemented
147 with 0.01% (v/v) Tween 20 for 5 min followed by three washes with BK-PEG by

148 centrifugation (700 g, 2 min). Control experiments were carried out by omitting either the
149 Kdo-N₃ or Fuc-N₃ during incorporation (Figure 2, step ②). Viable and growing labelled
150 pollen tubes were then observed under a confocal microscope. Pollen tube viability was
151 assessed based on the occurrence of active cytoplasmic streaming.

152

153 **Dynamics of pectins in living pollen tubes**

154 Interestingly, all confocal images revealed that cell wall labelling of pollen tubes showed
155 intensity fluctuations depending on the step at which the pollen tubes grew. As such,
156 pollen tubes that ceased growth during step 2 showed labelling of the cell wall at the tip
157 and the shank of the pollen tube (Figure 3A) whereas other pollen tubes that started to
158 germinate during step 2 and grew during step 3 revealed cell wall labelling in the pollen
159 tube emerging from the pollen grain and no labelling at the tip (Figure 3B). Lastly, a stop-
160 and-go labelling pattern of the pollen tube cell wall was observed. Pollen tubes that grew
161 during all three steps showed fluorescent labelling in a restricted area, which corresponds
162 to the pollen tube growth during step 2 (Figure 3C). Remarkably, increasing and
163 decreasing labelling intensities of the cell wall were observed at the beginning of steps 2
164 and 3, respectively whereas no labelling was observed in pollen tubes growing during
165 step 1 and after the washes in step 3 (Figure 3C). Negative controls without the addition
166 of a modified sugar did not show any fluorescence (Figure 3D). Finally, our data revealed
167 similar fluorescent labelling patterns in pollen tube walls that grew during the three steps
168 after incubation with Fuc-N₃ (Figure 3E).

169

170 **Clickable sugars do not affect pollen tube growth**

171 The effect of metabolic incorporation of azido sugars was assessed on pollen tube growth
172 by measuring the total length of viable pollen tubes at the end of the metabolic
173 incorporation, after SPAAC labelling and after the final washes before confocal
174 observations. Results are presented Figure 4A. In the control condition, the length of
175 pollen tubes after the 5.5 h of the experimental procedure (Figure 2, step 1 to 3 with the
176 30 min of washing steps) ranged from 88.4 μm to 374.8 μm with a mean value of 229.6
177 μm (Figure 4A). Mean lengths of pollen tubes incubated in Kdo-N₃ or Fuc-N₃ were

178 respectively 220.4 and 219.1 μm and were not significantly different from the control
179 condition (Figure 4A). The distribution of the length of pollen tubes ranged from 69.1 to
180 396.9 μm with Kdo- N_3 and from 113.1 to 316.3 μm with Fuc- N_3 (Figure 4A).

181 As no fluorescence was observed in the control condition (Figure 3D), the growth rate of
182 the pollen tube was not measurable in the control at each step of the protocol. However,
183 when pollen tubes were incubated with Kdo- N_3 or Fuc- N_3 , the growth rate corresponding
184 to the three steps of the protocol was assessed and is presented as growth speed ($\mu\text{m h}^{-1}$)
185 in Figure 4B. During the first hour of growth (Figure 4B, ❶), pollen grains were not
186 incubated with azido sugars. At this time, most pollen grains started to germinate (80%)
187 whereas a fraction of pollen grains did not germinate (20%) resulting in a distribution of
188 growth speed ranging from 0 to 115.9 $\mu\text{m h}^{-1}$ and 0 to 86.4 $\mu\text{m h}^{-1}$ for pollen tubes that
189 would further incorporate Kdo- N_3 or Fuc- N_3 , respectively (Figure 4B, ❶). During Step
190 2, the growth speed was similar between pollen tubes incorporating Kdo- N_3 or Fuc- N_3
191 with a respective mean growth rate of 43.5 $\mu\text{m h}^{-1}$ and 50.5 $\mu\text{m h}^{-1}$ (Figure 4B, ❷). The
192 mean growth rate during the last step of the protocol was not significantly different
193 between pollen tubes that had incorporated Kdo- N_3 or Fuc- N_3 , being 54 and 69 $\mu\text{m h}^{-1}$,
194 respectively (Figure 4B, ❸). No significant differences were observed during the three
195 steps across treatments, showing that neither azido sugars nor the SPAAC reaction with
196 cyclooctine Alexa 488 affected pollen tube growth.

197

198 **Metabolic labelling of RG-II in living roots**

199 As described above for pollen tubes, metabolic SPAAC labelling was carried out in living
200 root cells with Kdo- N_3 . Viability of the root cells was confirmed by observing the
201 cytoplasmic streaming in root hair cells. Under the control condition (Figure 5A,B), when
202 roots were not incubated with azido sugar but were submitted to the SPAAC with sDIBO
203 Alexa 488, no fluorescence was observable. In contrast, roots fed with Kdo- N_3 showed a
204 strong labelling of the cell walls in the early differentiation zone and particularly at the
205 apex of emerging root hairs as indicated by arrowheads (Figure 5C, D) as well as at the
206 apex of growing root hairs (Figure 5E, F). Simultaneous detection of cellulose using
207 Direct Red 23 and RG-II and using SPAAC labelling of Kdo- N_3 revealed the presence of
208 both polymers in the apex of emerging root hairs and in the periclinal cell wall of root

209 cells (Figure 5G-N). Direct Red 23 is the same molecule as Pontamine Fast Scarlet 4B,
210 S4B and label mostly cellulosic polymers (Anderson *et al.*, 2010). Interestingly, in this
211 region of the root, the differentiation zone, RG-II seemed to be abundant in the anticlinal
212 cell wall compared to cellulose (Figure 5H, L) as indicated with an asterisk.

213

214 **Conclusion and future trends**

215 To date, the imaging of cell wall polysaccharides relies on the use of dyes or antibodies
216 specific for various glycan epitopes. However, the immunolocalisation of these epitopes
217 within the cell walls generally requires sample fixation. As a consequence, although they
218 are efficient and informative about the localization of polysaccharides in the cell wall,
219 these imaging methodologies do not provide access to the dynamic imaging of these
220 polymers within living tissues. The recent development of click chemistry now offers an
221 alternative for the imaging of plant cell wall polymers. In this study, we have
222 demonstrated that the metabolic incorporation of azido sugars, Fuc-N₃ and Kdo-N₃, and
223 their subsequent localization by the Strain-Promoted Azide-Alkyne Cycloaddition
224 (SPAAC) permit visualization of the dynamics of cell wall polymers in living cells during
225 their elongation. In this strategy, the strained alkyne function linked to the fluorescent
226 probe is able to react in biological conditions with the azide group of modified
227 monosaccharides incorporated into cell wall polymers without any requirement of toxic
228 copper (I) ions for activation. Moreover, the method allowed for visualization of living
229 and growing cells under the confocal microscope. Here, we developed a protocol with
230 pollen tubes, one of the fastest tip-growing cells, as they can reach growth rates from 60
231 to 400 nm sec⁻¹, depending on the species (Cheung and Wu, 2008). This fast growth
232 implies an efficient coordination for synthesizing, delivering and modifying membrane
233 and cell wall material. This unique fast growing cell has already proven to be an effective
234 biological model to assess the developmental consequences of modification of the cell
235 wall *in vitro* with the use of mutant lines or enzymatic treatment (Hocq *et al.*, 2017, Hocq
236 *et al.*, 2020). Future work will be carried out to develop this technique of metabolic click-
237 mediated labelling on root cells and particularly on the elongation zone and on elongating
238 root hairs. SPAAC was previously used on *Arabidopsis thaliana* roots but without the
239 dynamic tracking of the labelling during growth (Zhu and Chen, 2017; Hoogenboom *et*

240 *al.*, 2016). This can be explained by the complexity of mounting living roots under the
241 confocal microscope without damaging the tissue. In order to test whether living and
242 growing roots can be labelled, we performed SPAAC labeling on root tissues. Our
243 labelling showed that RG-II was found in large amounts in the apex of root hairs (Figure
244 5) as previously shown (Dumont *et al.*, 2016). It also showed that RG-II was found at
245 high levels in anticlinal walls (Figure 5) in the differentiation zone. Previous work by
246 Anderson *et al.* (2012) using metabolic click labelling of RG-I with Fuc-alkyne showed
247 the same labelling pattern in this region of the root. This confirms that the use of metabolic
248 labelling and the possibility to label specific pectic domains can provide new insights into
249 the functions of pectin and particularly of RG-II in root growth. To do so the use of
250 microfluidic devices (Meier *et al.*, 2010 ; Grossmann *et al.*, 2011) together with sample
251 setups for vertical confocal imaging (Von Wangenheim *et al.*, 2017) will permit
252 researchers to follow elongating root cells along their natural gravity vector for long
253 periods. To date, the classical method of *in vitro* culture and sample mounting on glass
254 slides does not allow to take into account the effects of gravity on root elongation. The
255 development of metabolic SPAAC-labelling of plant cell wall polymers coupled with
256 super resolution microscopy such as Stimulated Emission Depletion (STED) will permit
257 investigation of the long-term dynamics of polysaccharides during their deposition,
258 remodeling and putative recycling by endocytosis in pollen tubes and Arabidopsis roots,
259 taking advantage of the availability of genetic mutants of Arabidopsis with defects in wall
260 polymer biosynthesis and exocytosis, cell wall remodeling enzymes, and salvage
261 pathways. Specifically, the question of how pectins are modified in the cell wall *in vivo*,
262 and whether endogenous enzymes that degrade these molecules are required for plant
263 growth and development, could be addressed. Finally, this metabolic click labelling offers
264 the advantage to control the time of incubation with the clickable substrate. As cells are
265 staying alive during the entire process, this technic can be used for experiments in which
266 plants are grown in control conditions then treated (pharmacological, enzymatic, nutrient
267 deficiency, heat or cold stress, ...) and grown again in control condition. If a clickable
268 substrate is added during the treatment and the labelling reaction performed just after,
269 thus the fluorescence will correspond to the cell wall synthesized or modified during the
270 period of the treatment. It will facilitate to correlate the influence of a specific treatment
271 on the cell wall biosynthesis and/or modifications.

272 **Experimental procedures**

273 Plant growth conditions

274 *Nicotiana tabacum* cv. Xanthi seeds were spread on the surface of sterilized soil. Plants
275 were grown with a photoperiod of a 16 h light/8 h dark cycle at 25 and 22 °C during the
276 light and dark period, respectively. Relative humidity was maintained at 60%, and plants
277 were watered every 2 days (Laggoun *et al.*, 2019).

278 *Arabidopsis thaliana* Col-0 seeds were surface sterilized in 70% (v/v) ethanol for 5 min
279 then in 30% (v/v) sodium hypochlorite for 2 min. Seeds were then washed 6 times in
280 sterile water. Sterilized seeds were deposited on Murashige and Skoog (MS 2.2 g/L)
281 mineral medium containing 1% sucrose and 0.8% plant agar. The plates were placed
282 vertically at 4°C for 48 h before being transferred in a growth chamber for 3 d at 24°C
283 under a 16 h light / 8 h dark cycle.

284

285 Pollen tube culture, incorporation of Kdo-N₃ or Fuc-N₃ and SPAAC labelling

286 Two fully open flowers were collected in the second row from the top of the primary and
287 secondary inflorescences. Anthers was cut and submerged in 1.7 mL of BK-PEG medium
288 composed of 1.5 mM H₃BO₃, 2 mM Ca(NO₃)₂ 4H₂O, 1.5 mM Mg(SO₄)₂ H₂O and 1 mM
289 KNO₃ (Brewbaker and Kwack, 1963) supplemented with 10% (w/v) polyethylene glycol
290 (PEG 6000) as an osmoticum (in 2 mL Eppendorf® plastic tubes). PEG was used instead
291 of sucrose to promote intake of the modified sugar. Tubes were shaken to release the
292 pollen grains from the anthers and anthers were removed with a pair of tweezers. Four
293 hundred µL of pollen suspension were then transferred in 1.5 mL Eppendorf® plastic
294 tubes and incubated for 1 h at 22°C in darkness. Pollen tubes were centrifuged (700 g, 2
295 min) and washed with BK-PEG three times before being incubated at 22°C in the dark
296 for 2 h in BK-PEG supplemented with 50 µM peracetylated 6-azido L-fucose (Fuc-N₃) or
297 200 µM Kdo-N₃. The synthesis of 6-azido L-fucose is described in the supporting
298 experimental procedure : Methods S1. Kdo-N₃ was synthesized as previously describes
299 in Dumont *et al.*, (2012). Pollen tubes were washed three times with BK-PEG by
300 centrifugation (700 g, 2 min) and transferred in BK-PEG containing 1 µM alkyne Alexa
301 Fluor 488 sDIBO (C20020, Invitrogen, Carlsbad, California, USA) for 1 h at RT before

302 being washed once in BK-PEG supplemented with 0.01% (v/v) Tween 20 for 5 min
303 followed by three washes with BK-PEG by centrifugation (700 g, 2 min). Control
304 experiments were carried out by omitting either the Kdo-N₃ or Fuc-N₃ during
305 incorporation. Pollen tubes were then covered with a cover slip and observed using a
306 Leica TCS SP5 scanning confocal microscope (Wetzlar, Germany) with a 490 nm
307 excitation laser and 500-550 nm emission detection. Microscope images were acquired
308 and measurements made using Leica microscope systems software (LAS AF Lite,
309 Wetzlar, Germany).

310

311 Incorporation of Kdo-N₃ and SPAAC labelling of Arabidopsis roots

312 Four-day-old light-grown seedlings were transferred from MS medium plates to 500 µL
313 of liquid MS ½ medium supplemented with 200 µM of Kdo-N₃ or with purified water
314 (negative control) for 48 h at 24°C under 16 h light/ 8 h night cycle. Seedlings were then
315 washed three times with liquid MS ½ medium and then transferred in 1 mL of 1 µM of
316 DIBO Alexa Fluor® 488 alkyne (Invitrogen, C20020) for 1 h at room temperature, in
317 dark and under gentle agitation. At the end of the incubation, four washes were performed
318 with liquid MS ½ containing 0.01% (v/v) Tween 20 for the first one and only with liquid
319 MS ½ for the three other washes. For the double labelling experiments, Direct Red 23
320 (Sigma Aldrich, 212490) was added at final concentration of 0.1 mg/mL during the first
321 wash step in liquid MS ½ containing 0.01% (v/v) Tween 20. After 30 min, three washes
322 were done using liquid MS ½. Roots were gently mounted between slides (Nunc Lab-Tek
323 II Chamber Slide, Thermo Fisher scientific, 154453) and glass coverslips (18X18,
324 Thermo Fisher scientific). Roots were mounted in liquid MS ½. Confocal microscope
325 observation was performed with a Leica TCS SP5 scanning confocal microscope
326 (Wetzlar, Germany) (490 nm and 561 nm excitation laser; 500-550 nm and 575-615 nm
327 emission laser respectively for DIBO Alexa Fluor 488 and Direct Red 23).

328

329 Statistical analyses

330 Significant differences were determined by one-way ANOVA followed by Bonferroni
331 multiple comparison test. Data are marked by different letters when significantly different
332 with respect to the control conditions (P-value < 0.05).

333

334 **Data availability**

335 All relevant data can be found within the manuscript and its supporting materials.

336

337

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346

347 **Author contributions**

348 MR and QH performed the experiments; AB, LF, MC, BV synthesized the 6-azido L-
349 fucose and the Kdo-N₃; MR, AL, JCM and PL wrote the manuscript; JCM and IB
350 acquired funding; JCM, PL and AL designed and supervised the experiments.

351

352 **Conflict-of-Interest**

353 The authors declare that they do not have competing interests

354

355 **Supporting Information**

356 **Supporting experimental procedures: Methods S1** : Synthesis of 6-Azido-6-deoxy-L-
357 galactose (Fuc-N₃)
358

359

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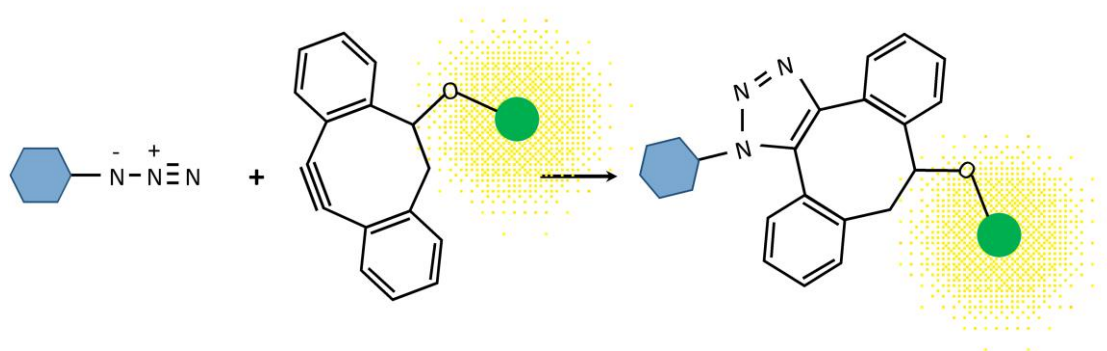
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442 **Figure**

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447 **Figure 1. Copper-free click-labelling (SPAAC).**

448 Covalent coupling of an azido-sugar and a strained dibenzocyclooctyne probe using the
449 Strain Promoted Azide-Alkyne Cycloaddition (SPAAC) reaction. The strained alkyne
450 function is able to react with azide groups in biological conditions without any
451 requirement of toxic catalysts for activation keeping cells alive during the labelling and
452 subsequent observation.

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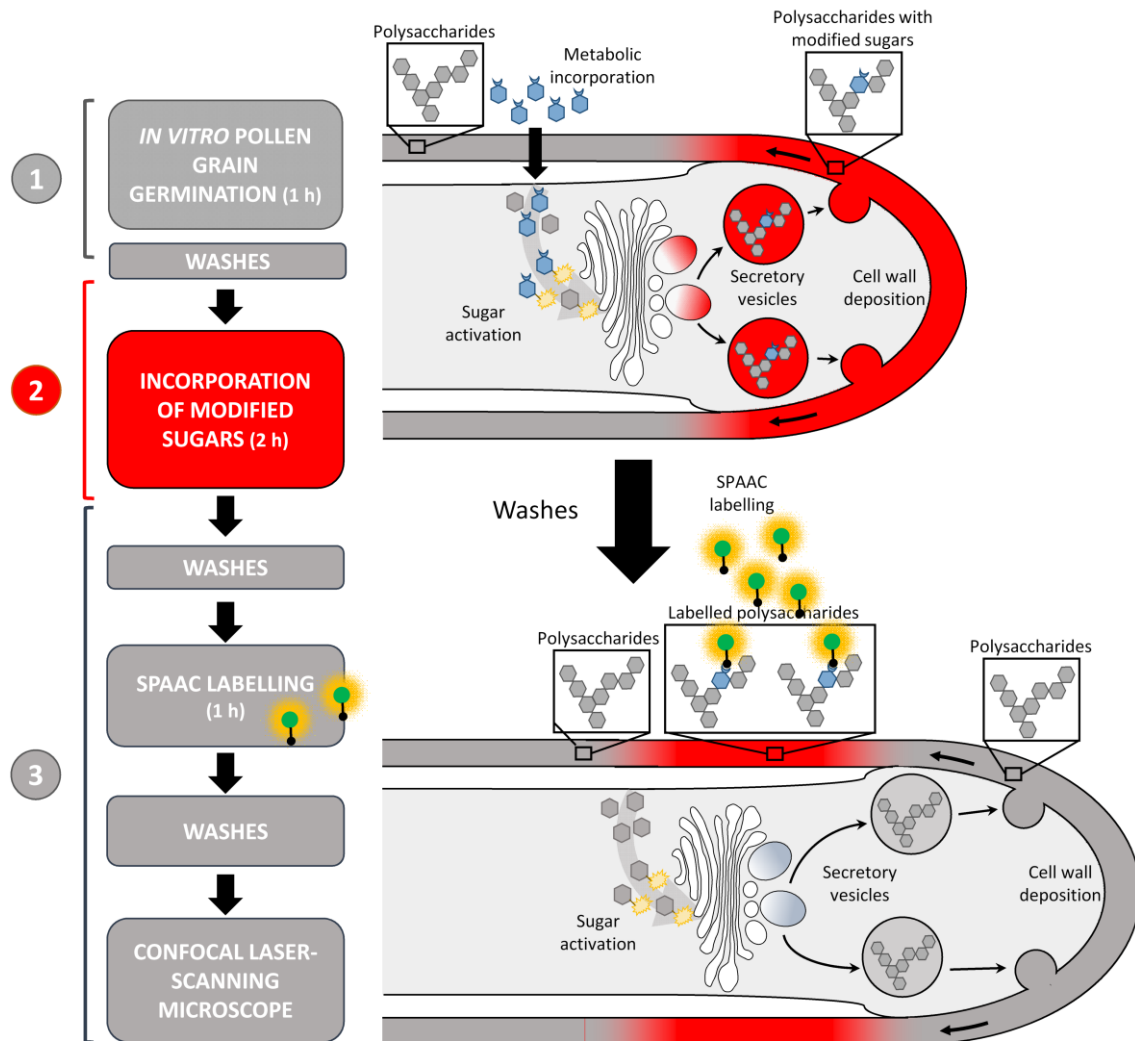
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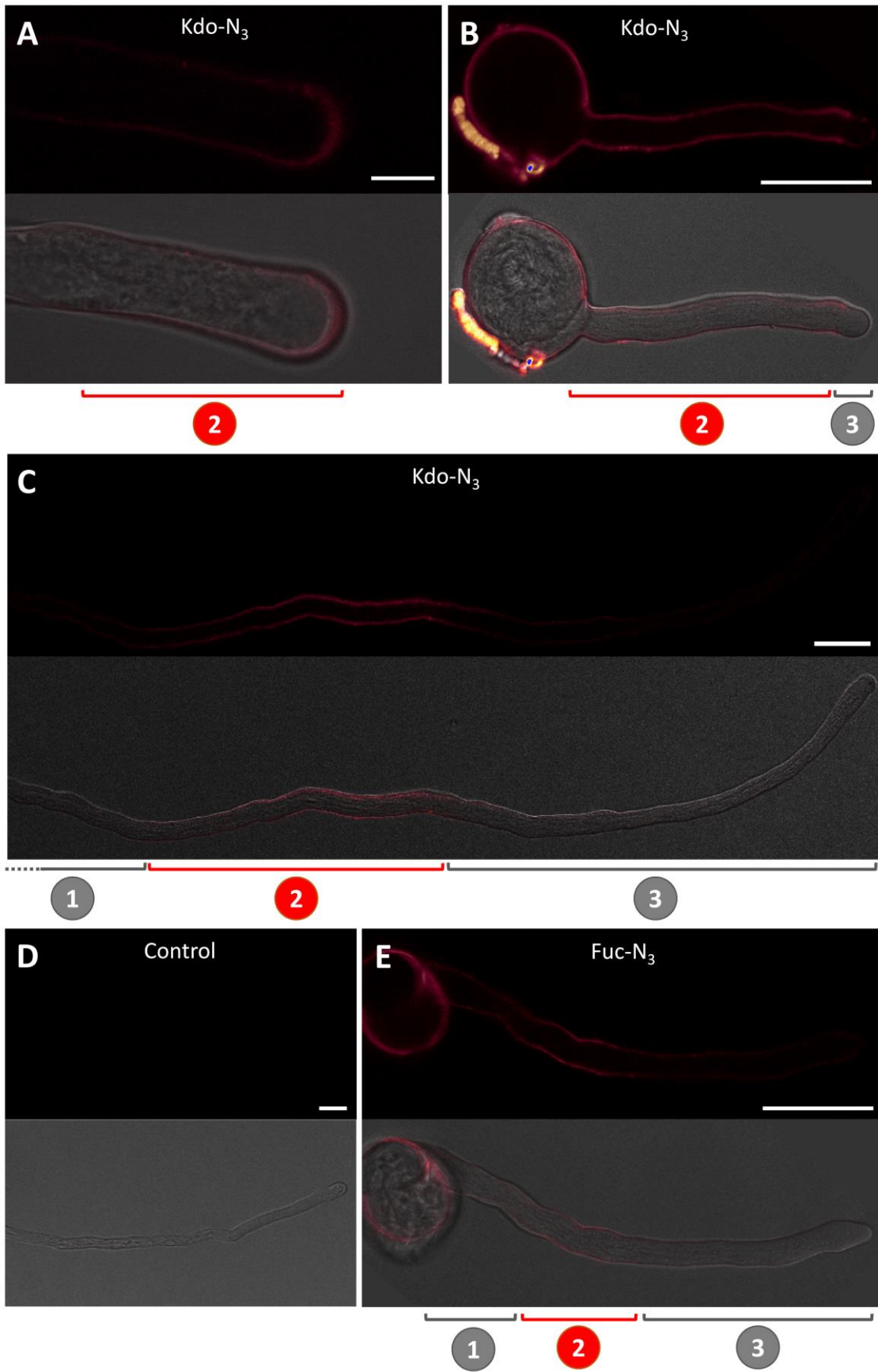
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465 **Figure 2. Protocol for the metabolic SPAAC labeling of living and growing pollen**
 466 **tubes.**

467 Schematic representation of the experimental procedure for pollen tube growth, metabolic
 468 incorporation of modified sugars and labelling as detailed in the material and method
 469 section. Key stages are numbered : **1** *in vitro* pollen grain germination for 1 h in
 470 germination medium at 22°C in darkness; **2** incorporation of Kdo-N₃ (200 μM) or Fuc-
 471 N₃ (50 μM) for 2 h at 22°C in darkness and **3** washes and click-labelling with 1 μM
 472 alkyne Alexa Fluor 488 sDIBO for 1 h at room temperature (RT) before confocal imaging.

473



475 **Figure 3. Dynamics of pectins during *Nicotiana tabacum* pollen tube growth.**

476 Note that Circled numbers correspond to the procedure described in Figure 2. ①, pollen
477 grain germination and pollen tube growth, ② metabolic incorporation of the modified
478 sugar, synthesis and deposition of clickable cell wall polysaccharides, ③ SPAAC
479 labelling and observation of viable pollen tubes. (A-E) Fluorescent and overlay confocal
480 images of pollen tubes that (A) ceased growth during incorporation (②) and (B) started
481 to germinate during incorporation (②) and grew during labelling (③). Note that the auto-
482 fluorescence of the pollen grain comes from the exine wall. (C) Fluorescent and overlay
483 confocal images of pollen tubes that expanded during the three steps. (D) Negative control
484 samples were submitted to steps ①, ② and ③ but without addition of Kdo-N₃ or Fuc-N₃
485 during the incorporation step (②). (E) Fluorescent and overlay confocal images of pollen
486 tubes that grew during the three steps after incorporation of Fuc-N₃ (②). Scale bars: (A)
487 7.5 μm and (B,C,D,E) 25 μm.

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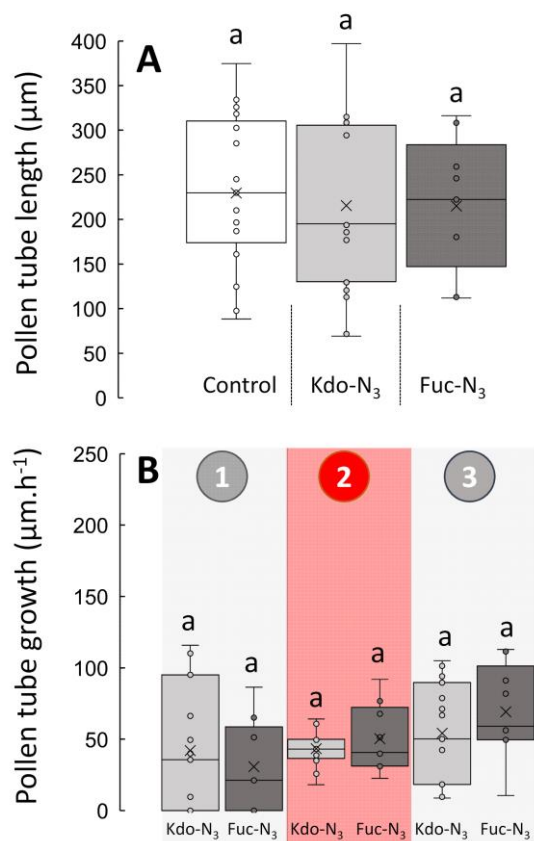
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495 **Figure 4. Effect of metabolic SPAAC labeling on pollen tube growth.**

496 A. Distribution of pollen tubes length after metabolic incorporation and SPAAC labeling

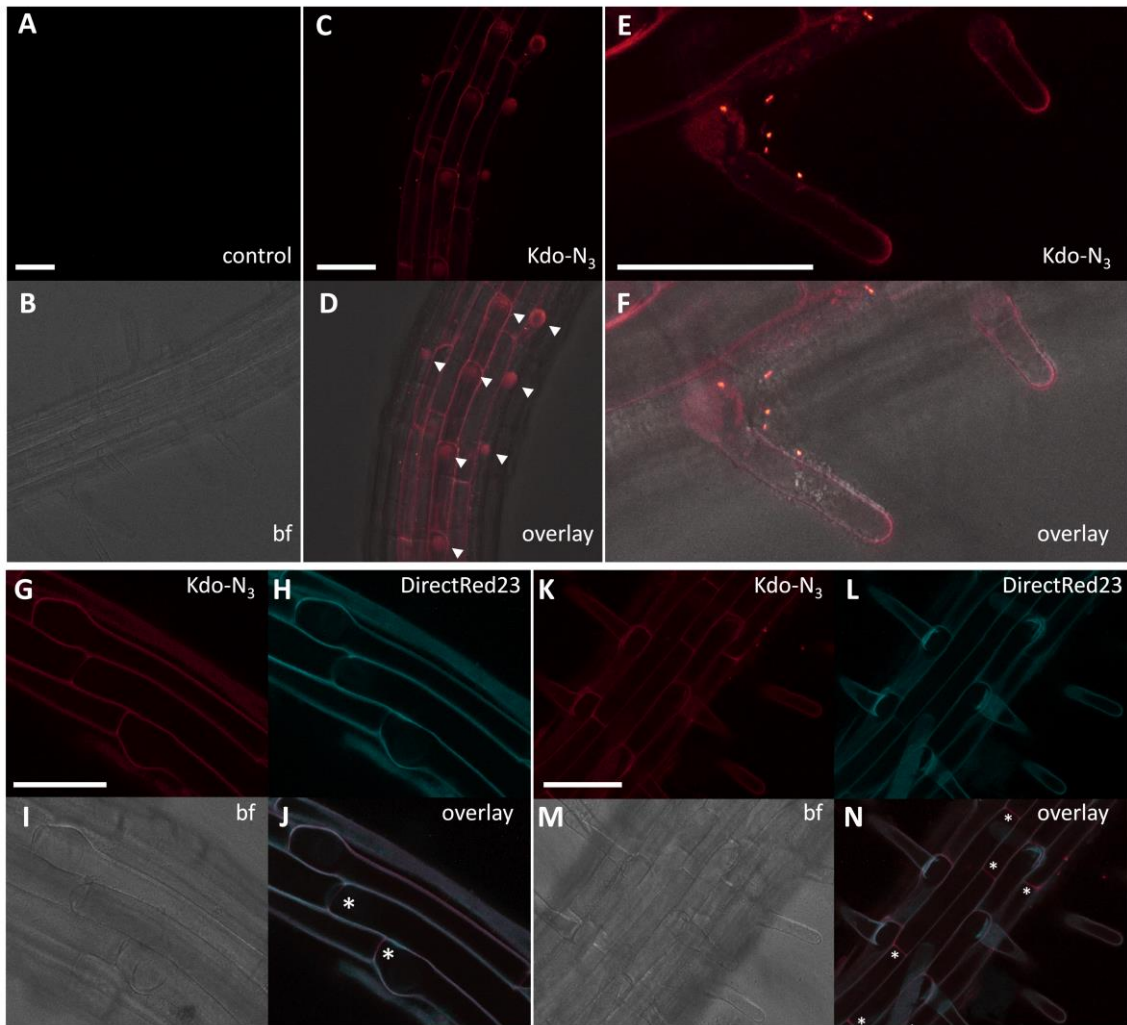
497 in control condition (n=17) or with Kdo-N₃ (200 µM, n=20) or Fuc-N₃ (50 µM, n=13).

498 B. Distribution of pollen tubes length before metabolic incorporation (❶), during

499 incorporation (❷) and during SPAAC labeling (❸) of Kdo-N₃ (n=15) or Fuc-N₃ (n=10).

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503 **Figure 5. Metabolic SPAAC labelling of living root of *Arabidopsis thaliana* with Kdo-**
 504 **N₃.**

505 (A, B) control condition without Kdo-N₃ during the incorporation step. (C, D) SPAAC
 506 labelling of RG-II in the differentiation zone of the root using Kdo-N₃. (E, F) SPAAC
 507 labelling of RG-II in root hairs using Kdo-N₃ (G-N) SPAAC labelling of RG-II with Kdo-
 508 N₃ and cellulose staining with Direct Red 23 in early differentiation zone (G-J) and in
 509 root hairs (K-N). Scale bars: 50 μm. arrowheads show the root hair apex; * marks the
 510 anticlinal cell wall.

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