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Summary

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

Introduction

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

The study of cell-surface glycans in vertebrates and bacteria has greatly benefited from the emergence of click chemistry. These experiments have mostly been performed by metabolic glycan labelling by feeding cells with monosaccharides containing a bio-orthogonal chemical reporter (Prescher *et al.,* 2004). In contrast, click chemistry has received limited attention in plant biology to date. An alkyne derivative of L-fucose has been used for cell wall labelling in *Arabidopsis thaliana* (Anderson *et al.,* 2012). In this pioneering study, the authors demonstrated that seedlings fed with the per-acetylated alkyne derivative of L-fucose incorporated this analogue into the cell wall, most likely in pectic rhamnogalacturonan-I (RG-I). Chemical ligation to a fluorescent azide-containing probe enabled efficient labelling of primary cell walls of the roots of Arabidopsis seedlings. This methodology was then successfully performed on Arabidopsis roots and tobacco cell-suspension cultures using 8-azido-3-deoxy-D-manno-oct-2-ulosonic acid, an azido derivative of Kdo (Kdo-N_3), a monosaccharide that is found in the cell wall in pectic rhamnogalacturonan-II (Dumont *et al.,* 2014; Dumont *et al.,* 2016). More recently, imaging of lignin in plants was performed using click-compatible derivatives of monolignols that were incorporated in lignin in the apoplast (Bukowski *et al.,* 2014; Lion *et al.,* 2017) and various clickable monosaccharide analogs have been developed for the glycan labeling of plant cell walls (Hoogenboom *et al.,* 2016; Wang *et al.,* 2016; Zhu and Chen, 2017).

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

Results and discussion

Procedure for labelling cell wall pectic polysaccharides

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

at the apical end of the cell, allowing for tip growth (Mollet *et al.*, 2013; Dehors *et al.*, 2019). For the dynamic labelling of pectins, pollen tubes were incubated with Kdo-N_3 (Dumont *et al.*, 2012), a monosaccharide derivatives that is incorporated into pectins through the endogenous biosynthetic machinery (Hoogenboom *et al.*, 2016; Dumont *et al.*, 2016). In addition, labelling of polysaccharides (pectin and xyloglucan) and glycoproteins containing fucose (Fuc) was monitored after incubation with 6-azido L-fucose (Fuc-N_3). Then, coupling to the fluorescent probe was achieved by using strained cyclooctyne probes (SPAAC) (Figure 1) that do not require toxic copper ions for activation (Zhu and Chen, 2017). Indeed, the strained alkyne function reacts with an azide group in biological conditions without any requirement of Cu^+ ions for coupling. This allows the pollen tube to remain viable after coupling to the fluorescent tag that occurs spontaneously in the culture medium.

The procedure is detailed in Figure 2 and consists of incubating germinating pollen grains and/or growing pollen tubes with modified sugars (Figure 2, step ❶). To do so, freshly harvested pollen grains were incubated in liquid germination medium for 1 h. The medium contained populations of germinated pollen grains with growing pollen tubes, plus ungerminated and germinating pollen grains. Germinated and germinating pollen grains were centrifuged and rinsed before being incubated in the dark in culture medium supplemented with 50 µM peracetylated 6-azido L-fucose (Fuc-N_3) or 200 µM Kdo-N_3 (Figure 2, step ❷). The metabolic incorporation and the subsequent synthesis and deposition of the clickable polymers in the cell wall occurred during a 2 h period while the clickable sugar was available for incorporation into newly synthesized cell wall material (Figure 2, step ❷). During this step ❷, the population of ungerminated pollen grains started germinating, producing a growing tube incorporating the clickable sugar from the first instant of growth. During the same time (Step ❷), already germinated pollen tubes started to incorporate clickable sugars in newly synthesized cell wall. At the end of the incorporation step, pollen tubes were washed three times with BK-PEG by gentle centrifugation (2 min, 700 *g*) in order to keep them alive. Pollen tubes were then transferred into a germination medium supplemented with 1 µM of the clickable fluorescent reporter (Alexa Fluor 488 sDIBO). Incubation with the fluorescent reporter lasted for 1 h (Figure 2, step ❸). Samples were washed once in BK-PEG supplemented with 0.01% (v/v) Tween 20 for 5 min followed by three washes with BK-PEG by

centrifugation (700 *g*, 2 min). Control experiments were carried out by omitting either the Kdo-N_3 or Fuc-N_3 during incorporation (Figure 2, step ❷). Viable and growing labelled pollen tubes were then observed under a confocal microscope. Pollen tube viability was assessed based on the occurrence of active cytoplasmic streaming.

Dynamics of pectins in living pollen tubes

Interestingly, all confocal images revealed that cell wall labelling of pollen tubes showed intensity fluctuations depending on the step at which the pollen tubes grew. As such, pollen tubes that ceased growth during step 2 showed labelling of the cell wall at the tip and the shank of the pollen tube (Figure 3A) whereas other pollen tubes that started to germinate during step 2 and grew during step 3 revealed cell wall labelling in the pollen tube emerging from the pollen grain and no labelling at the tip (Figure 3B). Lastly, a stop-and-go labelling pattern of the pollen tube cell wall was observed. Pollen tubes that grew during all three steps showed fluorescent labelling in a restricted area, which corresponds to the pollen tube growth during step 2 (Figure 3C). Remarkably, increasing and decreasing labelling intensities of the cell wall were observed at the beginning of steps 2 and 3, respectively whereas no labelling was observed in pollen tubes growing during step 1 and after the washes in step 3 (Figure 3C). Negative controls without the addition of a modified sugar did not show any fluorescence (Figure 3D). Finally, our data revealed similar fluorescent labelling patterns in pollen tube walls that grew during the three steps after incubation with Fuc-N_3 (Figure 3E).

Clickable sugars do not affect pollen tube growth

The effect of metabolic incorporation of azido sugars was assessed on pollen tube growth by measuring the total length of viable pollen tubes at the end of the metabolic incorporation, after SPAAC labelling and after the final washes before confocal observations. Results are presented Figure 4A. In the control condition, the length of pollen tubes after the 5.5 h of the experimental procedure (Figure 2, step 1 to 3 with the 30 min of washing steps) ranged from 88.4 µm to 374.8 µm with a mean value of 229.6 µm (Figure 4A). Mean lengths of pollen tubes incubated in Kdo-N_3 or Fuc-N_3 were

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

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

Metabolic labelling of RG-II in living roots

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

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

Conclusion and future trends

To date, the imaging of cell wall polysaccharides relies on the use of dyes or antibodies specific for various glycan epitopes. However, the immunolocalisation of these epitopes within the cell walls generally requires sample fixation. As a consequence, although they are efficient and informative about the localization of polysaccharides in the cell wall, these imaging methodologies do not provide access to the dynamic imaging of these polymers within living tissues. The recent development of click chemistry now offers an alternative for the imaging of plant cell wall polymers. In this study, we have demonstrated that the metablolic incorporation of azido sugars, Fuc-N_3 and Kdo-N_3, and their subsequent localization by the Strain-Promoted Azide-Alkyne Cycloaddition (SPAAC) permit visualization of the dynamics of cell wall polymers in living cells during their elongation. In this strategy, the strained alkyne function linked to the fluorescent probe is able to react in biological conditions with the azide group of modified monosaccharides incorporated into cell wall polymers without any requirement of toxic copper (I) ions for activation. Moreover, the method allowed for visualization of living and growing cells under the confocal microscope. Here, we developed a protocol with pollen tubes, one of the fastest tip-growing cells, as they can reach growth rates from 60 to 400 nm sec^{-1}, depending on the species (Cheung and Wu, 2008). This fast growth implies an efficient coordination for synthesizing, delivering and modifying membrane and cell wall material. This unique fast growing cell has already proven to be an effective biological model to assess the developmental consequences of modification of the cell wall *in vitro* with the use of mutant lines or enzymatic treatment (Hocq *et al.*, 2017, Hocq *et al.*, 2020). Future work will be carried out to develop this technique of metabolic click-mediated labelling on root cells and particularly on the elongation zone and on elongating root hairs. SPAAC was previously used on *Arabidopsis thaliana* roots but without the dynamic tracking of the labelling during growth (Zhu and Chen, 2017; Hoogenboom *et*

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

Experimental procedures

Plant growth conditions

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

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

Pollen tube culture, incorporation of Kdo-N_3 or Fuc-N_3 and SPAAC labelling

Two fully open flowers were collected in the second row from the top of the primary and secondary inflorescences. Anthers was cut and submerged in 1.7 mL of BK-PEG medium composed of 1.5 mM H_3BO_3, 2 mM $Ca(NO_3)_2$ $4H_2O$, 1.5 mM $Mg(SO_4)_2$ H_2O and 1 mM KNO_3 (Brewbaker and Kwack, 1963) supplemented with 10% (w/v) polyethylene glycol (PEG 6000) as an osmoticum (in 2 mL Eppenddorf® plastic tubes). PEG was used instead of sucrose to promote intake of the modified sugar. Tubes were shaken to release the pollen grains from the anthers and anthers were removed with a pair of tweezers. Four hundred µL of pollen suspension were then transferred in 1.5 mL Eppenddorf® plastic tubes and incubated for 1 h at 22°C in darkness. Pollen tubes were centrifuged (700 *g*, 2 min) and washed with BK-PEG three times before being incubated at 22°C in the dark for 2 h in BK-PEG supplemented with 50 µM peracetylated 6-azido L-fucose (Fuc-N_3) or 200 µM Kdo-N_3. The synthesis of 6-azido L-fucose is described in the supporting experimental procedure : Methods S1. Kdo-N_3 was synthesized as previously describes in Dumont *et al.,* (2012). Pollen tubes were washed three times with BK-PEG by centrifugation (700 *g*, 2 min) and transferred in BK-PEG containing 1 µM alkyne Alexa Fluor 488 sDIBO (C20020, Invitrogen, Carlsbad, California, USA) for 1 h at RT before

being washed once in BK-PEG supplemented with 0.01% (v/v) Tween 20 for 5 min followed by three washes with BK-PEG by centrifugation (700 *g*, 2 min). Control experiments were carried out by omitting either the Kdo-N_3 or Fuc-N_3 during incorporation. Pollen tubes were then covered with a cover slip and observed using a Leica TCS SP5 scanning confocal microscope (Wetzlar, Germany) with a 490 nm excitation laser and 500-550 nm emission detection. Microscope images were acquired and measurements made using Leica microscope systems software (LAS AF Lite, Wetzlar, Germany).

Incorporation of Kdo-N_3 and SPAAC labelling of Arabidopsis roots

Four-day-old light-grown seedlings were transferred from MS medium plates to 500 µL of liquid MS ½ medium supplemented with 200 µM of Kdo-N_3 or with purified water (negative control) for 48 h at 24°C under 16 h light/ 8 h night cycle. Seedlings were then washed three times with liquid MS ½ medium and then transferred in 1 mL of 1 µM of DIBO Alexa Fluor® 488 alkyne (Invitrogen, C20020) for 1 h at room temperature, in dark and under gentle agitation. At the end of the incubation, four washes were performed with liquid MS ½ containing 0.01% (v/v) Tween 20 for the first one and only with liquid MS ½ for the three other washes. For the double labelling experiments, Direct Red 23 (Sigma Aldrich, 212490) was added at final concentration of 0.1 mg/mL during the first wash step in liquid MS ½ containing 0.01% (v/v) Tween 20. After 30 min, three washes were done using liquid MS ½. Roots were gently mounted between slides (Nunc Lab-Tek II Chamber Slide, Thermo Fisher scientific, 154453) and glass coverslips (18X18, Thermo Fisher scientific). Roots were mounted in liquid MS ½. Confocal microscope observation was performed with a Leica TCS SP5 scanning confocal microscope (Wetzlar, Germany) (490 nm and 561 nm excitation laser; 500-550 nm and 575-615 nm emission laser respectively for DIBO Alexa Fluor 488 and Direct Red 23).

Statistical analyses

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

Data availability

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

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Author contributions

MR and QH performed the experiments; AB, LF, MC, BV synthesized the 6-azido L-fucose and the Kdo-N_3; MR, AL, JCM and PL wrote the manuscript; JCM and IB acquired funding; JCM, PL and AL designed and supervised the experiments.

Conflict-of-Interest

The authors declare that they do not have competing interests

Supporting Information

Supporting experimental procedures: Methods S1 : Synthesis of 6-Azido-6-deoxy-L-galactose (Fuc-N_3)

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Figure

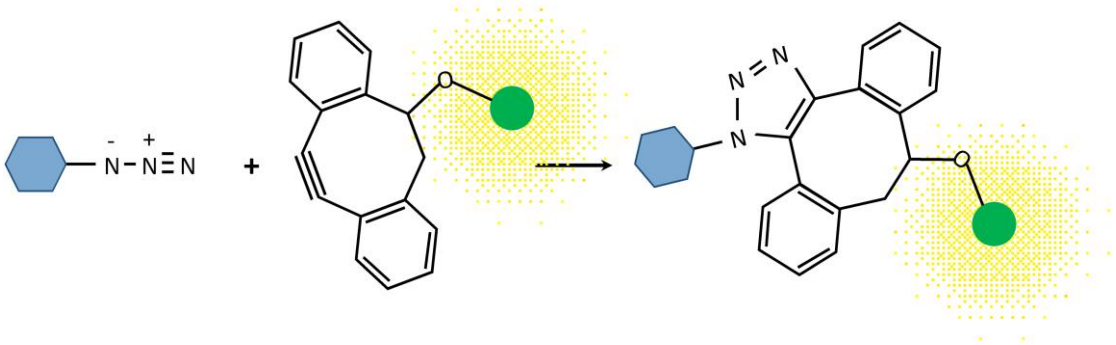

Figure 1. Copper-free click-labelling (SPAAC).

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

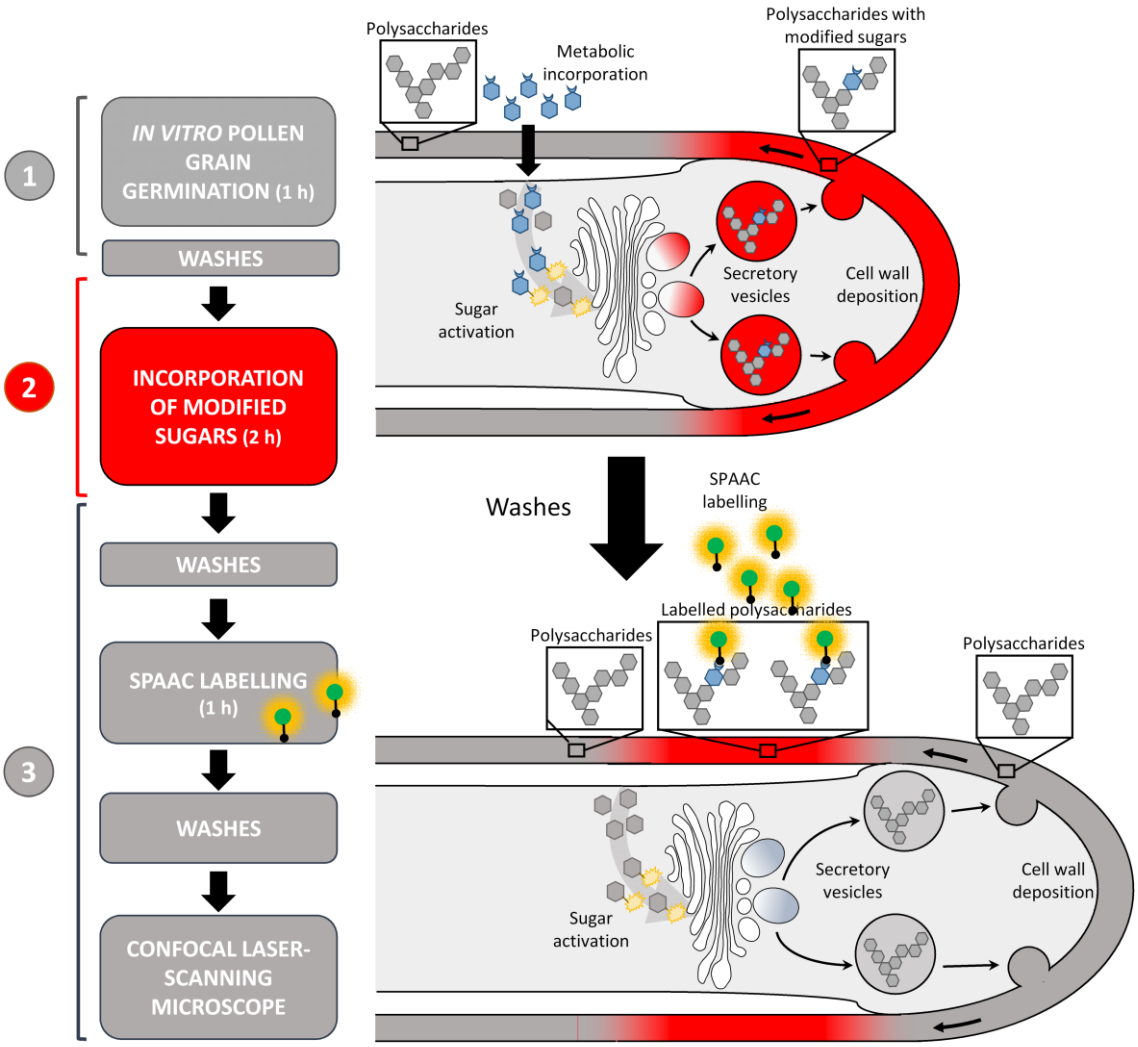

Figure 2. Protocol for the metabolic SPAAC labeling of living and growing pollen tubes.

Schematic representation of the experimental procedure for pollen tube growth, metabolic incorporation of modified sugars and labelling as detailed in the material and method section. Key stages are numbered : ❶ *in vitro* pollen grain germination for 1 h in germination medium at 22°C in darkness; ❷ incorporation of Kdo-N_3 (200 µM) or Fuc-N_3 (50 µM) for 2 h at 22°C in darkness and ❸ washes and click-labelling with 1 µM alkyne Alexa Fluor 488 sDIBO for 1 h at room temperature (RT) before confocal imaging.

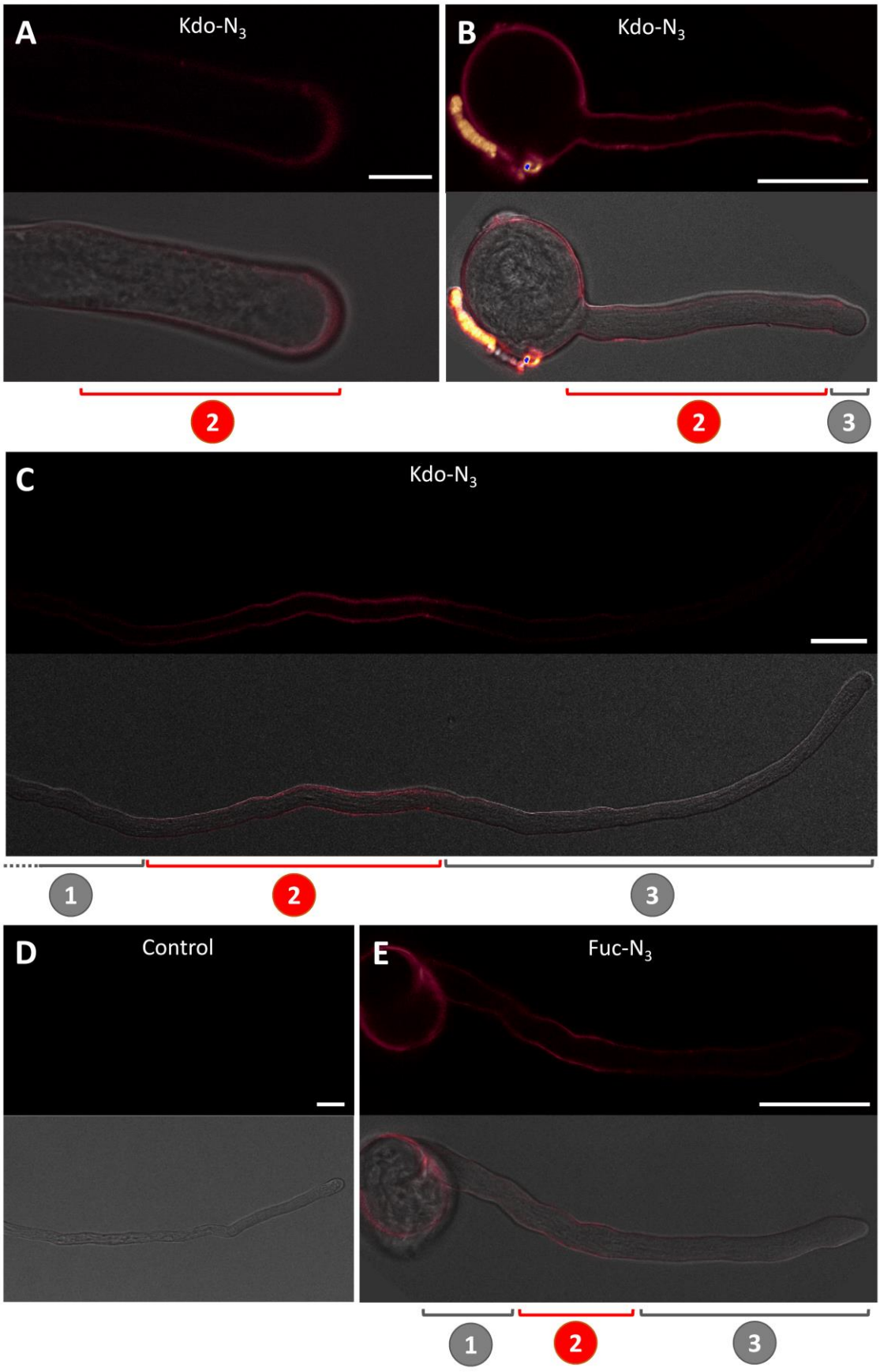

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Figure 3. Dynamics of pectins during *Nicotania tabacum* pollen tube growth.

Note that Circled numbers correspond to the procedure described in Figure 2. ❶, pollen grain germination and pollen tube growth, ❷ metabolic incorporation of the modified sugar, synthesis and deposition of clickable cell wall polysaccharides, ❸ SPAAC labelling and observation of viable pollen tubes. **(A-E)** Fluorescent and overlay confocal images of pollen tubes that **(A)** ceased growth during incorporation (❷) and **(B)** started to germinate during incorporation (❷)and grew during labelling (❸). Note that the auto-fluorescence of the pollen grain comes from the exine wall. **(C)** Fluorescent and overlay confocal images of pollen tubes that expanded during the three steps. **(D)** Negative control samples were submitted to steps ❶, ❷ and ❸ but without addition of Kdo-N_3 or Fuc-N_3 during the incorporation step (❷). **(E)** Fluorescent and overlay confocal images of pollen tubes that grew during the three steps after incorporation of Fuc-N_3 (❷). Scale bars: **(A)** 7.5 µm and **(B,C,D,E)** 25 µm.

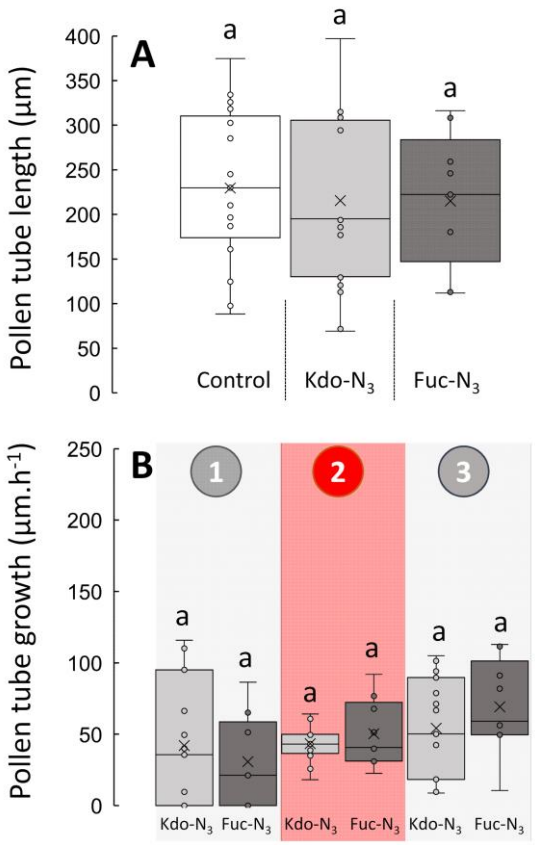

Figure 4. Effect of metabolic SPAAC labeling on pollen tube growth.

A. Distribution of pollen tubes length after metabolic incorporation and SPAAC labeling in control condition (n=17) or with Kdo-N_3 (200 µM, n=20) or Fuc-N_3 (50 µM, n=13). B. Distribution of pollen tubes length before metabolic incorporation (❶), during incorporation (❷) and during SPAAC labeling (❸) of Kdo-N_3 (n=15) or Fuc-N_3 (n=10).

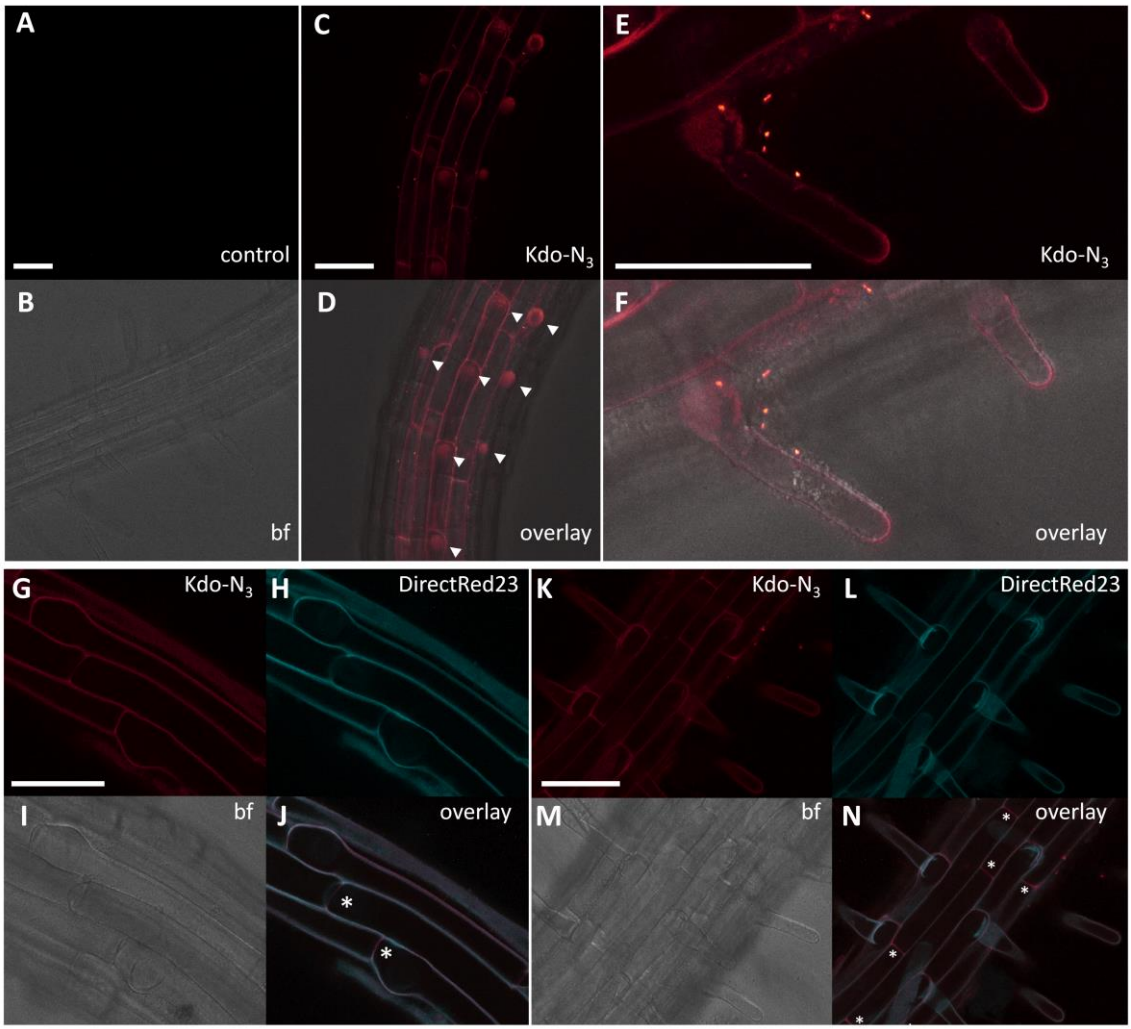

Figure 5. Metabolic SPAAC labelling of living root of *Arabidopsis thaliana* with Kdo-N_3.

(A, B) control condition without Kdo-N_3 during the incorporation step. **(C, D)** SPAAC labelling of RG-II in the differentiation zone of the root using Kdo-N_3. **(E,F)** SPAAC labelling of RG-II in root hairs using Kdo-N_3 **(G-N)** SPAAC labelling of RG-II with Kdo-N_3 and cellulose staining with Direct Red 23 in early differentiation zone **(G-J)** and in root hairs **(K-N)**. Scale bars: 50 µm. arrowheads show the root hair apex; * marks the anticlinal cell wall.