Bioimaging

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One, Two, Three: A Bioorthogonal Triple Labelling Strategy for Studying the Dynamics of Plant Cell Wall Formation In Vivo

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Abstract: *Reported herein is an in vivo triple labelling strategy to monitor the formation of plant cell walls. Based on a combination of copper-catalysed alkyne–azide cycloaddition (CuAAC), strain-promoted azide–alkyne cycloaddition (SPAAC), and Diels–Alder reaction with inverse electronic demand (DAR_{inv}), this methodology can be applied to various plant species of interest in research. It allowed detection of the differential incorporation of alkynyl-, azido-, and methylcyclopropenyl-tagged reporters of the three main monolignols into de novo biosynthesized lignin in different tissues, cell types, or cell wall layers. In addition, this triple labelling was implemented with different classes of chemical reporters, using two monolignol reporters in conjunction with alkynylfucose to simultaneously monitor the biosynthesis of lignin and non-cellulosic polysaccharides. This allowed observation of their deposition occurring contemporaneously in the same cell wall.*

Over the last two decades bioorthogonal reactions, combined with fluorescence microscopy, have proven to be a powerful tool for generating novel biological information in a wide variety of in vivo molecular imaging applications.[1–3] Many groups have focused on the use of reaction-based fluorescent probes for chemoselective bioimaging in mammalian living systems, but plants have generally received less attention despite the fact that they are complex multicellular eukaryotes. Plant cells are characterized by the presence of an extra-cellular matrix called the cell wall that is made up of different polymers (e.g., cellulose, hemicelluloses, pectins, lignin). To date, bioorthogonal approaches have been used in plants to investigate polysaccharide and lignin biosynthesis using tagged sugars or monolignols as reporters in in vivo labelling experiments,[4–9] as well as auxin.[10] Lignin plays vital roles in plants and is also of considerable industrial importance. It forms in the cell wall when hydroxycinnamyl alcohols (monolignols) are enzymatically oxidised by peroxidases and/or laccases and undergo polymerisation (Figure 1). In angiosperms, the three main monolignols, that is, *para*-coumaryl alcohol **1**, coniferyl alcohol **2**, and sinapyl alcohol **3**, give rise to the hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively, forming lignin.[11] Since lignin properties are influenced by monomer ratio and the nature of inter-unit bonds, improved knowledge of lignification would contribute to better understanding of plant physiology and factors affecting biomass quality.[12] We recently reported a dual labelling method using strain-promoted alkyne–azide cycloaddition (SPAAC) and copper-catalysed alkyne–azide cycloaddition (CuAAC) to detect incorporated azide-tagged H-monolignols ($\mathbf{H_{AZ}}$ **4**) and alkyne-tagged G-monolignols ($\mathbf{G_{ALK}}$ **5**).[13] The use of two different cycloaddition reactions is necessary for dual lignin labelling as the monolignol mimics **4** and **5** are incorporated into the same polymer. Because of their spatial proximity, using consecutive CuAAC ligations indeed leads to cross-linking reactions that compete with the desired probe ligation. To develop a triple labelling strategy for the three main monolignols, we tested whether the Diels–Alder reaction with inverse electronic demand (DAR_{inv}) could be used to label the lignin S-unit and successfully combined with SPAAC and CuAAC. Although there are various reports of dual labelling methods that combine two biorthogonal reactions both in vitro and in vivo,[6,7,13–19] the only instance where three such reactions were used concurrently within the same sample was based on a sequence of CuAAC, DAR_{inv}, and Staudinger–Bertozzi ligation in an activity-based protein profiling assay in solution.[20] Although three-color imaging using compatible reactions seems intuitively possible, to the best of our knowledge it has never been applied to track three distinct molecules within a single living sample. Herein, we demonstrate the feasibility of triple biorthogonal labelling in vivo by sequentially exploiting the major triad of biorthogonal reactions (DAR_{inv}, SPAAC, and CuAAC) to track the three main lignin monomers (Figure 1). Furthermore, we show that the same strategy can be used to conjointly monitor lignin and cell wall glycan biosynthesis.

We firstly designed a sinapyl alcohol surrogate bearing a suitable tag for DAR_{inv}. For this we opted for the reactive methylcyclopropenyl moiety[18,21,22] to allow fast kinetics of the DAR_{inv} without introducing a bulky *trans*-cyclooctene or norbornene tag that might hamper cell wall phenoloxidase-initiated oxidation. The new chemical reporter $\mathbf{S_{CP}}$ **6** was synthesized in seven steps from commercially available sinapic acid (see Scheme S1 in the Supporting Information) and its reactivity with the selected tetrazine-functionalized Cy5 fluorescent probe was evaluated by HPLC. Full conversion occurred rapidly at ambient temperature under aqueous conditions, and the cycloadduct was identified by MALDI-TOF mass spectrometry (see Figure S1).

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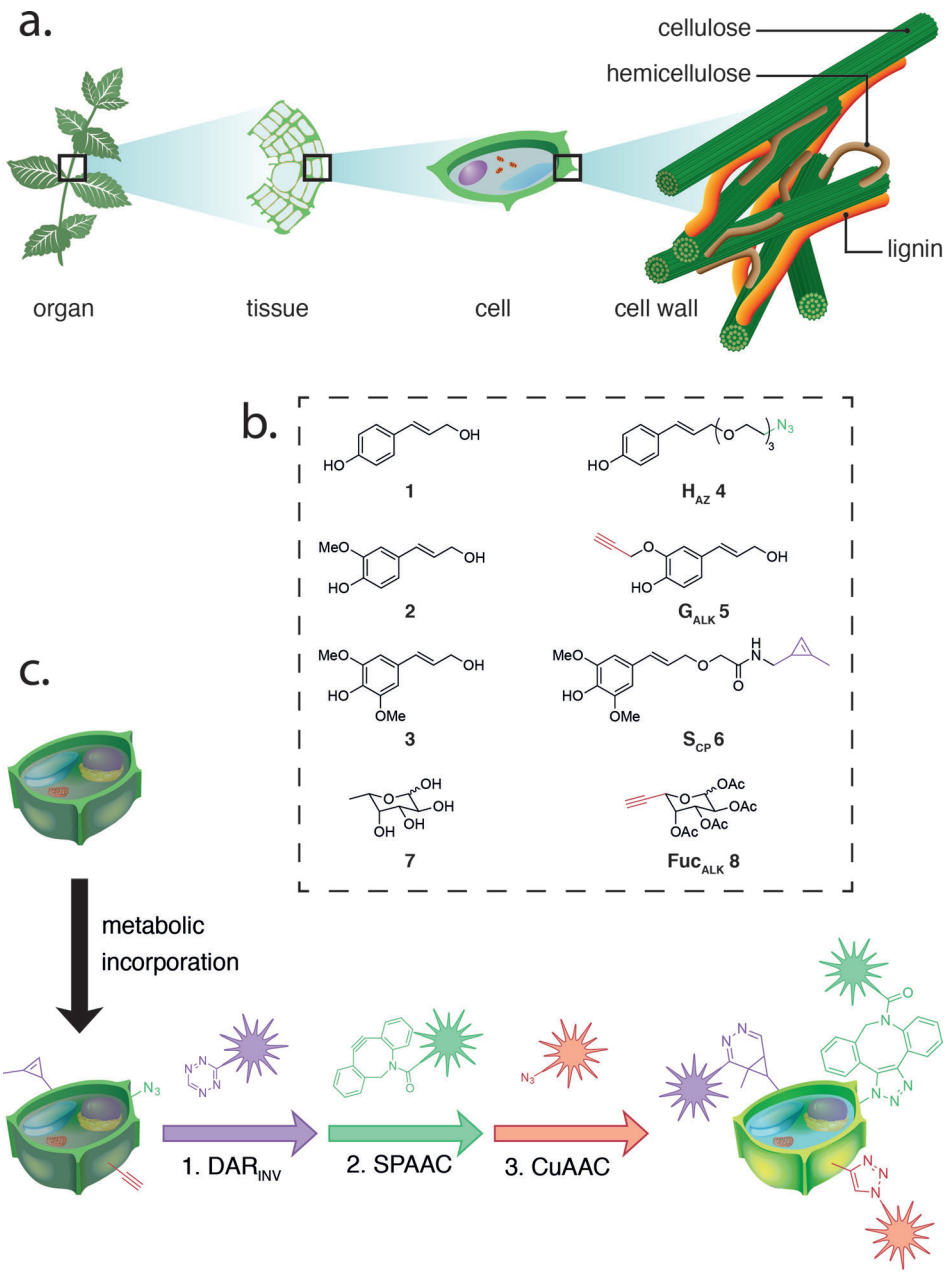

Figure 1. Overview of the bioorthogonal triple labelling strategy. a) General scheme of plant cell wall structure and localisation. b) Chemical structure of natural molecules of interest and corresponding tagged reporters. c) Chemical reporter strategy with triple labelling: DAR_{INV}, SPAAC and CuAAC.

After confirming its lack of toxicity on living flax seedlings (see Figure S2), we investigated whether $\mathbf{S_{CP}}$ **6** could be metabolically incorporated into the cell walls of flax plants. For this we established and fully optimized a single-labelling protocol in which the tetrazine-Cy5 probe is specifically ligated to metabolically incorporated lignin $\mathbf{S_{CP}}$ units by the DAR_{inv} reaction at ambient temperature.

Subsequent confocal fluorescence microscopy observation indicates that $\mathbf{S_{CP}}$ **6** is incorporated in a time- and dose-dependent manner into the lignifying walls of differentiating secondary xylem cells but is not/poorly incorporated into already lignified mature cell walls (Figure 2). This labelling pattern is in agreement with our previous observations of the alkyne- and azide-tagged G- and H-unit reporters **4** and **5**, respectively.[13,23,24] Additional experiments showed that $\mathbf{S_{CP}}$ incorporation is 1) abolished by competition with the natural S monolignol **3** and 2) almost completely eliminated in samples when the enzymatic machinery is heat denatured prior to incubation, indicating that $\mathbf{S_{CP}}$ incorporation is under enzymatic control (see Figures S3 and S4). These results also indicate that the introduction of the methylcyclopropenyl tag and spacer arm does not prevent either the phenoloxidase-mediated oxidation of the sinapylic moiety, or its radical polymerization.

Before incorporating the DAR_{inv} reaction into an in vivo triple labelling strategy we first evaluated the compatibility of

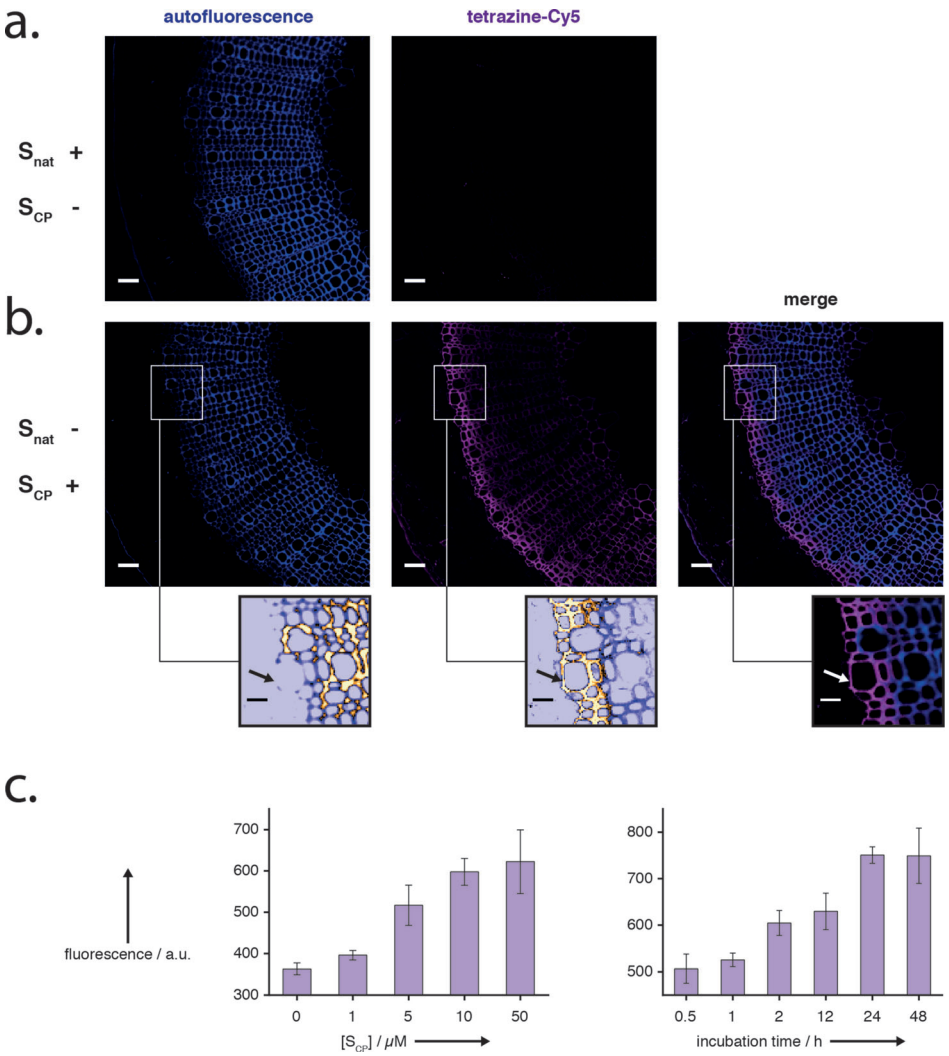

Figure 2. Incorporation of $\mathbf{S_{CP}}$ monolignol reporter into flax stem cell walls. a) Negative control incorporating unmarked **3**. b) Incorporation of tagged $\mathbf{S_{CP}}$ **6**. Lignin autofluorescence in blue, tetrazine-Cy5 fluorescence in magenta. Magnified zooms (left, middle) show intensity-dependant false colouring of differentiating xylem allowing direct comparison of the 3 images and illustrating the greater sensitivity of $\mathbf{S_{CP}}$ labelling compared to autofluorescence. c) $\mathbf{S_{CP}}$ concentration- (left) and time- (right) dependant fluorescence. Scale bars (a, b main figures = 50 μm, inserts = 20 μm).

the three click reactions in this context. DAR_{inv} specificity between $\mathbf{S_{CP}}$ **6** and the tetrazine-Cy5 probe was tested in the presence of $\mathbf{H_{AZ}}$ **4** and $\mathbf{G_{ALK}}$ **5** reporters in solution (see Figure S5). Results showed that DAR_{inv} is orthogonal to the other two reactions as $\mathbf{S_{CP}}$ is fully converted into the corresponding dihydropyridazine cycloadduct while $\mathbf{H_{AZ}}$ and $\mathbf{G_{ALK}}$ remain untouched. After full conversion of $\mathbf{S_{CP}}$, specific SPAAC cycloaddition between $\mathbf{H_{AZ}}$ and DBCO-PEG_4-Rhodamine Green occurs without degradation of either the $\mathbf{S_{CP}}$ cycloadduct or of the remaining $\mathbf{G_{ALK}}$ reporter in the mixture, and can in turn be linked to TAMRA-azide by CuAAC as previously reported.[13,23,24] Based on these results we developed an in vivo, sequential triple-labelling protocol to visualize lignification in flax plant cell walls. Samples were incubated for 20 hours with the reporters $\mathbf{H_{AZ}}$ **4**, $\mathbf{G_{ALK}}$ **5**, and $\mathbf{S_{CP}}$ **6** prior to: 1) DAR_{inv} labelling of incorporated $\mathbf{S_{CP}}$ units, 2) SPAAC labelling of $\mathbf{H_{AZ}}$ units, and 3) CuAAC labelling of $\mathbf{G_{ALK}}$ units. As expected, the CuAAC labelling of $\mathbf{G_{ALK}}$ units must be performed last, after removal of the excess cyclooctyne- and tetrazine-activated probes from the biological sample. This protocol avoids possible competitive $\mathbf{G_{ALK}}$-$\mathbf{H_{AZ}}$ cross-linking that may result in signal loss,[13,23,24] as well as side-reactions of the tetrazine-functionalized probe in the presence of copper sulfate that may result in strong background.[20] Our results (Figure 3; see Figure S6) show that the triple-labelling protocol can be successfully used to visualize H-, G-, and S-monolignol reporter incorporation into actively lignifying cell walls in differentiating flax secondary xylem using the two experimental systems we previously reported (i.e., flax stem sections as an easily implemented model, or entire flax stems as a relevant in vivo system). Crossed negative controls showed the chemoselective and specific nature of each reaction for its target reporter (see Figure S7). This strategy is also applicable to other plant organs, such as roots (see Figure S8).

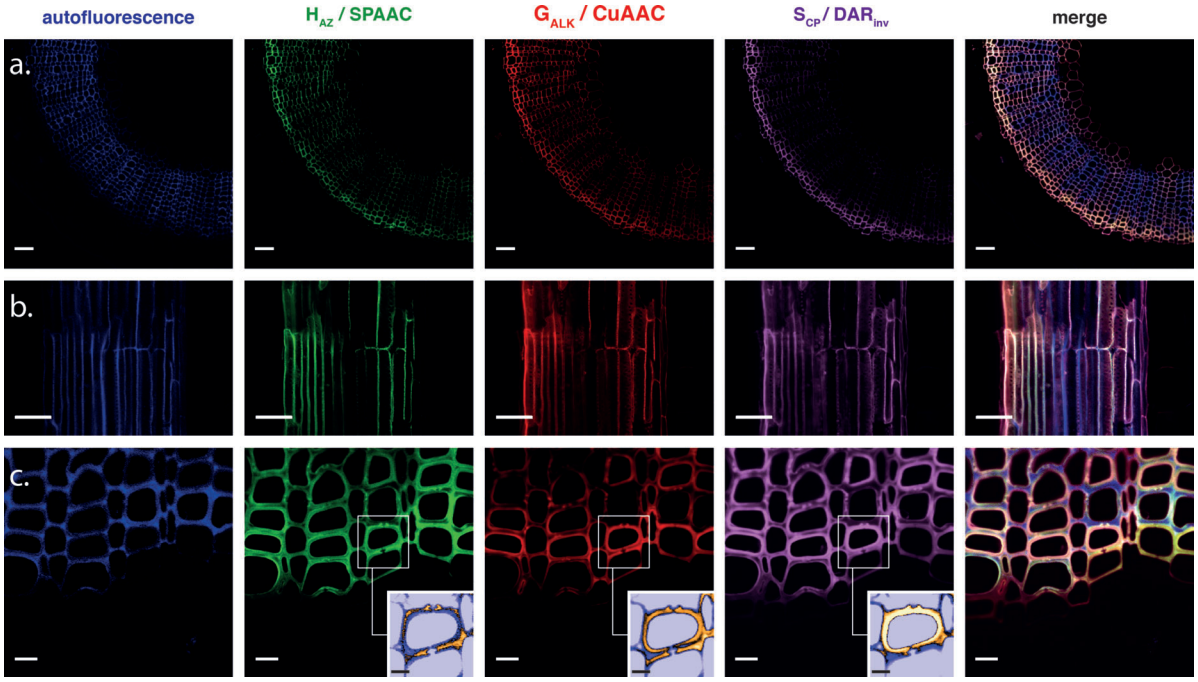

Figure 3. Bioorthogonal lignin triple (H, G, S) labelling in flax. Lignin autofluorescence (blue), incorporated $\mathbf{H_{AZ}}$ **4** (green, SPAAC/ DBCO-PEG_4-Rhodamine Green), $\mathbf{G_{ALK}}$ **5** (red, CuAAC/ Azide-fluor 545), $\mathbf{S_{CP}}$ **6** (magenta, DAR_{inv}/ tetrazine-Cy5), and merge channels. Incorporation of reporters in a) freshly cut transversal cross-sections of flax stems; b) freshly cut longitudinal sections of flax stems; c) living entire flax stems. Focus on cell walls of differentiating xylem: inserts show intensity-dependant false colouring image of cell walls illustrating differential incorporation of monolignol reporters in wall sub-layers. Scale bar = 50 μm (a,b), 10 μm (c) and 5 μm (insert).

Our triple labelling method offers a number of advantages for the dynamic study of lignification processes compared to more conventional approaches based on either autofluorescence, histochemistry, or immunolocalization. A first advantage is that this metabolic labelling enables us to distinguish between cell wall layers that are lignified, but not actively undergoing lignification, from cell wall layers that are still actively producing lignin (see higher reporter incorporation in differentiating xylem). The use of specific chemical reporters for the three main lignin units also allows us to analyse the capacity of different cell wall layers and/or domains to incorporate the different monolignols during development. Nevertheless, even if any potential reporter crosstalk is extremely unlikely, as reporters will be rapidly incorporated into cell wall lignin through the apoplastic route and will not enter the cytoplasm where enzymatic conversion might take place, future detailed quantitative studies would benefit from complementation by in-depth metabolomics. Although absolute quantitative data cannot be obtained by direct comparison between the three image channels due to varying acquisition parameters (e.g., different quantum yields or excitation conditions), layers showing preferential incorporation of different reporters can be identified. For example, Figure 3c clearly illustrates that, in the selected cell, $\mathbf{H_{AZ}}$ reporters are preferentially incorporated in the middle lamella and bordered pit compared to other cell wall regions. In contrast $\mathbf{G_{ALK}}$ reporters are preferentially incorporated into the primary cell wall/S1 secondary cell wall layer of this cell and the younger adjacent cell closer to the vascular cambium. Last, the $\mathbf{S_{CP}}$ reporter appears to be very heavily incorporated into the S1 and S2 secondary cell wall layers of the selected cell, but not in the wall of the younger adjacent cell. Although care should be taken when interpreting the biological significance of observed differences since, strictly speaking, labelling reflects the incorporation of monolignol mimics and not native molecules, our results are in agreement with previous studies using radioactively labelled molecules.[27] Altogether, this technique represents a powerful tool for investigating the heterogeneity in lignin cell wall composition.

To evaluate the triple labelling strategy for lignification in other species we tested three other commonly used plant models: *Arabidopsis thaliana*, *Nicotiana Benthamiana* (tobacco), and *Populus tremula x alba* (poplar). In all cases stem sections incorporated the three reporters into actively lignifying xylem cell walls (Figure 4). As observed in flax cell walls, labelling was generally more intense in the cell walls of young differentiating xylem cells compared to more mature cells. Lignified cell walls that were no longer undergoing active lignification could be distinguished from actively lignifying cell walls by their UV autofluorescence and absence of reporter signal. This observation was particularly evident in poplar, but could also be observed in *Arabidopsis* and tobacco. Reporters were also incorporated in lignifying interfascicular fibers (*Arabidopsis*) and sclerenchyma fibers (tobacco, poplar) that are more heavily lignified than flax fibers. A relatively strong labelling was also observed at the xylem/pith parenchyma junction in tobacco and *Arabidopsis*

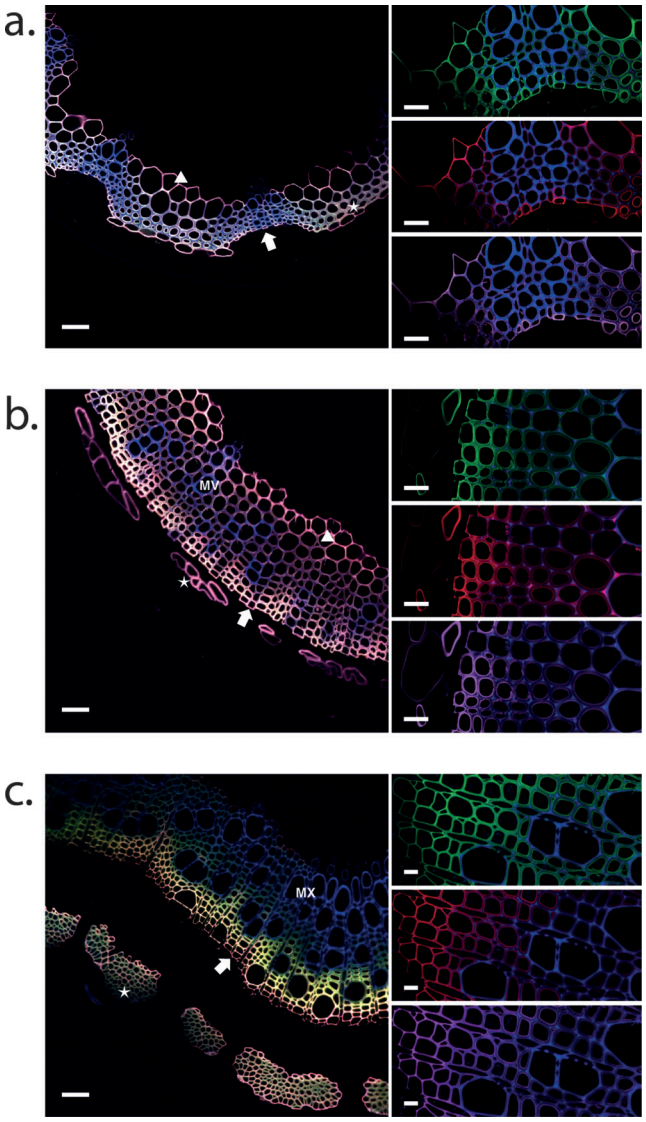

Figure 4. Bioorthogonal lignin triple H/G/S labelling in other plant species. $\mathbf{H_{AZ}}$ **4** incorporation in green (SPAAC/ DBCO-PEG$_4$-Rhodamine Green), $\mathbf{G_{ALK}}$ **5** in red (CuAAC/ Azide-fluor 545), $\mathbf{S_{CP}}$ **6** in magenta (DAR$_{inv}$/ tetrazine-Cy5), and autofluorescence in blue. Cross-section of a) *Arabidopsis thaliana* floral hemp; b) tobacco stem; c) poplar stem. Left panel: low power merge, scale bar = 50 µm; right panel: zoom of xylem vascular bundle (a) or differentiating xylem (b,c), scale bar = 20 µm. Differentiating xylem (arrow), (interfascicular) fibers (star), xylem/pith junction (triangle), mature vessel (MV), mature xylem (MX).

in comparison with a much weaker signal in poplar indicating a potential difference between lignification in woody and non-woody species. As observed in flax, certain cell wall layers/regions showed preferential incorporation of one (two) reporter(s) compared to the other(s) most likely reflecting heterogenity in the cell wall machinery and/or structure.

Cell wall formation in plants is a highly regulated process and lignin deposition is coordinated with the formation of other cell wall polymers. We therefore investigated whether this triple labelling approach could be used to simultaneously investigate the dynamics of both lignin and non-cellulosic polysaccharide (NCP) polymer deposition in flax stem cell walls. For this we used the $\mathbf{H_{AZ}}$ **4** and $\mathbf{S_{CP}}$ **6** monolignol reporters together with peracetylated alkyne-tagged fucose reporter $\mathbf{Fuc_{ALK}}$ **8**. Fucose (**7**) is present in both the side chains of xyloglucan hemicelluloses and in pectin rhamnogalacturonan I and II (RGI, RGII) motifs,[25,26] and previous reports have shown that $\mathbf{Fuc_{ALK}}$ **8** can be successfully used in a bioorthogonal reaction to label plant NCP cell wall polymers.[4,5] In contrast to the incorporation of monolignol reporters directly into the cell wall, NCP labelling requires that the reporter be metabolized into the cytoplasm and transported to the cell wall by the Golgi apparatus, and we therefore reasoned that the entire stem in vivo system might be more appropriate than the cross-section model. This approach was successful and our results (Figure 5) allowed us to distinguish three main zones according to precursor incorporation profiles: the marking pattern of cell walls in the phloem and vascular cambium (zone 1 in the Figure) indicated that fucose reporters had been incorporated into cell wall NCP polymers, but that these cells are not actively producing lignin. In the well-developed secondary xylem (zone 2) only $\mathbf{H_{AZ}}$ and $\mathbf{S_{CP}}$ monolignol reporters were incorporated into cell walls, indicating that NCP wall polymer biosynthesis had stopped in these cells. A third area, corresponding to the differentiating xylem (zone 3) incorporated both fucose and monolignol reporters in the same wall indicating that lignin and NCP polymer deposition occur contemporaneously in the same cell wall. Although different lines of evidence have for a long time suggested that such co-deposition could occur,[27,28] this is the first time, to our knowledge, that this has been experimentally demonstrated.

This data is the first report of the use of a SPAAC, CuAAC, and DAR$_{inv}$ triple labelling approach to successfully monitor three distinct molecules in vivo. We used this method to visualize the incorporation of the three main lignin monomers in the cell walls of different model plant species and showed that it can also be exploited to conjointly study the deposition of lignin and cell wall glycans in the same sample. The development of this powerful and versatile triple labelling strategy based upon the use of independent bioorthogonal reactions will greatly advance our understanding of cell wall formation in plants and should also become a valuable addition to the human and animal imaging toolbox. Furthermore, the combination of tags used in this study also theoretically allows the detection and quantification of up to three mimics by different non-imaging-based analytical techniques including fluorescence, mass spectrometry, magnetic resonance spectroscopies, and vibrational spectroscopies, thereby paving the way to many different applications. One exciting perspective would be to use the different emission fluorescence wavelengths to follow the dynamic release of the different lignin monomers during chemical/enzymatic degradation of the labelled lignin polymer in plant lignocellulose biomass.

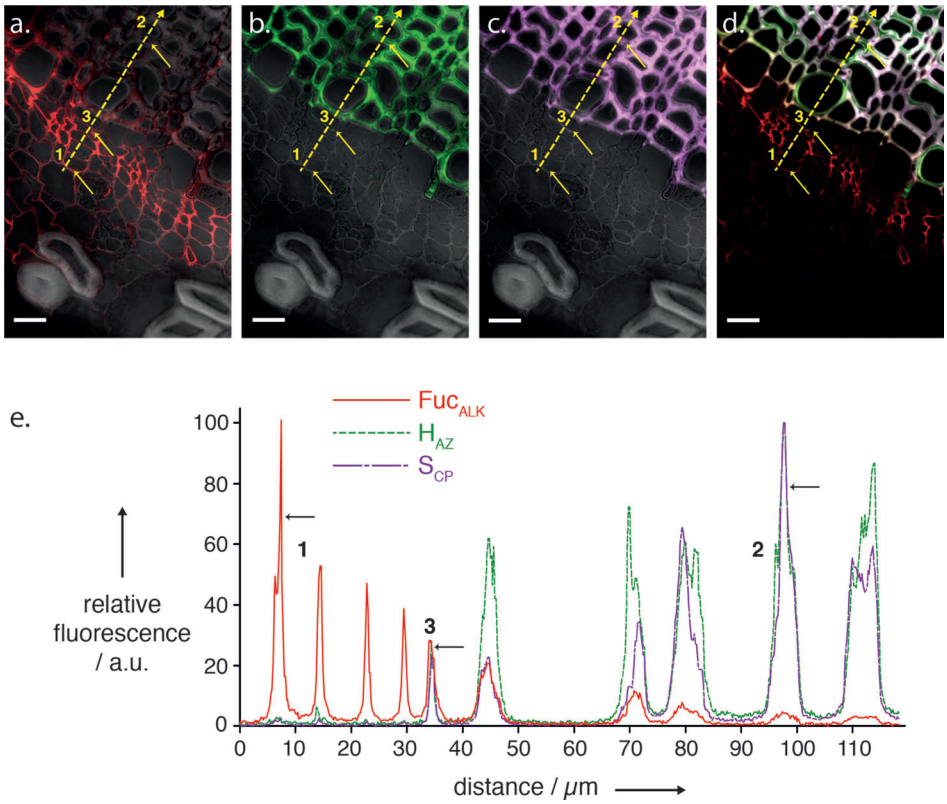

Figure 5. Bioorthogonal lignin and polysaccharide triple fucose/H/S labelling in flax. Incorporation of a) **Fuc$_{ALK}$ 8** (red, CuAAC/ Azide-fluor 545), b) **H$_{AZ}$ 4** (green, SPAAC/ DBCO-PEG$_4$-Rhodamine Green), c) **S$_{CP}$ 6** (magenta, DAR$_{inv}$/ tetrazine-Cy5) and d) merge in phloem/vascular cambium and differentiating xylem zones in entire living flax stem; e) intensity profile of reporter incorporation in cell walls in vascular cambium (1), maturing xylem (2) and young xylem (3). Dotted line = profile trace (a–d), arrows = indicated cell walls (a–d) and corresponding peaks (e). Scale bar = 20 µm.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: bioimaging · bioorganic chemistry · bioorthogonal chemistry · lignin · plant cell wall

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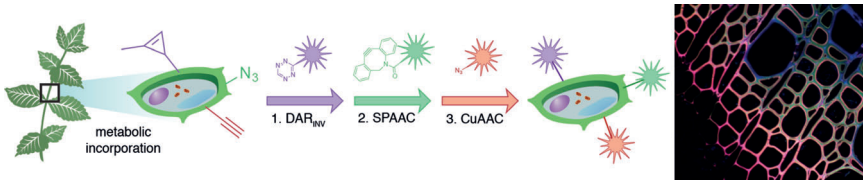

One, **two**, **three**: A triple bioorthogonal labelling strategy was developed to study the incorporation of three distinct monolignol reporters in lignin within plant cell walls. Alkynylfucose was also used in conjunction with monolignol reporters to show that non-cellulosic polysaccharide and lignin deposition can occur contemporaneously in the same cell wall. CuAAC: copper-catalysed alkyne–azide cycloaddition, SPAAC: strain-promoted azide–alkyne cycloaddition, DAR_{inv}: Diels–Alder reaction with inverse electronic demand.