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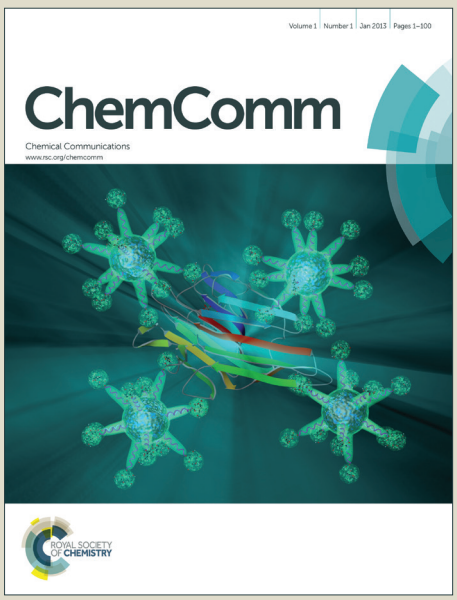

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A Click Chemistry Strategy for Visualization of Plant Cell Wall Lignification†

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Bioorthogonal click chemistry was commissioned to visualize the plant cell wall lignification process *in vivo*. This approach uses chemical reporter-tagged monolignol mimics that can be metabolically incorporated into lignins and subsequently derivatized via copper-assisted or copper-free click reactions.

Lignification, i.e., deposition of the phenolic lignin polymer in cell walls, is a crucial biological process that terrestrial plants undertake throughout various tissues. It most evidently occurs within vascular tissues such as xylem tracheids and structural fibers where lignin plays key roles in water conductivity, mechanical support, and pathogen defense.[1] Research on lignin biosynthesis and bioengineering have been a major focus particularly because, in addition to their fundamental importance in understanding plant life and evolution, lignin hinders many agro-industrial processes such as those that generate wood pulp and biofuels from plant biomass.[2] Lignin primarily derives from oxidative radical polymerization of *p*-hydroxycinnamyl alcohols, i.e., the monolignols. In the course of lignin biosynthesis, monolignols are first synthesized inside cells, and then transported to specific cell wall (CW) domains where they polymerize by the action of localized laccases and/or peroxidases. Whereas our knowledge of the genes and proteins controlling the synthesis of monolignols inside cells is relatively mature, little is known about how plants regulate the spatiotemporally specific transportation and polymerization of monolignols into CWs.[3]

Techniques to visualize the cell wall lignification processes *in vivo* are thus of particular importance in current lignin research, but are considerably limited. Unlike proteins, lignins and their direct precursors are not amenable to being genetically tagged for visualization. Fluorescence imaging using dye-tagged monolignol mimics has recently demonstrated success in providing spatio-temporal information in live plants; the intrinsic plasticity of lignification allows these synthetic probes to be metabolically incorporated into CW lignins.[4] However, attaching fluorophores directly to monolignols still suffers from several drawbacks. For example, the bulky and hydrophobic fluorescent tag may limit the probe's ability to accurately mimic the properties of a natural monolignol. There are also considerable limitations in obtaining effective dye-tagged monolignols, especially because the fluorophore must be carefully designed to not affect or be affected in the biological processes in which monolignols are involved.

The bioorthogonal chemical reporter approach has been recognized as a powerful strategy to visualize biomacromolecules in living systems.[5] In this strategy, biocompatible precursors modified with a simple chemical reporter group, such as an azide or an alkyne, are metabolically introduced into the target biomacromolecule where it is subsequently followed by derivatization *in vivo* via bioorthogonal chemistry with a detection tag for visualization. The copper-assisted azide-alkyne cycloaddition (CuAAC), often referred to as click chemistry,[6] has been effectively employed for bioorthogonal derivatization *in vivo*. In addition, Bertozzi and co-workers developed a copper-free variant, i.e., strain-promoted azide-alkyne cycloaddition (SPAAC), using activated cyclooctyne derivatives to execute the click reaction without the need for toxic metal catalysts.[7] These click labeling approaches have been extensively exploited in bacterial, mammalian, and yeast cells for imaging various biomolecules such as glycans, lipids, and nucleic acids.[5,6] More recently, Anderson et al.[8] used an alkyne-tagged sugar mimic to visualize plant CW pectins in *Arabidopsis thaliana*.

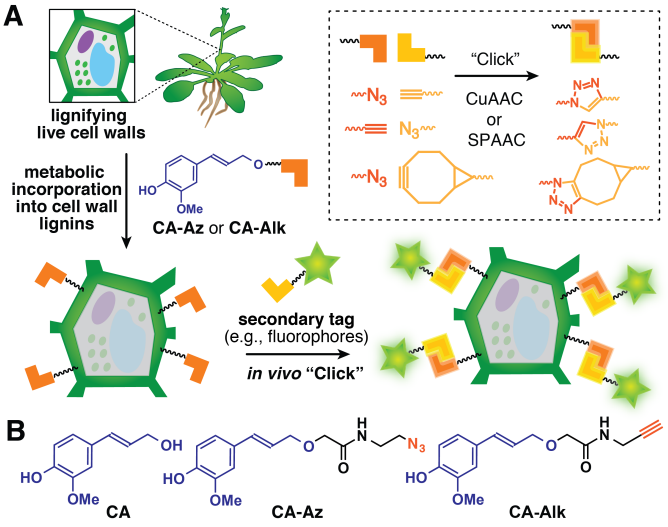

Fig. 1 (A) General strategy for metabolic labeling of plant cell wall lignins using the chemical reporter-tagged monolignols and introduction of secondary tags via *in vivo* click chemistry. (B) Natural coniferyl alcohol CA and its azide and alkyne-tagged mimics, CA-Az and CA-Alk.

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In this communication, we report the use of chemical reporter-tagged monolignol mimics for metabolic labeling of plant CW lignins (Fig. 1A). Compared to our previous approach using the dye-tagged monolignols, the use of chemical reporters has clear advantages in terms of their compact and inert structures as well as the flexibility in imaging as they can be attached to a variety of fluorescence and other detection probes (e.g., radioisotopes and affinity probes). Accordingly, coniferyl alcohol (CA) derivatives imbued with azide (CA-Az) and alkyne (CA-Alk) reporter functionalities (Fig. 1B) were synthesized (Fig. S1, ESI†), and the feasibility of using them to label and visualize CW lignins via click chemistry was tested through a series of *in vitro* and *in vivo* model experiments.

First, the compatibility of CA-Az and CA-Alk with lignin polymerization was tested *in vitro* by peroxidase-catalyzed dehydrogenative copolymerization of the probes with untagged CA (15:85, mol/mol, CA-Az:CA or CA-Alk:CA) under typical conditions used to model lignin polymerization *in vitro*.[9] The 2D HSQC NMR spectra of the azide- and alkyne-labeled guaiacyl synthetic lignins, GDHP-Az and GDHP-Alk, displayed the signals from the intact chemical reporter and alkyl spacer groups along with the signals from typical lignin aromatic (Fig. 2) and side-chain units (Fig. S2, ESI†).[9] The results demonstrate the integral incorporation of the probes into the lignin polymer chains, supporting our contention that the chemical reporter groups do not perturb a monolignol's ability to participate in lignin polymerization.

Next, to test the click reactivity of the labeled lignin polymers *in vitro*, we performed CuAAC reactions of both DHPs with alkyne- and azide-tagged nitrobenzofuran dyes, NBD-Az and NBD-Alk (Fig. S1, ESI†), with a typical $CuSO_4$/ascorbate system in the presence of tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) ligand. In the aromatic regions of HSQC NMR spectra of GDHP-Az conjugated with NBD-Alk and GDHP-Alk conjugated with NBD-Az, both the signals from NBD moieties as well as the triazol rings newly generated by the click chemistry are clearly visible (Fig. 2A,B). In the aliphatic regions of the spectra, subsets of the correlations from the chemical reporter and alkyl spacer groups experienced a complete shift upon the click reactions, whereas essentially no changes were observed for the lignin backbone signals (Fig. S2, ESI†). Thus, the CuAAC reactions proceeded efficiently and selectively. The fluorogenic NBD moieties attached to the DHPs were also evident from the characteristic absorptions and emissions

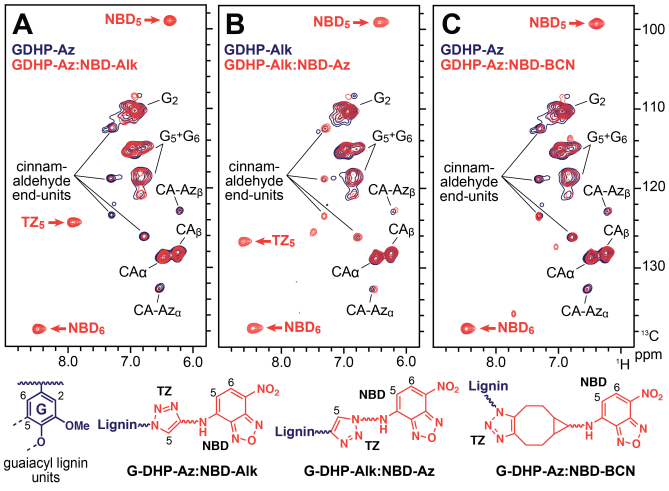

Fig. 2 Overlays of aromatic regions of ^{1}H–^{13}C HSQC spectra of synthetic lignins (DHPs) before and after click chemistry. (A) Azide-tagged DHP (GDHP-Az) derivatized with alkyne-tagged NBD (GDHP-Az:NBD-Alk), (B) alkyne-tagged DHP (GDHP-Alk) derivatized with azide-tagged NBD (GDHP-Alk:NBD-Az), (C) azide-tagged DHP (GDHP-Az) derivatized with BCN-tagged NBD (GDHP-Az:NBD-BCN).

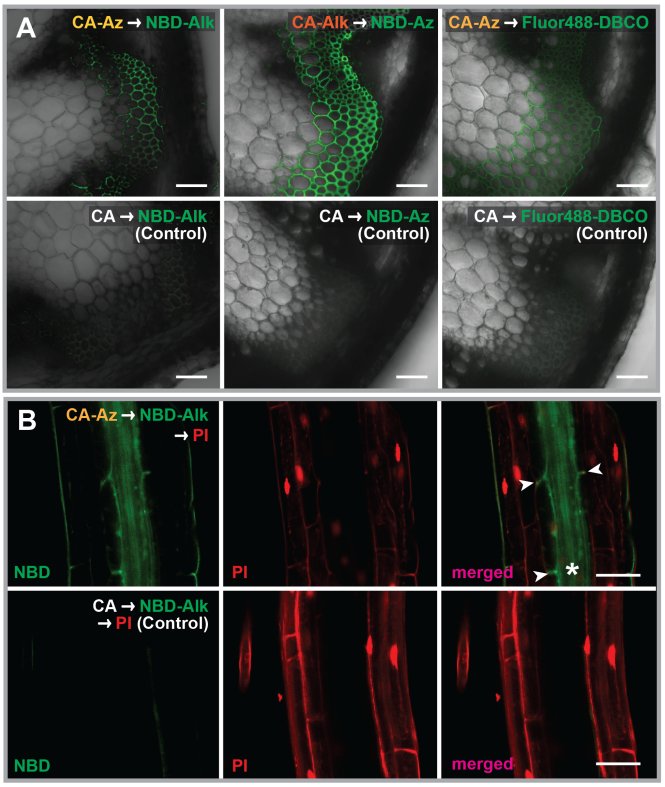

Fig. 3 (A) Arabidopsis stem sections treated with azide- or alkyne-tagged CAs, CA-Az and CA-Alk, and subsequently derivatized via *in vivo* click reactions with alkyne- and azide-tagged NBD, and DBCO-tagged Fluor488 dyes (upper), and control sections treated with non-tagged CA and NBD and Fluor488 dyes (lower). (B) Root differentiation zone of Arabidopsis seedlings labeled with CA-Az and subsequently derivatized with NBD-Alk (upper) and control root labeled with non-tagged CA and NBD-Alk (lower). Propidium iodide (PI) was used as an apoplastic tracer. Asterisks and arrowheads indicate where xylem and Casparian strip CWs are visualized. Scale bars denote 50 μm.

observed in UV-vis and fluorescence spectra (Fig. S3, ESI†).

In parallel, we also performed SPAAC reactions of GDHP-Az by simply mixing it with a bicyclo[6.1.0]nonyne-tagged NBD dye (NBD-BCN) in DMSO-water. The click linkage in the conjugated DHP was likewise confirmed by 2D NMR (Fig. 2C, and Fig. S3C, ESI†), and optical spectroscopy (Fig. S4, ESI†). Unlike what was observed for GDHP-Az:NBD-Alk prepared by CuAAC, the NMR signals from unreacted alkyl azide groups were still visible in the HSQC spectrum of GDHP-Az:NBD-BCN prepared by SPAAC (conversion, ~60%, based on the HSQC contour intensities) (Fig. S3, ESI†). It is likely that CuAAC is more efficient than SPAAC, whereas the latter clearly has an advantage of avoiding the use of toxic copper reagents that could be an issue especially in live-cell imaging; Anderson et al. described the toxicity of CuAAC reagents affecting the growth of *Arabidopsis thaliana*.[8]

At the onset of our imaging studies with the chemical reporter-tagged monolignols, we performed lignin labeling of the developing Arabidopsis stems; live stems were pre-sectioned and immediately treated with the probes for metabolic labeling and *in vivo* click chemistry. Freshly cut stems sectioned from *Arabidopsis thaliana* Col-0 ecotypes (12-week-old) were incubated with 100 μM CA-Az or CA-Alk for 1 h. For CuAAC labeling, the azide- and alkyne-tagged sections were incubated for 1 h with 1 μM NBD-Alk and NBD-Az in the presence of 1 mM $CuSO_4$, 1 mM sodium ascorbate, and 200 μM THPTA. In parallel, SPAAC labeling was performed by treating the azide-tagged sections with 1 μM Fluor488-dibenzocyclooctyn (Fluor488-DBCO, Sigma-Aldrich). The labeled sections were then directly analyzed by confocal laser scanning microscopy

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(CLSM) (Fig. 3A, and Fig. S5A, ESI†). As expected, intense fluorescent signals were specifically detected in the apoplastic compartment (cell corners, compound middle lamella, and CWs) of the vascular and interfascicular tissues where lignins are typically produced.[4a] In contrast, the control sections treated with non-tagged CA exhibited significantly weaker fluorescence under the same imaging conditions (Fig. S5B, ESI†). The results indicate that the fluorescence is primarily dependent on the click reactions, and that the contribution from non-specific binding of the click dye and/or autofluorescence from lignified CWs could be minimal. Administrating the probe in the presence of potassium iodide, a strong scavenger of the H_2O_2 that is required for peroxidase-mediated lignin polymerization,[10] substantially reduced the probe's incorporation (Fig. S6, ESI†), providing additional support for our contention that the monolignol mimics are incorporated into CWs only when and where lignification occurs. The fluorescence intensity can easily be quantified (Fig. S5C, ESI†), which allows closer analysis of lignification variations between different cell wall compartments, cell/tissue types, and developmental stages.

Lastly, we performed *in vivo* lignin labeling in growing Arabidopsis seedlings. Each seedling (3 days old) was supplied with a 0.1% agar droplet containing liquid MS medium supplemented with 100 μM CA-Az for 2 days, and subsequently treated with 1 μM NBD-Alk, 1 mM $CuSO_4$, 1 mM ascorbic acid, and 200 μM THPTA for *in vivo* click labeling. Propidium iodide was used to stain apoplastic compartments.[10] The seedlings labeled with CA-Az and NBD-Alk exhibited NBD fluorescence throughout root tissues. In the differentiation zone of the root labeled with CA-Az (Fig. 3B), the fluorescence was primarily observed within stele and, in particular, strong fluorescence was seen in the developing spiral xylem (indicated by asterisks) where lignins are actively produced. In addition, the labeling in Casparian strips where lignins are also specifically produced[10] was evident by the stub-like NBD fluorescence seen in the endodermal CW layers (indicated by arrow heads). A similar result was observed in our analogous experiments using fluorescence-tagged monolignols.[4a]

Overall, we have demonstrated the feasibility of using the new chemical reporter-tagged monolignols for facile metabolic labeling and visualization of lignins *in planta*. The possibility of using numerous detection tags through bioorthogonal click chemistry should greatly expand our ability to monitor cell wall lignification processes more accurately in complex biological systems. In combination with other biochemical and cell biological techniques, the current lignin imaging method should further improve our understanding of plant cell wall structure and development. In addition, azide- and alkyne-tagged monolignols as well as their lignin polymers synthesized in this study could be useful precursors for a new type of bioconjugate bearing monolignols or lignin molecules for various research purposes.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental protocols, spectroscopic data, and additional plant images. See DOI: 10.1039/x000000x/

1 N. D. Bonawitz and C. Chapple, *Annu. Rev. Genet.*, 2010, **44**, 337
2 (a) Y. Zeng, S. Zhao, S. Yang and S.-Y. Ding, *Curr. Opin. Biotechnol.*, 2014, **27**, 38; (b) C. G. Wilkerson, S. D. Mansfield, F. Lu, S. Withers, J. Y. Park, S. D. Karlen, E. Gonzales-Vigil, D. Padmakshan, F. Unda, J. Rencoret and J. Ralph, *Science*, 2014, **344**, 90; (c) A. J. Ragauskas, G. T. Beckham, M. J. Biddy, R. Chandra, F. Chen, M. F. Davis, B. H. Davison, R. A. Dixon, P. Gilna, M. Keller, P. Langan, A. K. Naskar, J. N. Saddler, T. J. Tschaplinski, G. A. Tuskan and C. E. Wyman, *Science*, 2014, **344**, 1246843; (d) N. D. Bonawitz, J. I. Kim, Y. Tobimatsu, P. N. Ciesielski, N. A. Anderson, E. Ximenes, J. Maeda, J. Ralph, B. S. Donohoe, M. Ladisch and C. Chapple, *Nature*, 2014, **509**, 376; (e) R. Vanholme, I. Cesarino, K. Rataj, Y. Xiao, L. Sundin, G. Goeminne, H. Kim, J. Cross, K. Morreel, P. Araujo, L. Welsh, J. Haustraete, C. McClellan, B. Vanholme, J. Ralph, G. Simpson, C. Halpin and W. Boerjan, *Science*, 2013, **341**, 1103; (f) N. D. Bonawitz and C. Chapple, *Curr. Opin. Biotechnol.*, 2013, **24**, 336
3 (a) E. Pesquet, B. Zhang, A. Gorzsas, T. Puhakainen, H. Serk, S. Escamez, O. Barbier, L. Gerber, C. Courtois-Moreau, E. Alatalo, L. Paulin, J. Kangasjarvi, B. Sundberg, D. Goffner and H. Tuominen, *Plant Cell*, 2013, **25**, 1314; (b) M. Schuetz, R. Smith and B. Ellis, *J Exp Bot*, 2013, **64**, 11; (c) R. A. Smith, M. Schuetz, M. Roach, S. D. Mansfield, B. Ellis and L. Samuels, *Plant Cell*, 2013, **25**, 3988; (d) S. Alejandro, Y. Lee, T. Tohge, D. Sudre, S. Osorio, J. Park, L. Bovet, Y. Lee, N. Geldner, A. R. Fernie and E. Martinoia, *Curr. Biol.*, 2012, **22**, 1207; (e) Y.-C. Miao and C.-J. Liu, *Proc. Natl. Acad. Sci.*, 2010, **107**, 22728.
4 (a) Y. Tobimatsu, A. Wagner, L. Donaldson, P. Mitra, C. Niculaes, O. Dima, J.-I. Kim, N. A. Anderson, D. Loque, W. Boerjan, C. Chapple and J. Ralph, *Plant J.*, 2013, **76**, 357; (b) Y. Tobimatsu, C. L. Davidson, J. H. Grabber and J. Ralph, *Biomacromol.*, 2011, **12**, 1752.
5 (a) M. Boyce and C. R. Bertozzi, *Nat. Methods*, 2011, **8**, 638; (b) E. M. Sletten and C. R. Bertozzi, *Acc. Chem. Res*, 2011, **44**, 666; (c) C. Wangler, R. Schirrmacher, P. Bartenstein and B. Wangler, *Curr. Med. Chem.*, 2010, **17**, 1092; (d) J. C. Jewett and C. R. Bertozzi, *Chem. Soc. Rev.*, 2010, **39**, 1272; (e) E. M. Sletten and C. R. Bertozzi, *Angew. Chem. Int. Ed.*, 2009, **48**, 6974; (f) M. D. Best, *Biochem.*, 2009, **48**, 6571; (g) J. A. Prescher and C. R. Bertozzi, *Nat. Chem. Biol.*, 2005, **1**, 13.
6 H. C. Kolb, M. G. Finn and K. B. Sharpless, *Angew. Chem. Int. Ed.*, 2001, **40**, 2004.
7 (a) P. V. Chang, J. A. Prescher, E. M. Sletten, J. M. Baskin, I. A. Miller, N. J. Agard, A. Lo and C. R. Bertozzi, *Proc. Natl. Acad. Sci.*, 2010, **107**, 1821;(b) S. T. Laughlin, J. M. Baskin, S. L. Amacher and C. R. Bertozzi, *Science*, 2008, **320**, 664; (c) J. M. Baskin, J. A. Prescher, S. T. Laughlin, N. J. Agard, P. V. Chang, I. A. Miller, A. Lo, J. A. Codelli and C. R. Bertozzit, *Proc. Natl. Acad. Sci.*, 2007, **104**, 16793.
8 C. T. Anderson, I. S. Wallace and C. R. Somerville, *Proc. Natl. Acad. Sci.*, 2012, **109**, 1329.
9 (a) Y. Tobimatsu, F. Chen, J. Nakashima, L. Jackson, L. L. Escamilla-Treviño, R. A. Dixon and J. Ralph, *Plant Cell*, 2013, **25**, 2587; (b) Y. Tobimatsu, S. Elumalai, J. H. Grabber, C. L. Davidson, X. Pan and J. Ralph, *ChemSusChem*, 2012, **5**, 676.
10 Y. Lee, M. C. Rubio, J. Alassimone and N. Geldner, *Cell*, 2013, **153**, 402.

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