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1. Supplementary Methods

1.1. Solution preparation

Firstly, Murashige and Skoog (MS) medium was prepared by dissolving 2.2 g MS basal medium and 0.6 g 4-morpholineethanesulfonic acid in 1 L sterile distilled water. Subsequently, alkynyl-labelled sinapyl alcohol (S_{ALK}) solutions with concentrations ranging from 0.01 to 1.00 mM were prepared using MS medium. Finally, CuAAC solutions were formulated with MS medium, containing either 2.5 mM ascorbic acid, 0.5 mM copper sulfate and 5 μ M Azide-fluor 545 or 25 mM ascorbic acid, 5 mM copper sulfate and 50 μ M Azide-fluor 545.

1.1. Cultivation of *Nerium oleander* stem sections with alkyne-tagged sinapyl alcohol at different concentrations

Two-month-flowered *N. oleander* plants were selected and stem sections more than 10 cm above the root were cut into approximately 200 μ m thick slices. These slices were then placed in cell culture plates containing MS solution for later use. In a 48-well cell culture plate, 300 μ L of pre-prepared S_{ALK} solutions with concentrations of 25, 50, 75 and 100 mM were added to each well. The stem sections were cultured under a cycle of 15 hr light and 9 hr darkness at 25 °C to investigate the absorption of *N. oleander* stem sections at different concentrations. Eight to ten *N. oleander* stem sections were placed in each well.

After cultivation, the S_{ALK} -containing monomer solution was aspirated using a pipette. Under room temperature in dark, the slices were washed eight times with 1 mL MS solution by shaking for 10 min each time. Subsequently, MS solution was removed and 300 μ L of CuAAC solution (containing 25 mM ascorbic acid, 5 mM copper sulfate and 50 μ M Azide-fluor 545) was added and shaken for 1 hr under the same conditions. After shaking, the CuAAC solution was removed and 1 mL of MS solution was added to repeat the initial washing operation twice. Then, the MS solution was removed and 500 μ L of 70 % (v/v) methanol-water solution was added to wash the unreacted CuAAC solution by shaking for 1 hr under room temperature. This step was repeated once for 30 min. After washing, the slices were stored in an antifade reagent. For detection, the slices were mounted on slides and observed under a confocal laser scanning microscope (LEICA SP8). The images were analysed using ImageJ software. When mounting, it is important to avoid the formation of bubbles under the cover slip. The prepared slides can be stored at low temperatures for several weeks.

1.2. Cultivation of *Nerium oleander* stem sections with alkyne-tagged sinapyl alcohol at different time intervals

The cultivation process was the same as above section, with 100 mM S_{ALK} solution. The cultivation times were 0.5, 1, 2, 12 and 24 hr (including 9 hr of darkness) and 48 hr (including 18 hr of darkness). Eight to ten *N. oleander* stem sections were added to the wells containing the monomer solution.

1.3. Fourier-transform infrared spectroscopy (FT-IR) detection

One milligram of sample was uniformly mixed with 100 mg of anhydrous potassium bromide that had been dried. The mixture was ground repeatedly in an agate mortar until it was well mixed. An

appropriate amount of the powder was pressed into a tablet at 20 MPa for 2–3 min. The sample was scanned using a FT-IR (Nicolet 6700, Thermofisher Nicolet) in transmission mode. The scanning range was 4000–5000 cm^{-1} , with a resolution of 2 cm^{-1} and 64 scans. The obtained spectra were processed using OMNIC software.

2. Results and Discussion

2.1. Structural analysis of 10-year-old *Nerium oleander* cellulase-enzymatic lignin

2.1.1. Fourier transform-infrared spectroscopy (FT-IR) detection

Infrared spectroscopy analysis can provide preliminary insights into the structural characteristics of cellulase-enzymatic lignin (CEL), thereby revealing key information about its functional groups and linkages. As shown in the FT-IR spectrum of CEL in Fig. S1, absorption peak at 3424 cm^{-1} was corresponded to stretching vibration of hydroxyl groups (-OH), indicating presence of abundant phenolic and/or alcoholic hydroxyl groups. The absorption peaks at 1590 cm^{-1} and 1500 cm^{-1} were attributed to aromatic ring (C=C stretching vibrations), further confirming the existence of aromatic rings. The peak at 1460 cm^{-1} was related to bending vibrations of methylene (-CH₂-) and methyl (-CH₃) groups, while the peak at 1425 cm^{-1} indicated the presence of methoxy (-OCH₃) stretching vibrations.

Moreover, absorption peak at 1325 cm^{-1} may be associated with bending vibration of hydroxyl groups (-OH), particularly phenolic hydroxyl groups. The peak at 1120 cm^{-1} was from stretching vibration of ether linkages (C-O-C), indicating presence of structural units connected by ether bonds in CEL. The peak at 1033 cm^{-1} was related to the C-O stretching vibration on aromatic ring. The absorption peak at 830 cm^{-1} indicated monosubstitution or para-substitution characteristics of aromatic ring, which was consistent with the typical structure of lignin. In summary, these characteristic peaks indicated that CEL has typical lignin structural features, including functional groups and linkages such as hydroxyl, aromatic rings, methoxy and ether bonds. These structural features were highly similar to those of natural lignin, further confirming chemical structural consistency between CEL and natural lignin.

2.1.2. Gel permeation chromatography (GPC) molecular weight detection

As shown in Table S1, weight-average molecular weight (M_w), number-average molecular weight (M_n) and polydispersity index (M_w/M_n , PDI) of CEL (CEL_{Blank}, CEL_{Control}, CEL₁, CEL₂, CEL₃ and CEL₄) were analysed to provide a comprehensive understanding of the molecular weight characteristics and distribution of each sample. It can be observed that there was no obvious difference in the molecular weight distribution among these samples. Specifically, M_w of CEL's ranged from 17615–22801 Da, M_n was from 6241–7904 Da, with PDI from 2.47–3.51. It was indicated that although there were some differences among different samples, overall molecular weight distribution range and dispersity were relatively close. Lignin is a complex high molecular weight polymer with a wide range of molecular weights. Moreover, the molecular weight distribution of lignin is influenced by various factors such as its source and extraction method (1). Overall, molecular weight distribution of CEL was close to that of natural lignin and molecular weight of CEL tended to decrease with increasing cultivation time.

2.1.3. Incorporation of alkyne-tagged lignin in *Nerium oleander* seedlings

The blue fluorescence at wavelength of 405 nm represents total natural lignin accumulated by the plant itself before sectioning and its fluorescence intensity remains almost unchanged. As shown in Fig. S2, with the increase of G_{ALK} and S_{ALK} concentrations, fluorescence intensity at wavelength of 552 nm gradually increased. The increase rate was fast in the initial stage (1–25 μM), while it slowed down in the later stage (50–100 μM) and reached the highest value at concentration of 100 μM . A similar phenomenon was also observed before of cultivating *Arabidopsis* with artificially synthesised lignin (2).

The labelled lignin precursors into *Arabidopsis* and it was observed that fluorescence signal intensity gradually increased with extension of cultivation time, reaching peak at 24 hr (3). As shown in Fig. S3, similar results were obtained using alkyne-tagged coniferyl alcohol and sinapyl alcohol and it was found that the fluorescence intensity also increased with the increase of cultivation time, reaching the highest level within 24 hr and then a lower fluorescence at 48 hr.

In plants, under the catalysis of enzymes, alkynes may form β -ether bonds and other structures through radical coupling reactions with quinone intermediates. With the extension of cultivation time, the number of these β -ether bond structures formed by alkynes increased, reducing the number of alkynes available for click chemistry reactions with AF545 and thus leading to a decrease in red fluorescence signal. This phenomenon indicated that the polymerisation and cross-linking reactions of alkyne-tagged lignin precursors in plants are dynamically changing and these changes might affect fluorescence signal intensity.

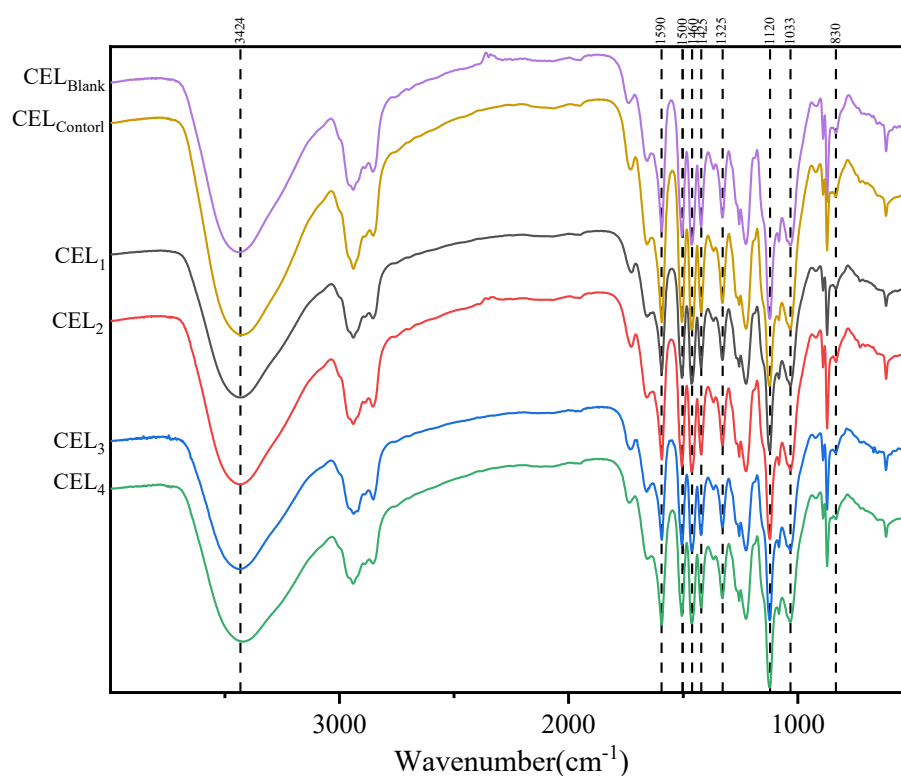
2.1.4. Incorporation of alkyne-tagged lignin in *Broussonetia papyrifera*

During investigation of lignification in *B. papyrifera*, it was found that lignin deposition was mainly confined to vessel elements. This difference resulted in a relatively lower fluorescence intensity of *B. papyrifera* compared with *N. oleander* in overall observation. Nevertheless, fluorescence variation trend of *B. papyrifera* in concentration and time-variable groups showed similarity to that of *N. oleander*. As shown in Fig. S4 and S5, overall fluorescence intensity of *B. papyrifera* sections gradually increased with increase of lignin precursors and reached a peak at concentration of 100 μ M. Furthermore, fluorescence intensity of G_{ALK} and S_{ALK} increased with extension of cultivation time and reached maximum at 24 hr. However, when the cultivation time was further extended to 48 hr, the fluorescence intensity decreased. This phenomenon may be related to the dynamics of lignin biosynthesis and deposition in plant cells. Within 24 hr, the deposition and polymerisation reactions of lignin precursors were relatively active, leading to an increase in fluorescence intensity; whereas after 48 hr, the fluorescence intensity may decrease due to changes in certain metabolic pathways or the consumption of lignin precursors. These observations were consistent with the findings, where a similar fluorescence variation trend was also observed (4).

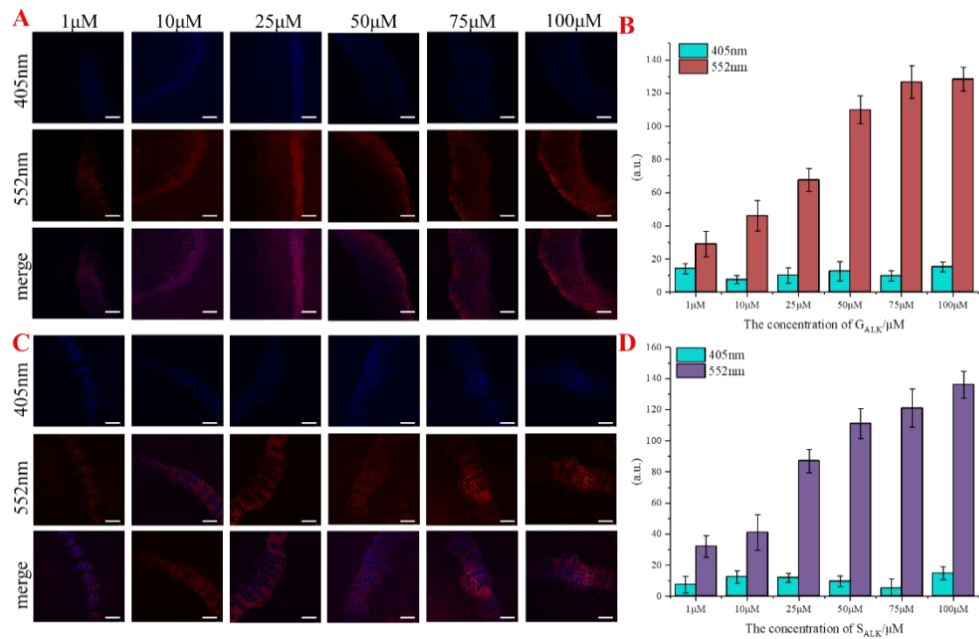
Compared with lignification pattern of fibre tracheids in *N. oleander*, *B. papyrifera* vessels exhibited a unique fluorescence variation pattern. Through fluorescence microscopic analysis, it was found that areas with red fluorescence were limited to vessels, while surrounding crystal cells and parenchyma cells showed no fluorescence signals. This indicated that lignin deposition was highly specifically confined to vessel structure, resulting in a significantly lower overall fluorescence intensity of *B. papyrifera* compared with *N. oleander*.

References

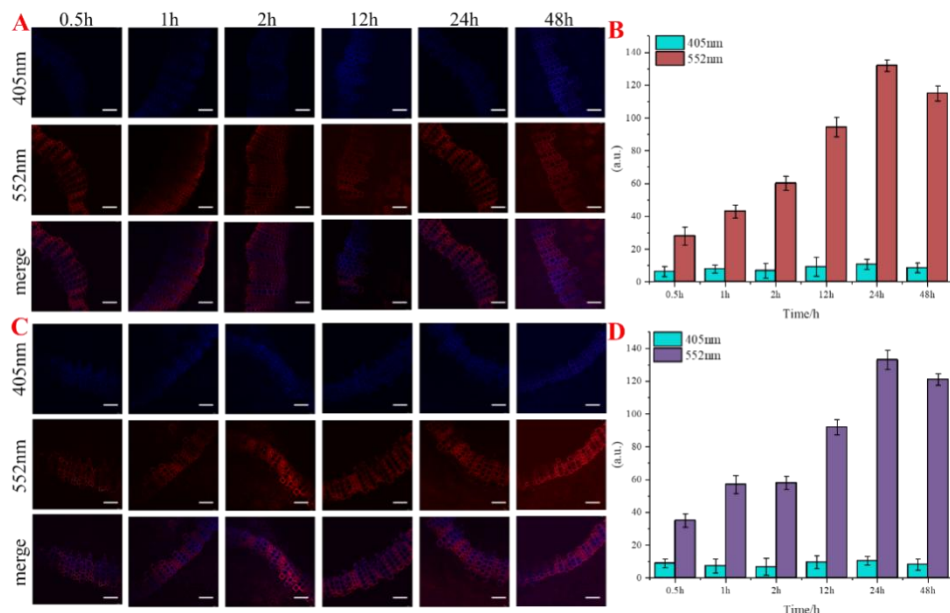
1. Lobbes JM, Fitznar HP, Kattner G. High-performance liquid chromatography of lignin-derived phenols in environmental samples with diode array detection. *Anal Chem.* 1999;71(15):3008–12. <https://doi.org/10.1021/ac981175i>
2. Lion C, Simon C, Huss B, Blervacq AS, Tirot L, Toybou D, et al. BLISS: A bioorthogonal dual-labeling strategy to unravel lignification dynamics in plants. *Cell Chem Biol.* 2017;24(3):326–38. <https://doi.org/10.1016/j.chembiol.2017.02.009>
3. Pandey JL, Kiemle SN, Richard TL, Zhu Y, Cosgrove DJ, Anderson CT. Investigating biochemical and developmental dependencies of lignification with a click-compatible monolignol analog in *Arabidopsis thaliana* stems. *Front Plant Sci.* 2016;7:1309. <https://doi.org/10.3389/fpls.2016.01309>
4. Simon C, Lion C, Spriet C, Baldacci-Cresp F, Hawkins S, Biot C. One, two, three: a bioorthogonal triple labelling strategy for studying the dynamics of plant cell wall formation *in vivo*. *Angew Chem.* 2019;57(51):16665–71. <https://doi.org/10.1002/anie.201808493>



Supplementary Fig. S1. Fourier transform-infrared spectroscopy spectra of cellulase-enzymatic lignin.

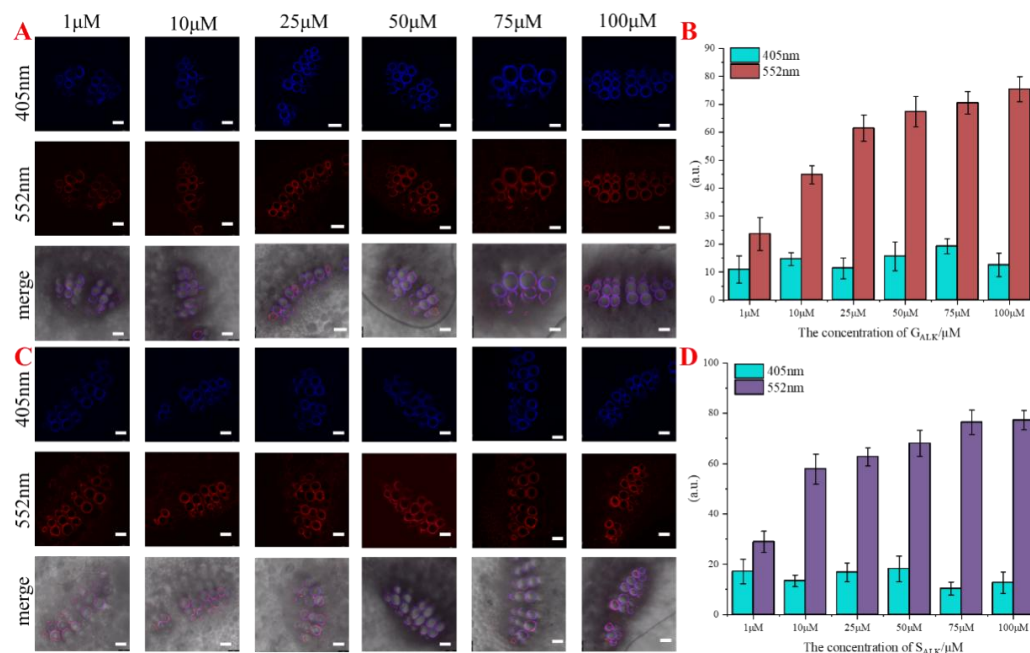


Supplementary Fig. S2. Fluorescence images of *Nerium oleander* seedlings treated with G_{ALK} and S_{ALK} precursors. (A) Fluorescence images of *Nerium oleander* seedling sections incubated in 1–100 μM G_{ALK} precursor solutions; (B) quantitative analysis of fluorescence intensity in (A); (C) fluorescence images of *Nerium oleander* seedling sections incubated in 1–100 μM S_{ALK} precursor solutions; (D) quantitative analysis of fluorescence intensity in (C). Scale bar: 100 μm .

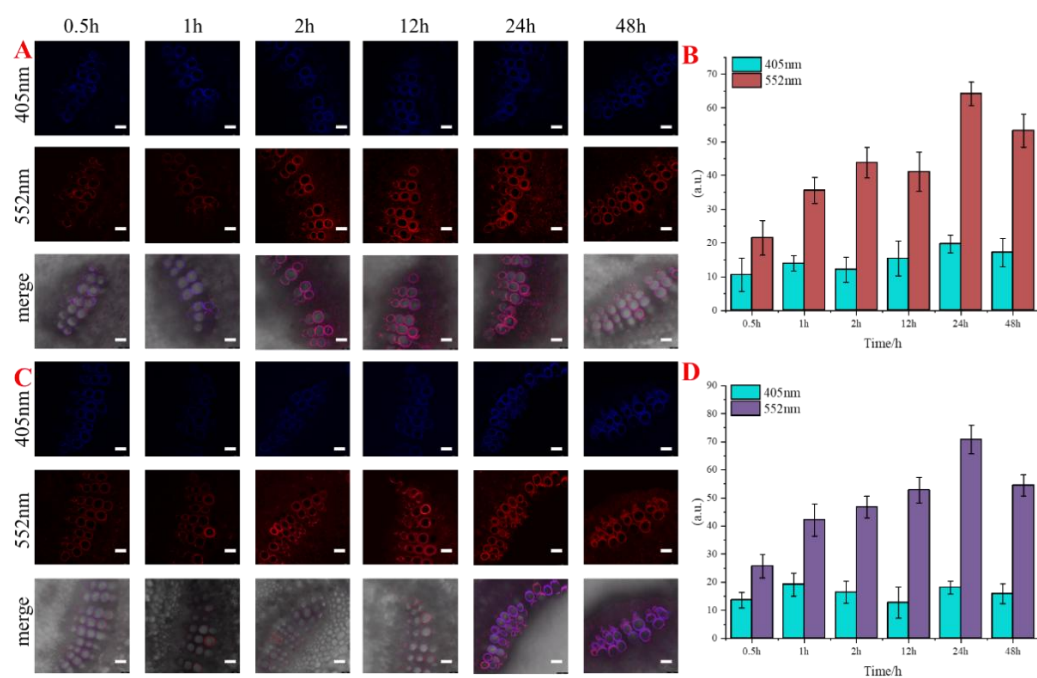


Supplementary Fig. S3. Fluorescence images of *Nerium oleander* seedlings treated with time gradient of G_{ALK} and S_{ALK} precursors. (A) Fluorescence images of *Nerium oleander* seedling sections incubated in G_{ALK} precursor solutions for 0.5–48 hr; (B) quantitative analysis of fluorescence intensity

in (A); (C) fluorescence images of *Nerium oleander* seedling sections incubated in S_{ALK} precursor solutions for 0.5–48 hr; (D) quantitative analysis of fluorescence intensity in (C). Scale bar: 100 μ m.



Supplementary Fig. S4. The overall fluorescence intensity changes in *Broussonetia papyrifera* stem section under different concentrations of G_{ALK} and S_{ALK} after 24-hr culture. (A, B) Fluorescence images and quantitative bar charts of G_{ALK} as precursor with concentration from 1 to 100 μ M; (C, D) fluorescence images and quantitative bar charts of S_{ALK} as precursor with concentration from 1–100 μ M. Scale bar: 100 μ m.



Supplementary Fig. S5. The overall fluorescence intensity changes in *Broussonetia papyrifera* stem section under different time points of G_{ALK} and S_{ALK} with a concentration of 100 μ M. (A, B) Fluorescence images and quantitative bar charts of G_{ALK} as precursor with time from 0.5–48 hr; (C, D) fluorescence images and quantitative bar charts of S_{ALK} as precursor with time from 0.5–48 hr. Scale bar: 100 μ m.

Supplementary Table S1. Molecular weights of cellulase-enzymatic lignin (CEL)

	CEL _{Blank}	CEL _{Control}	CEL ₁	CEL ₂	CEL ₃	CEL ₄
M_w /(Dal)	19786	22801	2186	1960	1761	1811
			4	2	5	2
M_n /(Dal)	6241	6491	7086	7904	7120	6825
M_w/M_n	3.17	3.51	3.09	2.48	2.47	2.66