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ARTICLE ADDENDUM

BLISS: Shining a light on lignification in plants

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ABSTRACT

Lignin is a polyphenolic polymer of the plant cell wall formed by the oxidative polymerization of 3 main monomers called monolignols that give rise to the lignin H-, G- and S-units. Together with cellulose and hemicelluloses, lignin is a major component of plant biomass that is widely exploited by humans in numerous industrial processes. Despite recent advances in our understanding of monolignol biosynthesis, our current understanding of the spatio-temporal regulation of their transport and polymerization is more limited. In a recent publication, we have reported the development of an original Bioorthogonal Labeling Imaging Sequential Strategy (**BLISS**) that allows us to visualize the simultaneous incorporation dynamics of H and G monolignol reporters into lignifying cell walls of the flax stem.[11] Here, we extend the application of this strategy to other plant organs such as roots and rapidly discuss some of the contributions and perspectives of this new technique for improving our understanding of the lignification process in plants.

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Together with chloroplasts and vacuoles, the presence of cell walls is one of the distinctive structural features that distinguish plant cells from animal cells. Cell walls of land plants are composed of cellulose, hemicellulose and pectins.[1] In certain specialized tissues (e.g., xylem, sclerenchyma) the cell walls become reinforced by a 3-dimensional phenolic polymer called lignin that can constitute up to 25% of the cell wall dry weight.[2] The lignin polymer is essential for plants as it plays a major role in mechanical support, water transport, wound healing and defense against pathogens, and has contributed to their successful colonization of the terrestrial habitat.[3] It is also an important parameter that influences the quality of plant-based bio-resources in many industrial areas including timber, paper and textile fiber production, animal feed, bio-fuels etc.[4,5]

Lignin is progressively assembled in the cell wall by an oxidative polymerization process in which the monomeric units (monolignols) are first enzymatically oxidized by peroxidases and/or laccases to form free radicals that then undergo spontaneous polymerization. In angiosperms, three main monolignols (*p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) are oxidized giving rise to the H (hydroxyphenyl) units, G (guaiacyl) units and S (syringyl) units of the lignin polymer, respectively. In gymnosperm lignin, S units are absent although recent work has shown that they can be induced by targeted genetic engineering.[2,6,7]

Given the biologic and industrial importance of lignin, its biosynthesis has long been studied by a combination of biochemical, molecular and genetic approaches. For imaging studies, lignin can be visualized *in situ* by a variety of different techniques including histochemistry, UV-autofluorescence, immunolocalisation, and spectroscopy (e.g., Infra-red, Raman, X-ray). While certain of these techniques can produce high-resolution images, they all present the disadvantage of not being able to distinguish newly deposited lignin from pre-existing lignin thereby making detailed studies of lignification dynamics complicated. However, thanks to the expansion of the chemical toolbox for biomolecular imaging applications, the plant biologist can now study this process quite easily.[8] Methods using bioorthogonal chemical reporters have been successfully developed and applied *in vivo*.[9,10,11] In these approaches chimeric monolignols bearing an azide or an alkyne are metabolically introduced into lignifying cell walls and specifically derivatized with a fluorescent dye by bioorthogonal click chemistry allowing incorporation to be followed *in vivo*. The facility of this technique promises to simplify cell wall studies compared with classical methods such as radioisotope labeling or indirect profiling using antibodies. In a recent article, we reported the dual labeling BLISS sequential combination of two well-known click reactions: the strain-promoted alkyne-azide cycloaddition (SPAAC) reaction and the copper-catalyzed alkyne-azide cycloaddition (CuAAC) reaction.[11] The major advantage of SPAAC and CuAAC is their operational simplicity, making the application of these bioorthogonal couplings extremely straightforward.

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Using this approach we incorporated the two different chimeric monolignols H_{AZ} and G_{ALK} (tagged with an azide and alkyne chemical handle, respectively) into lignifying cell walls of the flax stem. The BLISS methodology generates precise 3-color localization maps reflecting the differential incorporation of H_{AZ} and/or G_{ALK} chemical reporters into freshly synthesized lignin *vs.* preexisting lignin - the latter being observed by autofluorescence. It thus provides spatial information on the presence or absence of active lignification machinery between different tissues, between different cell types and within different wall layers of the same cell. Although lacking many of the genetic resources and tools that are available for Arabidopsis, flax is an economically relevant species and represents an interesting biologic model for evaluating the dual bioorthogonal lignin labeling strategy as the stem of this plant contains two distinct populations of cells with contrasted cell wall composition.[12] Xylem cells present in inner-stem tissues have walls that contain approximately 25% lignin whereas bast fibers, present in outer-stem tissues contain much lower amounts (typically 2–4%).[13,14]

As well as a proof of concept paper demonstrating the robustness of this technique, this work generated results that are of interest for the whole scientific community studying lignification,[11] as well as for researchers working on bast fiber cell wall biology in flax and other related species such as hemp, jute and ramie. Similar amounts of both H_{AZ} and G_{ALK} chemical reporters were incorporated into lignifying xylem cell walls when supplied in equal quantities despite the fact that 'natural' flax lignin, like that of most angiosperms, only contains low amounts of H-lignin. Furthermore, the relative incorporation of each reporter could be simply modified by changing the H_{AZ}/G_{ALK} ratio in the feeding medium. These observations indicate that lignin monomer composition is most likely regulated by monomer supply and not by the specificity of cell wall oxidizing enzymes for certain monolignol substrates. This is in agreement with numerous observations showing that targeted downregulation of specific monolignol biosynthesis genes modifies lignin structure. Nevertheless, the fact that H_{AZ}- and G_{ALK}-units did not always co-localize to the same subdomain of the cell wall would suggest that other factors such as the nature of the polysaccharide matrix and/or localization of the oxidizing enzymes could also play a part in regulating monolignol incorporation into lignin.[15,16]

The observation that H_{AZ}- and G_{ALK}-unit chemical reporter incorporation into bast fiber cell walls remains limited under the same conditions that allow substantial incorporation into xylem cell walls strongly suggests that bast fiber hypolignification results from an absence of cell wall machinery capable of oxidizing monolignols. Certainly, this hypothesis is in agreement with the observation that ectopic lignification in the flax *lbf1* mutant is associated with a significant increase in the accumulation of lignin-related peroxidase transcripts.[17]

In this Article Addendum we report that BLISS is also suitable for investigating lignification dynamics in other plant organs such as roots (Fig. 1) and discuss the new possibilities that this methodology opens up for the fine study of lignification dynamics. As previously observed in stems, the fluorescent signal in flax roots is most intense in the first few layers of the secondary xylem actively undergoing lignification. As shown by a more detailed analysis (Fig. 2),

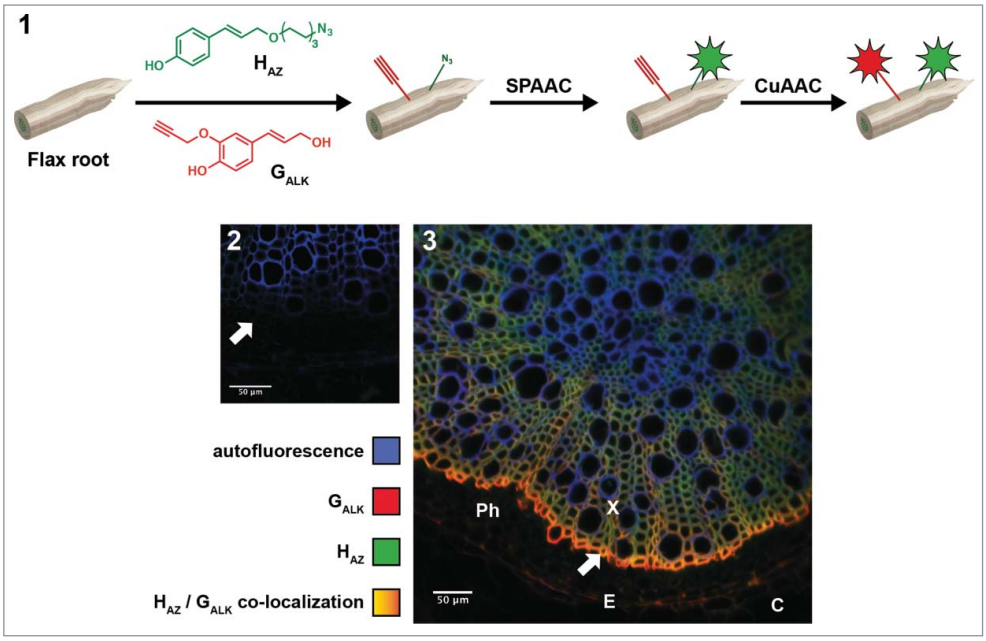

Figure 1. Bioorthogonal Labeling Imaging Sequential Strategy (**BLISS**) illustrating incorporation of monolignol chemical reporters into cell walls in 2-month-old flax roots. (1) Dual labeling strategy consisting of monolignol feeding followed by specific click ligation of fluorescent probes to incorporated H_{AZ}-units (SPAAC) and G_{ALK}-units (CuAAc) in freshly synthesized lignin. (2,3) View of BLISS-labeled hand section of flax roots previously incubated with native *p*-coumaryl and coniferyl alcohols as negative control (2), or with azide-labeled *p*-coumaryl alcohol (H_{AZ}) and alkyne-labeled coniferyl alcohol (G_{ALK}) monolignol reporters (3) and observed by confocal microscopy.[11] Blue channel: lignin autofluorescence (405 nm); green channel: H_{AZ}-unit fluorescence (526 nm); red channel: G_{ALK}-unit fluorescence (565 nm). Xylem (X), phloem (Ph), endodermis (E), cortex (C), differentiating xylem (arrow).

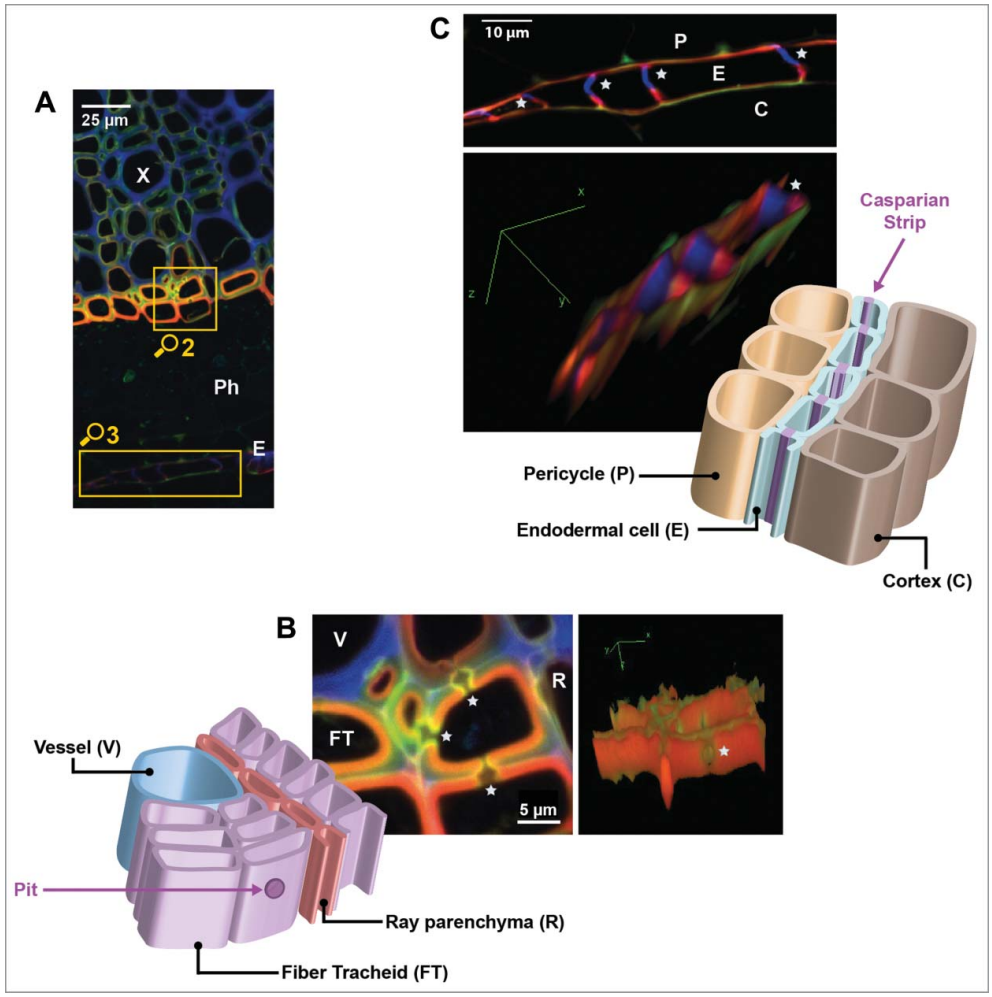

Figure 2. Confocal microscope imaging of monolignol chemical reporter incorporation into cell walls of 2-month-old flax roots. (1) View of BLISS-labeled root secondary xylem (X), phloem (Ph) and endodermis (E) with merged lignin autofluorescence channel (blue, 405 nm), H_{AZ} fluorescence (green, 526 nm) channel and G_{ALK} (red, 565 nm) channel. (2) 2D slice and 3D reconstruction of confocal z-stack zoom of a secondary xylem fiber-tracheid and neighboring cells in xylem differentiation zone. Note differential incorporation of chemical reporters *vs* autofluorescence highlighting cell wall layers of fiber tracheids (FT), vessels (V) or ray cells (R) as well as cell wall substructures such as pits (⋆). (3) 2D slice and 3D reconstruction of confocal z-stack zoom of root endodermis region showing incorporation of monolignol chemical reporters into radial and tangential cell walls of endodermal cells. The Casparian strip (⋆) only displays autofluorescence and does not incorporate H_{AZ} or G_{ALK}.

BLISS also provided evidence for the existence of specific (i.e., H_{AZ} vs G_{ALK}) monolignol-incorporation capacity in different wall domains of the same cell. For example, the bordered pits connecting adjacent cells in the differentiating xylem zone depicted in Fig. 2B can be clearly distinguished from the rest of the cell wall by their yellow color indicating H_{AZ} and G_{ALK} co-localization, whereas the orange/red color of the S2 secondary cell wall layer indicates that less H_{AZ} reporter is incorporated under the same conditions in this cell wall region.

Of interest is the fact that chemical reporters are also incorporated into the walls of cells in the endodermis (Fig. 2C). This cell layer forms an apoplastic transport barrier contributing to the selective uptake capacities of plant roots as well as restricting radial oxygen loss and microorganism access.[18] During the early stages of development the endodermal cell wall is characterized by the presence of a Casparian strip (CS) on the radial and transverse walls that can later spread to other cell walls. The H_{AZ} and G_{ALK} reporters are clearly incorporated into tangential walls and the parts of the radial walls where the CS is absent, but not at all in the CS itself. This indicates that the CS is mature at this stage of plant development while the other walls are still undergoing further polymer deposition. Endodermal cell walls contain both suberin and lignin and have been shown to possess the enzymatic machinery capable of oxidizing monolignols.[19,20,21]

Labeling is also observed (Fig. 3) in the walls of certain cells in the cortical layer immediately exterior to the endodermis (as well as in cell wall corners of certain pericycle cells) suggesting that the flax root cortex may well contain additional lignified/suberized cell layers as previously observed in rice.[22] The comparison of endodermal and cortical cell wall labeling in this region shows that these two cell types show very different patterns of monolignol reporter incorporation. Cortical cells show a marked preference for H_{AZ} reporters while endodermal cells preferentially incorporate G_{ALK} reporters suggesting the existence of cell-specific monolignol-oxidizing machinery and/or cell wall structure in these two neighboring cell types.

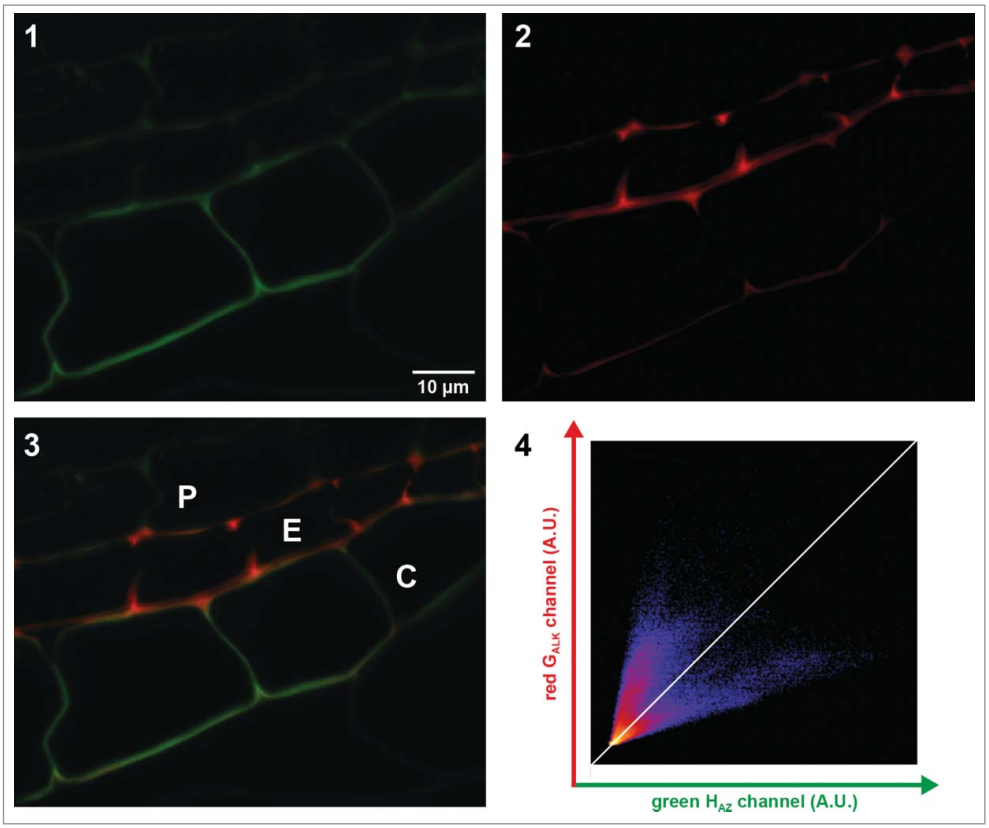

Figure 3. Monolignol chemical reporter incorporation into endodermal and inner cortical cell walls of 2-month-old flax roots. (1) H_{AZ} channel, green, 526 nm; (2) G_{ALK} channel, red, 565 nm; (3) merged H_{AZ} and G_{ALK} channels; (4) associated fluorogram illustrating the anti-correlation between G_{ALK} and H_{AZ} incorporation in this region. Indeed, few pixels are found on the co-localization diagonal except for the background while high green fluorescence is associated to low red signal (cortex) and vice-versa (endodermis). Pericycle (P), Endodermis (E), Cortex (C).

Conclusion

In conclusion, the development of a dual labeling technique that allows co-visualization by 2-color fluorescence detection at subcellular levels opens up exciting new perspectives for studying lignin biosynthesis in different plant organs and tissues. BLISS is capable of not only revealing differences in monolignol incorporation between different cell types, but is also able to detect differences between different cell wall layers/sub-structures within the same cell. Furthermore, such an approach can also be used for studying the dynamics of sugar incorporation into cell wall polysaccharides.[23,24,25] Such knowledge should ultimately contribute to better engineering and/or exploitation of lignocellulose biomass.

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