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Spotlight

Finding my way: The role of dirigent proteins in lignin assembly

Lignin emerged around 450 million years ago in early tracheophytes and played a pivotal role in the colonization of terrestrial ecosystems by plants. This aromatic biopolymer provides mechanical strength to supportive tissues and hydrophobicity to water-transporting vasculature and acts as a physical barrier against herbivores and pathogens (Renault et al., 2019). The deposition of lignin in cell walls for proper cellular functionalization involves several molecular steps, including the tight regulation of tissue- or cell-type-specific expression of lignin biosynthetic genes, the biosynthesis of distinct lignin monomers and their transportation to the apoplast, and the polymerization of monomer radicals to produce tissue- or cell-type-dependent polymers with distinct physico-chemical properties.

Although the process of lignin polymerization has been extensively characterized, several aspects of the mechanisms underlying lignin assembly remain unclear. Nevertheless, research efforts have systematically provided evidence that lignin polymerization occurs solely under chemical control via combinatorial radical coupling, without any proteinaceous control beyond the formation of lignin monomers radicals (Ralph et al., 2008; Tobimatsu and Schuetz, 2019). The discovery of dirigent proteins (DIRs), which are glycoproteins shown to mediate regio- and stereospecific coupling of monolignols during lignan formation (Davin et al., 1997), led to the proposition of an alternative hypothesis in which DIRs also guide regio- and stereospecificity during lignin assembly (Davin and Lewis, 2005). Although DIR epitopes have been frequently found in lignifying tissues, the lack of optical activity and the highly flexible chemical nature of lignin polymers (which can incorporate a vast array of alternative monomers) are inconsistent with a major role of DIRs in lignin polymerization.

Since their discovery in the late 1990s, DIRs from different plant species have been identified and characterized, but the genetic evidence for their involvement in lignification came only two decades later (Hosmani et al., 2013). Interestingly, studies aiming to unravel the molecular mechanisms underlying the formation of Casparian strips (CSs), lignin-based extracellular barriers deposited surrounding the root endodermis (Barbosa et al., 2019), were pivotal to identify novel players in lignin deposition. In a forward genetic screen of *Arabidopsis thaliana* for mutants with altered mineral nutrients, a genotype was found displaying defective CS formation, leading to a disrupted root diffusion barrier, compensatory lignification, and increased suberin biosynthesis (Hosmani et al., 2013). The mutant was named *enhanced suberin1* (*esb1*), and the causative gene was mapped to a DIR, thus establishing that a DIR protein was essential for the correct patterning of lignin deposition in CSs. A similar function in CS formation for a DIR protein in maize was subsequently demonstrated (Wang et al., 2022), showing that this mechanism is conserved among plants from different lineages. Despite these results, the precise biochemical role of DIRs in lignin deposition during CS formation was missing.

The recent study by Gao et al. (2023) is a major step toward understanding the involvement of DIRs in lignin assembly with the characterization of an endodermal family of DIRs associated with CS formation in Arabidopsis. Based on transcriptomic analyses, they first identified six candidate *DIR* genes among the 25 members of the family—*DIR9*, *DIR16*, *DIR18*, *DIR24*, *DIR25*, and *ESB1*—showing (i) a specific root endodermal expression pattern and (ii) down-regulation upon disruption of *MYB36* (a master regulator of CS formation). Based on the presence of characteristic protein domains, they were further classified into group I (ESB1 and DIR25), group II (DIR9 and DIR24), and group III (DIR16 and DIR18). The localization of the selected DIRs at the endodermis was confirmed using fusion with fluorescent proteins and confocal microscopy.

What is the functional role of these DIRs in lignin deposition during CS formation? Gao et al. addressed this question by generating single and higher-order knockout mutants among all candidate *DIR* members and analyzing their CS integrity. Except for the *esb1* mutant, single mutants of all other *DIR* genes showed normal CS lignin deposition, suggesting functional redundancy. Phenotypes compatible with impaired CSs, including gaps in the normally continuous lignin band and compensatory lignification at the endodermal cell corners, were observed for double mutants within groups (*esb1dir25* [group I], *dir9dir24* [group II], and *dir16dir18* [group III]). Confocal Raman microscopy showed that the CS lignin spectrum of the mutants was different from that found in control plants, suggesting that a chemically distinct polymer is synthesized at the CS of the mutants. Interestingly, higher-order mutants (i.e., from triple to sextuple) phenocopied the double mutants, suggesting that each group of DIRs perform specific functions to ensure localized lignin deposition at the CS and possibly work as multiprotein heterocomplexes. To test this possibility, the authors performed bimolecular fluorescence complementation and co-immunoprecipitation assays, which validated the physical interaction and showed that proteins from each group of endodermal DIRs can form heterotrimers.

To test the functional relationship between the endodermal DIRs and other CS-related proteins, Gao et al. first dissected how the loss of function of DIRs affects the localization of CS DOMAIN PROTEIN1 (CASP1), an integral plasma membrane protein involved in the localization and structuring of the CS. In this

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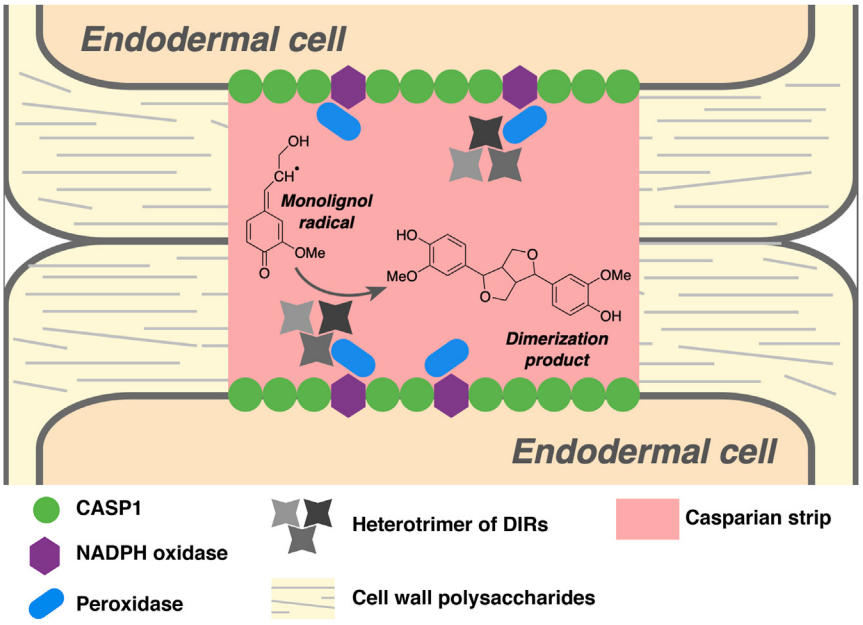

Figure 1. The role of DIRs in lignin deposition during Casparian strip (CS) formation.
CASP1 acts as a transmembrane protein scaffold for the localization of lignin-related enzymes. NADPH oxidase provides hydrogen peroxide to lignin-related peroxidases that oxidize monolignols to generate their corresponding radicals, which are used by DIR heterotrimers to produce dimerization products such as pinoresinol (8-8′ coupling products of coniferyl alcohol, as shown in the figure). The scheme represents two endodermal cells, between which the CS is formed.

way, they discovered that CASP1–GFP localization was clearly disrupted at the CS in higher-order mutants, indicating that endodermal DIRs are necessary for the proper localization of CASP1 to assemble a functional CS. The authors also evaluated whether the remaining lignin "islands" observed in the CS of the double *dir* mutants could be synthesized by a different lignin biosynthetic complex that requires the SCHENGEN pathway. The SCHENGEN (SGN) receptor module is a kinase signaling pathway that drives localized reactive oxygen species production and lignification at the endodermis (Fujita et al., 2020). The analysis of the triple mutants *sgn3esb1dir25*, *sgn3dir9dir24*, and *sgn3dir16dir18* showed that lignin deposition was practically abolished, with lignin coverage in the CS reduced to over 80%, compared to the 25% reduction found in the single *sgn3* mutant. These results show that the endodermal DIRs are responsible for the biosynthesis of most of the CS lignin, whereas the remaining lignin requires the SGN pathway.

Finally, the authors evaluated whether the endodermal DIRs are directly involved in lignin polymerization using recombinant enzymes in *in vitro* lignin polymerization assays. They tested three individual DIRs—DIR25, DIR24, and DIR18—and two heterocomplexes—ESB1/DIR9/DIR16 and DIR25/DIR24/DIR18—for which physical interactions were observed *in vivo*. High yields of different monolignol dimerization products were observed only when DIRs were added to the reaction. Additionally, more dimerized products were detected in the case of DIR heterocomplexes when compared to individual DIRs, further suggesting that these enzymes work as a complex during lignin formation at the CS. Altogether, these data suggest that the endodermal DIRs directly mediate lignin polymerization during CS formation. The authors propose that the presence of DIRs in the endodermis is essential to ensure lignin polymerization specifically at the CS (Figure 1), avoiding the diffusion of monolignol radicals to the cell corners, where they can polymerize spontaneously through the simple combinatorial radical coupling process.

The work reported by Gao et al. (2023) provides robust and integrative genetic and biochemical data to support the function of DIRs in lignin polymerization. Several questions emerge from these findings. First, is this function specific for the discrete lignification of the CS or can it be generalized to other lignifying plant cell types? For instance, the action of DIRs might help explain the distinct lignin chemistries found in different cell wall layers, which ensures specific cellular functionalization. In this scenario, different groups of DIRs might be involved in the lignification of different plant tissues or cell types. Second, is this function conserved across the different plant lineages? The identification of a DIR involved in lignin deposition at the endodermal CS domain in maize (Wang et al., 2022) advocates for a conserved mechanism, at least between eudicots and grasses. Nevertheless, although the overall role of DIRs in CS lignification might be conserved, differences in the underlying mechanism are also expected among different groups of plants. For instance, whereas group I DIRs are uniformly distributed throughout the CS, group II and III DIRs are concentrated at the edges, suggesting that the DIR heterotrimer should exclusively exist at the CS periphery and that group I DIRs may play a slightly distinct role compared to the other DIRs. Intriguingly, monocots are devoid of group I DIRs and employ the glycine/alanine/proline-rich domain protein GAPLESS to tether the CS plasma membrane domain to the CS, whereas GAPLESS orthologs are absent in Arabidopsis (Song et al., 2023). Thus, it is possible that group I DIRs might have an additional function in Arabidopsis and other eudicots, such as tethering the CS and CS plasma membrane domain. Third, to what extent is the activity of DIRs in lignin polymerization? Given the massive amount of data supporting the combinatorial radical coupling process, it is likely that DIRs function uniquely in initiating lignin polymerization or localizing lignin prepolymers in specific structures (e.g., the CS) or cell wall layers/domains, whereas polymer extension might occur via end-wise polymerization governed by chemical parameters.

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