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Review

A Molecular Blueprint of Lignin Repression

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Although lignin is essential to ensure the correct growth and development of land plants, it may be an obstacle to the production of lignocellulosics-based biofuels, and reduces the nutritional quality of crops used for human consumption or livestock feed. The need to tailor the lignocellulosic biomass for more efficient biofuel production or for improved plant digestibility has fostered considerable advances in our understanding of the lignin biosynthetic pathway and its regulation. Most of the described regulators are transcriptional activators of lignin biosynthesis, but considerably less attention has been devoted to the repressors of this pathway. We provide a comprehensive overview of the molecular factors that negatively impact on the lignification process at both the transcriptional and post-transcriptional levels.

Highlights

Negative regulators of lignin biosynthesis are promising tools to tailor biomass properties to meet human requirements.

The Mediator complex adds another level of transcription regulation to the several transcription factors that are known to repress lignification.

Targeted transcript or protein degradation, as well as protein–protein interactions, fine-tune the spatiotemporal pattern of lignification.

Understanding the interactions between genes, non-coding RNAs, and proteins opens new avenues towards understanding secondary cell wall formation.

Challenging Lignin

Lignin is a major cell wall component that fulfills fundamental functions in plant development as well as in defense against pests and pathogens. This heteropolymer impregnates the compound middle lamella and the secondary cell walls (SCWs) of cells genetically programmed to be lignified, such as xylem tracheary elements as well as xylem and phloem fibers. Lignin biosynthesis spans the phenylpropanoid and the monolignol pathways, from phenylalanine to *p*-coumaryl, coniferyl, and sinapyl alcohols. Monolignol homeostasis is regulated through a balance between the molecular factors that antagonistically regulate their biosynthesis. After monolignols are excreted into the apoplast, they are oxidized by the phenol-oxidoreductase laccases and/or by class III peroxidases, and are polymerized by radical coupling. A complex regulatory network involving phytohormones, transcription factors (TFs), and post-transcriptional events guide SCW deposition and lignification [1]. This network prevents lignin deposition in tissues undergoing active division or elongation such as apical meristems, vascular cambium, and immature xylem cells, as well as in non-lignified tissues such as stem pith and flax or hemp bast fibers that harbor gelatinous walls under normal developmental conditions. Similarly, this network negatively regulates lignification in xylem tissue formed in response to mechanical stresses such as, for instance, poplar tension wood that harbors hypolignified gelatinous walls.

The physical and chemical properties of lignin downgrade the value of plant feedstocks in several applications (Box 1). Lignin is known to impair pulping [2] and to reduce forage digestibility by ruminants [3,4]. It negatively impacts on the saccharification of plant biomass, therefore hampering biofuel production [5], even though lignin content and composition did not always correlate with saccharification potential [6,7]. In addition, a high lignin content in crops used in the human diet decreases their nutritional quality [8]. Various environmental stresses are known to induce lignin biosynthesis [9,10]. In the context of global climate change, a higher lignin content in crops and forest trees may be expected, thereby negatively affecting their chemical composition for various end-uses. Hence, a better understanding of the molecular mechanisms repressing lignification will be important to secure food quality and inspire biotechnological strategies for lignocellulosic biomass transformation. In light of these crucial societal needs, this review summarizes the main transcriptional events leading to the repression of lignification in eudicots. Examples of post-transcriptional events negatively regulating monolignol biosynthesis and lignin polymerization are also discussed. The regulation of lignification in monocots and/or grasses displays several divergences; these were recently reviewed [1,11] and will not be discussed in this article.

Transcriptional Repression of Lignin Biosynthesis

The roles of transcriptional activators promoting lignin biosynthesis have been well documented in various plants, such as thale cress (*Arabidopsis thaliana*) (reviewed in [12]) and tree species (reviewed

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Box 1. Handling Lignification

Lignin lowers the digestibility of several feed, food, and biomass products, resulting in detrimental effects that may worsen in the context of global warming.

Attempts to reduce lignin content in model plants through targeted gene expression modification have often resulted in impaired growth with a subsequent yield penalty.

Although several molecular factors can enhance lignin biosynthesis, those preventing/inhibiting this developmental process are currently under investigation.

Negative regulation of lignin biosynthesis is achieved through diverse mechanisms ranging from DNA accessibility to targeted proteolysis.

in [13,14]). The negative regulators of lignin biosynthesis and their underlying directing networks are tightly controlled.

NAC TFs, the Two Sides of SCW Regulation

Members of the NAC family act as first- and second-level master switches in the regulation of a battery of downstream TFs and SCW biosynthetic genes [15–18]. Among these, *VASCULAR-RELATED NAC DOMAIN*s (*VND*s) and *NAC SECONDARY WALL THICKENING PROMOTING FACTOR*s (*NST*s), also called *SECONDARY WALL-ASSOCIATED NAC DOMAIN*s (*SND*s), are positive regulators of SCW thickening (reviewed in [15]). In thale cress, VNDs induce **differentiation** (see Glossary) of vessels, whereas NSTs/SNDs regulate SCW deposition in fibers. The activities of these regulators are tightly controlled by different mechanisms [16–21].

XYLEM NAC DOMAIN 1 (XND1) blocks tracheary element differentiation through the negative regulation of *VND7* [19,20], preventing precocious deposition of the SCW (Figure 1, Key Figure). This repression is possibly linked to cell differentiation because XND1 physically interacts with the cell cycle and differentiation regulator RETINOBLASTOMA-RELATED via its LXCXE and LXCXE-mimic motifs [20].

VND-INTERACTING 2 (VNI2) is a transcriptional repressor reported to regulate the timing and spatial regulation of xylem cell development [21]. VNI2 acts as a passive repressor by forming **heterodimers** with VND proteins, preventing their positive regulation of vessel differentiation (notably through the expression of lignin-related *AtMYB46*). Because VNI2 displays an ethylene-responsive element binding factor-associated amphiphilic repression (EAR)-like motif [pdLNL(D/E)Lxi(G/S)], it might also actively repress the genes involved in xylem differentiation [21].

The SCW activator *NST2* is negatively transcriptionally regulated by WRKY12 (Figure 1), which binds to the W-box ***cis*-element** in the *NST2* promoter region (Table 1) [25]. WRKY12 and its functional poplar (*Populus trichocarpa*) ortholog *PtrWRKY19* were shown to be negative regulators of SCW deposition in the pith [25,28].

In poplar, the transcriptional activities of SNDs and VNDs are negatively regulated by dedicated **splice variants** with retained intron sequences [29]. An intron-retained (IR) splice variant PtrVND6-C1^{IR} negatively regulates the expression of *PtrMYB021* (a poplar ortholog of *AtMYB46*) by forming heterodimers with the full-size PtrVND6s, suppressing their positive transcriptional activity (Figure 1). In addition, PtrVND6-C1^{IR} downregulates the expression of five full-size *PtrVND6*s. Similarly, PtrSND1-A2^{IR} dimerizes with full-size PtrSND1s. Importantly, PtrVND6-C1^{IR} and PtrSND1-A2^{IR} cannot suppress their cognate TFs but can suppress all members of the other family, indicating that the splice variants from the PtrVND6 and PtrSND1 family may exert reciprocal crossregulation for complete transcriptional regulation of these two families in wood formation, providing a higher level of regulation to maintain homeostasis in plants to avoid abnormal growth and development [29].

Glossary

***Cis*-element**: a *cis*-element is a conserved nucleotide sequence (e.g., the G-box) that is generally found in the promoter region of the regulated gene, and is recognized by a specific family or subfamily of transcription factors (TFs; e.g., MYBs). These TFs are *trans*-regulating elements because they tune the expression of specific genes through intermolecular interactions. Mediator proteins are also *trans*-regulating elements.

Differentiation: a suite of incremental cellular and molecular modifications leading to the formation of specialized groups of cells from undifferentiated tissues, such as the shoot apical meristem or the vascular cambium. The process, the timing, and the location of differentiation are under stringent genetic regulation. Lignification is a key feature of the differentiation of vessels and fibers in xylem and phloem.

Heterodimer: in this review this term designates a complex of two different proteins, usually TFs. Heterodimer formation changes the properties of a protein relative to those of the isolated protein. Heterodimerization may for instance lead to higher transcriptional activity (either activation or repression), result in decreased activity of one of the proteins in the complex, or target one of the proteins to a specific cellular compartment (e.g., the nucleus or cytosol).

Non-coding RNA (ncRNA): an RNA that is not translated into protein. The functions of ncRNAs range from transcript degradation (microRNAs) to translation regulation, gene expression or RNA splicing. We here review some long non-coding RNAs (lncRNAs) and microRNAs mediating the degradation of *laccase* transcripts.

Semidominant mutant: an allele is said to be semidominant when the heterozygote displays an phenotype intermediate between that of the homozygote mutant (null mutant) and the wild type. Such mutants are useful to investigate the function of a gene whose null mutation results in a severe phenotype such as dwarfism or lethality. Semidominance is synonymous with 'Incomplete dominance'.

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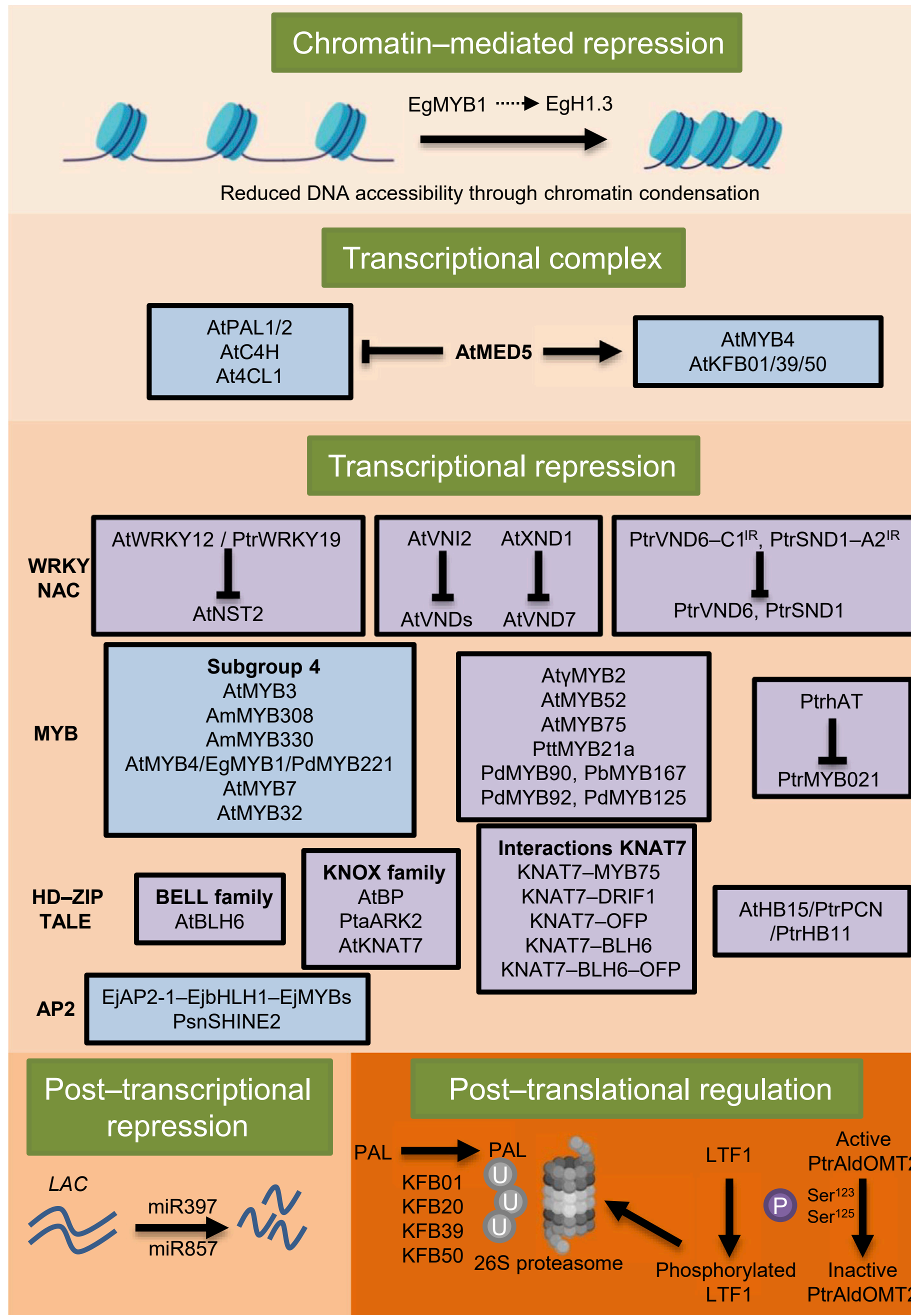
Key Figure

Molecular Regulation of Lignin Repression in Dicots at the Chromatin, Transcriptional, Post-Transcriptional, and Post-Translational levels (green boxes)



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Splice variants: these arise from alternative RNA splicing events, and display an mRNA sequence that differs from that of the regular transcript, resulting in a different protein. Alternative splicing allows the biosynthesis of several proteins from a single coding gene. In this review we describe alternatively spliced proteins regulating the expression of closely related coding genes.

Protein	Binding domain	Binding site	Refs
TALE	WFIN residue of homeodomain	KN-1 TGACAG(G/C)T	[22]
MYB subgroup 4	EAR motif pdLNL(D/E)Lxi(G/S)	AATAGTT	[23]
AP2/ERF	Acidic C-terminal domain	SNBE, SMRE, GCC-box	[24]
WRKY	WRKYGQK residue	W-box TTGACT/C	[25]
miRNA^{LAC}	Not applicable	Cu-oxidase domain	[26]
KFB^{PAL}	Kelch domain at the C-terminus	Unknown	[27]

Table 1. Binding Sites of Proteins Involved in the Repression of Monolignol Biosynthesis

R2R3 MYBs, the Gatekeepers of SCW Formation and Lignification

Some members of the R2R3-MYB TF family positively regulate gene expression of phenylpropanoid and lignin biosynthetic genes containing AC-rich *cis*-elements in their promoters [30], such as the 7 bp sequence ACC(A/T)A(A/C)(T/C), termed the secondary wall MYB-responsive element (SMRE) [31,32]. The importance of MYBs as repressors of phenylpropanoid metabolism has been highlighted in a recent review [33].

The first MYB factors shown to repress lignin biosynthesis were *AmMYB308* and *AmMYB330* from *Antirrhinum majus* [34]. Overexpression of these genes in tobacco caused a decrease in xylem lignin content by reducing the expression of phenylpropanoid and lignin biosynthetic genes (*C4H*, *4CL*, and *CAD*). The knockout of *AtMYB4*, a thale cress ortholog of *AmMYB308*, displayed increased amounts of sinapate esters through increased expression of *C4H* [35]. AtMYB4 belongs to subgroup 4 and, as the other proteins from this subgroup (AtMYB3, AtMYB7, and AtMYB32), contains an EAR-like repression motif in its C-terminus [36]. Members of subgroup 4 repress the phenylpropanoid pathway, the lignin pathway, and/or the biosynthesis of pigments (Figure 1) [35,37]. *AtMYB4* is down-regulated in thale cress ectopic lignification *de-etiolated 3*, *pom-pom 1*, and *ectopic lignification 1* mutants [38], suggesting that it could negatively regulate lignin biosynthesis. AtMYB32 negatively controls the expression of genes involved in phenylpropanoid and lignin biosynthesis, affecting the pollen-wall composition [39]. The repressive activity of subgroup 4 is tightly controlled. AtMYB7, AtMYB32, and AtMYB4 negatively regulate their own transcription as well as that of *AtMYB52* [40].

SENSITIVE TO ABA AND DROUGHT 2 (SAD2), an importin β-like protein, mediates nuclear trafficking of AtMYB4, AtMYB7, and AtMYB32, thereby increasing their repression of their target genes [41,42]. The interaction between SAD2 and members of subgroup 4 occurs through their SID domain GXXDFxxxG/DL, which is also a signature for protein degradation through the 26S proteasome pathway [43]. In buckwheat (*Fagopyrum tataricum*), jasmonates induce not only the expression of

Figure 1. The repressive activity of EgMYB1 increases when it is associated with EgH1.3. Some factors are devoted to specifically preventing monolignol biosynthesis (blue boxes), whereas others repress secondary cell wall (SCW) formation more widely (violet boxes). Proteins repressing SCW formation are essentially involved in the spatiotemporal regulation of specific tissue development, such as preventing SCW deposition in pith (AtWRKY12, AtHB15) or restricting the timing and localization of xylem cell development (AtVNI2). Abbreviations: 4CL, 4-coumarate-CoA ligase; AldOMT, 5-hydroxyconiferaldehyde *O*-methyltransferase; ARK, arborknox; BLH, BEL1-like homeodomain; BP, brevipedicellus; C4H, cinnamate-4-hydroxylase; DRIF, divaricata and radialis interacting factor; H1.3, linker histone variant; hAT, hobo activator Tam3 transposase; HB, homeobox; KFB Kelch F-box; KNAT, knotted1-like TALE homeodomain; LAC, laccase; LTF, lignin biosynthesis-associated transcription factor; MED, Mediator; NST, NAC secondary wall thickening promoting factor; OFP, ovate family protein; P, phosphorylation; PAL, phenylalanine ammonia lyase; PCN, popcorona; SND, secondary wall-associated NAC domain protein; U, ubiquitin; VND vascular-related NAC domain; VNI, VND interacting; XND, xylem NAC domain. This figure was created using BioRender (https://biorender.com/).

SAD2 and of five members of subgroup 4, but also the degradation of the corresponding proteins through the 26S proteasome pathway [23,43]. The subgroup 4 FtMYB11 binds to the AATAGTT motif to repress the expression of *FtPAL*, *FtC4H*, and *Ft4CL* (Figure 2 and Table 1). The repression mechanism of AtMYB3 is completely different from that of AtMYB4, AtMYB7, and AtMYB32 because it is directly regulated by the corepressors NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED 1 (LNK1) and LNK2, which could facilitate binding of AtMYB3 to the *C4H* promoter [37].

More direct evidence of the negative regulation of lignin biosynthesis by potential orthologs of *AtMYB4* has been reported in species other than thale cress (Figure 1). Overexpression of *Eucalyptus gunni EgMYB1* in thale cress and poplar negatively regulates SCW formation, including lignin biosynthesis [45]. When interacting with the linker histone variant EgH1.3, EgMYB1 displays stronger repressive activity, thereby preventing premature lignification of xylem cells during their early stages of differentiation [46]. The two orthologs of *EgMYB1* in *Populus deltoides* and *P. tomentosa* (*PdMYB221* and *PtoMYB156*) also reduce the SCW thickness of xylem fibers and the content of cellulose, lignin, and xylan [47,48]. Transgenic poplars overexpressing both *PtrMYB221* and *GA20-oxidase*, an enzyme involved in gibberellin biosynthesis, showed a twofold increase in plant biomass, a 16% reduction of lignin content, a higher amount of holocellulose, and an 8% improved saccharification efficiency compared to the wild type [49]. In addition, *PtoMYB156* represses phenylpropanoid biosynthetic genes, leading to a reduction in total phenolics and flavonoids [48].

During the formation of cotton ovule/fiber SCW, orthologs of *AtMYB4* are also highly expressed, supporting their roles in repressing lignification [50]. In hemp, a motif recognized by MYB3 was found in the promoter sequence of the *FASCICLIN-LIKE ARABINOGALACTAN* (*FLA*) genes that are highly expressed in mature phloem fibers. Those *FLA*s may be under a MYB3-driven regulatory circuit determining bast fiber hypolignification [51].

In loquat (*Eriobotrya japonica*), EjMYB2, a member of subgroup 4, represses the expression of genes belonging to the phenylpropanoid and lignin pathways (such as *HCT*, *CCoAOMT*, and *CCR1*), whereas EjMYB1 mediates their upregulation [52]. EjMYB2 interacts with EjbHLH1 and with a member of the AP2/ERF TF family, EjAP2-1, to repress cold-induced lignification (Figure 1) [53]. EjMYB1 and EjMYB2 both interact with EjAP2-1 to regulate the expression of *Ej4CL1*, leading to a stronger repressive activity of EjMYB2 and attenuation of the EjMYB1-mediated activation [54]. Although EjAP2-1 has two EAR motifs, it is unable to bind directly to the promoters of the lignin biosynthetic genes, suggesting that its repressive activity occurs via protein–protein interactions with MYB proteins [54]. On the other hand, overexpression of another member of the *AP2/ERF* family, *PsnSHINE2* from *P. simonii* × *P. nigra*, in tobacco represses the expression of *MYB* and *NAC* activators of the lignin pathway, leading to reduced lignin content in stems [24]. PsnSHINE2 binds not only to the AP2/ERF binding box but also to a secondary wall NAC binding element (SNBE, motif TCCTTTTCTCTCTAAGCAT) and to three MYB-binding elements (SMBEs; motifs ACCAAAT, ACCTACC, and ACCAACC) [24] that are present in the promoters of lignin, xylan, and cellulose biosynthetic genes (Table 1) [55]. AP2/ERF are therefore able to regulate the lignin biosynthetic pathway both directly and indirectly.

Other MYBs not belonging to subgroup 4 negatively regulate the expression of genes of the monolignol pathway (Figure 1). Several *Atmyb52* insertion lines show strong accumulation of lignin in interfascicular fibers, metaxylem vessels, and phloem cap cells [56]. *MYB52* is coexpressed with several TFs regulating SCW formation (*MYB85*, *SND2*, *XND1*) and genes involved in cellulose and xylan biosynthesis [56], highlighting the importance of monolignol homeostasis in lignifying tissues. Overexpression of the poplar xylem-specific TFs *PdMYB90*, *PdMYB167* (orthologs of *AtMYB52*), *PdMYB92*, and *PdMYB125* decreases the expression of xylan, lignin, and cellulose biosynthetic genes, modifying stem cell wall composition [57]. Downregulation of *PttMYB21a* (homolog to *AtMYB52*) by using an antisense strategy in transgenic aspen (*Populus tremula* × *P. tremuloides*) increased lignin content while decreasing the pool of structural carbohydrates in the bark [58]. However, another *PttMYB21a* antisense line showed a lower lignin content in wood, a decreased stem height and diameter, as well as increased glucose release during enzymatic digestion relative to the wild type [7], highlighting the

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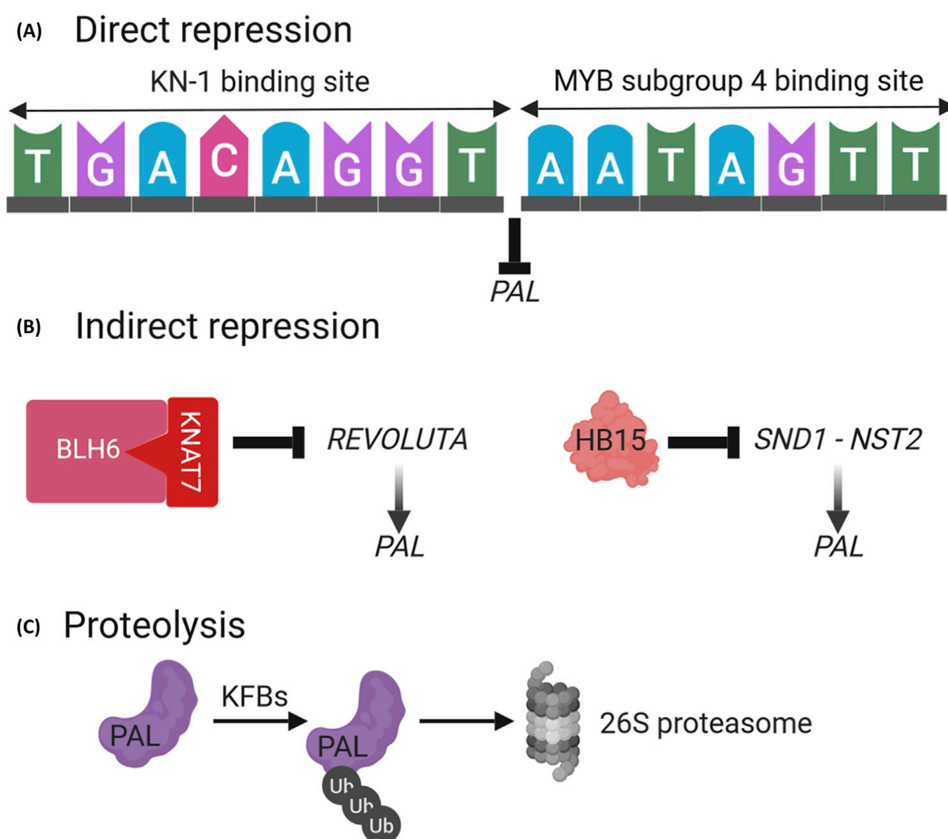

Figure 2. PAL as a Case Study of Multiple Mechanisms Regulating Gene Expression and Protein Abundance. (A) *PAL* is directly repressed through binding of various TALE family transcription factors (TFs; e.g., BREVIPEDICELLUS) to the DNA knotted 1 (KN-1) motif, as well as by binding of subgroup 4 R2R3 MYBs (e.g., MYB4) to the AATAGTT motif. (B) *PAL* is indirectly regulated through BLH6–KNAT7-mediated repression of the HD-Zip III TF *REVOLUTA*. HB15 represses the expression of the master switches *SND1* and *NST2*, which are activators of *PAL* expression. (C) Kelch F-box proteins ubiquitinate PAL to target it to the 26S proteasome for proteolysis. Other PAL regulatory pathways, including metabolic feedback repression (caffeic acid, cinnamic acid), environmental factors, and physical interactions with other phenylpropanoid enzymes (C4H) are beyond the scope of this figure but are reviewed in [44]. Abbreviations: BLH, BEL1-like homeodomain; HB, homeobox; KFB, kelch F-box; KNAT, knotted1-like TALE homeodomain; NST, NAC secondary wall thickening promoting factor; PAL, phenylalanine ammonia lyase; SND, secondary wall-associated NAC domain protein; Ub ubiquitin. This figure was created using BioRender (https://biorender.com/).

differential regulation of lignification in bark and wood. AtMYB75 induces the biosynthesis of anthocyanins, and represses lignin biosynthesis as well as the expression of cellulose and xylan biosynthetic genes in the SCW [59]. MYB75 physically interacts with KNAT7, negatively regulating SCW formation in stems and seed coats of thale cress [59,60].

The MYB coiled-coil (MYB-CC) family is a subset of the MYB superfamily that has emerging functions in plant development. Thale cress γMYB2 is a MYB-CC protein that governs SCW formation in stem and anther by repressing the expression of *MYB*s and *NAC*s directing cellulose, xylan, and lignin biosynthesis, such as *SND3*, *MYB46*, and *MYB63* [61]. In addition, γMYB2 represses the expression of the negative regulators *MYB4* and *MYB32*. Transgenic lines with constitutive *γMYB2* overexpression showed defective lignification of the anther endothecium layer, whereas

no obvious differences in lignin deposition were observed in the stems of any of the γ*MYB2* up- or downregulated lines. By contrast, cellulose strongly accumulated in lines with downregulated γ*MYB2* expression [61].

In addition to having repressive activity on lignin biosynthesis, a novel transcriptional regulatory mechanism for MYBs has been recently uncovered [62] that regulates the flow of carbon into the phenylpropanoid pathway and lignin biosynthesis through direct repression of *PtrMYB021* (Figure 1). Association mapping identified significant correlations between lignin content and one of the two paralogs of *5-enolpyruvylshikimate 3-phosphate synthase* gene (*PtrEPSP-TF*, Potri.*002G146400*). Compared to the wild type, transgenic poplars overexpressing *PtrEPSP-TF* showed ectopic deposition of lignin, accumulation of phenylpropanoids, differential expression of SCW biosynthetic genes, and higher sugar release during enzymatic saccharification, but without notable yield change in field trials [62,63]. Notably, PtrEPSP-TF harbors an additional N-terminal HTH DNA-binding motif that partially targets this protein to the nucleus, where it acts as a transcriptional repressor of its direct target *PtrhAT*, a hAT transposase family gene. PtrhAT is itself a transcriptional repressor of its direct target *PtrMYB021*. Through repressing *PtrhAT* expression, PtrEPSP-TF activates the expression of *PtrMYB021* and the phenylpropanoid pathway. The transcription of *PtrEPSP-TF* is under the control of the master regulator PtrWND1B, a homolog of AtSND1. Notably, the abundance of PtrEPSP-TF increases during the transition from primary to secondary growth in the stem [64], thus providing an elegant regulatory mechanism for the monolignol biosynthetic pathway.

KNOX, BELL, and Homeodomain: from Cell Division to Fiber SCW Thickening

Some members of the THREE AMINO ACID LOOP EXTENSION (TALE) family of homeodomain (HD) proteins may play a role in the repression of lignin biosynthesis (Figure 1). Two subgroups of TFs are found in the TALE family, namely KNOX and BEL-LIKE (BELL), which interact through binding of their HD to the DNA knotted 1 (KN-1) conserved motif (Table 1) [22,65]. BREVIPEDICELLUS (BP/KNOX1) regulates cell division and differentiation in the shoot apical meristem, as well as in the vascular cambium of stems [65,66], possibly by upregulating genes involved in cytokinin biosynthesis associated with a lower bioactive pool of gibberellins [67]. In addition, BP binds to the KN-1 motif in the promoters of lignin biosynthesis genes *PAL*, *C4H*, *4CL*, and *CAD*, and represses their expression (Figure 2) [22,68]. Accordingly, *bp* mutants show premature lignin deposition in interfascicular tissues of thale cress stems [68]. This negative regulation seems to be shared by both eudicots and monocots because overexpressing *KNOX1* in maize and tobacco leads to reduced lignin accumulation in plants, ending their vegetative growth [69]. The same altered lignification pattern has been described in poplar, where overexpression of *ARBORKNOX2* (*ARK2*) is negatively associated with lignin content and normal stem development [70].

KNOX forms heterodimers with BELL proteins and represses the expression of some lignin biosynthetic genes (*COMT1*, *CCoAOMT*, and *AtPRX2*) [65]. These heterodimers prevent recognition of BELL proteins by EXPORTIN 1A, a receptor for nuclear export that recognizes a leucine-rich nuclear export signal (similar to L–x2/3–L–x2/3–L–x–L, where x represents any amino acid). The cooperative heterodimer becomes completely contained in the nucleus, and the expression of the target genes is dramatically reduced relative to individual BELL or KNOX proteins [22,71].

The heterodimer KNAT7–BLH6 negatively regulates the commitment to SCW formation in interfascicular fibers of thale cress through repression of *REVOLUTA* (Figure 2), which encodes a HD-leucine zipper TF binding to the sequence GTAATNATTAC (Figure 1) [65,72]. The double loss-of-function *blh6 knat7* mutant has more lignin than the wild type, consistent with upregulation of most genes associated with lignin biosynthesis (*PAL2*, *C4H*, *HCT*, *CCoAOMT1*, *CCR1*, *F5H1*, and *LAC4*) [73]. The transcriptional repression by KNAT7 and BLH6 is enhanced by their interaction with other TFs, including OVATE FAMILY PROTEIN (OFP) 1 and 4, during SCW biosynthesis [74,75]. By analyzing different *knat7* mutants, KNAT7 was shown to regulate the expression of several xylan biosynthetic genes (*IRX8*, *IRX9*, *FRA8*) either positively [76] or negatively [77]. This result suggests that the xylan and lignin biosynthetic pathways do not share the same regulatory network, despite their common

activation by several MYB and NAC TFs [78]. In the cell wall, xylans are deposited before the onset of lignification [79]. KNAT7 may then regulate the first steps of SCW formation, favoring xylan biosynthesis over lignification. KNAT7 is functionally conserved in poplar [77]. Furthermore, poplar KNAT7 interacts with the DUF3755 domain within the MYB-like protein DIVARICATA AND RADIALIS INTERACTING FACTOR 1 (PtrDRIF1) [80,112]. PtrDRIF1 also interacts with PtrWOX13c, a WUSCHEL-RELATED HOMEOBOX TF whose coding gene is highly expressed in the xylem. These interactions compete, favoring either vascular cambium activity, commitment to differentiation, or the timing of lignification [80,112].

Repression of SCW formation in the pith is under the control of the class III HD-ZIP AtHB15 TF [81]. Indeed, the *athb15* mutant showed increased xylan and lignin contents in the pith as well as higher expression of SCW genes (Figure 2) [81]. Similarly, an ectopic lignification phenotype was reported in the *athb15* mutant [56]. Comparable results were found in poplar using a miRNA-guided degradation of *POPCORONA* (*PCN*) transcripts, the ortholog of *AtHB15* (Figure 1). Overexpression of a variant of *PCN* resistant to miRNA cleavage inhibits phloem fiber lignification, whereas overexpression of the same miRNA results in abnormal lignification of pith cells [82]. RNAi-mediated downregulation of *PtrHB11*, whose thale cress closest ortholog is *AtHB15*, resulted in increased glucose release after enzymatic hydrolysis, but without impacting on stem height and diameter [7].

Several *KNOX* and *BELL* genes are highly expressed in mature phloem fibers of hemp as well as in the pulling stem side of gravistimulated flax plants that deposit gelatinous cell walls in phloem fibers [83,84]. These genes likely play a role in the typical hypolignification of these tissues. Of note, *KNOX* are also part of the transcriptional network regulating the formation of tension wood in poplar [85] that is characterized by the presence of a thick, weakly lignified, cellulose-rich gelatinous layer. KNOX may therefore have a conserved role in the formation of the gelatinous cell wall.

Mediator, a Molecular Hub Coordinating Lignin Biosynthesis with Plant Growth

The 'mediator of RNA polymerase II transcription', or Mediator complex (MED), is essential to transduce signals (both positively and negatively regulating gene expression) to the transcription machinery via direct interactions with specific TFs [86]. The protein–protein interactions that underlie specific MED/TF recognition and subsequent signal transmission are poorly known so far [86]. Among the 27 MED subunits identified in thale cress [87], several negatively regulate the phenylpropanoid and monolignol biosynthetic pathways, contributing to the homeostasis of this family of secondary metabolites. The MED5 complex seems to be part of a system that senses the accumulation of phenylpropanoid pathway intermediates, resulting in feedback repression of genes involved in this pathway [88]. Double silencing of *MED5a* and *MED5b* increases the expression of genes such as *PAL1*, *PAL2*, *C4H*, and *4CL1* (Figure 1), and leads to hyperaccumulation of phenylpropanoids including glycosylated dilignols in thale cress [89]. In addition, the dwarf and reduced lignin content phenotypes of the *reduced epidermal fluorescence 8* (*ref8*/*c3h*) mutant is rescued in the triple *med5a*/*med5b*/*ref8-1* mutant [90]. However, the lignin monomeric composition is drastically modified in the triple mutant, consisting almost exclusively of H-lignin subunits (95% vs $<2\%$ in the wild type), suggesting that MED5a and MED5b are likely to have other functions [90]. This particular lignin pattern is associated with a substantial increase of glucose release during enzymatic digestion [90].

In addition, the **semidominant** *MED5b* mutation in thale cress leads to dwarfism as well as in strong upregulation of *AtMYB4* and of the *Kelch F-box* genes (*KFB01*, *KFB39*, and *KFB50*, described below) (Figure 1), accompanied by reduced phenylpropanoid accumulation [91]. Because ~3–4-fold more genes are downregulated than upregulated in the *med5ab* mutant, the repression of lignin biosynthesis, in this background, seems to be due to the upregulation of repressors rather than to the direct MED5-mediated repression of lignin biosynthetic gene expression [91]. Dolan and colleagues [91] have also demonstrated that the *MED5b* phenotype requires functional MED2, MED16, and MED23, which probably physically and functionally interact with MED5, as do their homologs in humans [92].

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Post-Transcriptional Repression of Monolignol Biosynthesis and Lignin Polymerization

In addition to the numerous mechanisms of transcriptional regulation that land plants have established to repress monolignol biosynthesis and hence lignification in different tissues and developmental stages, additional post-transcriptional mechanisms have been observed. Post-transcriptional modifications typically affect a restricted number of transcripts/proteins, allowing precise control of the output of a metabolic pathway such as lignin biosynthesis.

Non-Coding RNAs, Emerging Regulators for Genetic Control of Lignin Deposition

MicroRNAs (miRNAs) are small **non-coding RNAs** (ncRNAs) that post-transcriptionally regulate many aspects of plant development. Their expression is developmentally regulated and/or under the control of external stimuli such as abiotic stress or nutrient availability [93,94]. Over recent years the functions of specific miRNAs have been unveiled in the regulation of secondary growth, SCW formation, and lignification. Several miRNAs negatively regulate the lignification process by cleaving transcripts of *laccase* (*LAC*) genes in the conserved Cu-oxidase domain of the corresponding proteins (Figure 1 and Table 1). Overexpression of *ptr-miR397a* significantly reduces the expression of 17 of the 34 *LAC* found in poplar differentiating xylem, the global LAC activity of this tissue, and the lignin content of the whole plant [26]. In Chinese pear, *miR397a* targets 24 *LAC*, leading to decreased lignin content in the flesh and therefore to higher fruit quality [95].

Twenty conserved miRNAs, regulating 112 different targets genes, have been found in flax (*Linum usitatissimum*) [96]. The lignification pattern of the xylem in flax stems is probably linked to the expression pattern of *Lu-miRNA397* that cleaves *LuLAC1*, *LuLAC5*, *LuLAC9*, *LuLAC38*, *LuLAC39*, and *LuLAC44* [97]. Similarly, 18 conserved miRNAs targeting 80 genes were found in hemp, where they may have similar functions to flax miRNAs [98]. miRNAs specifically targeting a *LAC* transcript have been described in thale cress, where *miRNA857* and *miRNA397b* modulate the abundance of *AtLAC7* and *AtLAC4* transcripts, respectively [94,99]. The expression of *miRNA857* is downregulated by copper, suggesting that this gene is expressed at low copper concentrations, probably through binding of the SPL7 (SQUAMOSA promoter-binding protein like 7) TF to the GTAC motifs contained in the *miRNA857* promoter [94].

The characterization of those multiple miRNAs in several species also highlights the importance of the polymerization step in lignin deposition, whose regulation is far less understood than that of monolignol biosynthesis. The possibility to alter the quantity of one or several transcripts depending on a given miRNA opens a panel of possibilities to modulate the lignin content either in specific tissues (such as xylem vessels or fibers) or more widely. If the regulation of miRNAs can be elucidated, numerous industrial applications may be foreseen, such as overcoming recalcitrance for bioethanol production [26]. However, overexpression of *miRNA857* in thale cress reduces secondary growth and SCW thickness in response to decreased *AtLAC7* transcript quantity, resulting in lower lignin content, and potentially reducing plant yield [94].

The role of long non-coding RNAs (lncRNAs) in the regulation of lignification-associated genes seems to be similar to that of miRNA in both woody and herbaceous species [100,101]. These lncRNAs may be directly functional or serve as precursors for miRNA sequences such as *miR397* [101], and provide a further level of complexity in the regulation of lignin biosynthesis. Potential ncRNAs (lncRNAs and miRNAs) and TFs associated with lignin biosynthetic genes have been identified in *P. tomentosa* by association genetics and expression quantitative trait nucleotide (eQTN) mapping [102]. For instance, combined single-nucleotide polymorphisms in lncRNAs and genes related to lignin biosynthesis significantly impact on lignin content in a population of natural variants [102].

Protein Ubiquitination: the Signaling Wave to the Grave

PAL catalyzes the rate-limiting step of the phenylpropanoid pathway and thus constitutes an ideal target for regulating the flux of derived secondary metabolites. In thale cress, the abundance of various PAL isozymes is regulated by proteolysis via the ubiquitin–26S proteasome complex that is modulated by KFB proteins (Figures 1 and 2 and Table 1). The kelch motif in the C-terminal domain

is required for recognition and interaction between KFBs and their targets, whereas the F-box motif at the N-terminus is involved in the interaction with the SKP1 subunit of the E3 ligase complex [27].

Thale cress KFB01, KFB20, KFB39, and KFB50 physically interact with the four PAL isozymes, thereby regulating the biosynthesis of phenylpropanoids during plant development and in response to environmental stimuli [27,103]. The hemp ortholog of *KFB39* is upregulated in mature bast fibers, suggesting a role for KFBs in the hypolignification of this cell type [83]. KFB-mediated degradation of chalcone synthase, the first enzyme of the flavonoid biosynthetic pathway, has also been described in thale cress [104].

Switching On/Off Enzymatic Activity with Phosphorylation

Phosphorylation is a widespread post-translational modification which may impact on the lignification process. Monophosphorylation of PtrAldOMT2 (that catalyzes the methylation of 5-hydroxyconiferaldehyde to sinapaldehyde) at either Ser^{123} or Ser^{125} inhibits its activity (Figure 1) [105], in line with the observation that the pool of monolignol biosynthetic enzymes is usually not phosphorylated *in vivo* [106]. However, the biological significance of this switch remains unknown. Alternatively, phosphorylation may also constitute a signal for protein degradation through proteasome activity. By screening TFs binding to the poplar *4CL* promoter, Gui and colleagues identified a lignin biosynthesis-associated factor, LTF1, that represses several genes from this pathway (*PAL2*, *C4H1*, *C3H2*, *4CL1*, *CAld5H*, *COMT2*, and *CCoAOMT1*) and decreases lignin content in overexpressing lines [107]. After phosphorylation, LTF1 is targeted to the proteasome, thereby derepressing lignin deposition. This post-transcriptional regulation seems to be mediated by environmental factors such as wounding, and may trigger rapid lignification as a part of plant response to external stimulation [107].

Concluding Remarks and Future Perspectives

Further advances in synthetic and molecular biology combine with our growing knowledge about the molecular factors (mainly genes and proteins) driving SCW formation in various tissues and plant species to overcome the possible growth penalty of constitutive overexpression of genes repressing lignification (see Outstanding Questions). As proof of concept, lignin deposition in thale cress was restricted to vessels by controlling the expression of the monolignol biosynthetic gene *C4H* with the promoter of *VND6* – that encodes a TF regulating the formation of vessels in metaxylem [108]. Similarly, the dwarf thale cress *ccr1* mutant was rescued by driving the expression of *CCR1* in metaxylem and protoxylem vessels through a *proSNBE* promoter transcriptionally activated by VND6 and VND7 [109]. Targeted lignin biosynthesis repression may thus be achieved through temporal and/or spatial restriction of the activity of a selected gene using suitable promoters. For instance, *pNST1* could be used to restrict the expression of a lignin repressor to fibers, thus avoiding vessel collapse and the subsequent growth penalty. *Omics*-based predictive analysis of variables determining wood quality following targeted gene downregulation [110] constitutes a valuable tool to optimize strategies.

Future biotechnological tools to modify lignification may also rely on the understanding and valorization of epigenetic modifications. DNA methylation contributes to the regulation of cotton fiber development and can modulate the production of reactive oxygen species or the biosynthesis of lipids, flavonoids, and ascorbate [111]. The heritable nature of epigenetic modifications constitutes a valuable feature for the development of plants with such engineered characteristics (see Outstanding Questions).

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Outstanding Questions

How does MED associate with specific TFs to regulate monolignol biosynthesis? Which factors regulate *MED* expression?

Is there a conserved molecular pathway that controls the formation and the hypolignified pattern of gelatinous layers in tension wood and fiber crops? Are TALE proteins major players involved in these processes?

To what extent are ncRNAs involved in the negative regulation of lignification? How are their actions coordinated, from TFs to biosynthesis genes, to modulate and restrict lignification to the correct spatiotemporal sequence?

What is the best way to turn current knowledge of the regulation of lignification into practical applications to ensure the production of high-quality food and bioenergy feedstocks? Are genome-editing techniques and synthetic biology strategies promising tools as compared to breeding techniques?

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