

## Review

## Regulation of Cell Wall Thickening by a Medley of Mechanisms

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To provide physical support for developing structures and to withstand the pressures associated with water and nutrient transport, some cells deposit a secondary cell wall, a rigid matrix of polysaccharide and phenolic biopolymers. The biosynthesis and deposition of these materials and the patterning of secondary wall-forming cells is controlled by a network of transcription factors. However, recent work suggests that this network forms the core of a more complex, multi-level regulatory system. This expanded system includes epigenetic, post-transcriptional, and post-translational regulation, and is coordinated with other pathways controlling primary growth and responses to environmental stimuli. New findings expand the set of transcription factors identified as secondary cell wall regulators and reveal novel regulatory processes that further govern secondary wall biogenesis.

## Secondary Cell Wall

Plant cells are surrounded by a rigid wall structure composed of protein and polysaccharide biopolymers. Cells in some tissues, most importantly structural and vascular cell types, are further fortified through the development of a thick secondary cell wall deposited between the primary wall and the plasma membrane. While secondary wall composition differs between species, in general, cellulose microfibrils, unbranched structures of  $\beta$ 1,4 linked glucose monomers, comprise the majority of secondary cell wall. Progressively smaller fractions of hemicelluloses, cell wall polysaccharides with more diversity in their structure and composition, and lignin, a heterogeneous, radical-coupled aromatic polymer, contribute the remainder of cell wall biomass, surrounding and crosslinking microfibrils to form a rigid matrix with impressive mechanical strength.

Secondary wall deposition is tightly orchestrated to result in specificity across cell types and development. During **elongation growth** (see [Glossary](#)), plant cells produce a variety of expansins, hydrolases, and other wall-loosening machinery to relax the primary wall and allow plant cells to elongate [1]. No analogous process exists in the secondary wall, largely due to the number and diversity of linkages that arise from **radical coupling** of monolignols and the extensive crosslinking of polymers in the wall matrix. Therefore, secondary cell wall thickening is incompatible with further elongation [2]. Premature or ectopic thickening of secondary walls is associated with drastic disruptions of growth and body plan, requiring that biosynthesis is activated in some tissues and repressed in others, across the spatio-temporal process of plant development. Secondary wall materials also constitute a significant resource cost and, since secondary wall deposition is irreversible, tight regulation is required to avoid unproductive and therefore deleterious allocation of photosynthate.

Accordingly, secondary cell wall biosynthesis is controlled by a complex network of **transcription factors** and their DNA targets. Studies of this regulatory network have revealed the following: the composition of the *Arabidopsis thaliana* network, the regulation of enzymes required for the

## Highlights

Tight control of secondary cell wall development is essential in vascular plants. In the current model, secondary cell wall development is regulated by a complex network of mostly MYB and NAC transcription factors that bind DNA to influence the expression of cell wall genes and other transcription factors.

Recent work has identified a medley of novel regulatory processes that control wall thickening, including miRNA targeting, post-translational modification, protein–protein interactions, chromatin modification, and the involvement of new classes of transcription factors.

New findings contribute to a mechanistic understanding of previously observed environmental plasticity and stress responses in secondary cell wall development, particularly with temperature, abiotic stress, and light quality.

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synthesis of specific components, the evolutionary conservation of this network across a number of plant species, and the response of this network to abiotic stresses [3–9]. Briefly, this network consists of three ‘layers’ of transcription factors that engage in feed-forward loops, binding the promoter sequences of regulatory genes in lower layers of the network, but also directly regulating the expression of biosynthetic genes [3,9,10]. The first, most upstream layer of regulators is composed of NAC family genes, including the *VASCULAR NAC DOMAIN* transcription factors (*VND1–VND7*), the *NAC SECONDARY WALL THICKENINGS* (*NST1* and *NST2*), and the *SECONDARY WALL NAC DOMAIN* transcription factors (*SND1/NST3* and *SND2*). In *A. thaliana*, these genes positively regulate the MYB proteins of the second regulatory layer, *MYB46* and *MYB83*, which link upstream NAC expression in the first layer to a much more expansive suite of proteins that comprise the third and final layer. There, a variety of mostly MYB transcription factors target specific biosynthetic genes in a highly redundant fashion. Important target genes include the secondary wall-specific cellulose synthase genes, *CESA4*, *CESA7*, and *CESA8*, a set of *CELLULOSE SYNTHASE-LIKE* (*CSL*) genes that play a role in hemicellulose synthesis and deposition, and genes in the phenylpropanoid biosynthetic pathway, through which monolignols are produced, such as *FH5*, *CAD*, *CCR*, and *COMT*. Fundamental aspects of wall regulation are conserved across plant lineages; however, the current theoretical architecture of the core secondary cell wall regulatory network is largely based on experimental insights derived from work in *A. thaliana* [3,8]. While work devoted to expanding and further characterizing this network in *A. thaliana* is ongoing and critically important, this review will discuss progress in identifying secondary cell wall-associated regulatory processes that fall outside the canonical network. Recent work has expanded secondary cell wall regulation to include transcription factor families beyond the NAC and MYB genes that predominate the contemporary model, identified regulatory systems that control species-specific biochemical features of secondary walls, and exploded the number of regulatory processes implicated in wall thickening (Figure 1).

### Beyond the core Network: Novel Transcriptional Regulators in Secondary Cell Wall Biosynthesis

Several recent publications report regulatory functions by proteins that either have no direct homologs in *A. thaliana*, or that expand the set of known wall regulators beyond the NAC and MYB families that populate current models of secondary cell wall regulatory network.

In *Brachypodium distachyon*, the *SECONDARY WALL ASSOCIATED MYB* (*SWAM*) genes are members of the R2-R3 MYB subgroup G13, which includes several known wall regulating transcription factors. *SWAM1*, a member of this group is coexpressed with the wall genes *BdCESA4/7/8*, *BdCAD1*, and *BdCOMT6*, binds a conserved MYB binding motif, an **AC-element**, and functions as an activator of wall biosynthesis, with overexpression increasing secondary wall thickness and lignin deposition in the interfascicular fibers of the stem, and expression of *SWAM1* fused to a dominant transcriptional repressor producing opposite phenotypes (Box 1) [11,12]. The extent to which secondary cell wall transcriptional regulation is conserved between plant lineages is a key question in the field and critically, detailed phylogenetic analysis strongly supports the existence of a *SWAM* clade that includes both monocot and eudicot genes, but lacks any representatives from the Brassicaceae, suggesting the loss of an otherwise conserved set of regulators in the predominant model plant for secondary cell wall genetics, *A. thaliana* [11].

Beyond the additional genetic diversity afforded by work in alternative or nonmodel organisms, the grass secondary wall is chemically distinct from eudicots and non-commelinid monocots, particularly in its hemicellulose composition, and work with grasses like *B. distachyon* and *Sorghum bicolor* has been critical to the recent diversification of known cell wall regulators [13–15]. Present in both primary and secondary cell walls, mixed linkage glucans (MLGs) are a

### Glossary

**AC-element:** a set of AC-rich MYB transcription factor binding motifs associated with regulation of secondary cell wall target genes.

**Elongation growth:** the physical change in cell shape and size that, together with cell division at the meristem, increases the height or length of plant structures.

**Neofunctionalization:** the process by which duplicate or redundant genes acquire new functions.

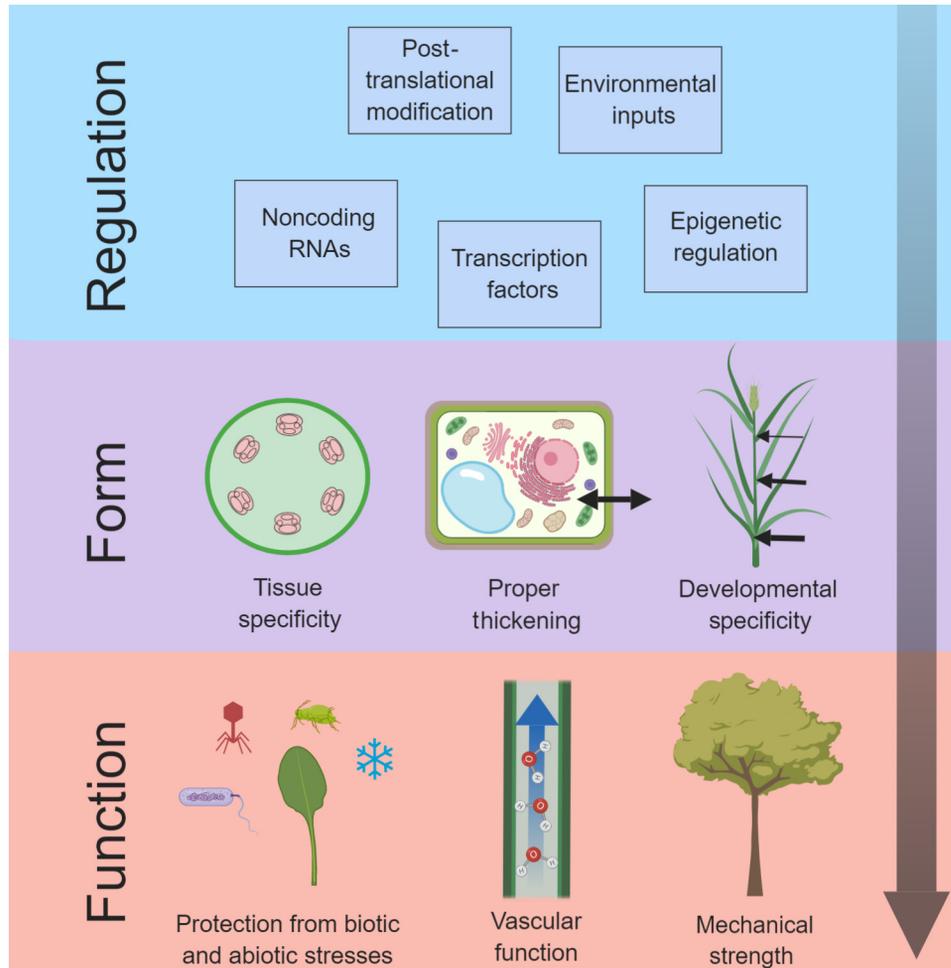
**Post-translational modification (PTM):** the covalent modification of proteins following translation from mRNA. Post-translational modifications can regulate the activity, localization, or turnover of proteins independent of their rate of expression and thus provide an additional layer of regulatory complexity.

**Radical coupling:** in lignin biosynthesis, monolignols are transported to the cell wall and enzymatically oxidized to generate highly reactive free radicals. Oxidative coupling between oxidized monolignols and the growing polymer results in the heterogeneous, unpredictable structure of lignin.

**Shikimate pathway:** a highly conserved metabolic pathway in archaea, bacteria, and plants. The shikimate pathway produces the aromatic amino acids phenylalanine, tyrosine, and tryptophan, as well as a diverse set of secondary metabolites. In plants, these processes take place in the chloroplast.

**Transcription factor:** regulatory proteins that bind DNA and regulate the transcription of genes. Transcription factors bind a DNA sequence motif defined by their protein structure and generally interact with basal transcriptional machinery, as well as other regulatory proteins, to activate or repress the transcription of RNA at a genomic locus.

**Ubiquitin:** a small regulatory protein tag. Ubiquitin occurs ‘ubiquitously’ in cells and is most commonly associated with degradation of specific ubiquitinated proteins. Canonically, proteins are ubiquitinated through step-wise interactions with E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes, and E3 ubiquitin ligases.



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**Figure 1. Tight Regulation of Secondary Cell Wall Biosynthesis Is Essential for Proper Plant Development.** Several distinct regulatory modes act in concert with this regulatory network to ensure spatio-temporal specificity and correct resource allocation throughout secondary wall development. Secondary cell walls provide a physical barrier that protect plants from pathogens and other biotic stresses, promote tolerance to abiotic stresses, and fortify cells to withstand the forces associated with water transport and the physical weight of plant structures. Created with BioRender.

class of hemicellulose exclusively found in grasses [13]. Like cellulose microfibrils, MLGs feature an unbranched glucose backbone. However, in addition to  $\beta$ 1,4 glycosidic bonds, MLGs feature single  $\beta$ 1,3 linkages at irregular intervals, causing a series of zig-zag bends along the length of the polymer. The newly identified *B. distachyon* transcription factor *BdTHX1* represents both the first identified regulator of MLG synthesis and the first trihelix family gene involved in secondary cell wall regulation [14]. *BdTHX1* binds an intronic region of the MLG synthesis gene *BdCSLF6*, and its tissue-specific expression overlaps with both *BdCSLF6* expression and MLG deposition in developing *B. distachyon* stems. Overexpression of *CSLF6* orthologs in other species is highly deleterious, potentially because it disrupts the coordination between MLG synthesis and the machinery required to integrate MLGs into the cell wall [14, 16]. Combined analysis of *BdTHX1* ChIP-seq binding peaks and transcriptomic data implicate *BdXTH8*, a glycoside hydrolase family 16 gene, as putative *BdTHX1* regulatory target, since it is both bound by and coexpressed with the trihelix transcription factor. Enzymatic assays identify *BdXTH8* as the first known enzyme in

**Box 1. Transcription Factor Characterization by DNA Affinity Purification Sequencing**

At the transcriptional level, secondary cell wall biosynthesis is regulated by a complex network of protein–DNA interactions, wherein transcription factors bind the *cis*-regulatory regions of other transcription factors as well as secondary cell wall biosynthesis genes. Understanding how protein–DNA interactions dictate the expression of downstream targets is key to developing models of secondary cell wall regulation capable of predicting the effects of specific perturbations of the regulatory network.

DNA affinity purification sequencing (DAP-seq) is a high-throughput, high-resolution technique to identify genome-wide transcription factor binding sites that has emerged as a tool to understand transcription networks [64]. In contrast to other DNA binding assays such as electrophoretic mobility shift assays, yeast one-hybrid, and chromatin immunoprecipitation PCR, DAP-seq does not require *a priori* selection of putative targets, but rather provides genome-wide binding information without the need to validate antibodies or generate transgenic plants. With DAP-seq, a DNA library is prepared by fragmenting genomic DNA and appending adaptor sequences to each fragment. Heterologously expressed affinity tagged transcription factors are bead-immobilized, incubated with the genomic DNA library, and washed such that only fragments that form a protein–DNA complex remain in the immobilized fraction. Elution and PCR amplification of this bound fraction further adds an indexed sequencing adaptor, allowing high-throughput sequencing of multiple affinity purification reactions that are mapped to a sequenced genome to identify enriched DNA fragments as putative binding peaks associated with individual transcription factors [64]. Analysis of the loci bound by a given transcription factor then allows for the deduction of transcription factor binding motifs by identifying overrepresented DNA sequences that co-occur with transcription factor binding peaks.

In the secondary cell wall network, analysis of DAP-seq data reported by O'Malley *et al.* (2016) facilitated the identification of a consensus binding motif for the layer 1 VND, NST, and SND NAC proteins. This motif, named the VNS (VND/NST/SND) element, consists of a simple palindrome flanking seven variable bases: C(G/T)TNNNNNNA(A/C)G [65]. Identification of a general *cis*-element associated with DNA binding by these top-level secondary cell wall regulators might be useful in efforts to discover the extent of their regulatory targets. DAP-seq analysis of SWAM1 DNA binding also allowed for the construction of a *de novo* binding motif, revealing that SWAM1 binding sites are enriched for the sequence (T/C)ACC(T/A)ACC [11]. This sequence is broadly consistent with the MYB AC element and the same sequence identified for a related *A. thaliana* cell wall regulator MYB61 [64].

grass species with significant MLG:xyloglucan endotransglucosylase activity. Both *BdTHX1* overexpression and dominant repressor fusion prevent the regeneration of transgenic plants from calli, complicating genetic approaches to conclusively establish direct regulation of *BdCSLF6* or *BdXTH8*. However, DNA binding data, coexpression analysis, and the close similarity in mispatterning observed in the abortive shoots of *BdTHX1-OE* calli and previous *CSLF6* overexpression lines support *BdTHX1* as an entirely novel secondary cell wall regulator and the first identified transcriptional regulator of MLG synthesis [14].

Two genes, *PtrEPSP* and *PtrhAT*, were recently reported as **neofunctionalized** wall regulators in *Populus trichocarpa* [17]. hAT- or SLEEPER-like genes are thought to be the product of molecular domestication, in which transposases acquire new functions [18,19]. *PtrhAT* binds the promoter of *PtrMYB021*, a *P. trichocarpa* ortholog of *AtMYB46*, and functions as a transcriptional repressor. *PtrhAT* is in turn repressed by *PtrEPSP*, with the net effect of de-repressing wall-activating gene expression. Transcriptional regulation by *PtrEPSP* is surprising, as this gene encodes a 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase, a critical enzyme in the **shikimate pathway** [20]. Evidence of a second, wall-related transcriptional function was first uncovered when single nucleotide polymorphisms in the *PtrEPSP* gene associated with variation in lignin content in a high-resolution linkage disequilibrium mapping study [21]. Overexpression of *PtrEPSP* in *P. trichocarpa* upregulated orthologs of *SND2*, *MYB46*, and *NST1* and produced trees with ectopic lignin deposition in epidermis, phloem fiber, and pith cells [17]. Consistent with partial neofunctionalization as a transcription factor, *PtrEPSP* localizes to the chloroplasts like other shikimate pathway genes, but also accumulates in the nucleus. Protein–DNA binding assays showed *PtrEPSP* directly binds to the *PtrhAT* promoter region and that affinity requires a putative helix–turn–helix domain, which is absent in the single other *P. trichocarpa* EPSP synthase gene. *PtrEPSP* retains EPSP synthase activity, although less than its single paralog. Despite

this evidence of functional novelty, PtrEPSP functions within the *P. trichocarpa* secondary cell wall network, as its expression is upregulated by *PtrWND1B*, an *SND1* ortholog, and it regulates downstream wall thickening [17].

### From Phenotype to Mechanism: Progress towards a Molecular Understanding of Secondary Cell Wall Environmental Responses

Environmental conditions, especially abiotic stresses, have long been known to influence wall phenotypes in both the primary and secondary cell walls [5,22]. With respect to the secondary cell wall, the regulatory mechanisms that enable these plastic responses have generally escaped characterization.

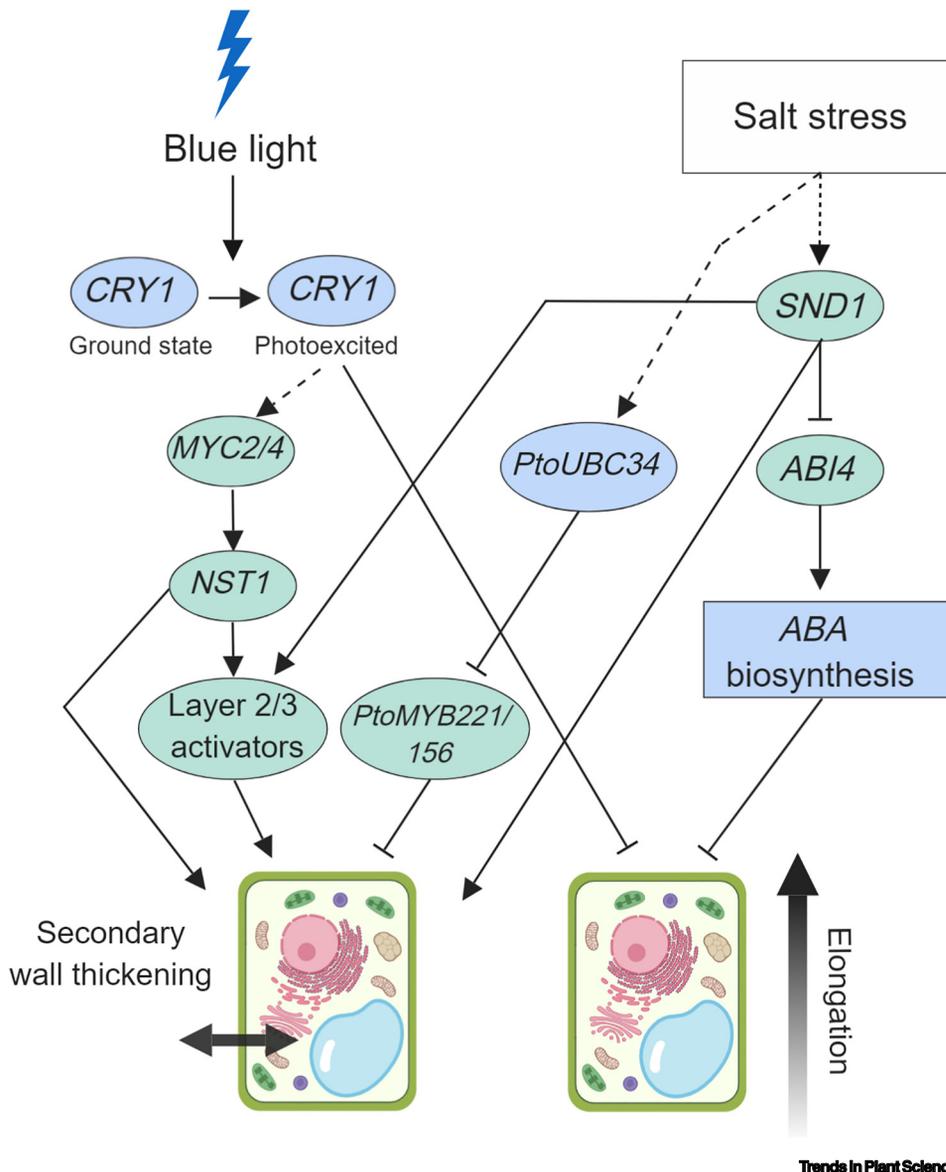
Long-term cold exposure induces wall phenotypic and transcriptional changes in the hybrid *Eucalyptus gunnii* X *Eucalyptus dalrympleana* [23]. Mimicking seasonal shifts in temperature, 46-day cold treatment induced the formation of thicker cell walls with enhanced lignification. At the transcriptional level, extended cold treatment stimulated a nonintuitive set of changes within the secondary wall regulatory network, increasing expression of an *SND3* ortholog, *EgNAC47*, but downregulating the *SND1* ortholog *EgNAC61*. However, within the third layer, cold treatment specifically enhances the expression of several activating transcription factors and represses a suite of repressors, explaining enhanced secondary wall deposition under prolonged cold [23].

*SND1*, an extensively characterized NAC protein in the first layer of the secondary cell wall network, was recently discovered to facilitate responses to salinity stress and, specifically, to coordinate wall deposition with altered phytohormone signaling under stress [24]. Salt treatment induces *SND1* expression, and *snd1* knockouts have reduced salt stress tolerance. The *snd1* plants also accumulate growth inhibiting phytohormone ABA, stimulating the discovery that *SND1* binds the promoter of the ABA regulator *ABI4*. This DNA binding activity and the observed hypersensitive upregulation of ABA synthesis in *snd1* plants under salt stress together suggest that *SND1* coordinates ABA inhibition of growth and secondary wall deposition under stress by activating downstream genes while also directly repressing *ABI4* (Figure 2).

In plants, cryptochromes (CRY) function as UV-A/blue light photoreceptors, undergoing a conformational change in response to photoexcitation of a covalently bound chromophore, which then enables transduction of light signals through a variety of protein–protein interactions with the excited CRY protein [25]. Mutants of *CRY1*, one of two *A. thaliana* CRY genes, display taller inflorescence stems with longer interfascicular fibers and reduced wall thickness, while *CRY1* overexpression reduces stem height and enhances fiber cell wall thickening, but not vessels [26]. These effects persist when plants are grown under blue light, but are eliminated by growth under monochromatic red, pinpointing blue light perception as the essential determinant of *cry1/CRY1-OE* secondary cell wall phenotypes. bHLH proteins have widespread involvement in transduction of plant photosensor signals, and Zhang *et al.* [26] identify MYC2 and MYC4 as novel bHLH activators of *NST1* expression. *MYC2* and *MYC4* expression and downstream *NST1* activation are blue light-responsive in a CRY1-dependent fashion, suggesting that *MYC2/4* are themselves regulated by CRY1 and serve as essential links between blue light perception and enhanced wall thickening. These findings lend mechanistic insight into previously observed UV and blue light mediated changes in mechanical strength and lignification [27,28].

### MicroRNA (miRNA) Regulation of Secondary Cell Wall-Associated Transcripts

Post-transcriptional miRNA regulation of transcripts is perhaps the most recognized and well characterized non-DNA-binding regulatory mechanism implicated in secondary cell wall regulation. miRNAs are a class of noncoding RNA that inhibit translation of proteins by partially pairing



**Figure 2. Environmental Stimuli That Influence Cell Elongation and Wall Thickening.** Cryptochrome (CRY) mediated blue light perception activates secondary cell wall deposition via transcriptional activation of two bHLH transcription factors, *MYC2* and *MYC4* [26]. Blue light signaling negatively regulates elongation growth, suggesting that blue light input coordinates secondary wall biosynthesis with primary growth and resource allocation. Salt stress stimulates biosynthesis of the phytohormone abscisic acid (ABA), which is generally antagonistic to primary growth. SND1, a layer 1 activator of secondary cell wall biosynthesis, is also induced by salt stress and promotes tolerance by preventing hypersensitive ABA accumulation [24]. In *Populus tomentosa*, salt stress stimulates *PtoUBC34* expression, which attenuates negative regulation of monolignol biosynthesis by *PtoMYB221* and *PtoMYB156* [53]. Unbroken and dashed lines indicate characterized and uncharacterized processes, respectively. Transcription factors are shown in green, environmental responses are indicated by blue. Created with BioRender.

with complementary regions of a transcript and inducing the cleavage of the mRNA molecule [29,30]. In *A. thaliana*, a family of five class III homeodomain-leucine zipper (HD-ZIP III) genes are required for correct xylem development and regulate the first layer of the secondary cell wall network [9,31]. This family, comprising *CORONA/HB15*, *REVOLUTA*, *PHAVOLUTA*,

*PHABULOSA*, and *HB8* are targets of miRNA165/166, and overexpression of these inhibitory RNAs misregulates vascular development [32]. miRNA targeting of HD-ZIP III transcripts serves to pattern the vasculature early in development, via a non-cell-autonomous gradient of miRNAs that controls HD-ZIP III protein levels across developing vascular and nonvascular tissues [33,34]. Separately, the *P. trichocarpa* miRNA, *ptr-miR397a*, represses lignification by targeting transcripts of laccase enzymes responsible for the polymerization of monolignols in the wall [35].

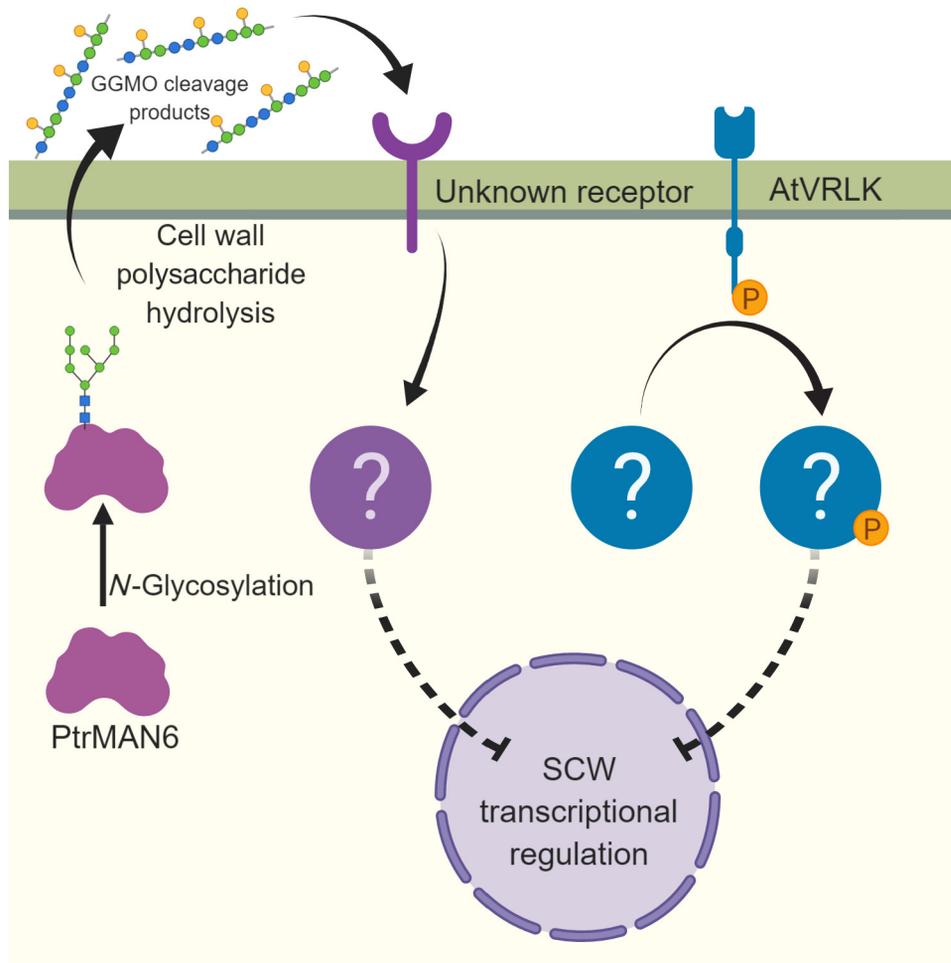
A recent report by Sun *et al.* [36] implicates both a novel miRNA, *miR319*, and a novel transcription factor, *TCP4*, in control of vascular patterning and secondary cell wall deposition. Overexpression of a cleavage-resistant *TCP4* allele increased the number of xylem vessel elements in *A. thaliana* hypocotyls and enhanced the thickness and lignin content of vessel walls, while lines overexpressing *miR319* had fewer vessels with thinner, less lignified walls. *TCP4* directly activates *VND7* expression in protoplasts and is capable of binding the *VND7* promoter *in vitro*. This activation is reflected in enhanced expression of cellulose and hemicellulose biosynthetic genes in stable *TCP4* overexpression lines, co-induction of these genes with *TCP4* in plants bearing an inducible *TCP4* construct, and their downregulation in *miR319* overexpressing plants [36].

### Post-translational Modifications Modulate Secondary Cell Wall Transcriptional Regulation

**Post-translational modification (PTM)** provides an additional mechanism to modulate the transcriptional effects of regulatory proteins and can add responsiveness and complexity to a regulatory system [37]. In cell wall biosynthesis, modifications that directly affect the activity of catalytic proteins have historically been well studied, with particular focus on phosphorylation of CESA proteins [38–40]. Recent work has identified a diverse array of post-translational modifications that act upstream of biosynthesis to regulate cell wall deposition.

Characterization of VASCULAR RELATED RECEPTOR-LIKE KINASE 1 (AtVRLK1) indicates that phosphorylation likely regulates both transcriptional and enzymatic activity in wall biosynthesis. AtVRLK1 is specifically expressed in vascular tissues [41]. Subcellular localization and *in vitro* enzyme experiments show that it has kinase activity and is localized to the plasma membrane, consistent with a putative function in PTM-mediated signal transduction. Although specific AtVRLK1 phosphorylation targets have not been identified, secondary cell wall activators and target genes are suppressed by AtVRLK1 overexpression and enhanced by expression of a dominant negative version of the kinase, with concurrent changes in wall thickness across vascular and mechanical cell types in the stem. A similar global effect on wall-associated gene expression is mediated by *N*-glycosylation of a xylem-specific *P. trichocarpa* endo-1,4- $\beta$ -mannanase, PtrMAN6. MAN proteins catalyze polysaccharide hydrolysis, promote seed germination, fruit ripening, and loosening of the primary cell wall [42]. *N*-glycosylation enhances PtrMAN6 hydrolytic activity and therefore its cleavage of wall polysaccharides. Both *PtrMAN6* overexpression and exogenous application of *PtrMAN6*-hydrolysis derived galactoglucomannan oligosaccharides are sufficient to downregulate secondary cell wall-associated genes and reduce wall deposition, suggesting the existence of a feedback system in which oligosaccharide molecules derived from the existing cell wall regulate additional wall deposition in a manner that depends on both *PtrMAN6* expression and *N*-glycosylation [41] (Figure 3).

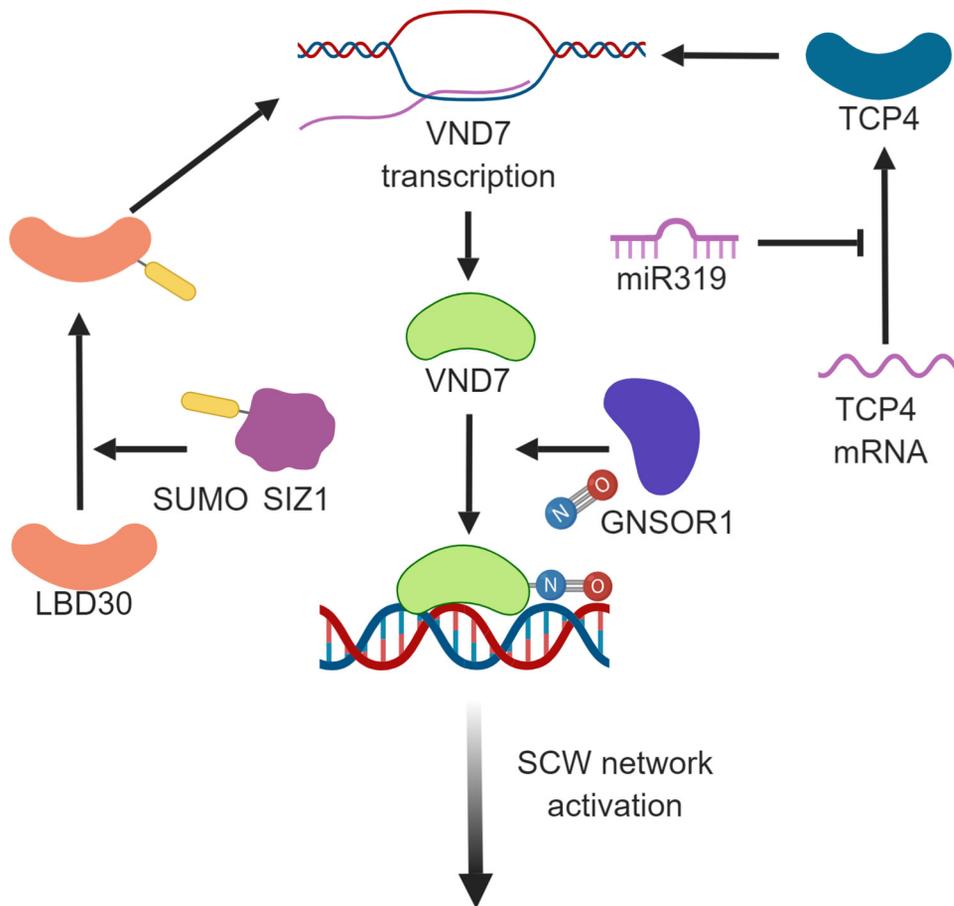
Specific secondary wall-associated targets of PTM have also been identified. LBD30 is one of a trio of lateral organ boundaries (LOB) domain-containing proteins known to activate *VND7* expression in *A. thaliana* [9,43,44]. This previously characterized function depends on a newly discovered protein–protein interaction with SIZ1, a well characterized small **ubiquitin**-related modifier (SUMO) E3 ligase, and the resulting sumoylation of LBD30 [45]. Sumoylation describes

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**Figure 3. Evidence of Post-translational Modification-Mediated Secondary Cell Wall Regulation by Extracellular and Membrane-Associated Components.** AtVRLK, a vasculature-expressed, membrane-localized receptor-like kinase represses secondary cell wall thickening. AtVRLK mutation and overexpression misregulate expression of transcription factors in layers 1 and 2 of the secondary cell wall regulatory network and biosynthetic genes, suggesting that an unknown, phosphorylation-dependent pathway acts on the top levels of the network to restrict wall deposition. P icon indicates AtVRLK autophosphorylation activity and phosphorylation of unidentified regulatory proteins. N-glycosylation, the covalent linkage of a glycan oligosaccharide to a protein, enhances the enzymatic activity of PtrMAN6, an endo-1,4- $\beta$ -mannanase capable of hydrolytic cleavage of galactoglucomannan hemicelluloses. Cleavage yields galactoglucomannan oligosaccharides (GGMO) that function as putative extracellular signaling molecules to repress secondary cell wall deposition through an unknown perception and transduction pathway. Abbreviation: SCW, secondary cell wall. Created with BioRender.

the enzyme-catalyzed conjugation of proteins with the diminutive SUMO protein at specific lysine residues and is implicated in a variety of processes, particularly in controlling subcellular protein localization [46,47]. Liu *et al.* [45] directly detect LBD30 sumoylation and show that the enhanced secondary wall deposition in LBD30 overexpression events depends on both the presence of the LBD30 K226 sumoylation target residue and SIZ1 activity.

VND7 itself is subject to a different PTM, S-nitrosylation, a covalent but reversible modification of cysteine residues by nitric oxide addition [48] (Figure 4). In a similar phenomenon, the presence of a PTM facilitating enzyme, here a S-nitrosogluthione reductase, GNSOR1 is a prerequisite for downstream activation of secondary cell wall genes by VND7. Kawabe *et al.* [48] identified

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**Figure 4. VND7 Gene Expression and Protein Activity Is Subject to Multiple Modes of Regulation.** VND7 is a layer 1 activator specifically associated with vascular differentiation and secondary cell wall (SCW) thickening. TCP4, an activator of VND7 expression, is regulated at the transcript level by *miR319* [36]. Separately, activation of VND7 by LBD30 depends on a post-translational modification of LBD30 protein, SIZ1-mediated SUMOylation [45]. VND7 function as a robust activator of wall biosynthesis further depends on GNSOR1-dependent S-nitrosylation [48]. VND7 protein levels and activity are key determinants of wall outcomes and these findings reveal that miRNA targeting, transcriptional and protein regulation work in combination to modulate the effects of a potent activator. Created with BioRender.

GNSOR1 regulation of VND7 using a forward genetics approach, mutagenizing *A. thaliana* carrying a DEX-inducible VND7 overexpression construct, and screening for mutations that disrupt the xylem transdifferentiation phenotype produced by VND7 induction [48,49]. VND7 can be S-nitrosylated *in vitro*, and serine substitution of two nitrosylation target cysteine residues reduces VND7 transcriptional activity *in planta*. Together these findings demonstrate that even well characterized aspects of wall regulation might be gated by protein-level phenomena like protein–protein interactions and post-translational modifications.

Changes to protein localization or turnover are common mechanisms by which PTMs control the regulatory functions of transcription factors [37,50]. In *Populus tomentosa*, PtoUBC34 is an endoplasmic reticulum (ER)-localized, E2 ubiquitin conjugating enzyme that, when coexpressed with two different transcriptional repressors of lignin biosynthesis, *PtoMYB221* and *PtoMYB156*, attenuates their negative regulation of target genes and triggers their accumulation in the endoplasmic reticulum, where they physically interact with PtoUBC34 [51–53]. While ubiquitinated

fractions of either MYB protein have not been isolated, this de-repressive effect is lost when the conserved active site of PtoUBC34 is mutated, suggesting that a specific protein–protein interaction with PtoUBC34 and subsequent ubiquitylation initiates ER-associated degradation of these proteins [53,54]. PtoUBC34 expression is strongly upregulated by salt and heat stress treatments, identifying ubiquitylation as a potential mechanism to fine-tune regulation in response to environmental cues.

### Epigenetic Regulation of Secondary Cell Wall Biosynthesis

Epigenetic regulation can have dramatic transcriptional consequences, gating the effects of other regulatory modes by controlling the accessibility of DNA binding sites [55]. While chromatin structure, DNA and histone modifications, and the functions of specific histone proteins have been extensively studied in development and stress responses, evidence that vascular patterning or wall deposition are regulated at the chromatin level has only recently emerged [15,56,57].

In *Eucalyptus grandis*, the histone EgH1.3 functions to regulate wall deposition and lignification in particular [57]. H1 histones bind DNA at the entry and exit sites of core nucleosomes, and these linker histones have multifaceted roles in chromatin organization and transcriptional regulation [58,59]. Orthologous to the *A. thaliana* histone AtH1.3, *EgH1.3* is expressed in the root vasculature early in development. However, in mature xylem layers, it is only strongly expressed in ray and parenchyma cells, tissues that are adjacent to vascular bundles, but which do not themselves undergo extensive wall deposition. This expression profile overlaps with *EgMYB1*, a direct repressor of phenylpropanoid pathway genes and lignin deposition [60]. *EgMYB1* and *EgH1.3* proteins interact to enhance *EgMYB1* activity as a transcriptional repressor [57]. Critically, the molecular mechanism of combinatorial transcriptional regulation by the *EgH1.3*–*EgMYB1* complex has not been elucidated. However the repressive activity of the *EgMYB1* conserved EAR domain is understood to involve recruitment of remodeling machinery to modify local chromatin structure, which might align with the general function of H1 histones in regulating chromatin stability [58,61] (Figure 5). The tissue-specific *EgH1.3* expression pattern may highlight yet another mechanism to ensure tight regulation of wall thickening, leading to differential effects by the same regulatory proteins between tissues.

Further implicating epigenetic processes in determining which tissues will undergo vascular differentiation and secondary cell wall thickening, Turco *et al.* [15] show that a suite of genes associated with *de novo* methylation and methylation maintenance are coexpressed with wall network genes in *S. bicolor* root vasculature [15]. Seedlings treated with the methyltransferase inhibitor 5-azacytidine displayed dramatically shorter roots, indicating a role for DNA methylation in *S. bicolor* root development. Methylation was sufficient to differentiate vascular and nonvascular tissues; however, combining inferences from transcriptomic and methylomic analysis, the relationship between DNA methylation and gene expression is apparently highly complex and suggests that methylation, particularly in the gene body, is especially potent in regulating genes that are generally lowly expressed [15]. Interestingly, a H1/H5 histone family gene, Sb07g020920, was among the genes found to be both differentially methylated and differentially expressed between vascular and nonvascular tissues, which may indicate that specific linker histone–transcription factor interactions are a conserved aspect of regulation.

### Concluding Remarks and Future Directions: A Multilevel Understanding of Secondary Cell Wall Regulation

The findings reviewed here represent early steps in establishing a new, multilevel paradigm of secondary cell wall regulation (Figure 6). This model incorporates a medley of novel processes that complement the function of the known regulatory network and reflect the expanded diversity

### Outstanding Questions

How do novel regulatory components and processes interact with the known secondary wall regulatory network? Are novel regulators like SWAM1 and BdTHX1 regulated upstream by known network components?

What aspects of wall regulation are common and which are distinct between herbaceous and woody eudicots and grasses? How are the unique morphological and biochemical aspects of grass secondary walls controlled?

How does the regulation of the secondary growth that generates wood (xylem) in trees interact with characterized components of the secondary cell wall network?

How does the secondary cell wall regulatory network mediate environmentally informed developmental plasticity, and what systems are involved in environmental perception and signal transduction?

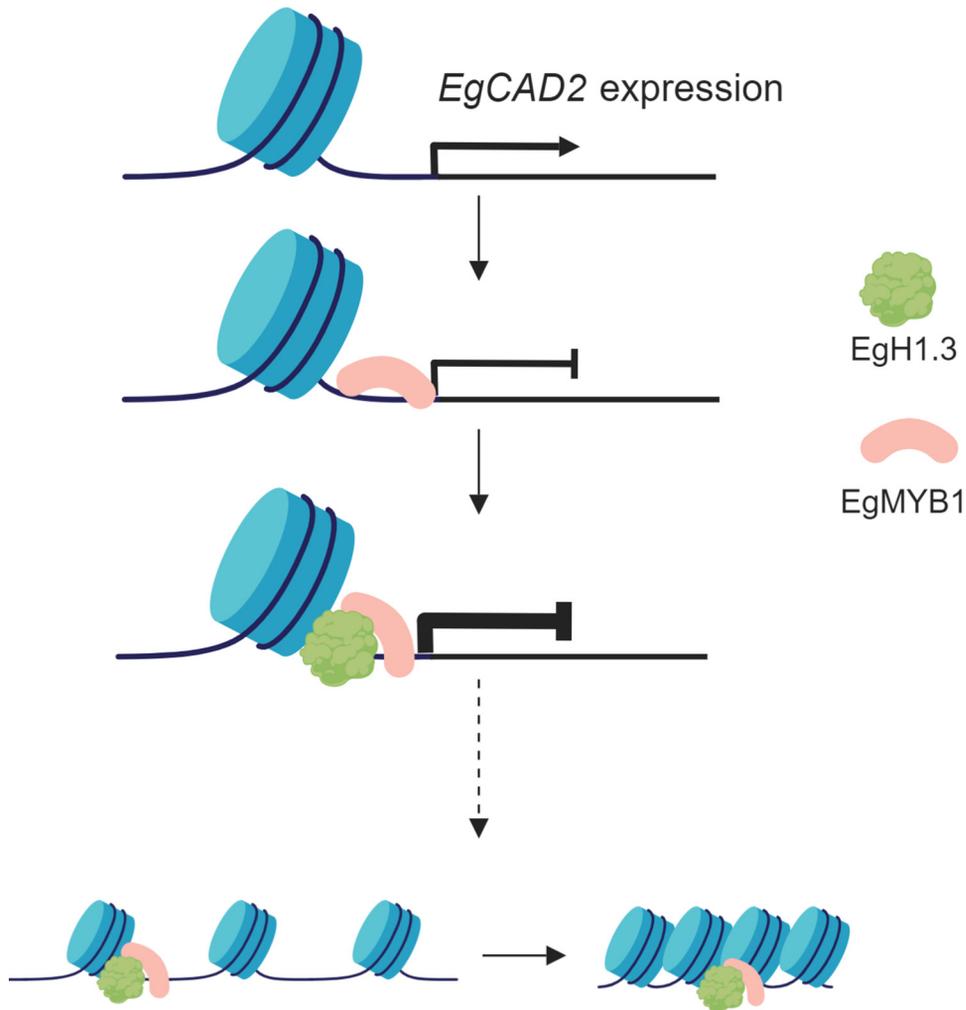
What receptors, signaling molecules, and transcription factors are involved in galactoglucomannan oligosaccharide-mediated repression of secondary cell biosynthesis?

What targets does AtVRLK1 phosphorylate? What other kinases are involved in phosphorylation-mediated secondary cell wall regulation?

What is the directional relationship between tissue-specific DNA methylation, vascular differentiation, and secondary cell wall thickening?

Are histone variants beyond EgH1.3 significant in secondary cell wall regulation? Does secondary cell wall regulation by histones occur in taxa beyond *Eucalyptus*, and how extensive is the diversity of secondary cell wall regulating histones?

What is the biological significance of protein-level regulatory processes in achieving tight spatio-temporal regulation or dynamic environmental responses?

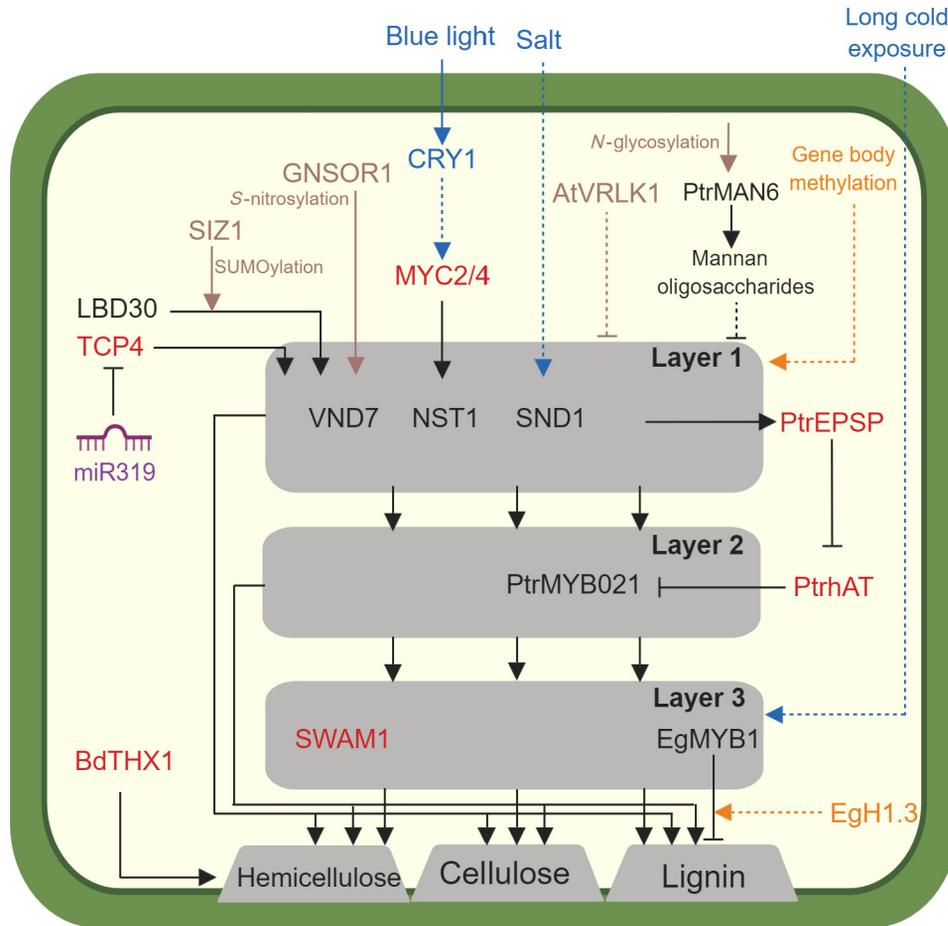


Trends in Plant Science

**Figure 5. Combinatorial Regulation by Histone and Transcription Factor Proteins Mediates Secondary Cell Wall Regulation.** EgMYB1 and EgH1.3 cooperatively repress expression of the monoglucanase gene *EgCAD2*. EgMYB1 is an EAR domain-containing MYB transcription factor that directly binds the *EgCAD2* locus and represses transcription. The linker histone protein EgH1.3 does not reduce *EgCAD2* expression, but co-overexpression of *EgH1.3* and *EgMYB1* synergistically represses *EgCAD2* and lignin biosynthesis. EgH1.3 and EgMYB1 engage in a protein–protein interaction in *planta* [57]. Both EAR domain-containing transcription factors and linker histones are understood to modulate transcription by controlling chromatin remodelling. Dashed line indicates the hypothesized mechanisms of combinatorial EgMYB1/EgH1.3 regulation. Created with BioRender.

of DNA binding. Technological advances and the continued proliferation of genomic resources in species beyond *A. thaliana* have also paved the way for a flurry of new discoveries, particularly from tree and grass species, that address questions specific to those lineages.

In our developing schema, DNA methylation is perhaps the most upstream known regulatory process to influence wall development, with a complex but potentially significant role in directing the tissue-specific expression of regulatory and biosynthetic machinery [15,62,63]. In cells fated to undergo secondary wall thickening by epigenetic or other mechanisms, the activation of layer 1 NAC transcription factors induces a feed-forward cascade of expression, in which transcription factors in layer 1 and 2 activate components of downstream layers and directly regulate



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**Figure 6. A Multilevel Network of Secondary Cell Wall Regulation.** Wall deposition is mediated by three layers of transcription factor regulation that engage in feed-forward activation to ensure strong expression of biosynthetic machinery. A suite of new findings expands this network and demonstrates that regulation by transcription factors is further modulated by epigenetic regulation, miRNA targeting, and post-translational modifications that affect protein function. For an expanded account of characterized proteins that comprise the three layers of the regulatory network, see Zhang *et al.* [9]. Shown here are novel components of this multimodal system and their interactions. Novel transcription factors are shown in red, environmental regulation in blue, epigenetic processes in orange, miRNAs in purple, and enzymes that facilitate post-transcriptional modifications are shown in brown. Unbroken and dashed lines indicate characterized and uncharacterized interactions, respectively. Created with BioRender.

biosynthetic machinery [3,9]. Further characterization of the novel transcription factors presented here could provide opportunities to determine if SLEEPER-like or EPSP synthase secondary cell wall transcriptional regulation exists outside of *Populus*, to better understand how SWAM1, TCP4, and BdTHX1 interact with other network components, and to further identify regulators of grass-specific hemicelluloses [11,14,17]. While the tissue specificity of wall deposition must be maintained to ensure adequate mechanical support and vascular function, environmental signals modulate the magnitude of transcriptional activation in fortified tissues. We review three publications that clarify the mechanisms that underpin these effects and that together highlight intriguing mechanistic differences in abiotic regulation of wall phenotypes. Jeong *et al.* [24] and Zhang *et al.* [26] show that blue light and salinity stress perception act on layer 1 genes, with blue light activating *NST1* via *CRY1* and *MYC2/4* and salt upregulating *SND1* [24,26]. In contrast, the transcriptional effects of cold treatment in a *Eucalyptus* hybrid are inconsistent with top-down

activation of the secondary cell wall network, as layer 1 genes are either downregulated or unaffected under cold treatment, but layer 3 activators are strongly expressed [23]. These differences in environmental perception and signal transduction, the emerging nexus of light and temperature signaling in plant biology, and the continued existence of wall-modifying abiotic stresses that have not been interrogated mechanistically suggests that future work will establish environmental regulation as a prominent aspect of an updated secondary cell wall regulatory model.

Beyond processes that govern the transcription of regulatory genes, post-transcriptional miRNA regulation of HD-ZIP III and other transcription factors provides the first illustration of an instance in which transcription factor–DNA binding regulation works in concert with a separate regulatory mode to more tightly control tissue-specific thickening of cell walls. This conceptual framework, wherein the expected regulatory effects of transcription factors further depend on the presence or absence of other components, constitutes the fundamental mechanism by which histone interactions, PTMs, and other protein-level regulatory modes reviewed here influence secondary cell wall development. It is intuitive that the more conditions that must be satisfied, such as the absence of inhibitory miRNAs, or the presence of PTM facilitating enzymes in addition to the expression of network transcription factors, the more the system is buffered against misregulation. This review captures the recent explosion of studies that describe individual instances where complementary modes of regulation modulate the function of network transcription factors. However, building a generalized model of regulation that incorporates these additional processes will require extensive future research, as it remains unclear the extent to which these novel systems are conserved outside of their species of discovery, how they might interact with each other, and how they interact with the secondary cell wall transcriptional network as it is currently understood (see Outstanding Questions).

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