Title: The formation of wood and its control

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Abstract

Wood continues to increase in importance as a sustainable source of energy and shelter. Wood formation is a dynamic process derived from plant secondary (radial) growth. Several experimental systems have been employed to study wood formation and its regulation. The use of genetic manipulation approaches and genome-wide analyses in model plants have significantly advanced our understanding of wood formation. In this review, we provide an update of our knowledge of the genetic and hormonal regulation of wood formation based on research in different plants systems, as well as considering the subject from an evo-devo perspective.

Introduction

Wood (secondary xylem) is mainly composed of tracheary elements (TEs, tracheids and vessels) and fibers, both of which have highly thickened secondary cell walls (SCWs); xylem parenchyma also forms a minor component. Patterning of the SCWs in xylem cells is adapted to their function. Fibers have thick and uniformly deposited walls to provide support and protection, whereas TEs have patterned walls to facilitate water transport. Protoxylem cell walls have annular or spiral patterning, providing elasticity to the elongating tissues. Metaxylem cells, on the other hand, have pitted or reticulate wall patterning, making them more rigid and durable. All of the types of xylem cells are derived from vascular cambium, the lateral secondary meristem. Wood formation is a dynamic and continuous process which includes cambial cell proliferation, xylem cell specification and expansion, secondary cell wall biosynthesis and programmed cell death. Each step is highly regulated by internal and external factors. In this review, we will first introduce various experimental systems and techniques

used in wood formation studies, followed by an update of our knowledge about the genetic and hormonal regulation of different developmental stages of wood formation from an “evo-devo” perspective.

Experimental models and approaches for studying wood formation

Several different experimental systems have been used to study wood formation. Important discoveries have been made using *Zinnia* cell cultures, including the identification of master regulators of TE differentiation and factors involved in SCW biosynthesis and PCD. In addition, *Arabidopsis* has emerged as an excellent model to study wood formation. *Arabidopsis* has a unique genetic resource for developmental studies: a cell-type specific gene expression map for the root vasculature [1]. Transcriptomics, combined with the genetic manipulation of *Arabidopsis*, has led to the identification of several key factors regulating wood formation (Figure 1) [2]. In several cases (see below), their tree orthologs have been shown to share a similar function, indicating that the control of wood formation is evolutionarily conserved between woody and herbaceous species (Figure 1).

Due to their massive capacity for wood production, trees are natural research models for this process. Researchers have taken advantage of the large size of the multilayered cambial meristem and wood forming tissues in a tree trunk; for example, transcriptomics and chemical profiling have been performed at a high spatial resolution across the wood-forming region of a *Populus* stem [3,4]. Tree genetics has enjoyed an increase in productivity thanks to the development of next generation sequencing technologies and the enormous genetic diversity of tree genomes. Genomes of several forest trees, including *Populus*, Eucalyptus [5], together with the first conifer genomes, Norway and white spruce [6¨, 7¨], have already been released. The spruce genome will provide a powerful platform to study wood formation, enabling researchers to conduct comparative genome-wide studies between angiosperms and gymnosperms. One difference between the two is the xylem, which is composed solely of tracheids in gymnosperms but of both fibers and TEs in angiosperms. Potentially reflecting this difference in xylem cell types, only two *VASCULAR RELATED NAC DOMAIN* (*VND*) genes (which are master regulators of xylem differentiation, see below) have been discovered in the spruce genome, compared with seven in *Arabidopsis*. This result suggests that expansion of the VND gene family was important in the development of angiosperm wood [6¨].

In addition, genomes of some “woody” monocot species, such as bamboo and oil and date palm trees, have been released recently [8,9,10]. Despite not having a vascular cambium, these

species can reach a large size by producing an extensive number of vascular strands during their growth. These genomic resources provide us novel opportunities to study the evolution of genes that are crucial for cambium initiation and wood formation.

Complementing the advances in genomics, a breakthrough has recently taken place in tree genetics research: the first specific, causative mutation behind a tree architectural trait has been identified. By mapping a segregating progeny population, Dardick *et al.* [11••] found that the *PpeTAC1* (*TILLER ANGLE CONTROL 1*) gene regulates branch angle in peach; a semidominant mutation of this gene is responsible for the standard, upright and pillar form of peach trees. The success of the mapping approach reaffirms the possibility of discovering the genes controlling any tree trait, including the regulation of their massive wood production in angiosperm trees. By taking advantage of an early flowering spruce mutant "acrocona" [12], in combination with spruce transformation technology [13], similar studies are now becoming possible also in gymnosperm trees.

Regulation of cambium activity and cell proliferation

Recent findings have identified a peptide-receptor-transcription factor signalling pathway, TDIF/CLE41/CLE44-TDR/PXY-WOX4, that controls cambium maintenance (Figure 1) [14,15]. The small peptide TDIF, that is processed from the translated products of *CLE41/CLE44* in *Arabidopsis*, is produced in the phloem; it interacts with its receptor, the receptor-like kinase TDR/PXY (TDIF RECEPTOR/PHLOEM INTERCALATED WITH XYLEM), which is expressed in (pro)cambium. *WOX4*, a *WUSCHEL HOMEOBOX RELATED* gene, mediates this ligand-receptor signalling to regulate the maintenance of (pro)cambium cells. Cambium activity is reduced in the hypocotyl and inflorescence stem of the *wox4* mutant, indicating that *WOX4* regulates, but is not required to establish, the meristem [14,16]. Another WOX-family gene, *WOX14*, acts redundantly with *WOX4* to regulate cambial cell proliferation [17]. The TDIF/CLE41/CLE44-TDR/PXY-WOX4 signalling pathway seems to be evolutionarily conserved between both woody and herbaceous species, as it has been described in both *Arabidopsis* and *Populus* [3].

Partners potentially interacting with TDR/PXY have recently been identified: the receptor-like kinases (RLKs) ERECTA (ER) and ER-LIKE1, together with their putative ligands EPFL4 and EPFL6 [17,18]. Their mutation enhances the *tdr/pxy* phenotype in vascular patterning. In addition, two other RLKs, MORE LATERAL GROWTH1 (MOL1) and REDUCED IN LATERAL GROWTH1 (RUL1), represent opposing regulators of cambial activity which probably act upstream of the TDR/PXY-WOX4 pathway. *MOL1* acts as a repressor and *RUL1*

as an activator of secondary growth in the inflorescence stem, which is enhanced in *mol1* and reduced in *rul1* [19].

Class I KNOX transcription factors (TFs) are important in maintaining the meristematic activity in the shoot apical meristem of *Arabidopsis*. Interestingly, KNOX genes are also expressed in the cambium region during wood formation in *Populus* [3]. When *ARK2*, the ortholog of *Arabidopsis BP/KNAT1* is overexpressed, the cambium region is expanded and xylem differentiation is inhibited; by contrast, in the knock-down lines, lignified xylem and fiber cells appear earlier than in wildtype, indicating a role for this KNOX gene in the regulation of secondary xylem differentiation (Figure 1) [20]. This is similar to the situation in *Arabidopsis*, where ectopic lignin deposition is found in *bp* mutant stems [21].

Plant hormones also play important roles to regulate wood formation (Figure 1). It has been known for a long time that auxin concentration peaks at the cambium zone in the tree stem. Perturbing auxin signalling by over-expressing a mutated *Populus IAA3* gene resulted in reduced cell proliferation in the cambium and thus less wood formation [22]. The TDR/PXY-WOX4 pathway appears to act downstream of auxin signalling in regulating cambial cell proliferation [16]. The role of cytokinin as an essential regulator of cambium activity has been demonstrated in both *Arabidopsis* and *Populus* [23,24]. Reduced cytokinin levels lead to impaired cambial activity in tree stems [24], and secondary growth does not occur in the *Arabidopsis* mutant *ipt1,3,5,7* where four genes encoding the cytokinin biosynthesis enzyme IPT are simultaneously mutated [23]. Consistent with this, secondary growth is enhanced when cytokinin signalling is increased [25].

Ethylene acts as a positive regulator of wood formation. In *Populus*, ethylene treatment promotes cambial cell proliferation [26]. In the *Arabidopsis* stem, ethylene appears to crosstalk with the TDR/PXY pathway. In the *tdr/pxy* background, a double mutant of two *ETHYLENE INDUCED RESPONSE FACTOR* genes (*ERF109* and *ERF018*) shows reduced secondary growth while an ethylene over-producing mutant displays enhanced growth [27]. Recently, a genome-wide screen for *Populus* ERFs led to the identification of *ERF* genes that modify secondary growth, wood properties and tension wood formation [28•], indicating that the ethylene pathway also regulates various aspects of wood formation in trees.

Recent findings have identified auxin-mediated basic helix-loop-helix (bHLH) transcription factor dimers as important factors regulating early vascular development, including *TARGET OF MONOPTEROS5* (*TMO5*), *LONESOME HIGHWAY* (*LHW*) and their closest homologs

[29•]. Vascular tissue differentiation was totally blocked in the roots of *TMO* quadruple mutants. By contrast, co-overexprsison of *TMO5* and *LHW* induced dramatic periclinal divisions within the vasculature of roots. During primary root vascular development in *Arabidopsis*, a cytokinin-auxin crosstalk loop has been shown to regulate procambium activity and xylem formation [30•]. It would be intriguing to find out how these factors interact to regulate secondary growth in different species.

Regulation of xylem specification and cell expansion

The *class III homeodomain leucine zipper* (*HD-ZIP III*) genes play important roles in xylem specification during primary growth (Figure 1). When expression of all five *HD-ZIP III* genes is reduced in *Arabidopsis*, procambium cells fail to differentiate into xylem cells [31]. Furthermore, the gradient of SHR and miR165/166 resulting from their bidirectional transport in root modulates *HD-ZIP III* levels to regulate protoxylem differentiation in the root [31]. Brassinosteroids can also activate *HD-ZIP III* expression and thus promote xylem differentiation [32]. The function of the *HD-ZIP III* genes during wood formation has been recently studied in *Populus*. Knockdown of *POPCORONA* causes abnormal lignification of pith cells, while overexpression of miRNA-resistant *POPCORONA* results in delayed lignification of xylem and phloem fibers [33]. On the other hand, when a microRNA-resistant form of *popREVOLUTA* was overexpressed, ectopic layers of cambium with reversed polarity were formed within cortical parenchyma [34].

Several NAC-domain transcription factors have been identified as master regulators of xylem differentiation. *VND7* induces protoxylem and *VND6* induces metaxylem differentiation, whereas *SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN 1*/ *NAC SECONDARY WALL THICKENING PROMOTING FACTOR3* (*SND1/NST3)*, together with *NST1*, promotes fiber differentiation (Figure 2) [reviewed in 2]. Similarly, in *Populus*, overexpression of the NAC domain genes *PtVNS/PtrWND* induces ectopic wood formation [35]. On the other hand, another NAC domain gene, *VND-INTERACTING 2* (*VNI2)*, acts as a repressor of *VND7* [36]. In *Populus*, galactoglucomannan oligosaccharides (GGMOs) have been identified as novel repressors of NAC TF expression [37]. Accordingly, over-expression of the endo-mannase gene *PtrMAN6* suppressed secondary cell wall thickening while its silencing had the opposite effect [37].

Although TDIF promotes cambial cell proliferation, it inhibits vessel differentiation in leaves and hypocotyls [14, 38]. Another group-A CLE peptide, CLE10, suppresses protoxylem vessel formation in roots by activating cytokinin-mediated pathway [39].

Unlike auxin, which is found in the cambium, gibberellic acid (GA) is observed in the differentiating xylem cells of tree stems [40]. When GA levels are increased by overexpressing GA-20 oxidase in *Populus*, an increase in both the number and length of xylem fibres is observed, indicating that GA promotes both cell division and xylem elongation (Figure 1) [41]. Recent analyses in *Arabidopsis* have revealed that xylem expansion in the hypocotyl is promoted by flowering-related GA transport [42].

Transcriptional regulation on secondary cell wall (SCW) biosynthesis and programmed cell death (PCD)

The NAC master regulators (VND6, VND7, NST1 and SND1/NST3) switch on the xylem differentiation program largely by inducing the expression of two MYB TFs, *MYB46* and *MYB83* (Figure 2) [43,44]. The *MYB46/83* node activates the expression of a plethora of other TFs and enzymes which are directly active in SCW biosynthesis (Figure 2) [43,44,45]. The induced TFs either promote biosynthesis of cellulose, hemicellulose, xylan and lignin, or alternatively act as negative feedback regulators of this process. In general, the xylem differentiation program functions through a robust multilevel feed-forward loop, with the *NAC*s and the *MYB*s acting as master switches which directly induce many of the same genes (Figure 2). This two-level master switch system of xylem cell differentiation appears to be evolutionarily conserved between woody and herbaceous species. Orthologs of the *Arabidopsis NAC* and *MYB* genes have been identified, and in some cases functionally verified, in several tree species, among them *Populus* (Figure 2), Eucalyptus and pine [46, 47].

The xylem cell-type specific pattern of SCW deposition is determined by cortical microtubules (MT) that direct the movement of cellulose synthase complexes [48]. Excitingly, several novel regulators of MT network patterning have been recently identified. Overexpression of two MT-ASSOCIATED PROTEIN 70 (MAP70) proteins promotes spiral cell wall patterning, whereas their silencing induces production of pitted walls [49]. By contrast, the MT-depolymerising protein MIDD1 promotes MT depolymerization at the forming pit regions; knock-down of MIDD1 produces pit-free walls [50]. Localization of MIDD1 at the plasma membrane takes place through ROP (Rho of plant) GTPase regulation, where local activation of ROP11 recruits MIDD1 at the forming pit [51•].

PCD represents the final stage of xylem differentiation. TEs undergo PCD via a fast autolysis mechanism that involves vacuolar collapse by tonoplast rupture, releasing digestive enzymes (nucleases and proteases) which degrade cell components [52]. Fibers undergo PCD through a slower pathway that requires DNA degradation and cellular dismantling before the vacuolar collapse [52]. In *Populus,* the 20S proteasome (20SP) was shown to be responsible for the caspase-3-like activity in secondary xylem development; inhibition of 20SP impairs PCD of TEs in poplar and *Arabidopsis* [53]. *VND6* induces the expression of the cysteine proteases XCP1 (XYLEM CYSTEINE PROTEASE 1) and XCP2 [54] which participate in autolysis during tracheary element PCD [55]. Cysteine protease METACASPASE 9 (AtMC9) has recently been shown to be important for efficient progression of autolysis during *Arabidopsis* vessel PCD [56]. The timing of PCD is regulated by the polyamine thermospermine, which is synthesised by ACUALIS5 (ACL5). Recent studies reveal that its ectopic overexpression in *Populus* delays xylem maturation [57].

Lignin is the last compound to be added to the SCW of xylem cells. Monolignols are stored within the vacuole and released during PCD to polymerize into the cell wall [58]. Lignification of TEs appears to be partly non-cell autonomous and continues even after the PCD [49•, 58]. Recently, miRNA control of lignification has been identified in *Populus; Ptr-miR397a* participates in post-transcriptional regulation of laccase genes [59•].

The recently published bamboo and palm genome sequences enable us to identify regulators of xylem formation also in “woody” monocot species. Although their vascular bundles consist of only primary xylem and phloem tissues, it is possible that the regulatory network of SCW formation is evolutionary conserved. The bamboo genome contains high copy numbers of genes that affect cell wall composition and structure, such as cellulose and lignin biosynthetic enzymes, similar as in *Populus* genome [8].

Conclusions and future Perspectives

The characterization of the TDIF/CLE41/CLE44-TDR/PXY-WOX4 signalling peptide-receptor-target module has greatly advanced our understanding of vascular regulation. However, as discussed above, cambium identity is not affected in the *wox4* mutant. Thus, the identification of upstream/downstream factors or novel regulators that are required for cambium identity will further reveal the mechanism of wood formation. Furthermore, investigation of these gene families in various plant species, including monocots and non-

vascular plants, can help us to understand the evolution of cambial development and diversity of wood formation.

It has also been demonstrated that there is no secondary growth in *ipt1,3,5,7* mutants; therefore, looking for new genes that act downstream of the cytokinin pathway may lead to the identification of master regulators for secondary growth. It is likely that wood formation is regulated by a gene regulatory network (GRN) consisting of various TFs. Previously, tissue-specific GRNs in the *Arabidopsis* stele have been mapped via systematic yeast one-hybrid and two-hybrid screens to discover protein–protein interactions between the selected TFs [60]. Also, recent comparative transcriptome analysis has able to identify several fundamental biological processes needed for vascular formation in Arabidopsis [61]. In this *in silico* investigation, 107 conserved vascular gene groups were identified and these gene groups may form a complex co-expression network with multiple functional connections. By combining genome-wide technology, *in silico* analysis and genetic manipulation, a vital GRN that regulates wood formation may be identified in the near future. Through comparative genetic analysis, this approach can be expanded to angiosperm and gymnosperm tree species, where we can identify GRNs specific for hardwood and softwood formation. This knowledge will provide a valuable resource for wood properties related tree breeding.

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Figure Legends

Figure 1. Illustration of transcriptional and hormonal regulation of wood formation. Cross sections of *Populus* stems are shown; secondary vascular tissues including phloem, cambium and xylem (wood) are displayed on the section. Different developmental stages of wood formation can be observed in the cross section. The functions of various transcriptional regulators and hormones (circled) in regulating cambium activity and xylem differentiation are presented, cross-talk among these regulators is also revealed. The source of the evidence is indicated using different font colors. Green: evidence obtained from *Arabidopsis*; Orange: evidence obtained from *Populus*; Blue: evidence obtained from both *Arabidopsis* and *Populus*. CK: cytokinin, GA: Gibberellin, BR: Brassinosteroid.

Figure 2. A **transcriptional regulatory network controlling secondary cell wall biosynthesis in** *Arabidopsis* **and** *Populus***.** *Arabidopsis* genes are presented in green and their *Populus* orthologs in orange. The NAC genes (blue boxes) function as first-level master switches; they induce expression of the second-level master switches, *MYB46* and *MYB83* (red box), which in turn activates a plethora of downstream TFs (yellow boxes), as well as many genes directly involved in secondary wall biosynthesis. The MYB target TFs promote the biosynthesis of lignin, cellulose, hemicellulose and xylan biosynthesis. A multilevel feed-forward loop structure is integrated in the transcriptional network: both NAC and MYB master switches directly induce expression of many of the same genes (dashed arrows).

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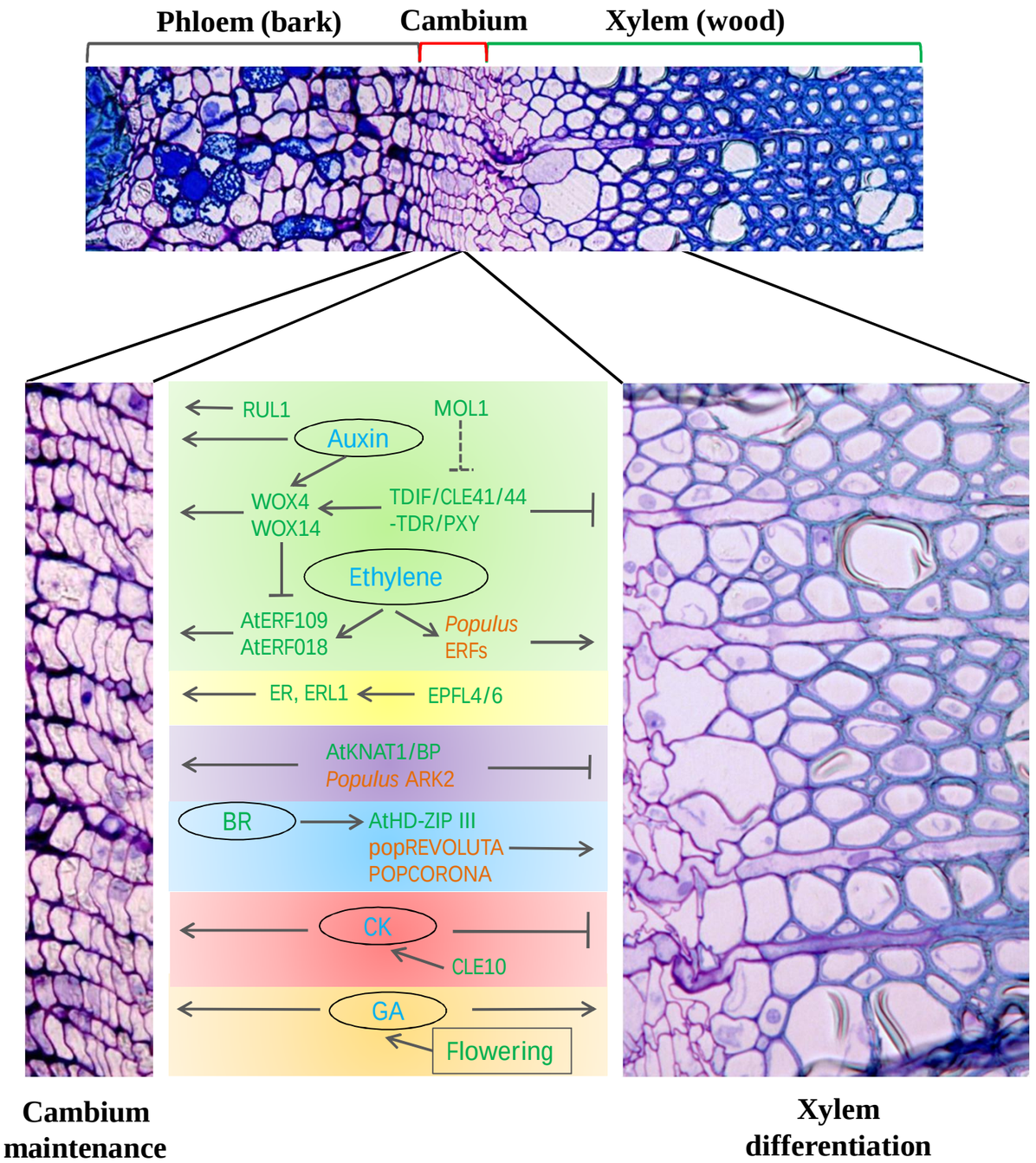

Figure 1. Illustration of transcriptional and hormonal regulation on wood formation.

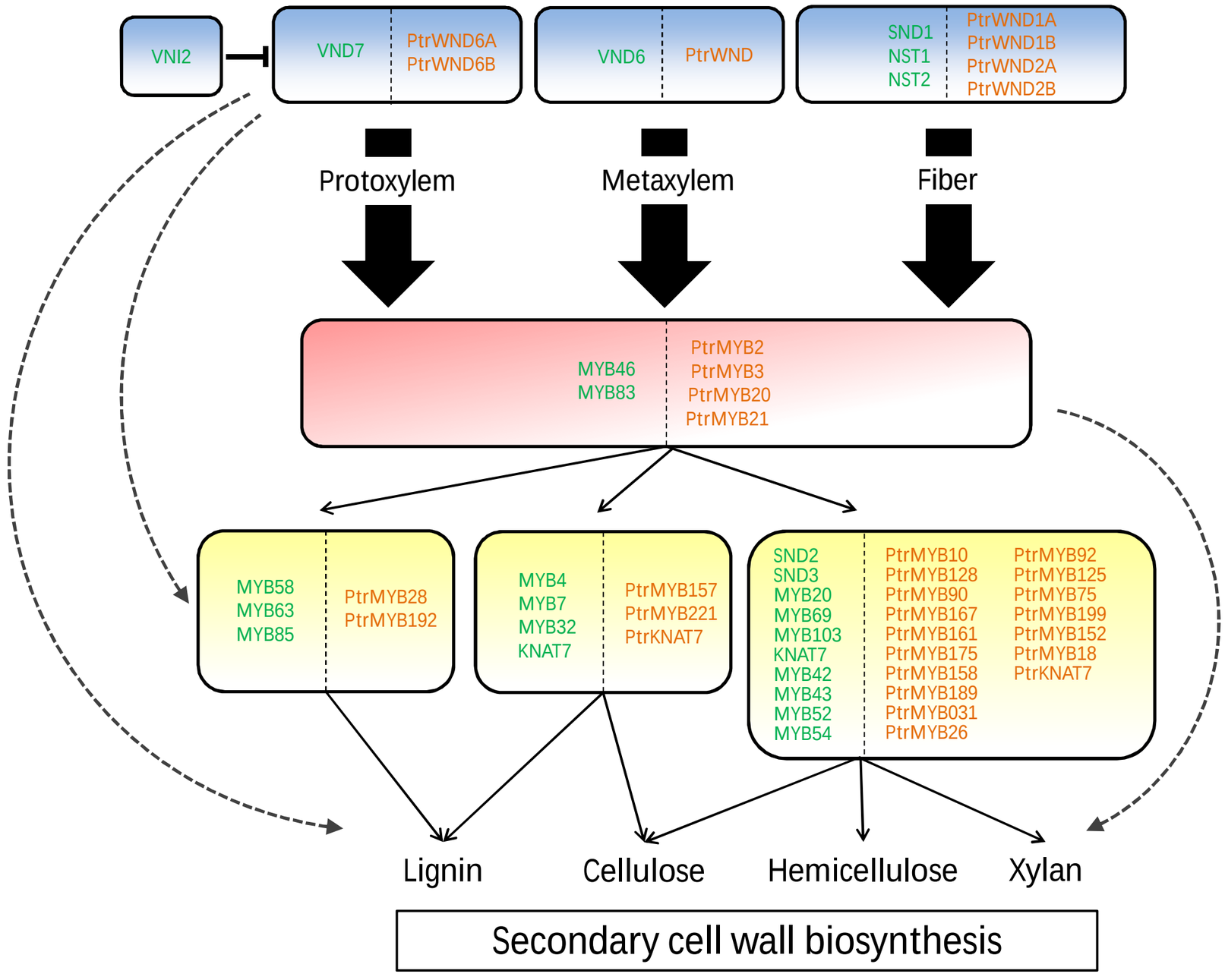

Figure 2: Transcriptional network of biosynthesis of secondary cell wall.