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Invited Expert Review

Transcriptional Regulation of Secondary Growth and Wood Formation

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Abstract

Secondary growth and wood formation are products of the vascular cambium, a lateral meristem. Although the mechanisms have only recently begun to be uncovered, transcriptional regulation appears increasingly central to the regulation of secondary growth. The importance of transcriptional regulation is illustrated by the correlation of expression of specific classes of genes with related biological processes occurring at specific stages of secondary growth, including cell division, cell expansion, and cell differentiation. At the same time, transcription factors have been characterized that affect specific aspects of secondary growth, including regulation of the cambium and differentiation of cambial daughter cells. In the present review, we summarize evidence pointing to transcription as a major mechanism for regulation of secondary growth, and outline future approaches for comprehensively describing transcriptional networks underlying secondary growth.

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Introduction

Secondary growth is the radial growth of stems that occurs after elongating growth. This process, as typified by extant forest trees, is supported by a lateral meristem, the vascular cambium. Evidence from classical studies identify initials within the cambium (Murmanis 1970), which divide to produce daughter cells that subsequently divide and expand prior to differentiating as mature, functional cell types within either secondary xylem (wood) or secondary phloem (bark). The cambium is an amazingly dynamic meristem, responding to and even anticipating seasonal and environmental changes, replacing lost initials, and balancing the rate of periclinal and anticlinal divisions (Larson 1994). In the case of long-lived forest trees, the cambium can remain active for thousands of years (Lanner 2007).

There are important scientific motivations to study secondary growth, as it presents striking examples of meristem activity, tissue patterning, and cell differentiation. All of these developmental processes are influenced by environmental cues and are extremely plastic in nature. Although the mechanisms regulating secondary growth have only recently begun to be defined, the pace of discovery is accelerating rapidly. Better defining the genes and mechanisms regulating the cambium will address important questions regarding the evolutionary origins of secondary growth, as well as its variations in extant plants. This knowledge will further our understanding of a major innovation in land plant evolution that gave rise to gymnosperm and angiosperm forests that currently cover roughly 30% of land, worldwide (Mygatt 2006).

There are also practical incentives to better understand secondary growth. The total forested land is estimated to have

shrunk by some 40% due to anthropogenic effects (Mygatt 2006), and deforestation is a major contributor to climate change. While the land base available for forests is shrinking, the need for forest trees for fuel wood, biofuels, lumber, and forest products is increasing. Although the solutions to deforestation and meeting the needs for forest products are complex, one proven strategy is to develop trees for industrial forestry that can optimize production on a limited land base. Currently, less than 5% of the world's forest area is in managed plantations, yet such forests contribute almost 35% of the annual wood harvest (Mygatt 2006). Because trees are largely undomesticated and characterized by large genetic diversity, there is great potential for increasing yields, decreasing losses to disease and insect pests, and tailoring trees for specific end uses. Tree breeding has been successful in modifying wood properties tailored for specific end uses, but because of the relatively slow and costly process associated with traditional breeding, there is a desire to accelerate modifications using biotechnology. The application of biotechnologies such as molecular aided breeding require better understanding of the genes that can be manipulated to engineer desirable growth characteristics (Groover 2007).

The cambium and secondary growth present attractive attributes for study. The radial organization of the cambium and derived tissues provide unique opportunities and approaches not found in apical meristems. Notably, the woody tissues of secondary xylem present an historical record of development, whereby cambial activity and subsequent cell differentiation is cemented in place by the long lasting, lignified cell walls of fibers and tracheary elements of the secondary xylem (Carlquist 2001). The radial organization allows for the progressive longitudinal sectioning from older to younger bark tissues, through the cambial zone, and into progressively older regions of secondary xylem. As discussed later, this approach has been exploited in combination with microarrays to catalog global gene expression across the different tissues and developmental stages of secondary growth (Schrader et al. 2004). Radial organization also facilitates the harvest of large amounts of secondary vascular tissues for biochemical or other analyses requiring significant amounts of tissue. For example, in the springtime during active cambial divisions, the relatively thin cell walls of the cambial initials and immediate derivatives make the cambial zone relatively fragile. The bark can be peeled off the stem, separating at the cambial zone. Large amounts of cambium, secondary xylem, and secondary phloem can then be scraped from the stem for biochemical analysis.

While there is an extensive classical literature characterizing the anatomical features of woody tissues produced in a large number of species (Carlquist 2001), the genes responsible for regulating secondary growth have only recently begun to be identified. Importantly, full genome sequence (Tuskan et al. 2006) and attendant molecular and genomic tools are available for the model genus *Populus*, and are rapidly being developed for other woody species (e.g. eucalyptus). Genome-supported genomic tools are well suited to the study of secondary growth and minimize many of the limitations traditionally associated with genetic studies in woody perennials (Groover 2007). The ability to transform *Populus* (Han et al. 1996, 1997) has supported the functional characterization of individual genes, provided genotypes with unique attributes (e.g. reporter genes), and supported gene tagging approaches (Busov et al. 2003; Groover et al. 2004). At least some mechanisms of cell differentiation are similar among diverse species and are shared between primary and secondary vascular tissues (e.g. tracheary element differentiation), allowing judicious extrapolation of some findings from herbaceous model plants (notably *Arabidopsis*) to the study of secondary growth (Nieminen et al. 2004). Excitingly, genomic approaches are now available that provide the technical ability to undertake studies that can comprehensively describe transcriptional and biological networks underlying secondary growth.

It is difficult to assign the relative importance between transcriptional regulation during secondary growth and other types of regulation. Indeed, secondary growth is known to involve regulation by phytohormones (Nilsson et al. 2008), and likely involves significant regulation at the posttranscriptional and posttranslational levels. However, as described within this review, there is a striking correlation among the classes of genes expressed with the developmental processes associated with different stages and tissue types in secondary vascular tissues (Schrader et al. 2004), supporting the hypothesis that transcription is a key point of regulation. Functional analysis of transcription factors has also demonstrated the fundamental importance of transcription to secondary growth regulation. Importantly, transcription is arguably more tractable for study using current technologies: transcripts can be readily quantified using quantitative real time polymerase chain reaction (PCR), microarrays, or high-throughput sequencing; their expression patterns can be visualized using *in situ* hybridization; their cognate genes can be readily identified and mapped to genetic loci; and new computational methods are being developed to identify complex transcriptional networks.

In the present review, we summarize multiple lines of evidence that underscore the importance of transcriptional regulation of secondary growth and wood formation. Global gene expression studies are addressed first, followed by examples of individual transcription factors that influence specific aspects of cambium functions and cell differentiation. We conclude with a description of new systems and computational biology approaches that can describe the complex transcriptional networks underlying secondary growth.

A Brief Overview of Secondary Growth

Secondary growth is the developmental process driving the radial expansion of woody stems and is supported by a lateral meristem, the vascular cambium (**Figure 1**). Vascular cambium originates from the procambium in the shoot apex (Esau 1977), and in mature stems forms a continuous cylinder of meristematic tissue. The cambium contains initials –cells that divide without differentiation to supply daughter cells, which in turn divide before differentiating into specialized cell types of secondary vascular tissues (Murmanis 1970). Cambial initials include two types; ray initials and fusiform initials (Larson 1994). The ray initials are nearly isodiametric, small cells that occur often in groups. They produce the radially-orientated ray cells found in most woody plants, which serve radial transport and storage functions (Esau 1977). The fusiform cambial initials are oriented longitudinally relative to the stem, and undergo periclinal divisions that produce two types of daughter cells, phloem mother cells to the outside of the stem and xylem mother cells to the inside of the stem. Phloem and xylem mother cells undergo additional rounds of periclinal division to form transit cell populations that ultimately undergo terminal differentiation within secondary xylem (wood) or secondary phloem (bark). Cells undergoing differentiation in secondary xylem undergo predictable and progressive stages of expansion, elongation, secondary cell wall formation and programmed cell death.

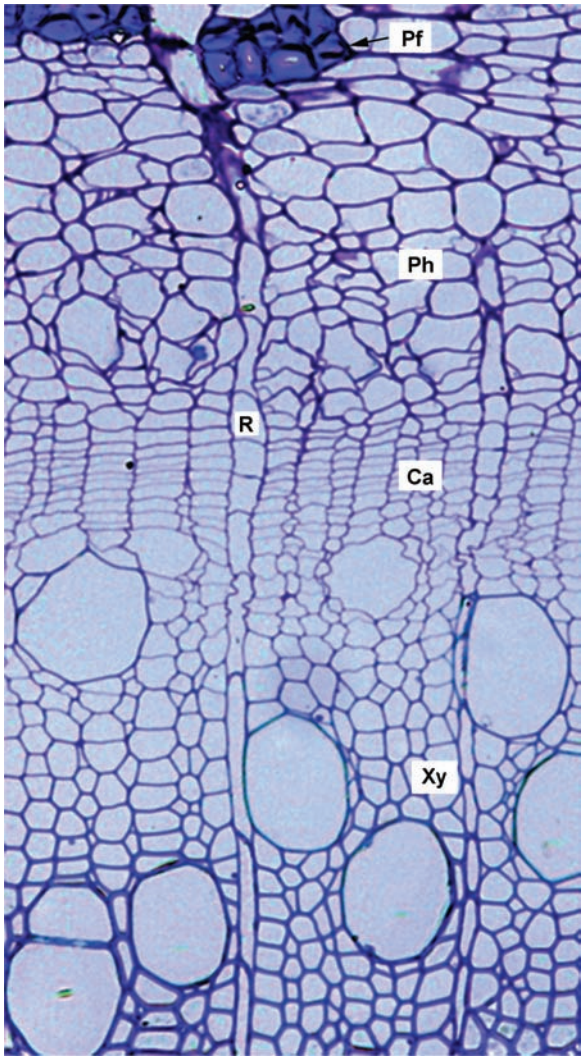

Figure 1. Transverse section through a stem of Chinese white poplar (*Populus tomentosa*), showing the radial organization of vascular tissues.

The cambial zone (Ca) is relatively wide in this stem, indicating active growth. The fusiform initials of the cambium produce longitudinally-oriented cells that differentiate within secondary xylem (Xy) and secondary phloem (Ph). *Populus* produces pronounced groups of phloem fibers (Pf). The cambium also contains initials that produce cells of rays (R), which are responsible for lateral transport and storage across secondary vascular tissues.

Global Gene Expression during Secondary Growth

Significant insights into the importance of transcriptional regulation have been gained through microarray profiling of longitudinal sections from *Populus* stems undergoing secondary growth. As previously mentioned, the radial organization of stems presents the opportunity to harvest cells at specific points in development by successive longitudinal sectioning through secondary vascular tissues. This property was exploited to determine global gene expression profiles across the cambium zone and into different developmental stages of secondary xylem and phloem (Schrader et al. 2004).

In general, for a given developmental stage, there is very good correlation between the classes of genes expressed and the associated developmental stage. For example, traveling from the cambium region to the terminally differentiating cells of secondary xylem, there is a progressive shift in expression from gene classes involved in cell division, to cell expansion, to cell differentiation. This reflects the associated developmental events across the same regions including division of cambium daughters, followed by cell expansion (most notably by vessel precursors), followed by the synthesis of lignified, secondary cell walls and cell death (for example of tracheary elements). Notably, the regions of gene expression for both gene classes and individual genes are relatively broad (**Figure 2**). This could be a reflection of technical challenges of the sampling scheme, heterogeneity in the developmental stage of the differentiation of cells sampled, or slow decay of transcripts. It is also possible that there simply are not sharply defined zones or boundaries

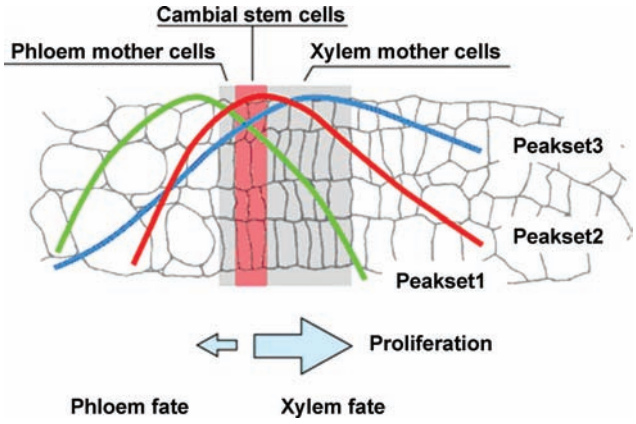

Figure 2. Average expression profiles of genes clustering within three different peaksets defined by the collective expression of genes that show differential expression across the cambial zone.

Peakset 1 contains genes whose maximal expression is associated with phloem and phloem mother cells. Peakset 2 includes maximal expression overlapping the presumed cambial initials. Peakset 3 shows maximal expression within zones of cell division and expansion within secondary xylem. Although these profiles reflect averages of multiple genes, it is noteworthy that no individual genes sharply demarcated a narrow cell layer (e.g. cambial initials) either. Reproduced from Schrader et al. (2004) with permission.

within secondary vascular tissues but rather gradations of differentiation.

That no tightly-defined boundaries or compartments have been defined for secondary vascular tissues is especially relevant to the comparison of cambia to primary meristems, where the dividing stem cells are associated with an organizing center. In the *Arabidopsis* shoot apical meristem, an organizing center is sharply defined by a small number of *WUSCHEL* (*WUS*) expressing cells, which underlie the stem cell population. *CLAVATA3* (*CLV3*)-mediated signaling between the organizing center and stem cells regulates the dynamics of the size of the organizing center and stem cell niche (Scheres 2007). Similarly, a *WUSCHEL*-like gene (*WOX*), *WOX5*, has been shown to be expressed in the organizing center (the quiescent center) of the root apical meristem (Sarkar et al. 2007) and a *CLV3* homolog, *CLE19*, functions to regulate the size of the root meristem (Casamitjana-Martinez et al. 2003). It is not immediately obvious that there are similar tightly defined functional centers within the cambium based on anatomical features or gene expression patterns published to date. One possibility would be that the cells termed initials actually serve as an organizing center, with xylem and phloem mother cells acting as the true stem cells of the meristem. While *WOX* and *CLE* genes are known to be expressed in the cambial zone (Schrader et al. 2004), none have been functionally characterized to date, nor have precise expression patterns been determined with *in situ* hybridizations. Thus, whether cambia use WOX-CLE signaling or utilize an organizing center is uncertain.

Examination of the expression of specific genes encoding transcription factors and proteins known to be involved in the regulation of primary meristems does point to significant overlap of genes and mechanisms regulating primary meristems and cambia (Groover 2005). As discussed below, the *Arabidopsis* Class I KNOX genes *SHOOTMERISTEMLESS* (*STM*) and *BREVIPEDICELLUS* (*BP*) are major regulators of stem cell maintenance and cell differentiation in shoot apical meristems. *Populus* orthologs of *STM* and *BP* are expressed both in the shoot meristem as well as in stems undergoing secondary growth (Groover et al. 2006; Du et al. 2009). Similarly, *Populus* homologs of *Arabidopsis* Class III HD Zip genes, *KANADI* genes, *PINHEAD*, and *AINTEGUMENTA*, which play key roles in regulating primary vascular development, shoot apical meristems and organ primordia derived from them (Floyd et al. 2006) are also expressed in stems undergoing secondary growth (Schrader et al. 2004), further demonstrating that major genes and mechanisms regulating shoot meristems have been directly co-opted during the evolution of secondary growth.

The relationship between root apical meristems, and vascular cambia and apical meristems is less clear. There are important regulatory genes that overlap in expression among root meristems and cambia. For example, Class III HD ZIPs play regulatory roles in root and shoot apical meristem functions (Hawker and Bowman 2004), and are also expressed in the cambial zone of *Populus* (Schrader et al. 2004). But there are also significant differences between root and shoot meristems. For example, the SHORT-ROOT (SHR) transcription factor acts non-cell autonomously and physically interacts with the SCARECROW (SCR) transcription factor, to specifying tissue identity in root meristems (Cui et al. 2007). Putative *Populus* homologs of *SHR* are expressed in the cambial zone, while *Populus SCR* homologs are not (Schrader et al. 2004). Further experiments are needed to define the expression and function of genes associated with primary meristems during secondary growth, towards fully resolving the relationships among cambia and primary meristems. Quite likely, a complex story will emerge showing various degrees of co-option and recycling of mechanisms from primary growth during the evolution of secondary growth.

Transcription Factors that Regulate Secondary Growth

For an understanding of the evolution and regulation of cambia and secondary growth, it is necessary to undertake detailed

functional characterizations of individual transcription factors. Because of traditional difficulties associated with working directly with forest trees, much of our knowledge about gene function comes from non-tree model species, most notably *Zinnia elegans* and *Arabidopsis thaliana* (Ko et al. 2004; Nieminen et al. 2004; Demura and Fukuda 2007). However, increasing numbers of transcription factors are being characterized in *Populus*, using transgene constructs that either misexpress or knock down the expression (e.g. with RNA interference) of target genes. We discuss below notable examples of transcription factors that have been shown to regulate specific aspects of vascular development relevant to secondary growth.

Transcription factors regulating meristem maintenance and tissue differentiation

Class I KNOX genes regulate core functions of the shoot apical meristem (SAM). The *Arabidopsis* Class I KNOX homeobox gene *STM* is first expressed in several cells of the globular embryo and is later downregulated in the regions surrounding the central SAM (Lincoln et al. 1994). Loss of function *stm* mutants lack a functional SAM due to differentiation of stem cells during embryogenesis (Long et al. 1996). A *Populus* ortholog of *STM*, *ARBORKNOX1* (*ARK1*), is expressed not only in the SAM but also in the cambial zone of *Populus* (Groover et al. 2006). *ARK1* expression in the stem is primarily in the cambial zone. Overexpression of *ARK1* in transgenic *Populus* results in an extended delay of secondary growth, and stems with highly reduced phloem fibers and secondary xylem. Whole-genome microarray analysis carried out on such plants showed that in the stem a large number of genes involved in cell wall biosynthesis and lignification were misexpressed (Groover et al. 2006). The stems from *ARK1* overexpressing plants also have slightly increased total lignin, consistent with upregulation of a number of genes encoding enzymes in the lignin biosynthetic pathway. These transgenic plants also have altered lignin monomer ratios, which is reflected in the downregulation of a *Populus FAH1* ortholog, encoding an enzyme (F5H) responsible for shunting lignin precursors destined to produce either *p*-hydroxyphenyl (H), guaiacyl (G), or syringyl (S) monolignols to the S-specific pathway. Taken together, analysis of *ARK1* transgenic lines revealed that *ARK1* appears to regulate specific aspects of cambial functions and cell differentiation during secondary growth in *Populus*, including regulation of cell wall biosynthesis (Groover et al. 2006).

Similarly, the *Arabidopsis* gene *BREVIPEDICELLUS* (*BP*), is expressed in the peripheral zone of the SAM as well as in cortical and vascular tissues, and in phloem cells adjacent to the inflorescence stem cortex (Lincoln et al. 1994; Venglat et al. 2002; Douglas and Riggs 2005). In *Arabidopsis*, *BP* overexpression plants have impaired lignin deposition in inflorescence stems during primary growth, while *bp* plants show ectopic lignification in the interfascicular region of the inflorescence stem (Mele et al. 2003). Importantly, the expression of key cell wall synthesis-related genes show negative correlation with *BP* expression levels, and the BP protein binds to the promoters of some of these genes *in vitro* (Mele et al. 2003). A *Populus BP* ortholog, *ARBORKNOX2 (ARK2)*, is expressed not only in the cambial zone, but also in lignifying cells (**Figure 3**) (Du et al. 2009). Transgenic *Populus* overexpressing *ARK2* plants have a wider cambial zone and delayed cambial daughter cell differentiation (**Figure 4A, B**). The expression of numerous genes encoding proteins involved in lignin and cellulose biosynthesis are negatively correlated with *ARK2* expression, which is reflected in reduced carbohydrate and lignin content in stems of *ARK2* transgenic lines (Du et al. 2009). Conversely, expression of a synthetic miRNA that targets *ARK2* transcripts results in enhanced secondary growth, including premature development of secondary xylem and phloem fibers (**Figure 4C, D**). Lignin and cellulose synthesis genes are upregulated, which is reflected by increased carbohydrate and lignin content in *ARK2* knockdown stems. *ARK2* regulates secondary growth and wood phenotypes in part by negative regulation of genes involved in cell wall biosynthesis (Du et al. 2009).

Transcription factors regulating tracheary element differentiation and secondary cell wall synthesis

Tracheary elements are a major cell type in wood, and undergo a complex process of differentiation that includes synthesis of elaborately patterned secondary cell wall, followed by programmed cell death (Groover and Jones 1999). The end result is a hollow cell corpse which, by end to end association with other tracheary elements, creates water conducting vessels. *NAM*/*ATAF*/*CUC* (*NAC*) family genes are expressed in developing woody tissues and differentiating tracheary elements (Demura and Fukuda 2007; Zhong and Ye 2007a). *Arabidopsis VASCULAR-RELATED NAC DOMAIN6* (*VND6*) and *VND7* encode NAC domain proteins expressed in metaxylem and protoxylem vessels, respectively. Spectacularly, misexpression of *VND6* induces transdifferentiation of mesophyll and other cell types into tracheary elements with secondary cell walls similar to metaxylem vessels in both *Arabidopsis* and *Populus* (**Figure 5**). Similarly, misexpression of VND7 results in transdifferentiation of cells into tracheary elements with secondary cell walls similar to protoxylem elements (**Figure 5**). These and related results (Kubo et al. 2005) demonstrate that simple transcriptional switches may be involved in the differentiation of a major cell type of wood.

Secondary cell wall synthesis is one of the most defining events of wood formation, and is a major determinant of wood properties (Mellerowicz and Sundberg 2008). Biosynthesis of secondary cell wall requires a coordinated transcriptional activation of genes responsible for secondary cell wall

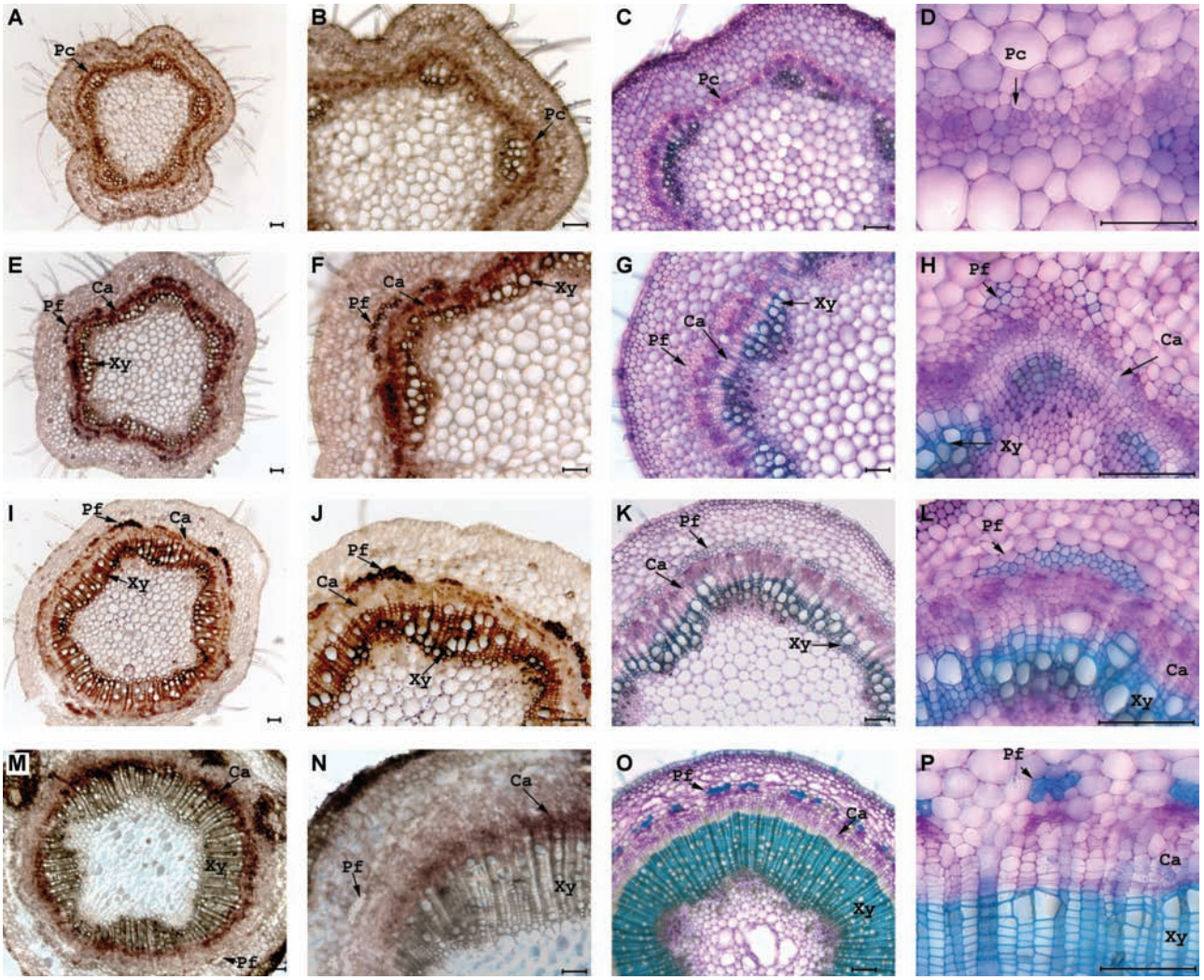

Figure 3. Whole mount *in situ* hybridization of *Populus* stems, showing expression of *ARK2* during secondary growth.

(A) Section from first elongating internode hybridized with antisense *ARK2* probe. *ARK2* is expressed in procambium tissue in the first elongating internode during the primary growth. Arrow indicates the location of procambium.

(B) Higher magnification of first elongating internode hybridized with antisense *ARK2* probe. Arrow indicates the location of procambium.

(C) Section from first elongating internode and stained with toluidine blue (TBO) to show the anatomy. Arrow indicates the location of procambium.

(D) Higher magnification of (C). Arrow indicates the location of procambium.

(E) Section from third internode hybridized with antisense *ARK2* probe. Arrows indicate *ARK2* expression in cambium zone, differentiating secondary xylem cells and differentiating secondary phloem fiber cells during the transition to the secondary growth.

(F) Higher magnification of (E). Arrows indicate *ARK2* expression in cambium zone, differentiating secondary xylem cells and differentiating secondary phloem fiber cells.

(G) Section from third internode and stained with TBO to show the anatomy. Arrows indicate cambium zone, differentiating secondary xylem cells and differentiating secondary phloem fiber cells.

(H) Higher magnification of (G). Arrows indicate cambium zone, differentiating secondary xylem cells and differentiating secondary phloem fiber cells just start form secondary cell wall.

(I) Section from fifth internode hybridized with antisense *ARK2* probe. Strongest *ARK2* expression is associated with differentiating secondary xylem and secondary phloem fibers, in which cell walls are undergoing lignifications. Lower expression is detected in the cambium region. Arrows indicate the location of the cambium zone, differentiating secondary phloem fibers and secondary xylem cells.

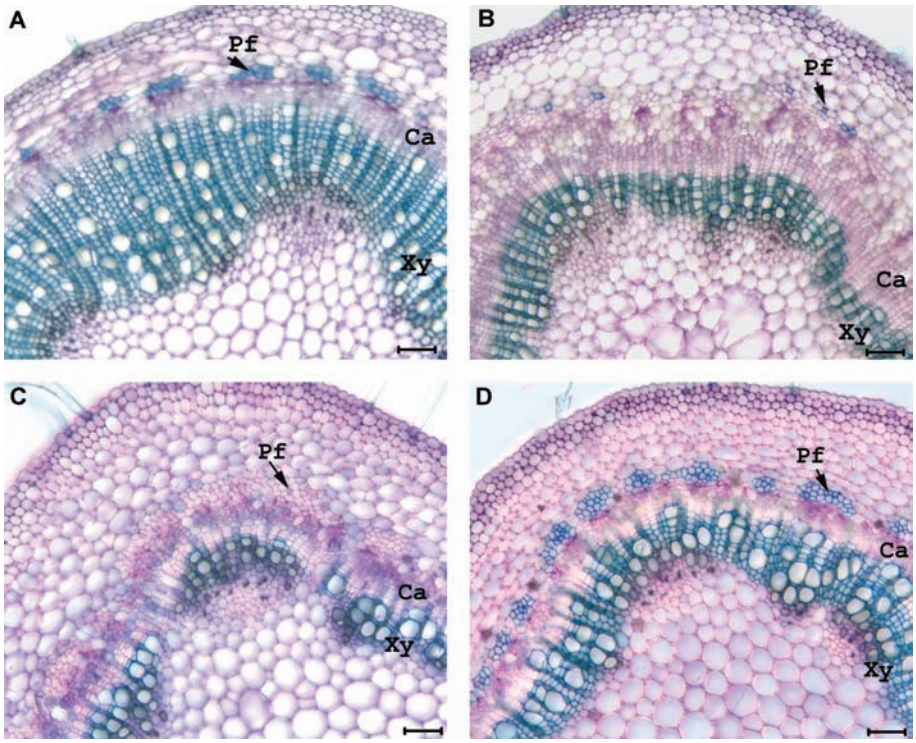

Figure 4. Comparison of secondary growth in wild-type *Populus* versus *ARK2* transgenic stems. Transverse sections stained with toluidine blue.

(A) Bottom internode of wild type *Populus* showing well developed phloem fibers and extensive secondary xylem.
(B) Bottom internode of *35S:ARK2* trasgenic plant line, which lacks well-developed bundles of phloem fibers and has decreased secondary xylem.
(C) Section from the seventh internode of a wild type *Populus* stem, showing onset of secondary growth and lacking fully developed, lignified phloem fibers.
(D) Section from the seventh internode of an *ARK2*-knockdown (a-miRNA:*ark2*) transgenic stem, with precocious secondary phloem fibers and more abundant secondary xylem.
Ca, cambium; Pf, phloem fiber; Xy, secondary xylem. Images taken from Du et al. (2009).

←

(cont.)
Figure 3.
(J) Higher magnification of (I). Arrows indicate location of the cambium zone, differentiating secondary phloem fibers and secondary xylem cells.
(K) Section from fifth internode and stained with TBO to show the anatomy. Arrows indicate the location of the cambium zone, differentiating secondary phloem fibers and secondary xylem cells.
(L) Higher magnification of (K).
(M) Section from base internode hybridized with antisense *ARK2* probe. Arrow indicates *ARK2* expression is largely limited to the cambium region.
(N) Higher magnification of (M). Arrow indicates *ARK2* expression is largely limited to the cambium region.
(O) Section from base internode and stained with TBO to show the anatomy. Arrows indicate the location of the cambium zone, differentiated secondary phloem fibers and secondary xylem cells with thicker and lignified secondary cell wall.
(P) Higher magnification of (O).
Ca, cambium; Pf, phloem fiber; Xy, secondary xylem. Images from Du et al. (2009).

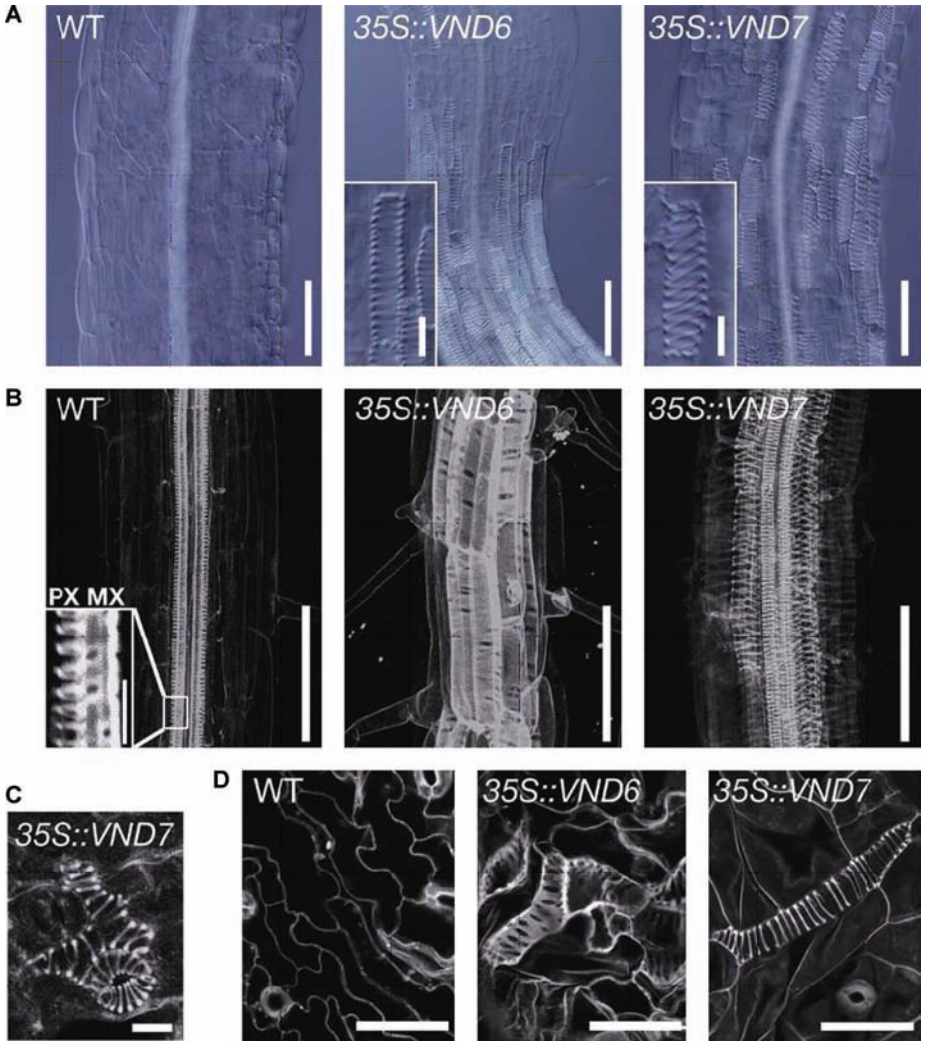

Figure 5. Phenotypes of *Arabidopsis* and *Populus* plants misexprexpressing the NAM/ATAF/CUC (NAC) domain transcription factors VND6 and VND7.

(A) Differential interference contrast (DIC) images from *Arabidopsis* hypocotyls showing ectopic formation of tracheary elements in transgenic VND6 and VND7 overexpression plants.
(B) Confocal laser scanning microscope images of *Arabidopsis* roots. Note that VND6 overexpressing plants produce ectopic tracheary elements with secondary cell wall characteristics of metaxylem, whereas VND7 overexpressing plants produce protoxylem-like tracheary elements.
(C) Confocal image showing ectopic tracheary element formation in *Arabidopsis* leaf epidermis of transgenic VND7 overexpressing plants.
(D) Confocal images of epidermis in *Populus* leaves, showing ectopic tracheary element formation in response to VND6 and VND7 overexpression. Note the metaxylem-like elements formed in VND6 overexpressing cells, versus protoxylem-like cells formed in VND7 overexpressing cells.
PX, protoxylem; MX, metaxylem. Reproduced from Kubo et al. (2005) with permission.

components, such as cellulose, xylan and lignin. Ultimately the biological networks regulating secondary cell wall synthesis need to be elucidated, and the results from molecular genetic and genomic studies have already allowed for a first approximation of a transcriptional regulatory network involved in secondary wall biosynthesis in tracheary elements and fibers (Zhong and Ye 2007b). *Arabidopsis* NAC domain transcription factors, including *SECONDARY WALL–ASSOCIATED*

NAC DOMAIN PROTEIN1 (*SND1*), *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1* (*NST1*), *NST2*, *VND6*, and *VND7*, have been placed in a putative transcriptional network along with MYB transcription factors that regulate secondary cell wall synthesis in lignified cell types (Zhong and Ye 2007a; Zhou et al. 2009). However, evidence for this transcriptional network is still incomplete, and additional levels of supporting data will be required to fully reconstruct the interrelationships of transcription factors and target genes. In addition, it is unknown what the relationship might be among transcriptional networks regulating cell wall biosynthesis in *Arabidopsis* and other species. It is intriguing to note that MYB transcription factors in both a gymnosperm (*Pinus taeda*; PtMYB1 and PtMYB4) and an angiosperm (*Eucalyptus grandis*; MYB2) have been shown to be expressed during secondary xylem development in wood and bind to AC cis-elements (Patzlaff et al. 2003a, 2003b; Goicoechea et al. 2005). This suggests that at least some homologous genes, or even regulatory networks, may control fundamental aspects of cell wall synthesis in diverse plant taxa.

Reconstructing Transcriptional Networks

Ultimately, a complete understanding of secondary growth will require reconstruction of the complex transcriptional networks underlying cambial regulation, tissue patterning, and cell differentiation. This can be accomplished through application of computational approaches that can turn complex, interdependent genomic data into diagrams of gene regulatory interactions (Filkov 2005). These approaches integrate different data types into networks with fewer false positives than those obtained from individual data types, and identify follow-on experiments that will best elucidate the hidden network (Davidson et al. 2002; Long et al. 2008). Numerous examples exist where these computational approaches have been used to reveal the regulatory interactions between genes in bacteria, yeast, metazoa, and plants (e.g. Filkov and Shah 2008). The most complete networks can be obtained by combining different data types together: for example, precise perturbation expression data, time-course data, protein-DNA binding, protein–protein binding, etc. (Tegner et al. 2003; Materna and Oliveri 2008).

Secondary growth is now well suited to transcriptional network reconstructions. As previously mentioned, secondary vascular tissues can be harvested for gene expression and biochemical analysis. Various expression array platforms are now available for *Populus* and have been used to catalog gene expression in secondary growth in both wild type plants and in transgenic lines misexpressing transcription factors (Schrader et al. 2004; Groover et al. 2006; Nilsson et al. 2008; Du et al. 2009). One of the most direct means of establishing initial transcriptional networks is by determining the binding sites for key transcription factors. This can be done genome-wide in *Populus*, using chromatin immunoprecipitation followed by sequencing (ChIP-seq). The process involves harvesting cambium or secondary vascular tissues, followed by cross linking of proteins to chromosomal DNA. The DNA is then sheared, followed by immunoprecipitation using an antibody recognizing a transcription factor or DNA binding protein of interest. As shown in **Figure 6**, ChIP-Q-PCR has been established for *Populus* cambium samples, showing the technical feasibility of the approach. The next extension is to sequence the isolated DNA using massively parallel sequencing (e.g. Solexa), and map the sequence reads back to the reference *Populus* genome. The resulting analysis reveals the binding sites, and thus genes regulated by the transcription factor. Such data, combined with expression data and protein–protein interaction data, could be used to build robust models of how

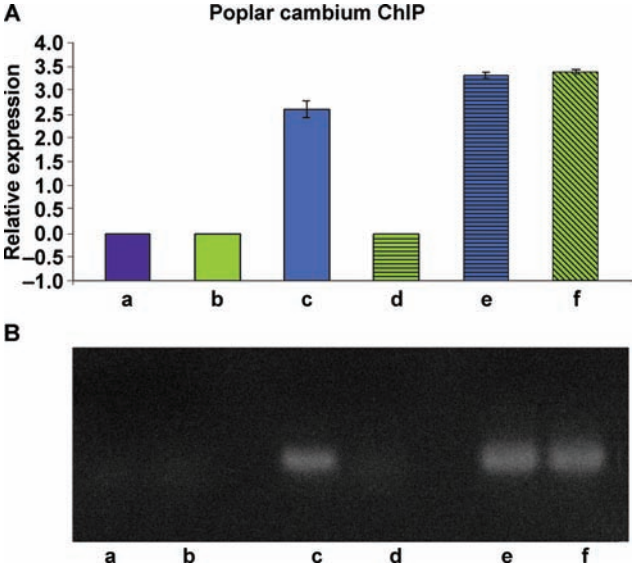

Figure 6. Chromatin immunoprecipitation of cambium samples of *Populus trichocarpa*, followed by quantitative reverse transcription-polymerase chain reaction amplification of purified chromosomal DNA fragments (ChIP-Q-RT-PCR).

(A) Quantification of PCR products resulting from ChIP and amplification using the following antibodies and controls for ChIP, with the indicated primers.
Lane a, Immunoglobulin G (IgG) mock-ChIP amplified with tubulin primers as a negative control.
Lane b, IgG mock-ChIP amplified with negative control primers.
Lane c, RNA POLII ChIP amplified with tubulin primers.
Lane d, RNA POLII ChIP amplified with negative control primers.
Lane e, Input amplified with tubulin primers as positive control.
Lane f, Input amplified with negative control primers.
(B) Horizontal agarose gel electrophoresis of PCR products amplified as described in (A).

the cambium is regulated, and guide future research in the evolution and developmental biology of secondary growth.

Concluding Remarks

It is an exciting time to study secondary growth. New genomic technologies are well suited to the task of identifying the basic biological networks underlying cambial functions and wood formation, and promise to yield interesting insights into the biology of one of the most important, but least understood plant meristems. At the same time, practical applications addressing problems ranging from bioenergy, deforestation, and climate change will all rely, to some degree, on increasing our basic knowledge of secondary growth, the cambium, and the development of those wonderful plants called trees.

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