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REVIEW

Hormone interactions in xylem development: a matter of signals

Ana Milhinhos · Célia M. Miguel

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Abstract Xylem provides long-distance transport of water and nutrients as well as structural support in plants. The development of the xylem tissues is modulated by several internal signals. In the last decades, the bloom of genetic and genomic tools has led to increased understanding of the molecular mechanisms underlying the function of the traditional plant hormones in xylem specification and differentiation. Critical functions have been assigned to novel signaling molecules, such as thermospermine. These signals do not function independently, but interact in a manner we are only now beginning to understand. We review the current knowledge of hormone signaling pathways and their crosstalk in cambial cell initiation and maintenance, and in xylem specification and differentiation.

Keywords Cambium · Xylem · Auxin · Cytokinin · Ethylene · Thermospermine · HD-Zip III · ACAULIS5 (ACL5)

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A. Milhinhos · C. M. Miguel (✉)
Instituto de Biologia Experimental e Tecnológica,
Apartado 12, 2781-901 Oeiras, Portugal
e-mail: cmiguel@itqb.unl.pt

A. Milhinhos
e-mail: milhinho@itqb.unl.pt

A. Milhinhos · C. M. Miguel
Instituto de Tecnologia Química e Biológica,
Universidade Nova de Lisboa, Av. da República,
2780-157 Oeiras, Portugal

Introduction

Xylem and phloem are the conducting vascular tissues that connect the plant body. While phloem functions to transport and distribute the photoassimilates produced in the shoot, xylem transports water and minerals taken up by the roots and provides structure and girth to the plant. In the past years, our knowledge of how vascular tissues are formed has significantly increased, in great part due to the use of the *Arabidopsis* root model system and *Zinnia elegans* xylogenic cultures. Several studies have also used more complex systems, such as tree stems, for which *Populus* has been the model system of choice. New insights into how signaling molecules direct phloem and xylem differentiation are coming to light. Hormones are important signals in this process, being recognized and integrated into responses during vascular development. Auxin, cytokinin, gibberellins and ethylene have been recognized for some time as regulators of vascular development (Sachs 1981; Mähönen et al. 2000; Eriksson et al. 2000; Savidge 1988). More recently, brassinosteroids and nitric oxide were identified as also being involved in the process (Choe et al. 1999a; Gabaldón et al. 2005). Other important signals include thermospermine, H_2O_2 and small peptides (Vera-Sirera et al. 2010; Ros Barceló et al. 2002; Ito et al. 2006). Even though signaling pathways for some of these compounds are quite well characterized (such as for auxin, cytokinin, ethylene, gibberellin and brassinosteroids), the exact molecular mechanism underlying their control of vascular development is not fully understood.

Vascular cells are produced from the apical meristems, located in the shoot and root apices, and from the vascular meristem. The meristems contain the stem cell niches, composed of a few undifferentiated cells that give rise to the different cell types present in the plant body.

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Procambial and cambial cells are the vascular stem cells that retain meristematic activity, and develop into xylem and phloem precursor cells (Fig. 1; Schrader et al. 2004; Matte Risopatron et al. 2010; Aichinger et al. 2012). Xylem precursor cells differentiate into the different xylem cells: tracheary elements, xylem parenchyma cells and xylem fibers. Phloem precursor cells differentiate into sieve elements, companion cells, phloem parenchyma cells and phloem fibers.

During primary growth, procambium promotes growth of vascular tissues in the apical directions. As the plant matures, radial files of cambial cells form a continuous cylinder around the plant stem, the vascular cambium, leading to secondary growth (Fig. 1). Periclinal divisions of the vascular cambial cells and their differentiation into phloem and xylem thus promote lateral growth. Previous studies demonstrated that the dynamics of shoot and root apical meristems (RAM) is regulated by similar molecular

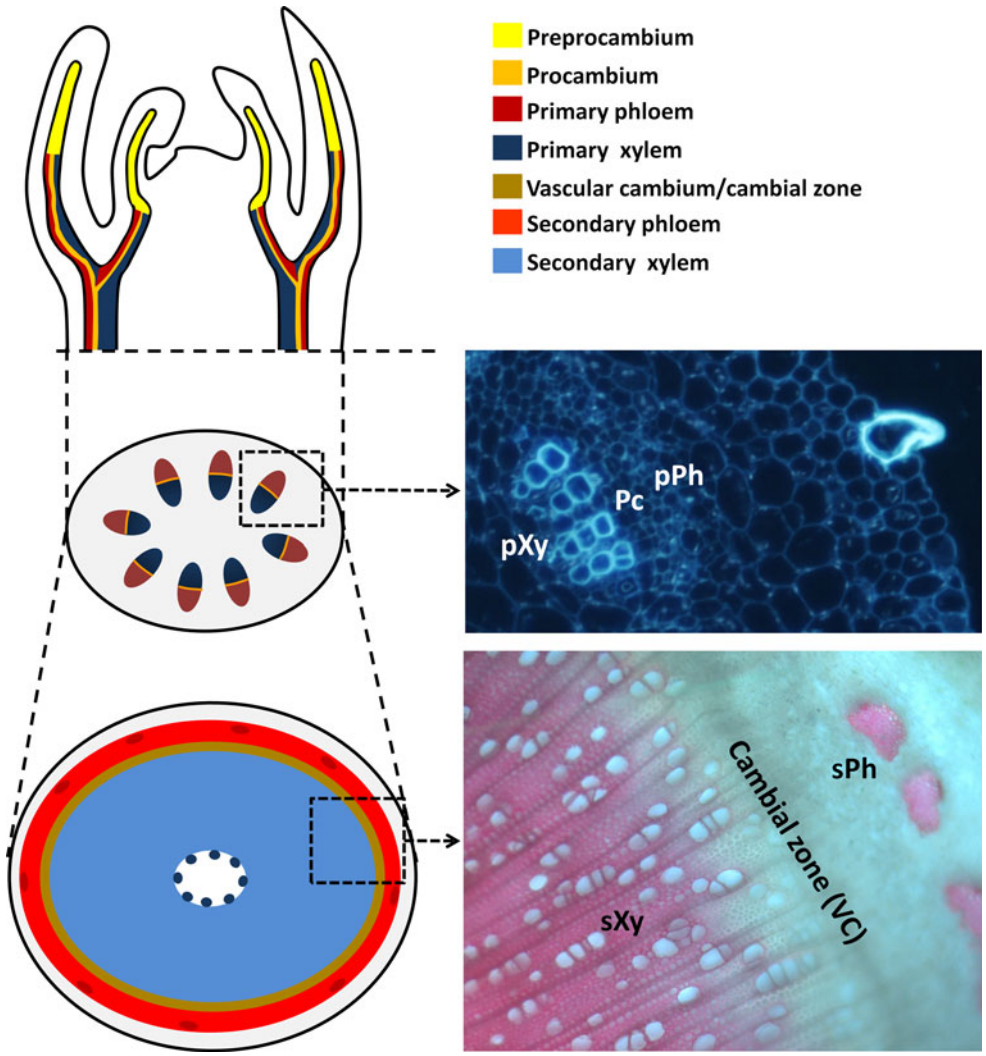

Fig. 1 Schematic representation of vascular tissue organization during primary and secondary growth in higher plants. *From the top* sections represent the shoot apical meristem, where pre-procambial/provascular cells precede vascular development. At the cellular level, the formation of the venation pattern begins in the leaf primordium with the siting of procambial cells among equivalent provascular cells, which are cells in an uncommitted meristematic state with vascular potential (Clay and Nelson 2002). Procambium strands result from longitudinal divisions that give rise to aligned elongated cells. The vascular strands include primary xylem and phloem tissues that differentiate from the procambium cells in opposite directions. The transition from primary to secondary growth involves the formation of fascicular cambium, which arises within vascular bundles, and interfascicular cambium that arises between vascular bundles (not shown, reviewed in Sanchez et al. 2012). Secondary growth of vascular tissues shows secondary phloem and xylem that derive from the vascular cambium, a layer of meristematic/cambial cells. The cambial cells at the vascular cambium undergo cell divisions that develop xylem precursor cells (toward the interior of the stem) and phloem precursor cells (toward the exterior of the stem). Xylem precursor cells then differentiate into different xylem cell types (such as tracheary elements, like vessels and tracheids, xylem fibers and ray cells; detailed in Fig. 3). Primary and secondary development is also shown in the corresponding *Populus tremula* × *Populus tremuloides* stem cross sections (on the *right*)

mechanisms (Sarkar et al. 2007; Stahl 2009). The apical meristem and the vascular cambium are also thought to be controlled by similar regulators (Sanchez et al. 2012; Aichinger et al. 2012). For instance, Yordanov et al. (2010) showed that the cambium zone is a boundary region that is likely under the same type of regulation as the one described in the shoot apical meristem (SAM) that separates the stem cell niche from the emerging lateral organ primordia. The authors reported that, in poplar, the LATERAL ORGAN BOUNDARIES1 (PtLBD1) transcription factor is expressed in the phloem side of the cambial zone and regulates secondary phloem production. The expression analysis of putative PtLBD1 targets suggested that this function is likely mediated through suppression of genes that promote meristem cell identity (such as *KNOXI*, *class I KNOTTEDLIKE HOMEOBOX*) and the activation of genes that trigger differentiation of phloem (such as *APL*, *ALTERED PHLOEM DEVELOPMENT*). *LATERAL ORGAN BOUNDARY* (*LBD*) target genes have similar expression patterns in the cells neighboring the cambium (in *Populus*) and in the SAM (in *Arabidopsis*), supporting their similar function in both types of meristem (Yordanov et al. 2010). The same kind of mechanistic resemblance between SAM and vascular cambium has been identified for the *ARBORKNOX* genes in *Populus* and *SHOOTMERISTEMLESS* (*STM*)/*BREVIPEDICELLUS* (*BP*) in *Arabidopsis* (Groover et al. 2006; Du et al. 2009), as well as in the monocotyledonous maize and rice plants, suggesting that *KNOX* genes are conserved mediators of meristematic potential (Scofield and Murray 2006).

Xylem development is a process of terminal cell differentiation that includes initial cell division, followed by cell expansion, secondary cell wall formation and programmed cell death (PCD). However, since the mechanisms that determine vascular identity begin earlier on, we start by reviewing how auxin promotes the transition of undifferentiated cells into procambial cells and which signals maintain procambial/cambial cell identity and prevent or promote their development into xylem cells (see Fig. 2). Given the similarities between mechanisms modulating meristem activity, we integrate data from the study of procambium in the shoot and root meristems, and the inflorescence stems of *Arabidopsis*, along with data on lateral growth and emerging knowledge from studies in *Arabidopsis*, *Zinnia elegans* xylogenic cultures, and the wood-producing *Populus* trees. We present an integrated view of the current knowledge on hormone signaling and xylem development, from preprocambial cells to fully differentiated xylem. A particular focus is given to how these signals interact with one another to produce a response in the context of xylem differentiation.

Auxin role in cambial cell identity: initiation and keeping it cambial

Auxin is a key regulator of almost any aspect of plant development and vascular development is no exception. It has long been known that the initiation of pre-procambial cells depends on auxin signaling and transport. According to the auxin canalization theory, auxin creates its own transport channels as a result of an initial auxin flux from an auxin source to an auxin sink. Thus, canalization of auxin flow initiates vascular cells, which also promote further canalization of auxin into these channels. The preferred channels inhibit further canalization in the surroundings and thus differentiate as narrow vascular strands (Sachs 1981). Evidence of auxin transport and signaling components functioning in vascular specification, together with pharmacological studies of auxin transport inhibitors, has supported the fundamental role of auxin in the induction of vascular bundles (Galweiler et al. 1998; Mattsson et al. 1999; Sieburth 1999).

Earlier studies have also shown the involvement of auxin in initiation and promotion of vascular cambium growth. Auxin supply from the SAM is required for cambial cell proliferation (Aloni 1987; Shininger 1979). Increased auxin concentrations have been found in the cambial stem cells of *Pinus* (Uggla et al. 1996). Also, in transgenic *Populus* trees, a decrease in auxin levels was shown to diminish cell division in the xylem (Tuominen et al. 1997; Nilsson et al. 2008). Baba et al. (2011) have demonstrated that, during dormancy, cambial cell division ceases due to a decrease in cambium responsiveness to auxin. The auxin gradient, with its maxima in the cambial zone, is thought to be essential for cambial proliferation, but only recently have the molecular mechanisms underlying this dependence of vascular development on auxin gradients started to be unveiled.

Auxin is perceived by the family of F-box domain receptors TRANSPORT INHIBITOR RESPONSE1 (TIR1) (Dharmasiri et al. 2005; Kepinski and Leyser 2005). TIR1 is part of an ubiquitin ligase complex that targets the degradation of AUXIN/INDOLE ACETIC ACID (Aux/IAA) transcriptional regulators by the 26S proteasome in an auxin-dependent manner (Gray et al. 1999, 2001). The *Aux/IAA*s are auxin-responsive genes that repress the transcriptional activities of the AUXIN RESPONSE FACTOR (ARF) family members (Fig. 2; Kieffer et al. 2010; Mockaitis and Estelle 2008). Thus, the Aux/IAA–ARF system modulates the developmental responses to auxin, since ARFs are the elements that transcriptionally activate or repress the downstream developmental genes. In the early stages of *Arabidopsis* vascular development, initiation of procambium in the embryo relies on the auxin flow that promotes the degradation of Aux/IAA proteins,

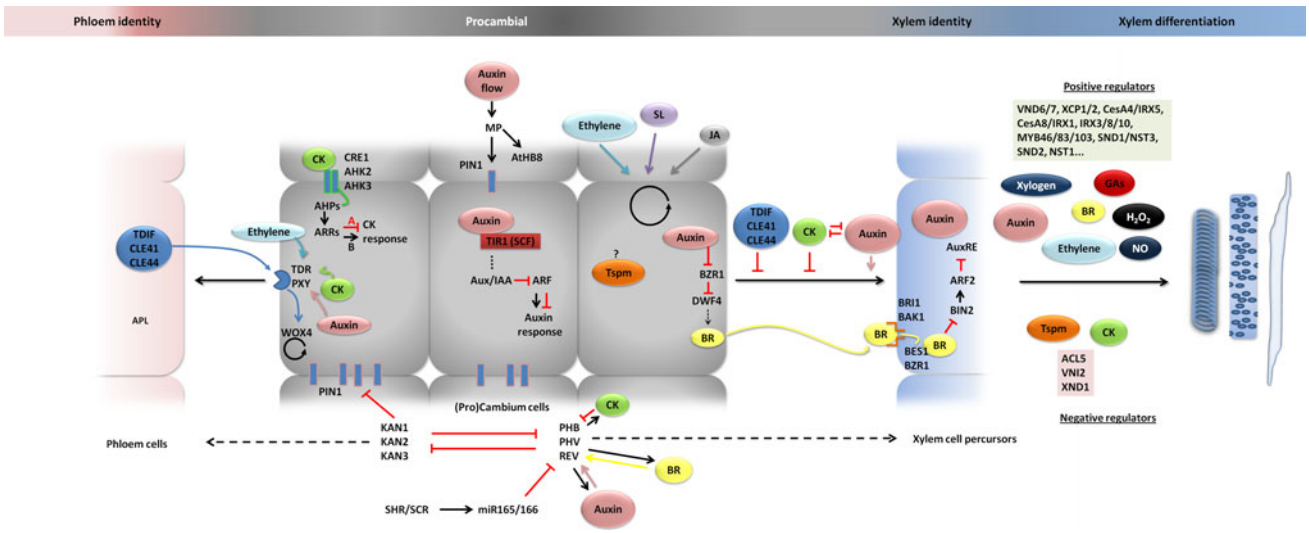

Fig. 2 Schematic representation summarizing hormone action and regulatory mechanisms involved in cambial cell identity determination, maintenance of cambial cell pool and differentiation into the different xylem cell types

thus relieving MONOPTEROS/AUXIN RESPONSE FACTOR 5 (MP/ARF5) from repression. The *MP* gene is essential in establishing procambium cells, as shown by the lack of the central provascular cylinder in *mp* embryos (Berleth and Jurgens 1993; Hardtke and Berleth 1998) and the decreased auxin sensitivity in *mp* mutants (Mattsson et al. 2003). It has been also shown that MP/ARF5 confers procambium identity, possibly by activating the *CLASS III HOMEODOMAIN-LEUCINE ZIPPER 8* (HD-Zip III *AtHB8*) gene transcription in *Arabidopsis* leaf veins (Donner et al. 2009). In response to increased auxin levels, the *MP* gene becomes transcriptionally activated (Wenzel et al. 2007). Reports also show that MP may regulate the expression of *PIN-FORMED1* (*PIN1*), a major auxin efflux carrier protein encoding gene (Sauer et al. 2006; Wenzel et al. 2007; Schuetz et al. 2008). PINs are polarly localized transmembrane proteins fundamental for directional cell-to-cell auxin transport, that is, for polar auxin transport (PAT) (Leyser 2005). Prior to procambium identity definition, there is an increase in the expression of *PIN1* (Scarpella et al. 2006; Wenzel et al. 2007). This increase in auxin flow is what determines pre-procambial cell state acquisition. *ATHB8* is necessary to stabilize pre-procambial cell specification against auxin transport perturbations (Donner et al. 2009) and probably acts by reducing the sensitivity to auxin, in terms of *PIN1* expression, and thereby confining procambium precursor cell state acquisition to narrow regions in the developing leaf (Scarpella et al. 2006; Wenzel et al. 2007; Donner et al. 2009; Ohashi-Ito and Fukuda 2010). Since procambial cell formation in the *athb8* null mutant does not deviate from the pattern observed in the wild type, it is also possible that MP acts on several other key components to promote procambium identity (Baima et al. 2001; Prigge et al. 2005). Curiously, while ATHB8 was found to be activated during interfascicular cambium formation, PIN1 and MP were not detected, suggesting the existence of an alternative mechanism of *ATHB8* activation, as in the procambium (Agusti et al. 2011a).

CLE peptides: keep it cambial and suppress xylem differentiation

The maintenance of cambial cell identity and activity has been shown to involve another signal: the tracheary element differentiation inhibitory factor (TDIF). TDIF is a CLAVATA3/ENDOSPERM SURROUNDING REGION (CLE)-family peptide produced by the activity of the *CLE41* or *CLE44* genes and is involved in short-range signaling and cell-to-cell communication (Ito et al. 2006; Fukuda et al. 2007). TDIF also inhibits the transdifferentiation of tracheary elements in *Zinnia* cell cultures and promotes proliferation of the procambium cells in *Arabidopsis* hypocotyls and leaves (Hirakawa et al. 2008). The receptor of TDIF is a leucine-rich repeat receptor kinase (LRR-RLK) named TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR RECEPTOR (TDR). This receptor is also called PHLOEM INTERCALATED WITH XYLEM (PXY) because it was initially cloned from the *pxy* mutant that showed vascular patterning defects in the positioning of xylem and phloem (Fisher and Turner 2007).

Several studies have demonstrated that *WUSCHEL-RELATED HOMEOBOX* (*WOX*) gene family members cooperate with *CLAVATA* (*CLV*)/*CLE* genes to organize

initial cell populations during development (Brand et al. 2000; Schoof et al. 2000; Ji et al. 2010). For instance, it has recently been found that *WOX4* is transcribed in the procambium of developing vascular bundles in the root and shoot lateral organs of *Arabidopsis* and tomato, and that the downregulation of this gene reduces vascular development and increases accumulation of undifferentiated ground tissue (Ji et al. 2010). This discloses an essential role for WOX4 in promoting cambium activity in the lateral meristem. TDIF/PXY signaling targets *WOX4*, which maintains proliferation of the cambial cells in response to the TDIF signal (Fig. 2; Hirakawa et al. 2010; Ji et al. 2010). Suer et al. (2011) further revealed that the auxin-dependent cambium stimulation requires *WOX4* and its upstream regulator, TDR/PXY (the receptor of TDIF). The application of an inhibitor of auxin transport, 1-*N*-naphthylphthalamic acid (NPA), to the bottom internodes of *Arabidopsis* inflorescences, stimulated cambial activity above the treated area, likely due to accumulation of the basipetally transported auxin in the wild type, but not in *wox4* mutants (Suer et al. 2011). These observations suggest that WOX4 is essential in the translation of the basipetal auxin transport into cambium activity. It has also been shown that a TDIF peptide signal is produced in the phloem cells and perceived in procambial cells by TDR/PXY, leading to the upregulation of *WOX4* in the vascular cambium cells (Hirakawa et al. 2008; Matsubayashi 2011). TDR/PXY is thus required for the correct polar patterning of xylem and phloem tissues. The disclosure of this TDIF–PXY system of communication is remarkable as it shows a crosstalk between phloem and xylem, whereby the peptide signals produced in the phloem cells activate a signaling cascade perceived by receptors in the plasma membrane of the procambial cells, which results in the stimulation of procambial cell proliferation and the inhibition of xylem differentiation.

The mechanisms behind establishment and maintenance of stem cell populations in the SAM and RAM show many similarities (Sarkar et al. 2007). This suggests that the same type of mechanisms that regulate stem cell homeostasis may well exist in the lateral meristem—vascular cambium. In fact, the molecular mechanism involved in the TDIF/CLE41/CLE44–TDR/PXY–WOX4 module closely resembles the signaling module comprising CLAVATA3 (CLV3) peptide and the receptor components CLAVATA1 (CLV1) and CLAVATA2 (CLV2) that operate in the control of stem cell maintenance in the SAM. Despite the differences, surprising parallels can be found in the molecular regulation of the apical and lateral meristems: both pathways comprise a CLE peptide, an LRR-RLK, and a WOX transcriptional regulator. However, the CLV pathway limits the expression of *WUS* or *WOX5*, whereas TDIF promotes the expression of the *WUS-like* gene *WOX4* to function in stem cell maintenance in the meristems (Hirakawa et al. 2010).

A parallel action of TDR/PXY and ethylene signaling in the control of cambial cell division has also been proposed. *pxy* loss-of-function *Arabidopsis* mutant does not exhibit a drastic reduction in vascular cell number, indicating that a compensatory pathway may be activated in the absence of TDR/PXY. Blocking ethylene signaling aggravates the typical defects of the *pxy* mutant, suggesting that ethylene signaling, *WOX4*, and TDR/PXY work in parallel to regulate cell divisions during *Arabidopsis* vascular development (Etchells et al. 2012). CLE peptides have been shown to inhibit protoxylem vessel formation in the *Arabidopsis* root via the cytokinin signaling pathway, suggesting crosstalk between CLE peptide signaling and cytokinin signaling in protoxylem vessel formation (Kondo et al. 2011). It would be interesting to further explore the hormone signaling crosstalk to dissect this TDIF/PXY regulation module.

Cytokinin signaling: keep it cambial and suppress xylem differentiation II

Cytokinin (CK) signaling is important in the maintenance and proliferation of cambial cells and in cambial cell specification. CK perception involves a family of CK receptors CYTOKININ RESPONSE1/WOODEN LEG/ARABIDOPSIS HISTIDINE KINASE4 (CRE1/WOL/AHK4) and ARABIDOPSIS HISTIDINE KINASE2 (AHK2) and AHK3 that activate a phosphorylation cascade whereby histidine phosphotransfer proteins (AHPs), in the nucleus, activate type-B ARABIDOPSIS RESPONSE REGULATORS (type-B ARRs), which in turn activate CK responses (Fig. 2; reviewed in Bishopp et al. 2006; Kieber and Schaller 2010). Type-B ARR proteins are also known to activate transcription of type-A *ARR*s that in turn negatively regulate back CK signaling (Dello Ioio et al. 2008a).

The expression of the key CK catabolic gene *CYTOKININ OXIDASE 2* (*CKX2*), in the *Populus* cambium, leads to a decrease in CK concentration and results in reduced radial growth (Fig. 3; Nieminen et al. 2008). In a similar manner, in *Arabidopsis*, the disruption of four genes encoding CK biosynthetic isopentenyltransferases results in plants unable to form cambium and with reduced stem and root thickenings (Matsumoto-Kitano et al. 2008). These studies have confirmed that CKs are important cambial regulators. Furthermore, CK receptors are highly expressed in dividing cambial cells. Hejátko et al. (2009) have shown that the histidine kinase CYTOKININ-INDEPENDENT1 (CKI1), AHK2 and AHK3 are important for vascular development by regulating procambium

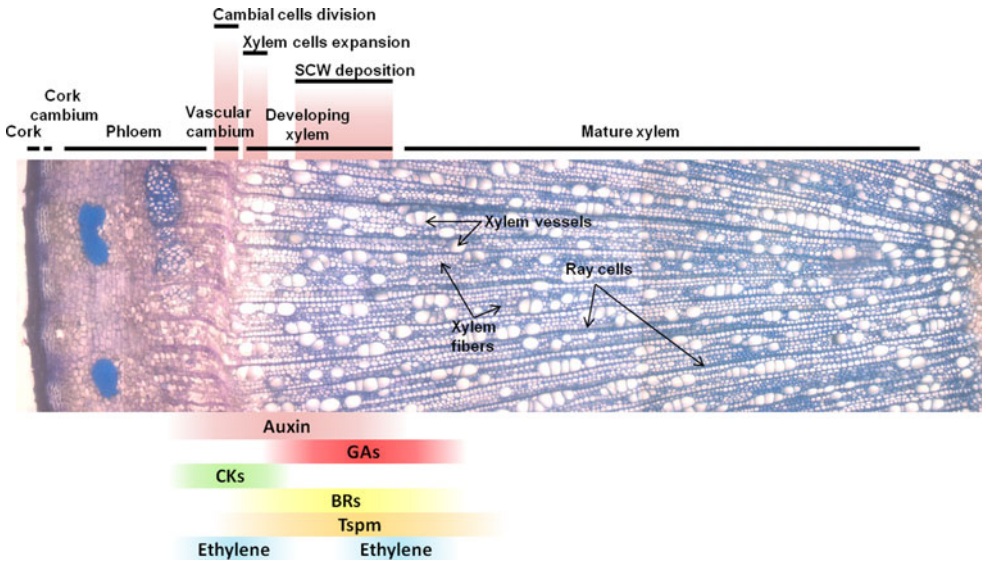

Fig. 3 Cross section of hybrid aspen (*Populus tremula* × *Populus tremuloides*) stem, showing the stages of xylem differentiation and the spatial context of hormones action. The vascular cambium, a secondary lateral meristem in tree stems, produces secondary phloem toward the outside and secondary xylem (wood) toward the inside. In the middle of the vascular cambium reside the stem cells that are the meristematic cells from which the phloem and xylem precursor cells originate. Since the exact location of the stem cells is not known, this region is also commonly named cambial zone. Xylem development initiates with active cambial cell division, followed by rapid cell expansion and deposition of secondary cell wall and programmed cell death. The xylem cell types in *Populus* wood (ray parenchymatic living cells, xylem vessel elements and xylem fibers) are indicated. The cork cell layers cover the surface of the stem, which are produced by the activity of the cork cambium, the other secondary lateral meristem in tree stems. The known or possible function domains of *auxin*, gibberellin (*GAs*), cytokinin (*CK*), brassinosteroids (*BRs*), thermospermine (*Tsmp*) and *ethylene* across the *Populus* cambial zone are shown. *Auxin* (Tuominen et al. 1997) and *GA* (Israelsson et al. 2005) *labels* reflect the concentration of the bioactive hormones and expression pattern of auxin and GAs signaling genes (Moyle et al. 2002; Israelsson et al. 2005). *CK label* depicts the peak of expression of cytokinin signaling genes in the phloem side of the cambial zone (Nieminen et al. 2008). *Ethylene label* depicts its stimulatory effect on cambial cell division (Love et al. 2009; Pesquet and Tuominen 2011). *Tspm label* depicts place of action on developing xylem, delaying xylem cell death (Muñiz et al. 2008; Milhinhos et al. submitted for publication). BRs are currently not studied in *Populus*, thus the *BR label* depicts current knowledge from *Arabidopsis* and *Zinnia* showing that BRs are produced in procambial cells, perceived in the xylem precursor cells to induce xylem differentiation (see main text)

proliferation and/or the maintenance of its identity in *Arabidopsis* shoots. Mutations in the CK-induced receptors AHK2 and AHK3 result in defects in vascular tissue formation in the inflorescence stem that are partially rescued by overexpression of CKI1 (Hejátko et al. 2009). The *wol* mutants that are defective in *CRE1/WOL/AHK4* gene have reduced cell proliferation and cell files within the pericycle layer only differentiate into protoxylem (Scheres et al. 1995; Mähönen et al. 2000, 2006). A concrete mechanism was unveiled when Mähönen et al. (2006) found that CK negatively regulates protoxylem specification and that AHP6 (a histidine phosphotransfer protein lacking the histidine residue necessary for phosphorelay) counteracts CK signaling, thus having a positive effect on protoxylem formation. On the other hand, CK signaling negatively regulates the spatial domain of *AHP6* expression. The hypothesis is that this balance between proliferation and differentiation of cell lineages directs vascular development in early embryogenesis (Mähönen et al. 2006). Furthermore, auxin was also brought to this equation.

Auxin and cytokinin interplay in protoxylem differentiation

Bishopp et al. (2011) further elucidated the AHP6-mediated crosstalk between auxin and CK signaling in vascular patterning. The authors showed that, in the *Arabidopsis* root vasculature, the cells fated to be protoxylem exhibit high auxin and low CK signaling, whereas the procambial cells exhibit high CK but low auxin signaling. A mutually inhibitory mechanism was proposed wherein high CK signaling in the procambial cells promotes the expression of *PIN*s and the lateral localization of PIN proteins. This CK-dependent PIN activity forces a lateral flow of auxin from the procambial cells, to the meristematic cells from which protoxylem will form. The increase in auxin signaling in these cells promotes their specification into protoxylem and the transcription of *AHP6*, which in turn inhibits CK signaling (Bishopp et al. 2011). Moreover, a piece of this story has been linked to the mobile GRAS family transcription factor SHORT-ROOT (SHR) required

for cell specification and patterning of the *Arabidopsis* root (Helariutta et al. 2000; Nakajima et al. 2001). Because CK levels are found elevated in *shr* mutant and SHR directly regulates a cytokinin oxidase (*CKX3*) that is preferentially expressed in the protoxylem, it has been proposed that SHR controls vascular patterning by controlling CK homeostasis (Cui et al. 2011). This is, when SHR is functional it is thought to impose low CK levels to the cell and therefore promote xylem differentiation. On the other hand, when *SHR* is disrupted (*shr*) or in the presence of exogenous CK, this CK minimum is perturbed, and as a consequence, the high CK levels therefore present would suppress xylem cell fate (Cui et al. 2011).

Yet another mechanism of auxin–CK crosstalk is present in the *Arabidopsis* root meristem. On the one hand, type-B ARRs (ARR1 and ARR12), which are the end points of CK signaling as mentioned above, inhibit the *Aux/IAA* gene *SHORT HYPOCOTYL 2* (*SHY2/IAA3*). On the other hand, SHY2/IAA3 inhibits *PIN* gene expression but also feeds back to repress CK biosynthetic genes. IAA has also been shown to repress back *SHY2/IAA* expression. These loops that feed back to hormone synthesis (Ruzicka et al. 2009; Dello Ioio et al. 2008b; Jones et al. 2010), the time and space of action, as well as the involvement of other hormones, such as gibberellin and brassinosteroids (Depuydt and Hardtke 2011), further increase the complexity of this crosstalk.

HD-Zip III family, KANADIs and hormonal interactions: keeping it cambial and triggering xylem cell fate

The onset of vascular tissue differentiation from cambial cells also involves the interaction between two other well-characterized genes families, class III homeodomain-leucine zipper transcription factors (HD-Zip III) and GARP transcription factors KANADI (KANs), as well as their effect on auxin flow. In *Arabidopsis*, the HD-Zip III protein family includes five members: PHABULOSA (PHB), INTERFASCICULAR FIBERLESS1/REVOLUTA (IFL/REV), PHAVOLUTA (PHV), CORONA (CNA/ATHB15) and ATHB8 (Baima et al. 1995; McConnell et al. 2001; Ohashi-Ito and Fukuda 2003; Ohashi-Ito et al. 2005; Otsuga et al. 2001; Prigge et al. 2005). The patterns of expression of *HD-Zip III*s have been intensively studied in *Arabidopsis*, in *Zinnia elegans* and, more recently, in *Populus*. In *Arabidopsis* and in *Zinnia*, *AtHB15/ZeHB13* is predominantly expressed in procambial cells and proposed to be a regulator of procambium formation (Ohashi-Ito and Fukuda 2003). Kim et al. (2005) reported that repression of *AtHB15*, in the *Arabidopsis* inflorescence stems, actually accelerates xylem cell differentiation from the procambial cells. Therefore, ATHB15 could have a role in maintenance of cambial cell identity. However, in *Populus*, the *AtHB15* ortholog *POPCORONA* was not found to be restricted to provascular cells or primary xylem, but was also found in secondary vascular tissues, namely ray xylem cells and secondary xylem, which could imply that HD-Zip IIIs are involved in new roles in xylem formation in trees (Du et al. 2011). The *AtHB8* domain of expression coincides with tracheary element precursors and *ATHB8/ZeHB10* gain-of-function plants have increased production of tracheary elements in the vascular bundles (Ohashi-Ito et al. 2005; Baima et al. 2001). In *Populus*, the *AtHB8* homolog *PttHB8* has increased expression that coincides with the auxin radial concentration gradient in the cambial cells of the stem, thus suggesting a conserved role in promoting xylem specification (Nilsson et al. 2008). The *IFL/REV/ZeHB11/ZeHB12* was found expressed in procambium and xylem parenchyma cells, and increased *REV* expression led to increased production of xylem precursor cells, but not to increased differentiation into tracheary elements (Emery et al. 2003; Ohashi-Ito et al. 2005; Zhong and Ye 2004). In *Populus*, the *REV* ortholog *POPREVOLUTA* (*PRE/PtaHB1*) was found localized in cambial cells and has been suggested to have a role in secondary growth, perhaps in the transition from primary to secondary growth, since transgenic poplar for *PRE* constitutive expression showed reverse polarity of xylem and phloem after abnormal cambial cells were produced from cortical parenchyma cells (Ko et al. 2006; Robischon et al. 2011). These observations mean that REV may function both in cambial maintenance and in xylem specification.

While *HD-Zip III*s are mainly expressed in procambial and xylem precursor cells and thought to promote xylem differentiation, *KAN*s are mainly expressed in phloem and seem to act antagonistically on vascular specification (Fig. 2; Eshed et al. 2001; Kerstetter et al. 2001; Ilegems et al. 2010). However, *KAN* loss-of-function *Arabidopsis* mutants develop phloem cells, indicating that these genes are not essential for phloem specification. HD-Zip IIIs and KANs also control tissue polarity in the vascular bundles. In the *Arabidopsis* shoot, gain-of-function *HD-Zip III* mutations and lack of *KAN*s expression in the triple mutant *kan1 kan2 kan3* result in a shift from the typical collateral vascular bundle arrangement to an amphivasal one, wherein xylem surrounds the phloem. Vice versa, *HD-Zip III* loss-of-function mutants result in an amphicribral arrangement, wherein phloem surrounds xylem (Emery et al. 2003; McConnell and Barton 1998; McConnell et al. 2001; Zhong and Ye 2004). The loss of function of all five *HD-Zip III*, observed in the quintuple *phb phv rev cna athb8* mutant, and ectopic *KAN1* expression result in no vascular development at all. In fact, in the *phb phv rev cna athb8* mutant, there is a suppression of the differentiation

of procambial cells into xylem cells and a subsequent increase in procambium cells proliferation (Carlsbecker et al. 2010).

Expression of *KAN1*, driven by the *AtHB15* promoter, has a negative effect on expression and polar localization of PIN1 proteins resulting in the inhibition of procambium formation in early stages of *Arabidopsis* embryogenesis (Ilegems et al. 2010). It is well known that *HD-Zip III* genes have an overlapping pattern of expression with the pattern of auxin distribution (Izhaki and Bowman 2007), which likely indicates that auxin interplays with the *HD-Zip III* genes. In fact, the expression of *ATHB8*, *REV*, *PHV* and *CNA*/*ATHB15* is known to be induced by auxin, and auxin flux is modulated by HD-Zip III (Baima et al. 1995; Zhou et al. 2007; Izhaki and Bowman 2007; Yoshida et al. 2009; Ilegems et al. 2010). This suggests that KAN proteins control cambial activity by negatively acting on auxin transport, whereas HD-Zip III promotes xylem differentiation by having a role in the canalization of auxin flow (Ilegems et al. 2010).

Mobile signals and HD-Zip IIIs: a matter of signal dosage?

HD-Zip III genes are suppressed by the expression of microRNA 165/166. miRNA 165/166 are known to target *HD-Zip III*, as mapped in the *HD-Zip III Arabidopsis* gain-of-function mutations (Emery et al. 2003; Kim et al. 2005; Zhong and Ye 2004). A link to SHR and SCARECROW (SCR) proteins has been established showing that both are involved in root vascular patterning as transcriptional activators of miRNA 165/166 (Fig. 2; Carlsbecker et al. 2010). These authors described how crosstalk between the vascular cylinder and the surrounding endodermis is mediated by the cell-to-cell movement of SHR in one direction and miRNAs in the other. SHR, produced in the vascular cylinder, moves into the endodermis to activate SCR and together these transcription factors activate mir165/166. The miRNA 165/166 in turn migrates from the cells where they are produced, in the endodermis of the *Arabidopsis* root, into the stele periphery where they act on *HD-Zip III PHB* levels (Carlsbecker et al. 2010). Thus, this non-cell autonomous and dose-dependent action of miRNA165/166 modulates the PHB gradients in the stele, controlling xylem differentiation: where a high dosage of PHB specifies metaxylem, while a low dosage of PHB specifies protoxylem differentiation (Carlsbecker et al. 2010; Miyashima et al. 2011). Furthermore, SHR is thought to regulate miR165/166 through its effect on CK homeostasis, since high CK levels repress xylem specification in the *shr* mutant (Cui et al. 2011). Another regulatory model for the interplay between PHB and miRNA165 that also involves CK has been proposed for the *Arabidopsis* root meristem, wherein PHB induces CK biosynthesis by activating the biosynthetic *ISOPENTENYL TRANSFERASE 7* (*IPT7*) gene, thereby promoting cell differentiation, but CK feedback represses both *PHB* and mirRNA165, thus negatively regulating both its activator and the activator repressor. This almost *non-sense* regulatory circuit is proposed to be a mechanism of balancing the division and differentiation of stem cells during root growth (Dello Ioio et al. 2012). Miyashima et al. (2011) recently suggested that miRNA165/166 might act as morphogens, given that they are emitted from a local source, affecting the neighboring tissues and their *HD-Zips III* targets dosage is read by each receiver cell for different cell fates. Morphogen-like action in plants may not follow the exact criteria as defined in animal systems; therefore, it has been suggested that a "morphogenetic trigger is a factor or signal that induces, through unequal distribution of its activity, acquisition of a new developmental fate in a cell or a group of cells" (Dubrovsky et al. 2008; Benková et al. 2009). IAA could, therefore, also be considered a morphogenetic trigger in several contexts, as its local maximum acts like an instructive signal for initiation of organ formation, such as lateral root initiation in *Arabidopsis* (Benková et al. 2009; Dubrovsky et al. 2008) or the developmental gradient of secondary xylem cell specification found in *Pinus* and *Populus* that coincides with the auxin concentration gradient (Uggla et al. 1996; Tuominen et al. 1997). *MP* (discussed above) is also a genetic switch triggered in response to auxin in a threshold-dependent manner (Lau et al. 2011). *MP*, which likely regulates SAM (Zhao et al. 2010), links auxin signaling and meristem function. Morphogenetic triggers or morphogen-like action is receiving increased attention, as RNAi-derived small RNAs and auxin embody the required mobile signals (Bhalerao and Bennet 2003; Benková et al. 2009; Skopelitis et al. 2012). Vascular differentiation may involve yet more substances with morphogen type of action.

Brassinosteroids: are we closer to xylem identity?

Brassinosteroids (BRs) are also involved in *HD-Zip III* regulation. BR-deficient *Arabidopsis* mutants produce extreme dwarf plants, with reduced amounts of xylem (Szekeres et al. 1996; Choe et al. 1999b). The BRs are synthesized in the procambial cells and are perceived by receptors in xylem precursor cells [LRR receptor kinases: BR INSENSITIVE 1(BRI1)/BR RECEPTOR LIKE 1 and 3 (BRL1 and BRL3)], inactivating the negative regulator BR INSENSITIVE 2 (BIN2), thus allowing the un-phosphorylated forms bri1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE RESISTANT 1 (BZR1) to promote

xylem differentiation by increasing *HD-Zip III* genes expression (Fig. 2; Caño-Delgado et al. 2004; Ohashi-Ito and Fukuda 2003; Ohashi-Ito et al. 2002; Fukuda 2004). Indeed, in *Zinnia* cell cultures, *AtHB8* and *REV* homologs are repressed by inhibitors of BR biosynthesis, but restored by exogenous application of BR (Ohashi-Ito et al. 2002). *Zinnia AtHB15* homolog expression is also induced by BR, which suggests that *HD-Zip III* genes function in vascular differentiation also in response to BR signaling (Ohashi-Ito and Fukuda 2003). In addition, BR perception is promoted by HD-Zip III, as increases in *HD-Zip III* genes expression induce BRL3 and BRI1-associated receptor kinase-1 (BAK1) (Ohashi-Ito et al. 2005).

Interestingly, BRs and auxin converge in a set of common target genes, suggesting coordinated signaling pathways. For instance, BIN2 kinase regulates the *AUXIN RESPONSE FACTOR 2* (*ARF2*) that inhibits transcription of auxin-responsive genes (Fig. 2; Vert et al. 2008), while auxin inhibits the binding of the transcriptional repressor BZR1 to the promoter of the BR biosynthesis gene *DWARF4* (*DWF4*), implicating auxin in BR biosynthesis (Chung et al. 2011). The overexpression of *BR-RELATED ACYLTRANSFERASE1* (*BAT1*), a gene encoding a putative acyltransferase, renders a typical BR-deficient phenotype. Additionally, auxin also highly induces *BAT1*, suggesting that the conversion of brassinolide intermediates into acylated-BR conjugates is promoted by auxin (Choi et al. 2012). Thus, auxin seems to be involved in the control of BRs homeostasis, while BRs repress the inhibition of auxin-responsive genes transcription, acting synergistically in vascular development. Moreover, it has been suggested that PAT is enhanced by BRs, possibly by modulation of *PIN* genes expression (Li et al. 2005). The number of vascular bundles is also enhanced by BRs, which have been predicted to modulate the procambial cell number required to set the number of auxin maxima at the shoot vasculature, suggesting that PAT acts in coordination with BR signaling (Ibañes et al. 2009). In sum, BRs and auxin overlap in their transcriptional control of common target genes, and both hormones exert effects on each other's signaling and perception. However, how these crosstalks are mechanistically integrated into xylem differentiation is still largely unknown.

Xylogen: a mobile signal toward xylem

Xylogen, an extracellular arabinogalactan protein (AGP), is a mobile signal found in procambium and xylem cells that promotes xylem cell differentiation in the vascular tissues (Motose et al. 2004). It was first isolated from *Zinnia elegans* xylogenic culture medium and found to be secreted from differentiated xylem cells to promote differentiation of uncommitted cells into tracheary elements (Motose et al. 2004). Two *Arabidopsis* genes, *XYLOGEN PROTEIN 1* and 2 (*AtXYP1* and *AtXYP2*), and 13 xylogen-type genes (*XYLP*) have been identified. *AtXYP2* has been suggested to be the best candidate as the *Arabidopsis* counterpart to the *Zinnia* xylogen gene, *ZeXYP1*, responsible for the production of the xylogen peptide. Both *AtXYP1* and *AtXYP2* are expressed in the vascular tissues and the *xyp1 xyp2* double mutant, disrupted in xylogen function, displays discontinuous xylem (Motose et al. 2004; Kobayashi et al. 2011). However, the xylogen mode of action in xylem is not yet understood. It is possible that it is a coordinator molecule, secreted from differentiating vascular cells, that induces xylem differentiation in neighboring uncommitted cells. As for hormonal interactions, it has been shown that the expression of *ZeXYP1* is induced by auxin, and that auxin and cytokinin induce the accumulation of xylogen, suggesting that both hormones act synergistically as positive regulators of xylogen, although by unknown mechanisms (Motose et al. 2004).

Ethylene has dual roles: keeping it cambial but also differentiating xylem

ETHYLENE RESPONSE 1 (ETR1) was the first hormone receptor to be identified, but other ethylene receptors have been identified since then (Chang et al. 1993; for detailed reviews on ethylene signaling see Alonso and Stepanova 2004; Stepanova and Alonso 2009). Downstream of the ethylene receptors is CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), a negative regulator of ethylene signaling, and downstream of CTR1 is the positive regulator ETHYLENE-INSENSITIVE2 (EIN2). EIN3, a transcription factor that mediates responses to ethylene, is downstream of EIN2 and is shunt to the proteasomal degradation pathway (Kieber et al. 1993; Alonso et al. 1999). Recent work in transgenic *Populus* trees with disrupted ethylene perception has demonstrated that ethylene has a stimulatory effect on cambial cell division, at least in trees that are mechanically stimulated (Fig. 3; Love et al. 2009). Previously, in the *Arabidopsis* root meristem, it had already been indirectly shown that the over accumulation of ethylene by the loss of ETHYLENE OVERPRODUCER1 (ETO1) function had a stimulatory effect on cell division at the core of the stem cell niche, the quiescent center that during normal growth scarcely undergoes cell division (Ortega-Martinez et al. 2007). However, the work by Pesquet and Tuominen (2011) suggested that ethylene has a dual function in vascular development, one stimulating the rate of tracheary elements differentiation and another controlling stem cell pool size during secondary growth *in planta*.

Gibberellins: late players in xylem differentiation

The discovery of the gibberellin (GA) receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1) allowed further understanding of the molecular mechanisms involved in GA signaling (Ueguchi-Tanaka et al. 2005). DELLA proteins are central repressors of the GA signaling pathway by acting immediately downstream of GID1 receptor. Binding of GA to GID1 causes binding of GID1-GA to DELLAs and leads to their degradation via the ubiquitin–proteasome pathway (for specific reviews on GA signaling pathway, see, for instance, Sun 2011; Schwechheimer 2012). The effects of GAs in vascular differentiation suggest that GA is essential for xylem proliferation. Indeed, the overexpression of *GIBBERELLIN 20-OXIDASE1* (*GA20ox*), a GA biosynthetic gene, in *Populus* results in increased growth and xylem fiber length (Eriksson et al. 2000). *GA20ox* mRNA and bioactive GAs have also been demonstrated to accumulate in the expansion zone of developing xylem (Israelsson et al. 2005). Moreover, Mauriat et al. (2011) showed that decreased GA precursor levels and reduced bioactive GA levels result in reduced secondary growth. Altogether, these results suggest a role for GAs in xylogenesis.

Crosstalk between GA and auxin pathways has been demonstrated in *Populus* stems: Björklund et al. (2007) found that GA stimulates auxin transport, and exogenous application of GAs and auxin to decapitated trees had a positive synergistic effect on cambial growth. The application of gibberellin to decapitated *Populus* trees did not trigger xylogenesis, but instead disrupted the meristematic identity of the cambial cells, again showing that the auxin maxima in cambium cells is indispensable for cambial activity, whereas GA acts later in xylem formation. In this same study, GA treatment induced the expression of *PIN1*. This coincides with observations made by Willige et al. (2011) demonstrating that *Arabidopsis* GA biosynthesis and signaling-deficient mutants have reduced PAT. The GA-deficient plants showed a reduced abundance of PIN1, PIN2 and PIN3, but all PIN protein levels recovered to wild-type levels after GA treatment. This suggests that, for normal PIN protein accumulation, GA promotes the degradation of DELLA proteins via the GID1 pathway (Willige et al. 2011).

Applying gibberellic acid-3 (GA3) to *Zinnia* xylogenic cultures increased the differentiation of tracheary elements and their lignin content, whereas GA biosynthesis inhibitors decreased tracheary elements differentiation (Tokunaga et al. 2006). Ragni et al. (2011) recently proposed that GA is actually the mobile shoot-derived signal that triggers xylem expansion upon flowering initiation in *Arabidopsis*. This work, however, reports that auxin does not appear to be limiting for increased xylogenesis, evidencing discrepancies between *Arabidopsis* hypocotyls and inflorescence stems and *Populus* stems.

Xylem differentiation transcriptional network

The final steps of xylem development involve secondary cell wall formation and PCD (Fig. 3). An intricate regulatory network of transcriptional factors involved in differentiation of xylem cells has been identified (Kubo et al. 2005). By analysis of transdifferentiating tracheary elements in *Arabidopsis* cell culture, Kubo et al. (2005) isolated several *VASCULAR RELATED NAC DOMAIN* (*VND1–VND7*) transcription factor genes. In particular, *VND6* and *VND7* were found to be the key regulators of xylem cell specification, as ectopic expression of *VND6* induced metaxylem cell-type specification, whereas *VND7* induced protoxylem differentiation (Kubo et al. 2005; Yamaguchi et al. 2010a). Investigation of hormonal control of such transcription factors is lacking. Nevertheless, the combined application of auxin, cytokinin and brassinosteroids to hypocotyls of wild type and seedlings carrying transgenic promoter GUS fusions of *VND6* and *VND7* led to increased expression of *VND6* and *VND7*, suggesting that these transcription factors act downstream of the hormone signaling pathways (Kubo et al. 2005). Yoshida et al. (2009) also found that *VND* genes are overexpressed shortly after NAA application to differentiating *Zinnia* cell cultures. BRs also promote expression of *VND*s and PCD stages in *Populus* and *Arabidopsis* xylem tracheary elements by controlling other regulatory proteins, such as GTP-binding RabG3b protein (Kwon et al. 2010, 2011). So far, no hormonal regulatory mechanism of this transcriptional network has been unveiled.

Several regulatory elements from another NAC family of transcription factors are involved in the terminal stages of xylem development. *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1* (*NST1*), *NST2* and *SECONDARY WALL-ASSOCIATED NAC-DOMAIN 1* (*SND1*/*NST3*) are key regulators of secondary cell wall thickening, particularly in *Arabidopsis* fibers (Zhong et al. 2006, 2007a; Mitsuda et al. 2007). Examples of characterized transcription factors from *Arabidopsis*, poplar, pine and eucalyptus suggest that the NAC-mediated transcriptional regulation of secondary wall biosynthesis is a conserved mechanism throughout vascular plants (Zhong et al. 2010a). A number of MYB transcription factors function downstream of SND1 to upregulate the synthesis of secondary cell wall components, such as cellulose and lignin (Zhong et al. 2007b; McCarthy et al. 2009). Hussey et al. (2011) also observed that *Arabidopsis SND2* regulates genes involved in secondary cell wall development in

Arabidopsis fibers, while overexpression of *AtSND2* in *Eucalyptus* increases fiber cell area.

A recent work by Yamaguchi et al. (2011) has dissected the possible direct targets of VND transcriptional action in xylem vessel differentiation. These authors showed that VND7 directly regulates both secondary cell wall thickening and PCD by revealing that recombinant VND7 protein binds to the promoter sequences of downstream genes involved in both xylem developmental processes. The direct targets of VND7 include genes encoding cellulose synthase subunits *CesA4/IRX5* and *CesA8/IRX1*, but also encoding to their MYB regulators *MYB46*, *MYB83* and *MYB103*, as well as *XYLEM CYSTEINE PROTEASE1* (*XCP1*) and *XCP2* genes (Fig. 2; Zhong et al. 2007b, 2008; McCarthy et al. 2009; Yamaguchi et al. 2011). The *XCP1* and *XCP2* function could be traced by analysis of *xcp1* and *xcp2* mutations in *Arabidopsis* roots showing delayed autolysis during xylogenesis (Avci et al. 2008).

The transcriptional network in control of secondary cell wall deposition and PCD includes members of the LOB DOMAIN PROTEIN/ASYMMETRIC LEAVES LIKE (LBD/ASL) protein family. For example, the overexpression of *LBD30/ASL19* and *ASL20* genes induces transdifferentiation of *Arabidopsis* cells from non-vascular tissues into tracheary element-like cells, similar to those induced upon *VND6/7* overexpression (Soyano et al. 2008). Moreover, VND7 transcription factor has been shown to directly target *LBD30/ASL19* and *LBD15/ASL11* genes (Yamaguchi et al. 2011), whereas the LBD proteins ASL20/LBD18 and ASL19/LBD30 are part of a positive feedback loop that amplifies the expression of *VND6* and *VND7* (Soyano et al. 2008). These observations reveal an intricate transcriptional regulatory network, but also indicate that most of the regulatory factors involved in secondary cell wall deposition are also implicated in PCD during development.

Two negative regulators of xylem formation have been described in *Arabidopsis*: VND-INTERACTING2 (VNI2) that acts to suppress the VND7 capacity to activate transcription; and a gene encoding *XYLEM NAC DOMAIN1* (*XND1*), whose overexpression causes the complete suppression of vessel secondary wall biosynthesis and PCD, suggesting that it negatively regulates xylem vessel differentiation (Fig. 2; Yamaguchi et al. 2010b; Zhao et al. 2008). Curiously, both VNI2 and XND1 are suggested to be targeted for proteasomal degradation by 20S proteasome (20SP). The 20SP is thought to be part of the ubiquitin/26SP proteolytic system, and it possesses caspase-3-like activity, characteristic of animal cell apoptosis. 20SP may degrade VNI2 and XND1 to induce tracheary elements differentiation in *Arabidopsis* and *Populus* (Han et al. 2012). Yet another signal, thermospermine, acts as a negative regulator of xylem differentiation, as discussed below.

Thermospermine: preventing a troubled premature death

Polyamines share common substrates with the ethylene biosynthetic pathway. *S*-adenosylmethionine decarboxylase (SAMDC) is an example of a cross-point between polyamine biosynthesis and ethylene biosynthetic pathway, as both pathways need *S*-adenosylmethionine (SAM) as substrate. The *bushy and dwarf 2* (*bud2*) *Arabidopsis* mutant, disrupted in the SAMDC4 enzyme, displays enlarged vascular tissues and high lignin content, demonstrating a link to vascular development (Ge et al. 2006). Curiously, a resembling phenotype was found in a polyamine biosynthesis mutant, *acaulis5* (*acl5*), which produces dwarf plants with impaired stem elongation and develops vascular bundles with great amounts of differentiated cells with thickened walls (Hanzawa et al. 1997, 2000; Clay and Nelson 2005). *ACL5* gene codes for thermospermine synthase, an aminopropyltransferase that transfers an aminopropyl residue from the decarboxylated SAM to an amine acceptor on spermidine producing the tetra-amine thermospermine, a structural isomer of spermine (Tspm in the figures; Knott et al. 2007). But contrary to spermine, thermospermine has a role in xylem development: *ACL5* is specifically expressed in procambial and xylem vessels of *Arabidopsis* and prevents the premature cell death of the xylem elements (Muñiz et al. 2008). Thus, the *acl5* mutant shows a complete lack of xylem fibers, immature vessels, and disrupted secondary growth suggesting that the timing of xylem differentiation was inappropriate, leading to stunted plants in which xylem differentiation proceeded too fast. The precise mechanism by which this happens is still unknown, but some regulatory factors have been described. The disruption of a basic-helix-loop-helix (bHLH) transcription factor, named SUPPRESSOR OF ACAULIS5 1 (SAC51), restores the wild-type phenotype in the absence of thermospermine (Imai et al. 2006). The *SAC51* mRNA contains five upstream open reading frames (uORFs) and the *sac51-d* allele has a mutation in one of these uORFs, creating a stop codon and leading to the production of a truncated SAC51 polypeptide (Imai et al. 2006). In the *acl5* mutant, the translation of the SAC51 main ORF is suppressed. In the double mutant *sac51-d acl5-1*, which shows restored stem elongation, the inhibition of the *SAC51* translation is suppressed and a functional SAC51 is overproduced. This suggests that ACL5 or thermospermine activates the translation of SAC51 by inhibiting this uORF and preventing it from negatively regulating *SAC51* main ORF translation (Imai et al. 2006). Thus, the effect of thermospermine might be to bypass the inhibition of the *SAC51* uORF (Yoshimoto et al. 2012; Takano et al. 2012). Furthermore, VND7 is thought to induce *SAC51*. Therefore,

a more complex network could be functioning to balance the repression and induction of xylem differentiation, in a timely manner, involving the action of NAC domain transcription factors (Zhong et al. 2010b; Bollhöner et al. 2012).

Thermospermine is increasingly viewed as a novel plant growth regulator (Kakehi et al. 2010; Yoshimoto et al. 2012; Takano et al. 2012; Milhinhos et al. submitted for publication). It has recently been suggested that thermospermine interacts with auxin in differentiating xylem (Vera-Sirera et al. 2010). Yoshimoto et al. (2012) demonstrated that thermospermine is required to suppress the auxin-inducible xylem differentiation. 2,4-Dichlorophenoxy acid (2,4-D) and other auxin synthetic analogs were shown to induce excessive xylem vessel differentiation in cotyledons of *acl5 Arabidopsis* mutant, but not in the wild type, suggesting a rather high threshold for auxin-inducible xylem formation. Furthermore, in the double mutant *sac51-d acl5-1*, the application of 2,4-D did not induce xylem differentiation (Yoshimoto et al. 2012). This means that, while auxin exerts a positive effect on xylem differentiation, thermospermine acts as a limiting factor in differentiation and that the effect of auxin on xylem differentiation may be mediated through SAC51 (Yoshimoto et al. 2012). Interestingly, we have found *ACL5* to negatively affect endogenous auxin levels and endogenous auxin to positively affect *ACL5*, in a mechanism that maintains steady-state levels of thermospermine in *Populus* xylem tissues (Milhinhos et al. submitted for publication). These results are in line with recent findings by Cui et al. (2010) showing that *BUD2* is induced by auxin and that the expression of auxin-inducible genes is reduced in *bud2* mutants, suggesting that polyamine levels or encoding transcripts might play a role in regulating auxin levels or responses to auxin.

Polyamines, ethylene and NO signals for death

Other polyamine catabolism and biosynthesis products have been shown to influence xylem differentiation. Tisi et al. (2011) demonstrated that, after spermidine supply in *Zea mays* and PAO overexpression in tobacco plants, the H_2O_2 derived from polyamine catabolism behaves as a signal for secondary wall deposition and for induction of developmental PCD. Waduwara-Jayabahu et al. (2012) also observed that the recycling of 5′-methylthioadenosine (MTA), a by-product of polyamine and ethylene biosynthesis, is essential to maintain normal vascular development in *Arabidopsis*. Thermospermine and other polyamines share common substrates with the biosynthetic pathway of ethylene, and thus it would be interesting to know if there is crosstalk in xylem development. Some hints are emerging. For instance, polyamines were shown to modulate genes involved in ethylene biosynthesis and signaling pathways during olive mature fruit abscission (Parra-Lobato and Gomez-Jimenez 2011). This work described polyamines playing a positive role in NO production and discovered an inverse correlation between nitric oxide (NO) and ethylene presence in the abscission tissue. Also, the presence of NO in the xylem, proximal to the abscission zone of olive fruit, is indicative of the involvement of NO in xylem cell wall lignification and differentiation (Parra-Lobato and Gomez-Jimenez 2011). NO, in other contexts, could thus be a linking signal molecule between polyamines, ethylene, and xylem cell death. Gabaldón et al. (2005) have shown that NO production is assigned to the vascular tissues of *Zinnia elegans*. In this work, the spatial NO gradient was inversely related to the degree of xylem differentiation. The authors observed a NO burst associated with a single cell layer of pro-differentiating thin-walled xylem cells. The treatment of transdifferentiating *Zinnia* cells with a NO scavenger inhibited the tracheary element differentiation and increased cell viability, suggesting that the NO production is related to the early processes of xylem differentiation, which take place immediately before the late processes of secondary cell wall formation and cell autolysis (Gabaldón et al. 2005). Elucidating the crosstalk among these signals and signaling pathways will be crucial to understand the trigger mechanism of cell death in xylem differentiation. Figure 3 integrates the place of action of hormones during xylem differentiation cellular events in *Populus*.

Conclusions and future perspectives

Many hormone signaling pathways have been identified in the last 15 years increasing our knowledge of the crosstalks between hormone signaling pathways. The developmental context of these crosstalks is also being elucidated. Many mechanisms of cambial state maintenance have been proposed and new players, like thermospermine, but also jasmonic acid and strigolactones, are being brought to an already complex scenario: jasmonic acid was suggested as a possible cambium regulator by triggering cell division (Sehr et al. 2010); and plants with reduced strigolactones signaling or biosynthesis show reduced cambium activity and, conversely, treatment with synthetic strigolactone enhances cambial growth in the *Arabidopsis* inflorescence stem (Agusti et al. 2011b). More recently, strigolactone signaling was found to trigger PIN1 depletion from xylem parenchyma cells and inhibit bud outgrowth in the stems by counteracting the bud-activating auxin fluxes (Shinohara et al. 2013), suggesting that molecular crosstalk between strigolactones and auxin could be taking place during secondary growth. One important feature is that many regulatory mechanisms that function in apical meristems

are proved similar in lateral cambium development. This provides great hints on which components to search for in the study of the molecular mechanisms underlying the vascular cambium formation in trees and how these relate to novel candidate genes obtained from the plethora of work reporting genome-wide transcriptomic analysis. Thus far, most functional studies use transgenic approaches that increase or repress hormone signaling components or downstream transcriptional regulators in the whole plant. It would be important to use cambial-specific promoters that do not affect the apical meristem to determine the effect of manipulating hormone signaling components in a tissue-specific manner. It is evident that the increase in cambial division results in increased xylem biomass production. This is valuable information from a biotechnological point of view, given the high demands for improved biomass yields. The downstream cell cycle intervenients underlying increased cambial cell division are now starting to emerge. Recently, Fujii et al. (2012) succeeded in inducing cell proliferation of cambium and enlargement of secondary xylem region by *AtcycD* overexpression in tobacco plants, and a checkpoint kinase, *WEE1*, has been shown to protect cells against premature vascular differentiation, by arresting cell cycle progression upon replication stress (Cools et al. 2011). It would be interesting to connect the observations of hormonal cambial cell division control with the downstream cell cycle molecular events. A profusion of transcriptional regulators of xylem differentiation has been discovered recently, but there is a hiatus between hormone signaling and this transcriptional regulation of secondary cell wall deposition and cell death in xylem. Another missing link is the transition from primary to secondary growth and what part hormones play in triggering these events, which define tree growth. In the future, it will be challenging to use this knowledge for breeding purposes, by selecting candidate genes for transgenic manipulation or selecting trees with increased/decreased expression of the genes of interest from a natural gene pool. Given the complexity of the hormone and transcriptional network involved, this task will be a challenge. The fact that xylem development is controlled by multiple hormones and downstream factors is likely an indication that, during the evolutionary history of plants, several mechanisms emerged to develop this vascular tissue that conferred plants a highly successful adaptive feature to life on land.

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