Review

Stem cell function during plant vascular development

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The plant vascular system, composed of xylem and phloem, evolved to connect plant organs and transport various molecules between them. During the post-embryonic growth, these conductive tissues constitutively form from cells that are derived from a lateral meristem, commonly called procambium and cambium. Procambium/cambium contains pluripotent stem cells and provides a microenvironment that maintains the stem cell population. Because vascular plants continue to form new tissues and organs throughout their life cycle, the formation and maintenance of stem cells are crucial for plant growth and development. In this decade, there has been considerable progress in understanding the molecular control of the organization and maintenance of stem cells in vascular plants. Noticeable advance has been made in elucidating the role of transcription factors and major plant hormones in stem cell maintenance and vascular tissue differentiation. These studies suggest the shared regulatory mechanisms among various types of plant stem cell pools. In this review, we focus on two aspects of stem cell function in the vascular cambium, cell proliferation and cell differentiation.

The EMBO Journal (2013) 32, 178–193. doi:10.1038/emboj.2012.301; Published online 20 November 2012

Subject Categories: development; plant biology

Keywords: cambium; secondary development; stem cell; vascular tissue

Introduction

A stem cell is a self-renewing cell whose progeny has a competence to differentiate into diverse ranges of specialized cell types. By classical definition, a stem cell is characterized by asymmetric cell division in which one daughter cell retains the characteristics of an undifferentiated mother cell and the other acquires a specific cell fate, which is triggered by intrinsic and extrinsic signals. Stem cells maintain their

pluripotent status in special microenvironments, called ‘stem cell niche’, together with niche cells (Spradling *et al*, 2001). Niche cells send out short-range signals that are required for stem cell maintenance. The concept of stem cell niche was first proposed in mammalian haematopoietic tissue and has been extended to various types of stem cells (Fuchs *et al*, 2004; Li and Li, 2006).

Plants continue to grow in apical and lateral directions, forming new cells and organs throughout their life cycle. Cell division that promotes these growth activities is concentrated in meristems, specialized domains that maintain actively dividing undifferentiated cells. Meristems provide a microenvironment that protects the stem cell niche, thereby acts as a central place for growth and development (reviewed by Scheres, 2007). The shoot apical meristem (SAM) and the root apical meristem (RAM), located at the apices of main and lateral shoots and roots, produce cells for lateral organ formation and tip growth (Figure 1A, B and D). The number of stem cells within SAM and RAM is tightly maintained by mechanisms that balance between cell division and cell differentiation. The SAM is divided into three different zones: the peripheral zone (PZ), the central zone (CZ) and the rib zone (RZ) (Figure 1A). In the CZ, pluripotent stem cells and the organizing centre (OC) together form a stem cell niche (Figure 1A). These stem cells continuously produce daughter cells by asymmetric cell division, and their daughter cells, subsequently displaced to the PZ, are recruited into lateral organ primordia (Tucker and Laux, 2007). In the RAM, the stem cells are present as a single cell layer that surrounds the quiescent centre (QC), and their daughter cells are committed into specific cell fates depending on their position (Figure 1B; Van den berg *et al*, 1995). In apical meristems, the OC and QC act as niche cells that maintain adjacent stem cells (Laux *et al*, 1996; Mayer *et al*, 1998; Fletcher *et al*, 1999; Schoof *et al*, 2000; Sarkar *et al*, 2007; Stahl *et al*, 2009).

In addition to stem cells in the SAM and RAM, vascular stem cells, or more commonly called procambium and cambium are formed in vascular plants (Figures 1C and 2A). This pool of stem cells continuously produces xylem and phloem, major plant vascular tissues. Plant vascular tissues provide physical strength to plant bodies and transport water, nutrients and other substances required for growth and defense. They interconnect all the plant body parts by their conductive function, from the root tip to the various organs in the shoot. Xylem is the main tissue for transporting water and solute minerals, whereas phloem is the route for distributing photosynthetic products and various signalling molecules. Vascular stem cells generate these two conductive tissues via asymmetric periclinal cell division (Eames and MacDaniels, 1947; Esau, 1965).

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Received: 28 May 2012; accepted: 16 October 2012; published online: 20 November 2012

Figure 1 Regulation of stem cells and their niches in Arabidopsis. (A–C) The organization of stem cell niche in shoot apical meristem (SAM) (A), root apical meristem (RAM) (B) and vascular cambium (C), and their WUS-CLV regulatory mechanism in each meristem. In SAM and RAM, the stem cells are adjacent to the niche cells, which maintain their pluripotency (A, B). In RAM, daughter cells of stem cells are committed into specific cell fates depending on their position (B). (D) The location of SAM and RAM is indicated in 5-day-old Arabidopsis seedling.

Gymnosperms and many dicotyledons undergo two distinct phases of growth and development (Eames and MacDaniels, 1947; Esau, 1965). The primary growth increases plant biomass mainly in the apical direction while the secondary growth does in the lateral direction. During primary growth, xylem and phloem differentiate from cells that are derived from intervening procambium. Once the primary vascular tissues are established, cambium is generated from procambium and its neighbouring cells in stems and roots where it promotes the secondary growth. Cell proliferation in the cambium is usually more active than the one in the procambium (Eames and MacDaniels, 1947; Esau, 1965). An increase in the amount of vascular tissues mediated by cambium is one of the characteristics that distinguish dicotyledons and gymnosperms from monocots. In most monocots, all the procambial cells appear to differentiate into vascular tissues during primary growth. As a result, the cambium does not form and the secondary growth does not occur in these plants (Esau, 1965). The procambium and the cambium may be considered as the same set of vascular stem cells at two developmental stages, because their basic characteristics are very similar. For instance, both procambium and cambium are the source of xylem and phloem. Furthermore, the morphology of procambial cells gradually becomes similar to cambial cells' as both become vacuolated (Esau, 1965). Currently, however, we lack an understanding of the precise developmental transition from procambium to cambium.

Cell division and differentiation in the cambium lead to the thickening of stems and roots and thereby increase the biomass. Since the secondary growth massively occurs especially in woody plants, its research has been traditionally focusing on tree species. However, the secondary growth is

Figure 2 Organization of vascular tissues in Arabidopsis root and Populus stem. (A) A schematic cross-section of Arabidopsis root showing the vascular organization during the primary development. (B, C) Cross-sections of Arabidopsis root (B) and Populus stem (C) during the secondary development.

Figure 3 The initiation and formation of procambium cells is regulated by the auxin-mediated positive feedback loop. (A) Schemes of longitudinal median sections during early embryogenesis in Arabidopsis. (B) The polar localization of PIN1 and the expression pattern of MP during early Arabidopsis embryogenesis. (C) The auxin-mediated regulatory loop controlling the vascular initiation during leaf vein growth.

also observed in many herbaceous plants, and it results in the tissue organization very similar to that in tree species (Figure 2B and C). With several advantages such as genomic resources, Arabidopsis has emerged as a useful model for investigating the secondary growth. In particular, Arabidopsis root is an excellent model system for studying vascular development in the primary and the secondary growth because it is simpler and more predictable than the vascular development in other organs (Mahonen *et al*, 2000, 2006a; Matsumoto-Kitano *et al*, 2008; Carlsbecker *et al*, 2010).

With the advancement in genomics and other molecular tools, our knowledge regarding the vascular development, such as the formation of vascular stem cells and subsequent differentiation into xylem and phloem, has rapidly expanded in recent years. Among them, significant progress has been made in studies on the role of phytohormones during the vascular development. Several phytohormones, such as auxin, cytokinin, gibberellins (GAs), ethylene and brassinosteroids, have been shown to regulate various aspects of vascular morphogenesis (Tuominen *et al*, 1997; Scarpella *et al*, 2006; Nilsson *et al*, 2008; Matsumoto-Kitano *et al*, 2008; Ibañez *et al*, 2009; Mauriat and Moritz, 2009). Recent molecular genetic studies in Arabidopsis and Populus and cellular studies with Zinnia xylogenic cell culture have started to reveal the molecular mechanisms underlying phytohormone action in vascular development (Ohashi-Ito *et al*, 2002; Schrader *et al*, 2004b; Mahonen *et al*, 2006a; Scarpella *et al*, 2006). In addition, several transcription factors involved in the vascular stem cell maintenance have been identified. These findings suggest

the existence of conserved regulatory programs among different plant stem cell pools (reviewed by Sablowski, 2011).

Vascular stem cells: initiation and primary development

The initiation of vascular stem cells during embryogenesis is well characterized in Arabidopsis. At the early globular stage of Arabidopsis embryos, cells inside the protoderm divide into distinct layers, the ground tissue precursors and vascular stem cell initials (procambium) (Figure 3A; Mansfield and Briarty, 1991; Lau *et al*, 2010). At the late globular stage, four procambial cells divide periclinally, giving rise to the pericycle and vascular stem cells. During the late globular, heart and torpedo stages, the number of cell files in the procambium continues to increase by further periclinal cell divisions resulting in the radial vascular pattern identical to that of a post-embryonic primary root (Scheres *et al*, 1994; Mahonen *et al*, 2000).

Simultaneously, a network of vascular stem cells arises in cotyledons as well. Although a continuous procambial tissue extends in an apical-basal axis without exhibiting any feature of differentiated vascular elements in a mature embryo, several vascular tissue-specific markers are reported to be expressed at this developmental stage, indicating that vascular cell fate decisions are already made during embryogenesis (Bonke *et al*, 2003; Mahonen *et al*, 2006a).

Over the years numerous genetic studies have revealed several genes involved in the radial patterning during

Table I List of genes that regulate vascular stem cells

Gene Name	Description	Function	References
RECEPTOR-LIKE PROTEIN KINASE1 (RPK1)	LRR-receptor kinase	determination of procambium position in Arabidopsis embryo	Nodine <i>et al</i> , 2007
TOADSTOOL2 (TOAD2)	LRR-receptor kinase	determination of procambium position in Arabidopsis embryo	Nodine <i>et al</i> , 2007
MONOPTEROS (MP)	auxin responsive transcription factor	auxin response, vascular stem cell initiation	Hardtke & Berleth, 1998; Scarpella <i>et al</i> , 2006; Donner <i>et al</i> , 2009
PIN-FORMED1 (PIN1)	auxin efflux carrier	polar auxin transport, vascular stem cell initiation	Hardtke & Berleth, 1998; Scarpella <i>et al</i> , 2006; Donner <i>et al</i> , 2009
ARABIDOPSIS THALIANA HOMEBOX 8 (ATHB8)	class III homeodomain-leucine zipper	xylem formation, targeted by microRNA165/166	Baima <i>et al</i> , 2001; Carlsbecker <i>et al</i> , 2010; Donner <i>et al</i> , 2009; Hardtke & Berleth, 1998; Scarpella <i>et al</i> , 2006;
SCARFACE (SFC)	ARF-GAP protein	PIN protein recycling; affects vein growth	Deyholos <i>et al</i> , 2000; Koizumi <i>et al</i> , 2005; Sieburth <i>et al</i> , 2006
Xylogen (XYP1/2)	proteoglycan-like protein	affects the continuity of xylem networks	Motose <i>et al</i> , 2004
OCTOPUS (OPS)	membrane localized protein	affects the continuity of phloem networks	Truernit <i>et al</i> , 2012
PHABULOSA (PHB)	class III homeodomain-leucine zipper	xylem formation, targeted by microRNA165/166	Carlsbecker <i>et al</i> , 2010
PHAVOLUTA (PHV)	class III homeodomain-leucine zipper	xylem formation, targeted by microRNA165/166	Carlsbecker <i>et al</i> , 2010
REVOLUTA (REV)	class III homeodomain-leucine zipper	formation of interfascicular fibers, xylem formation, targeted by microRNA165/166	Carlsbecker <i>et al</i> , 2010
CORONA (CNA)	class III homeodomain-leucine zipper	xylem formation, targeted by microRNA165/166	Carlsbecker <i>et al</i> , 2010
KANADI (KAN)	GARP family protein, putative transcription factor	antagonistic role to class III homeodomain-leucine zipper	Emery <i>et al</i> , 2003; Ilegems <i>et al</i> , 2010
SHORT ROOT (SHR)	GRAS type transcription factor	endodermis formation, regulatin microRNA165/166 in root	Carlsbecker <i>et al</i> , 2010
SCARECROW (SCR)	GRAS type transcription factor	endodermis formation, regulatin microRNA165/166 in root	Carlsbecker <i>et al</i> , 2010
VASCULAR-RELATED NAC-DOMAIN 6/7	NAC transcription factor	vessel formation	Kubo <i>et al</i> , 2005
ALTERED PHLOEM DEVELOPMENT (APL)	MYB coiled-coil-type transcription factor	phloem differentiation	Bonke <i>et al</i> , 2003
HIGH CAMBIAL ACTIVITY 2 (HCA2)	Dof type transcription factor	cell proliferation and phloem formation in cambium	Guo <i>et al</i> , 2009
Pta LBD1	LBD/ASL family protein	cell proliferation in cambium	Yordanov <i>et al</i> , 2010
ARK1/2	class I KNOTTED1-like homeobox (KNOX) transcription factor	cell proliferation in cambium	Du <i>et al</i> , 2009; Groover <i>et al</i> , 2006
WUSCHEL-like HOMEBOX 4 (WOX4)	homeobox transcription factor	stem cell maintenance in cambium	Hirakawa <i>et al</i> , 2010b; Suer <i>et al</i> , 2011
CLAVATA3/ESR-related 41/44 (CLE41/44)	peptide ligand	stem cell maintenance in cambium	Hirakawa <i>et al</i> , 2008; Whitford <i>et al</i> , 2008
PHLOEM INTERCALATED WITH XYLEM (PXY)/TDIF RECEPTOR (TDR)	LRR-receptor kinase	stem cell maintenance in cambium	Fisher & Turner, 2007; Hirakawa <i>et al</i> , 2008
MORE LATERAL GROWTH 1 (MOL1)	LRR-receptor kinase	regulating cell proliferation in interfascicular cambium	Agusti <i>et al</i> , 2011b
REDUCED IN LATERAL GROWTH 1 (RUL1)	LRR-receptor kinase	regulating cell proliferation in interfascicular cambium	Agusti <i>et al</i> , 2011b
CYTOKININ RESPONSE 1 (CRE1)/WOODEN LEG (WOL)/ARABIDOPSIS HISTIDINE KINASE (AHK)	Histidine kinase, cytokinin receptor	cytokinin receptor, stem cell maintenance in procambium/cambium by inhibiting xylem differentiation	Mahonen <i>et al</i> , 2000
AHP6	a pseudo-phosphotransfer protein	cytokinin signalling, protoxylem specification	Mahonen <i>et al</i> , 2006a
ARR1, 10, and 12	Type B ARRs	cytokinin signaling, inhibit the differentiation of procambium into xylem	Argyros <i>et al</i> , 2008; Hutchison <i>et al</i> , 2006; Ishida <i>et al</i> , 2008; Yokoyama <i>et al</i> , 2007
ISOPENTENYLTRANSFERASE (IPT)	ISOPENTENYLTRANSFERASE	cytokinin biosynthesis, promote cambium activity	Matsumoto-Kitano <i>et al</i> , 2008
BRI1, BRL1-3	LRR-receptor kinase	brassinosteroid receptor, promote xylem development	Cano-Delgado <i>et al</i> , 2004; Nakamura <i>et al</i> , 2006; Yamamoto <i>et al</i> , 2001; Yamamoto <i>et al</i> , 2007
GA 20-oxidase	GIBBERELLIN 3-OXIDASE	gibberellin biosynthesis, promote a cambium activity and the formation of xylem fibers	Israelsson <i>et al</i> , 2005
GA 3-oxidase	GIBBERELLIN 20-OXIDASE	gibberellin biosynthesis, promote a cambium activity and the formation of xylem fibers	Israelsson <i>et al</i> , 2005
JASMONATE ZIM-DOMAIN (JAZ) 7 and 10	jasmonate ZIM-domain protein	jasmonates signaling, jasmonates receptor, repress cambial activities by inhibiting MYC2	Chini <i>et al</i> , 2007; Sehr <i>et al</i> , 2010; Thines <i>et al</i> , 2007
CORONATINE INSENSITIVE1 (COI1)	F-box protein	jasmonates signaling, jasmonates receptor	Chini <i>et al</i> , 2007; Sehr <i>et al</i> , 2010; Thines <i>et al</i> , 2007
MYC2	MYC related transcription factor	induced by jasmonates, promotes cambial activities	Chini <i>et al</i> , 2007; Sehr <i>et al</i> , 2010; Thines <i>et al</i> , 2007
ERF104	ETHYLENE RESPONSE FACTOR	promote interfascicular cambium initiation	Bethke <i>et al</i> , 2009

Arabidopsis embryogenesis (reviewed by Jenik *et al*, 2007 and Lau *et al*, 2012). Among them, a pair of LRR receptor-like kinases, *RECEPTOR-LIKE PROTEIN KINASE1* (*RPK1*) and *TOADSTOOL2* (*TOAD2*), seems to be involved in the determination of the procambium position during early embryogenesis (Table I; Nodine *et al*, 2007). Both of these genes start to be expressed in the early globular stage. Embryo development in the *rpk1 toad2* double mutant is arrested at the late globular stage. Interestingly, in the *rpk1 toad2* embryo, the expression of procambial marker genes expands beyond procambium cells into ground tissue initials and protoderm, while the expression of markers for protoderm and ground tissue is severely attenuated. This indicates that these two LRR receptor-like kinases regulate

positioning and patterning of the early procambium in the embryo (Nodine *et al*, 2007).

The importance of auxin in vascular stem cell initiation has been demonstrated in several studies using Arabidopsis as a model system (Table I; reviewed by Reinhardt, 2003). An auxin responsive transcription factor, MONOPTEROS (MP)/AUXIN RESPONSE FACTOR5 (ARF5), plays a critical role in the specification of vascular stem cells (Hardtke and Berleth, 1998). During early embryogenesis, MP is expressed in the procambial cells and its expression is rapidly upregulated by auxin (Figure 3B). In *mp* knockout mutant embryos, procambium completely fails to form and weak alleles display irregular vascular development (Berleth and Jurgens, 1993; Hardtke and Berleth, 1998). Such a defect seems to be related

to the lack of polar auxin transport during embryogenesis. During early embryogenesis, PIN-FORMED1 (PIN1) proteins, major auxin efflux carriers, are polarly localized in the inner cells of pro-embryo before they turn into procambial cells (Figure 3B; Friml, 2003). *PIN1* expression is dramatically reduced in a loss-of-function *mp* mutant, suggesting that MP might regulate its transcription (Wenzel *et al*, 2007).

In early stages of leaf development, preprocambial cells, from a sub-epidermal layer of leaf primordia, are organized into continuous strands to form veins, networks of vascular tissues (Eames and MacDaniels, 1947). Unlike stem cells in the SAM or RAM, vascular stem cells in developing leaves are constantly formed to produce vein networks. Therefore, the proper vein growth requires the same molecular processes involved in the vascular stem cell formation in an iterative manner. One key question in the vein growth is how procambial cells signal from one to the other to form continuous vascular strands. The auxin-canalization hypothesis has been proposed to explain this (Sachs, 1981; Rolland-Lagan and Prusinkiewicz, 2005). According to this hypothesis, the vein growth is promoted via a positive feedback regulation of auxin transport. More specifically, the auxin maxima established in procambial cells enhance the machinery that transports auxin to adjacent cells in a polar manner. The adjacent cells that subsequently perceive a high level of auxin turn into procambium and repeat the same process to the next neighbours.

The auxin canalization theory was supported by the findings of the auxin efflux carrier protein, PIN1. In *pin1* mutant stems, the arrangement of vascular bundles is abnormal, similar to when the auxin transport inhibitor is applied (Gälweiler *et al*, 1998). *PIN1* expression was further characterized in developing leaves (Scarpella *et al*, 2006). This study showed that *PIN1* starts to be expressed in the vascular stem cells before they become morphologically discernible. Then, expression domains of *PIN1* expanded to the future veins as leaf primordia emerged and grew. When the veins were about to bifurcate, PIN1 localization became bipolar in the bifurcating cell. These suggest that polar auxin transport is vital for the proper organization of vascular stem cells.

The auxin canalization theory proposes that the positive feedback regulation of auxin transport is required for the auxin maxima to be established in the preprocambial cells of growing veins. Recent genetic studies revealed the molecular basis of such regulation (reviewed by Scarpella *et al*, 2010). One of the earliest expressing transcriptional regulators in the preprocambial cells is the *ARABIDOPSIS THALIANA* *HOMEODOMAIN* *ATHB8*, a class III homeo-domain leucine zipper (HD-ZIP III) transcription factor family gene (Table I; Donner *et al*, 2009). It turned out that the transcription of *ATHB8* is activated directly by MP. *ATHB8* subsequently directs the formation of preprocambial cells and induces the expression of *PIN1* (Figure 3C; Scarpella *et al*, 2006). These processes establish a positive feedback loop of auxin-MP-*ATHB8*-PIN1 in the initiation and specification of vascular stem cells. When this positive feedback regulation was disrupted in the *athb8* mutant, the expression domain of a preprocambium marker (J1721) expanded. This supports the importance of positive feedback regulation for spatially restricting preprocambial cell specification (Donner *et al*, 2009).

PIN proteins cycle between plasma membranes and endosomes via the activity of ARF-GEF protein, GNOM (Geldner *et al*, 2003). The importance of polar auxin transport in vein growth and patterning was further evident from mutations in components of PIN localization machinery. For example, *scarface* (*sfc*)/*van3* generates dense but fragmented vein islands on leaves and cotyledons. *SFC* encodes an ADP ribosylation factor GTPase activating protein (ARF-GAP), a modulator of ARF-GEF, involved in the vesicle trafficking (Deyholos *et al*, 2000; Koizumi *et al*, 2005; Sieburth *et al*, 2006). In the *sfc*, intracellular trafficking of PIN1 was abnormal, suggesting that SFC might affect the endosomal cycling of PIN1 (Sieburth *et al*, 2006). Other factors involved in vascular continuity have also been reported (Table I). Among them, Xylogen, a large proteoglycan-like protein (Motosé *et al*, 2004), is polarly localized in the cell walls of differentiating tracheary elements to function as an intercellular signal molecule. The Arabidopsis genome contains two genes encoding Xylogen, *AtXYP1* and *AtXYP2*. When these two are knocked out xylem strands develop in a discontinuous manner (Motosé *et al*, 2004). OCTOPUS, a polarly localized membrane-associated protein in the phloem, has been reported to regulate phloem continuity in Arabidopsis (Truernit *et al*, 2012).

Besides *ATHB8*, the Arabidopsis genome contains four other *HD-ZIP III* genes *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA/INTERFASCICULAR* *FIBERLESS1* (*REV/IFL1*), *CORONA* (*CNA*)/*ATHB15* (Table I) (Talbert *et al*, 1995; Baima *et al*, 2001; McConnell *et al*, 2001; Green *et al*, 2005; Prigge *et al*, 2005). All of them are expressed in vascular tissues in somewhat overlapping fashion, suggesting a likely redundancy in their function (Prigge *et al*, 2005; Carlsbecker *et al*, 2010). A quadruple knockout mutant for *ATHB8*, *CNA*, *PHB* and *PHV* was reported to exhibit enhanced cell proliferation in procambium (Carlsbecker *et al*, 2010). Furthermore, a quintuple mutant, *athb8-11 cna-2 phb-13 phv-11 rev-6*, completely lacked xylem, highlighting their involvement in the vascular cell type specification (Carlsbecker *et al*, 2010). The *KANADI* (*KAN*) genes, a subclass of GARP family transcription factors, have been shown to play an antagonistic role to *HD-ZIP III* genes in dorsoventral patterning of lateral organs (Emery *et al*, 2003). In the vascular tissue of a quadruple loss-of-function *KANADI* mutant (*kan1 kan2 kan3 kan4*), the cell proliferation in procambium was strikingly enhanced (Ilegems *et al*, 2010). On the contrary, ectopic expression of *KAN1* under the *CNA/ATHB15* promoter suppressed *PIN1* expression in procambium during embryogenesis and thereby repressed the vascular stem cell initiation (Ilegems *et al*, 2010). This finding indicates that *KAN* genes might antagonize *HD-ZIP III* genes through the modulation of *PIN1* expression. Interestingly, *KAN* genes are expressed in the phloem, thus they might control vascular stem cell development in a non-cell autonomous manner (Emery *et al*, 2003). In summary, a positive feedback loop of auxin-MP-PIN1 mediated by *HD-ZIP III* and *KAN* genes primarily controls the initiation and specification of vascular stem cells.

Establishment of vascular cambium during the secondary development

The cambium or vascular cambium represents a pool of stem cells arranged in radial series and gives rise to xylem and

Figure 4 Schematic illustration of the primary and secondary stem anatomy in Arabidopsis. The primary stem exhibits disconnected vascular bundles with procambium. In the secondary developmental phase, this procambium turns into a fascicular cambium and the cells between bundles become an interfascicular cambium. Fascicular and interfascicular cambia interconnect to each other and establish a cambium in a circular form.

phloem cells during the secondary phase of plant development (Figures 2 and 4; Eames and MacDaniels, 1947; Esau, 1965). In stems, the procambium derived from apical meristem usually resides within the bundles (Eames and MacDaniels, 1947). During the secondary growth phase, the procambial cells become the ‘fascicular cambium’, the cambium derived from a vascular bundle of primary development (Figure 4). The clusters of fascicular cambium become interconnected by the ‘interfascicular cambium’, which is formed from parenchyma cells between the vascular bundles (Eames and MacDaniels, 1947), thereby completing the radial arrangement of cambium (Figure 4). The development of xylem in Arabidopsis can be divided into two distinct phases, based on which cell types differentiate from cambial cells. In an early phase, only vessel elements differentiate, and the rest of the xylem cells remain as parenchyma. At a later phase, both vessel elements and lignified fibre cells are produced from cambial cells, resulting in a stem anatomy comparable to wood of an angiosperm tree (Chaffey *et al*, 2002). Although numerous histological analyses in several plant species have revealed the dynamic morphological changes during the secondary development (Esau, 1965; Eames and MacDaniels, 1947), little is known about the molecular mechanisms underlying these processes. A loss-of-function mutation in *REV* leads to the disrupted development of xylem fibres and vessel elements in Arabidopsis, indicating a role for *REV* during the secondary phase of vascular development (Zhong and Ye, 1999).

The vascular cambium enlarges its circumference through anticlinal cell divisions (occur perpendicular to an adjacent cell layer), while it continues to undergo periclinal cell divisions (occur parallel to an adjacent cell layer) to produce mother cells for xylem and phloem (Figure 4). The vascular cambium contains two types of cells: elongated cells with tapering ends, the fusiform initials (spindle-shaped initials), and the ray initials, which are nearly isodiametric and

relatively small in size. The fusiform initials upon differentiation give rise to xylem and phloem, while the ray initials give origin to the radial strands of cubical cells that play a role in nutrient transport and storage (Vanbel, 1990).

Just before the formation of cambium, there is a transitional period during which cell divisions occur in multiple planes in the procambial cells (Eames and MacDaniels, 1947). In Arabidopsis root, periclinal cell divisions first take place in the procambium during the transitional period. This occurs even before the primary vessel members complete their differentiation (Baum *et al*, 2002). This along with sequential periclinal divisions in the procambial derivatives lead to an oblong shape stele with constrictions at xylem poles (Baum *et al*, 2002). The periclinal cell divisions in the pericycle adjacent to the xylem pole extend the pericycle away from xylem poles and produce cells that eventually form a continuous cylinder of cambium cells (Baum *et al*, 2002). The pericycle cells then keep dividing periclinal to form cork cambium, the second layer of lateral meristem that produces the protective outer layer or cork (Baum *et al*, 2002). During this process, the epidermis and ground tissue are gradually stretched and peeled off from the expanding root.

As described above, a *stem cell niche* is a cellular micro-environment providing intercellular signals that maintain pluripotency of stem cells. Several recent studies have revealed the molecular regulatory mechanisms underlying stem cell maintenance in SAM and RAM (Figure 1A and B; Sablowski, 2011). Even though substantial progress has been made in understanding stem cell functions in apical meristems, our knowledge about vascular stem cells and their niche in lateral meristem is still limited. The current opinion holds cambium includes undifferentiated ‘initial cells’ (stem cells), with the first committed derivatives on each side being phloem and xylem mother cells (Esau, 1965). Although the existence of pluripotent stem cells in cambium

is generally acknowledged, the spatial distribution and identity of niche cells in cambial zone is still unclear. The initial cells in cambium are the only cells in cambial zone that can give rise to both phloem and xylem by periclinal divisions and produce new cell files by anticlinal divisions. It has been shown that anticlinal cell divisions in cambial zone are concentrated in a few cell layers in the vascular cambium in *Populus* (Schrader *et al*, 2004b). This implies that the identity of initial cells might be restricted to these cell layers in the cambial zone. The subdivision of cambium into initial cells and mother cells has mainly been a conceptual one. This is largely due to the failure in distinguishing these two cell types under a microscope as they are anatomically more or less uniform. Furthermore, there are no molecular markers currently available that can define the initial cells in the cambial zone.

In order to establish the molecular tools, which mark a stem cell population in cambial zone, a high-resolution transcription profile analysis was performed in poplar cambium using tangential thin sections. This study provides evidence that anatomically indistinguishable cells in the cambial zone actually differ in their transcription profiles (Schrader *et al*, 2004b). Interestingly, several homologues of known SAM regulators showed cambium-specific expression patterns (*PttCLV1*, *PttANT* and *PttKNOXs*), suggesting the existence of similar regulatory networks in the poplar cambium and SAM. Although this transcription map provided molecular support for a compartmentalization of the cambial zone and identified several distinct layers in the poplar cambium, the resolution is not high enough to differentiate molecular events at the level of a single cell layer (Birnbaum *et al*, 2003; Brady *et al*, 2007). However, these data serve as a rich source for developing new molecular cambium markers.

Transcriptional and post-transcriptional regulations in the specification and proliferation of vascular tissues

Soon after the germination, xylem and phloem strands differentiate in the defining domains in an organ-specific manner. In *Arabidopsis* post-embryonic roots, 4–5 cell files of xylem vessels develop in the central axis, and the two poles of phloem develop perpendicular to the xylem axis (Figure 2A; Scheres *et al*, 1994; Mahonen *et al*, 2000). Two types of xylem vessels develop in the root. Protoxylem strands with lignins deposited in a spiral pattern develop in the periphery of xylem axis and metaxylem strands with lignins in a reticulate pattern develop in the centre (Figure 2A). Between xylem and phloem, vascular stem cells maintain their undifferentiated state and later give rise to cambium at the secondary development stage (Figure 2A and B) (reviewed by Elo *et al*, 2009). As mentioned above, all the *HD-ZIP III* genes are expressed in *Arabidopsis* vascular tissues. In *Zinnia* mesophyll cell culture, the expression of *HD-ZIP III* genes (*ZeHB-10*, *-11* and *-12*) is induced during xylogenesis (Demura *et al*, 2002; Ohashi-Ito *et al*, 2002; Ohashi-Ito and Fukuda, 2003), suggesting their involvement in xylem differentiation. In *Arabidopsis* root, a loss of all the five *HD-ZIP III*s results in the failure of xylem vessel development (Carlsbecker *et al*, 2010). On the contrary, over-

expression of *AHTB8* causes enhanced xylem differentiation (Baima *et al*, 2001), indicating that *HD-ZIP III*s are *de novo* regulators of the xylem vessel formation.

Levels of *HD-ZIP III* transcription factors are post-transcriptionally controlled by *microRNA 165/6* (Emery *et al*, 2003; Mallory *et al*, 2004). Recently, it was discovered that this *microRNA*-mediated post-transcriptional regulation is a critical process in specifying and patterning proto- and metaxylem vessels in roots (Figure 5; Table I; Carlsbecker *et al*, 2010; Miyashima *et al*, 2011). *HD-ZIP III*s specify these two xylem vessel types in a dosage-dependent manner. A high level of *HD-ZIP III*s specifies metaxylem and a low level does protoxylem. For xylem vessels pattern properly in the root, *HD-ZIP III*s should be differentially distributed in a way that their levels are high in the centre of xylem axis but low in the periphery. Such a differential distribution of *HD-ZIP III*s in the xylem precursors turned out to be regulated by *SHORT ROOT* (*SHR*) and *SCARECROW* (*SCR*), two GRAS family transcription factors, in a non cell-autonomous manner. *SHR* proteins are generated in the vascular cylinder and then move into the endodermis layer and QC where they activate *SCR* (Cui *et al*, 2007; Nakajima *et al*, 2001). In the endodermis, it was found that *SHR* and *SCR* together activate two genes that encode *microRNA 165/6*. When either *SHR* or *SCR* was knocked out, all the xylem strands were specified and differentiated into metaxylem. Conversely, *microRNA 165/6* expressed in the ground tissue layer in *shr* or *scr* mutant was able to recover the gradient distribution of *HD-ZIP III*s and wild-type xylem patterning. These results suggest that *microRNA 165/6* acts as mobile signals that establish and facilitate the dosage-dependent regulation of *HD-ZIP III*s. Further supporting the role of *HD-ZIP III*s in determining xylem cell types, the gain-of-function mutants *phb-d* (*phb-1d* and *-7d*), in which the *microRNA 165/6* target site is disrupted, exhibited ectopic metaxylem vessel formation at the position where protoxylem would normally differentiate (Carlsbecker *et al*, 2010).

A study in *Populus* also supports that *HD-ZIP III* transcription factors are key regulators of vascular stem cell proliferation and xylem formation. When a *microRNA*-resistant *Populus REVOLUTA* was ectopically expressed, ectopic cambium formed in cortex layers (Robischon *et al*, 2011). In addition, these transgenic plants formed xylem towards the stem periphery, further highlighting the importance of relative levels and spatial distribution of *HD-ZIP III*s in vascular tissue patterning.

NAC domain transcription factors VASCULAR-RELATED NAC-DOMAIN6 (*VND6*) and *VND7* also play an essential role in xylem vessel differentiation in both *Arabidopsis* and *Populus* (Table I). It has been first reported in *Arabidopsis* that *VND6* promotes the differentiation of metaxylem and *VND7* induces protoxylem fate (Kubo *et al*, 2005). Further analyses including ectopic expression studies in leaves demonstrated that *VND6* and *VND7* respectively act as central regulators of metaxylem and protoxylem vessel fates (reviewed by Ohashi-Ito and Fukuda, 2010).

In summary, pathways that integrate transcriptional and post-transcriptional regulation and cell-to-cell communication govern the xylem specification and patterning. Furthermore, factors involved in the xylem formation are evolutionally conserved among various vascular plants (Floyd and Bowman, 2004; Prigge and Clark, 2006; Floyd and Bowman, 2007), raising the possibility that afore-

Figure 5 Cell type patterning in Arabidopsis roots is controlled by the bidirectional cross-talk mediated by SHR and *microRNA* 165/166 that are mobile between cells. SHR, generated in the stele, travels to the endodermis and activates the expression of *microRNA* 165/6. *MicroRNA* 165/6 from the endodermis travels to the vascular cylinder and degrades mRNAs of HD-ZIP III transcription factors in the endodermis and periphery of vascular cylinder, resulting in a graded distribution of HD-ZIP IIIs. A high level of HD-ZIP IIIs specifies metaxylem and a low level specifies protoxylem.

mentioned xylem regulatory programs might have arisen in early lineages of vascular plants.

Unlike xylem development, our knowledge about the molecular control of phloem formation is still relatively poor. An MYB transcription factor, ALTERED PHLOEM DEVELOPMENT (APL), which is expressed in developing phloem cells, has been identified as a key regulator of phloem development. Knocking out *APL* gene results in the absence of phloem sieve elements and companion cells (Table I; Bonke *et al*, 2003). In addition, xylem vessel-like cells are formed where phloem cells normally develop. On the contrary, ectopic expression of *APL* under the control of *WOL* promoter inhibits xylem development, suggesting that *APL* possesses a dual role: one is to promote phloem differentiation and the other is to repress xylem differentiation during root vascular development (Bonke *et al*, 2003).

Recently, two other transcription factors that also affect vascular stem cell activities and phloem formation were identified. HCA2 (HIGH CAMBIAL ACTIVITY2) was isolated among activation tagging lines in Arabidopsis (Table I; Guo *et al*, 2009). This activation tagging line promoted cambium formation and phloem proliferation. The phenotype was found to be due to ectopic expression of *At5g62940*, a gene that encodes a Dof family transcription factor. *HCA2* is highly expressed in the cambium, phloem and interfascicular parenchyma in wild-type background, suggesting that it might promotes the formation of cambium and asymmetric cell division that forms phloem cells at the very early stage. This idea was further supported by the finding that the ectopic expression of *HCA2* upregulates phloem and cambium marker genes, but downregulates xylem marker genes. *HCA2* seems to function mainly as a transcriptional activator to promote the formation of interfascicular cambium. When plants were introduced with modified *HCA2*, which was fused with EAR-motif repressor domain, they exhibited phenotypes opposite to the ectopic expression lines. In these transgenic plants, the formation of interfascicular cambium was

significantly inhibited; however, no major effect on the phloem development was observed.

Another transcription factor that is actively involved in the cambial activities is a *Populus* homologue of LBD1 (Pta LBD1). Pta LBD1 promotes the lateral stem growth, by increasing phloem proliferation (Yordanov *et al*, 2010). In parallel, Pta LBD1 was found to repress two important cambium regulators, *ARBORKNOX1* (ARK1) and *ARK2*, class I KNOTTED1-like homeobox (KNOX) transcription factors. *ARK1* is an orthologue of *SHOOT MERISTEMLESS* (*STM*) and *ARK2* is an orthologue of *BREVIPEDICELLUS* (*BP*) in Arabidopsis. These two proteins were also suggested to promote vascular stem cell maintenance in *Populus* (Groover *et al*, 2006; Du *et al*, 2009). The repression of *ARK2* by Pta LBD1 is reminiscent of the repression of *BP* by *ASYMMETRIC LEAVES2* (*AS2*), an LOB domain transcription factor, in Arabidopsis. In Arabidopsis, *AS2* (Iwakawa *et al*, 2002) and *AS1* (Byrne *et al*, 2000) form a complex and repress *BP* expression (Guo *et al*, 2008). These again suggest the existence of conserved regulatory programs between Arabidopsis and *Populus*.

Peptide-receptor signalling pathways in the specification and proliferation of vascular tissues

Stem cell populations are maintained by balancing cell proliferation and differentiation. A key mechanism in the plant stem cell maintenance is the signalling contributed by the interaction between small mobile peptides and receptor like kinases (Sablowski, 2011). In the SAM, dodecapeptide CLAVATA3 (*CLV3*) (Fletcher *et al*, 1999; Kondo *et al*, 2011) diffuses from the dividing stem cells to the underlying cell layer and interacts with *CLV1* (Clark *et al*, 1997; Ogawa *et al*, 2008), a Leucine-Rich Repeat (LRR) Receptor-Like Kinase (RLK), or a receptor complex composed of *CLV2*, a receptor-like protein, and *CORYNE*, a membrane localized Serine/

Threonine kinase (Figure 1A; Jeong *et al*, 1999; Muller *et al*, 2008). A signal produced by this interaction represses the expression of WUSCHEL (WUS), a homeobox transcription factor that specifies niche cells and maintains the stem cell population through cell-to-cell movement (Mayer *et al*, 1998; Yadav *et al*, 2011). Recently a similar regulatory program was also identified in the RAM (Figure 1B; Sarkar *et al*, 2007; De Smet *et al*, 2008). Analogous to SAM, this signalling module also comprises a small peptide ligand, CLE40 (CLV3 homologue) and an LRR-RLK protein ARABIDOPSIS CRINKLY4 (ACR4) (Stahl *et al*, 2009). CLE40 regulates distal stem cell differentiation in a dose-dependent manner with high concentrations promoting differentiation. Like WUS in the SAM, WOX5 acts in the QC to maintain the stem cell pool while the CLE40-ACR4 ligand-receptor complex restricts its function and triggers distal stem cell differentiation.

Like in the SAM and RAM, the interaction between CLEs and RLKs controls vascular stem cells (Figure 1C; Table I; Hirakawa *et al*, 2010a). In a study using the xylogenic culture system of *Zinnia mesophyll* cells, CLV3 promoted the differentiation of tracheary elements (or xylem vessels) whereas another peptide, named as TDIF (Tracheary Element Differentiation Inhibitory Factor), which is identical to CLE41/44 in Arabidopsis, inhibited this process (Ito *et al*, 2006). Consistent with the finding in *Zinnia*, overexpressing CLE41 or 44 in Arabidopsis also inhibited the differentiation of tracheary elements and resulted in the discontinuous xylem strand formation. Furthermore, it promoted division of vascular stem cells in the hypocotyl (Hirakawa *et al*, 2008; Whitford *et al*, 2008). Therefore, CLE41 and 44 have two roles: one is to act as positive signals for cell proliferation and the other is to act as negative signals for xylem differentiation. These signals were found to be perceived via the interaction between CLE41/44 and PXY (PHLOEM INTERCALATED WITH XYLEM), also named as TDR (TDIF RECEPTOR) (Figure 1C; Hirakawa *et al*, 2008). PXY was originally identified from an Arabidopsis mutant with an aberrant organization of xylem and phloem in the stem (Fisher and Turner, 2007). In the absence of PXY/TDR, plants became insensitive to high levels of CLE41/44 in the cell proliferation and xylem differentiation.

Similarly to the CLV3-CLV1 regulation in the SAM, CLE41/44 and PXY/TDR are expressed in different cell types that are adjacent to each other. CLE41 and 44 are produced in the phloem and diffuse towards the vascular stem cells where PXY/TDR is expressed. Further investigation suggests that the spatial restriction of CLE41/44 in phloem cells is important for proper vascular tissue patterning in the stem (Figure 1C; Etchells and Turner, 2010). When CLE41 was expressed ubiquitously or on the xylem side, the organization of vascular tissues in the hypocotyl was perturbed dramatically and formed mixed populations of xylem and phloem. Furthermore, ectopic expression of CLE41 suppressed the PXY/TDR expression. When such a negative feedback regulation was completely broken by the ectopic expression of both PXY and CLE41, a dramatic increase in the size of vascular stem cell population was observed. This suggests that the vascular stem cell population is maintained by the negative feedback regulation of PXY/TDR, which is triggered by the interaction between CLE41/44 and PXY/TDR.

An interaction between CLE41/44/TDIF and PXY/TDR induces the expression of WOX4, a homologue of WUS, in

vascular stem cells (Hirakawa *et al*, 2010b; Ji *et al*, 2010). WOX4 expression was rapidly induced when a high dose of TDIF was treated to Arabidopsis. However, such an induction failed to happen in the absence of PXY/TDR. This positive regulation of WOX4 by TDIF-PXY/TDR complex is opposite to what CLV3-CLV1 complex does to WUS. Further investigation suggested that although WOX4 regulates the proliferation of vascular stem cells, it does not influence the differentiation of tracheary elements in response to TDIF-TDR interaction. In *wox4* mutant, exogenously applied TDIF still caused the intermittent xylem strand formation, but did not promote vascular stem cell proliferation any more. When *wox4* mutant plants were grown for a long term, the secondary growth of hypocotyls was significantly reduced, suggesting that WOX4 is required to maintain the cell proliferation activity of vascular stem cells (Hirakawa *et al*, 2010b). In addition, a recent study has reported the positive effect of auxin on WOX4 expression in the cambium, suggesting that various inputs integrated into WOX4 are controlling the cambium activity (Suer *et al*, 2011).

TDIF-TDR-WOX4 pathway seems to be an evolutionarily conserved regulatory program. Transcription profiling in the *Populus* cambial zone suggested that *Ptt-HB3*, a homologue of WOX4, and *Ptt-RLK3*, a homologue of CLV1, are highly expressed in the vascular stem cells (Schrader *et al*, 2004b). Ectopic expression of WOX4 orthologue in tomato, which has bicollateral vascular organization in stems (phloem is formed in both inside and outside the xylem), also promoted the vascular cell proliferation (Ji *et al*, 2010).

In addition to TDR/PXY, two LRR-RLKs were shown to regulate vascular stem cell activities (Agusti *et al*, 2011b). In this study, cells that are in the process of interfascicular cambium formation were isolated from Arabidopsis stem segments in a time course using the laser capture micro-dissection technique and global mRNA expression was profiled in those cells. Two genes encoding LRR-RLK, *MOL1* (*MORE LATERAL GROWTH1*) and *RUL1* (*REDUCED IN LATERAL GROWTH1*), are highly induced during the interfascicular cambium formation (Table I). As their names indicate, the cell proliferation related to interfascicular cambium activity was enhanced in the *mol1* mutant, whereas it was suppressed in *rul1*. Interestingly, the *mol1* mutations enhance the expression of WOX4 and TDR/PXY. Thus, MOL1 seems to regulate cell proliferation in the interfascicular cambium through the TDR/PXY-WOX4 pathway.

Vascular stem cell regulation by plant hormones

Auxin

Auxin plays a crucial role in the specification of vascular stem cells (procambium) as explained above and so it does in the cambium activities (Figure 7). Analysis of the auxin distribution across the cambial region in hybrid aspen trees showed a radial auxin gradient reaching a peak level in the cambial zone or at the border between the cambial zone and the expansion zone towards the xylem (Uggla *et al*, 1996; Tuominen *et al*, 1997). This auxin distribution pattern is very similar to what was found in a gymnosperm, *Pinus sylvestris*, suggesting an evolutionary conserved function of auxin in cambium activities (Uggla *et al*, 1996). When a stem is wounded, the orientation of xylem growth changes and

grain patterns are altered. It turned out that such changes correlate with changes in the auxin distribution in response to wounding, which subsequently affect the orientation of cambial initials and derived cells of secondary xylem (Kramer *et al*, 2008). Auxin function in the secondary growth was further tested in *Populus* by perturbing auxin signalling. In this study, a stabilized form of PttIAA3, which has a mutation in an amino acid that is important for the auxin-mediated degradation of IAA, was ubiquitously expressed (Moyle *et al*, 2002; Nilsson *et al*, 2008). Suppressing auxin responsiveness significantly reduced cambial cell division activities and the radial growth. Such reduction in cambial cell divisions affected the growth of xylem more significantly than the phloem. Recently, strigolactones, a carotenoid-derived group of plant hormones were shown to act downstream of auxin-dependent activation of cambium activities and secondary growth in *Arabidopsis* (Agusti *et al*, 2011a).

In trees, rates of radial stem growth change seasonally. When the day length becomes shorter than the one that permits growth, cell division stops in the cambium (dormant). Consistently, cambium recovers cell division activities if plants are exposed to long days again. Comparison of genome-wide expression profiles in the active and dormant *Populus* cambia revealed significant repression of auxin efflux carriers *PttPIN1* and *PttPIN2* in the dormant cambium (Schrader *et al*, 2003, 2004a). During active phases of growth, expression of these polar auxin transporters was reactivated in response to the increase in auxin level via the positive feedback loop mechanism (Schrader *et al*, 2003; Vieten *et al*, 2005). A recent study suggests that auxin signalling pathway is significantly disrupted during cambium dormancy. This seems to be due to the perturbation of upstream auxin signalling pathways, one of which is the SCF^{TIR} activity and ARF regulation (Baba *et al*, 2011). Dormancy of cambial activity and other growth responses to seasonal changes have been shown to be signalled through FLOWERING LOCUS T (FT), which is regulated by CONSTANS and PHYA (Böhlenius *et al*, 2006). In *Populus*, two *FT* gene duplicates were found to be sub-functionalized and induced under different environmental conditions (Hsu *et al*, 2011). An *FT* gene that is induced under conditions of a warm temperature and long day promotes the radial stem growth. It remains to be identified whether the growth regulation of FT is mediated via auxin signalling pathways.

Cytokinins

Cytokinins, a key phytohormone along with other factors, maintain stem cell pools by regulating cell differentiation (Figures 6 and 7; Table I). The role of cytokinin in vascular stem cell maintenance first emerged from a finding of the *wooden leg* (*wol*) mutant that has a severely reduced number of procambial cell files in embryos and roots (Scheres *et al*, 1995). In addition, the vascular cylinder of the *wol* primary root consists solely of protoxylem (Mahonen *et al*, 2000). The *WOL* gene encodes a two-component histidine kinase protein, one of the three cytokinin receptors in *Arabidopsis* (Mahonen *et al*, 2000). The *wol* mutation mimics the cytokinin-unbound state and confers constitutive phosphatase activity, removing phosphate from histidine-containing phosphotransfer (HPT) protein. This blocks the total phospho-load and cytokinin signalling (Mahonen *et al*, 2006b). Similarly to the *wol*

mutant, a triple loss-of-function mutant of cytokinin receptors (*ahk2 ahk3 ahk4*) exhibited ectopic protoxylem formation, accompanied by a reduction in vascular cell files (Mahonen *et al*, 2006a). AHP6, a pseudo-phosphotransfer protein, has been reported to act as a negative regulator of cytokinin signalling by interacting with the phosphorelay machinery (Mahonen *et al*, 2006a). AHP6 is specifically expressed in the protoxylem cells, and its loss-of-function mutation results in sporadic differentiation of protoxylem (Mahonen *et al*, 2006a). The expression analysis of *ARR15* as cytokinin signalling reporter revealed that cytokinin signalling is ectopically activated at sites where protoxylem would normally differentiated in *ahp6* mutant. This suggests that AHP6 counteracts cytokinin signalling and thereby allows cells to differentiate into protoxylem identity (Mahonen *et al*, 2006a). Therefore, the pluripotency of vascular stem cells and identity of protoxylem seem to be determined by spatially distributed regulatory interactions between cytokinin signalling and its inhibitor AHP6 in the *Arabidopsis* root.

Recently, it has been reported that a mutually inhibitory feedback loop between auxin and cytokinin regulates the vascular patterning in *Arabidopsis* primary root (Figure 6; Bishopp *et al*, 2011). In the post-embryonic *Arabidopsis* root, vascular tissues are organized in a bisymmetric pattern, in which the first plane of symmetry runs through a central xylem axis and the second plane through the phloem poles. Along with this bisymmetric vascular pattern, the signalling maxima of two major phytohormones, cytokinin and auxin, is established in a bisymmetric manner. Cytokinin signalling maximum is detected in the intervening procambium cell files. On the other hand, auxin signalling maximum is observed in the xylem axis, overlapping with AHP6 expression domain which is positioned in the protoxylem pole. AHP6 expression is promoted by auxin through ARF binding sites in the AHP6 promoter, suggesting that AHP6 is targeted as a primary auxin response gene. When plants are treated with the auxin transport inhibitor 1-naphthylphthalamic acid (NPA), both auxin signalling maximum in the xylem axis and AHP6 expression in the protoxylem position disappear. Consistently, a loss-of-protoxylem phenotype is observed in NPA-treated plants similar to that of *ahp6* mutant. This suggests that the active auxin transport facilitates the formation of auxin signalling maximum in the xylem axis, which in turn promotes AHP6 expression in the protoxylem position. Among eight *PIN* genes, the expression of *PIN3* and *PIN7* exhibits a non-radially symmetric pattern in the root stele. *PIN3* is predominantly expressed in the pericycle cells touching the protoxylem pole and *PIN7* is present in all the intervening procambium cells and phloem initials. In *pin3 pin7* double mutant, the robust bisymmetric expression patterns of *DR5* and AHP6 become more random than in the wild type, suggesting that these PINs are required for creating a stable auxin maximum in the xylem axis. Interestingly, the expression of these PINs is significantly downregulated in the *wol* mutant where the cytokinin signalling is reduced. In contrast, exogenous cytokinin treatment results in the expansion of *PIN7* domain into the protoxylem position and the shift of *PIN3* domain away from the protoxylem-associated pericycle cells. These suggest that cytokinin signalling might regulate the expression of PINs in *Arabidopsis* vascular tissues. Furthermore, auxin signalling maximum is expanded throughout the vascular cylinder in the *wol* mutant. Taken together, cytokinin signalling restricts the

Figure 6 The regulatory loop between auxin and cytokinin controls vascular patterning in the *Arabidopsis* primary root. Cytokinin signalling restricts the auxin signalling maximum to the xylem axis by promoting the bisymmetric localization of PINs. Consequently, auxin activates the expression of *AHP6* in the protoxylem position, and in turn *AHP6* counteracts cytokinin signalling, thereby allowing cells to differentiate into protoxylem.

auxin signalling maximum to the xylem axis by regulating the bisymmetric localization of PINs. Auxin then promotes the expression of *AHP6* in the protoxylem precursor so that it can counteract cytokinin signalling and allow for cells to differentiate into protoxylem.

Other genetic studies also suggested that cytokinin is required to maintain the vascular stem cells by preventing differentiation process. When the cytokinin signalling was abolished via mutations in *AHPs* or type-B *ARABIDOPSIS RESPONSE REGULATORS* (*B-ARRs*), vascular stem cells underwent differentiation into xylem (Table I; Hutchison *et al*, 2006; Yokoyama *et al*, 2007; Argyros *et al*, 2008; Ishida *et al*, 2008). In mutants of *ARR1*, *10* and *12*, or *AHP 1*, *2*, *3*, *4* and *5* all the vascular cells in *Arabidopsis* primary roots differentiated into xylem. Recently, exogenous application of CLE peptides was reported to inhibit protoxylem formation in *Arabidopsis* roots through a *CLV2*-dependent pathway (Kondo *et al*, 2011). These CLEs were found to enhance cytokinin signalling through the repression of *A-ARRs*, and thereby suppress the protoxylem cell fate.

Cytokinins are also required for promoting cell proliferation activities in the vascular stem cells during the secondary growth. When the cytokinin level was significantly reduced in the quadruple mutant of *IPTs* (*ipt1 3 5 7*), secondary growth did not occur (Matsumoto-Kitano *et al*, 2008). A similar approach was made by expressing cytokinin oxidase, an enzyme that breaks down an active form of cytokinin, in the vascular stem cells of *Populus* (Nieminen *et al*, 2008). The low level of cytokinin significantly reduced the secondary growth of *Populus* stems. Consistent with the effects of a cytokinin level on the vascular stem cell maintenance, perturbing the cytokinin signalling also affected the secondary growth of *Arabidopsis* stems. Mutating two cytokinin receptors, *AHK2* and *AHK3*, caused significant reduction in the secondary growth of vascular tissues (Hejatko *et al*, 2009). Similar effects were also observed when modified CKI1, which has a mutation in Histidine residue, was ectopically expressed (Hejatko *et al*, 2009). CKI1 does not bind cytokinin *in vitro*, however, possesses the phospho-transfer activity-like AHKs. Mutation in Histidine residue of CKI1 perturbs phospho-transfer activity and results in phenotypes similar to cytokinin receptor mutants. This and other aforementioned studies suggest that balancing phospho-transfer activities in the cytokinin signalling plays a crucial role for controlling

Figure 7 Hormonal regulation in the vascular stem cells.

downstream regulators in the maintenance and activities of vascular stem cells.

Brassinosteroids

During xylogenesis in *Zinnia*, brassinosteroid (BR) promotes the formation of tracheary elements by triggering the secondary cell wall formation and cell death (Figure 7; Yamamoto *et al*, 1997). Consistent with its involvement in the differentiation of tracheary elements, an increase in the BR levels and expression of genes involved in BR biosynthesis was observed during *in vitro* xylogenesis (Yamamoto *et al*, 2001, 2007). When BR synthesis was inhibited by Brassinazole treatment, dramatic decrease in

the secondary xylem proliferation was observed in Cress plants. This suggests that BR also regulates the cell proliferation activity of cambium (Nagata *et al*, 2001).

BR is perceived by BRI1, a membrane-localized LRR-RLK (Table I). There are three BRI1 homologues in Arabidopsis (BRL1–3) and one of them, VASCULAR HIGHWAY 1 (VH1) or BRL2, regulates the procambial cell formation in developing leaves (Clay and Nelson, 2002). However, this protein does not functionally substitute BRI1 or bind BR. By contrast, BRL1 and 3 bind to BR and complement *bri1* when they are expressed under the *BRI1* promoter. Unlike *BRI1*, which is expressed broadly in Arabidopsis, *BRL1* and 3 are only expressed in the vascular tissues. When *BRL1* was knocked out, phloem domain expanded at the expense of xylem. This suggests that BR positively regulates the xylem development (Cano-Delgado *et al*, 2004). *BRI1*, *BRL1* and *BRL3* seem to have diverged before the divergence of monocot and eudicot species. Putative orthologues of these three genes are present in rice and like in Arabidopsis, phloem formation was promoted in rice when BR signalling was perturbed (Nakamura *et al*, 2006).

In addition to being involved in xylem development, BR coordinates with auxin to spatially regulate the formation of vascular bundles (Ibañez *et al*, 2009). Mathematical modelling combined with mutant analyses suggests that auxin maxima generated by the auxin efflux transporters (PINs) may promote vascular bundle formation. However, it is the BR signalling pathway that seems to regulate the spacing of auxin maxima and vascular bundles, likely by influencing cell division activities.

Gibberellins

Analysis of the distribution of GAs in the main stems of *Populus* showed that GA levels are usually low in the vascular cambium but high in the region where xylem cells expand (Figure 7; Table I; Israelsson *et al*, 2005). Similar patterns were observed for the expression of *GA 20-oxidase1* and *GA 3-oxidase*, genes that are involved in generating biologically active GAs. Consistent with these data, two GA responsive genes, *DELLA-like1* and *GIP-like1*, were found highly induced in expanding xylem cells.

However, when GA levels were increased in transgenic *Populus* by ectopically expressing *GA 20 oxidase*, there was a significant increase in plant growth rate (Eriksson *et al*, 2000). These transgenic plants also exhibited an enhanced vascular stem cell activity and an increase in the xylem fibre number and length. Consistent with this study, an increase in cambial cell division activity was observed when GA was locally applied to the stems of other angiosperm tree species (Funada *et al*, 2008). Interestingly, xylem fibres generated under a high level of GA showed characteristics of the tension wood that is formed under the mechanical stress. Therefore, GAs might not be the main driver of cell division activities of vascular cambium under an unstressed condition, but might be under the mechanical stress. In a more recent study, GA was found to act as a mobile signal that promotes xylem expansion in Arabidopsis hypocotyls (Ragni *et al*, 2011).

Jasmonate and ethylene

Jasmonate (JA) and its signalling are known to regulate several aspects of plant stress responses (Wasternack, 2007). During the establishment of interfascicular cambium, cells in that region undergo dramatic reorganization. A recent study suggests that

genes in JA signalling pathways are actively involved in the formation and activity of interfascicular cambia (Sehr *et al*, 2010). In JA signalling pathway, JASMONATE ZIM-DOMAIN (JAZ) proteins act as repressor of JA signalling by directly binding to a key transcriptional regulator MYC2 (Table I; Chini *et al*, 2007; Thines *et al*, 2007). Once JA binds to its receptor, CORONATINE INSENSITIVE1 (COI1), JA-COI1 elicits the degradation of JAZ proteins, by which MYC2 is released from JAZ and becomes transcriptionally active. Among 12 genes that encode JAZ proteins in Arabidopsis, the expression of *JAZ7* and *10* is induced by JA. Genome-wide expression profiling of stem segments undergoing interfascicular cambium formation showed upregulation of *JAZ7* and *10* and several other genes in JA signalling pathway (Sehr *et al*, 2010). In the knockout mutant of *JAZ10*, the radial growth and interfascicular cambium formation in stems became more active than in the wild type. Consistently, the stem radial growth was reduced by about 25% when the *COI1* or *MYC2* was knocked out.

Similarly, ethylene, which is also involved in plant stress responses, plays an important role in vascular stem cell activities that forms tension wood, which has a high level of cellulose and a low level of lignins and microfibrils, in *Populus* (Love *et al*, 2009). When *Populus* was treated with ethylene, xylem growth significantly increased in comparison to non-treated plants. This increase in xylem growth was from the enhancement in the cambial cell division rate, not in the cell expansion. Transgenic plants that cannot sense ethylene properly failed to form tension wood in the leaning stem, suggesting that ethylene is a key signalling molecule that initiates tension wood formation in the cambium. In addition, transcriptional profiling indicated alterations in the touch-inducible ethylene signalling component *ETHYLENE RESPONSE FACTOR 104* expression (Table I; Bethke *et al*, 2009). Further analysis of its knockout line revealed a decrease in the interfascicular cambium initiation in the acropetal direction. Taken together, these findings suggest active involvement of stress-related hormones such as JA and ethylene in the interfascicular cambium formation.

Conclusions and future perspectives

Extensive progress has been achieved in understanding the molecular mechanisms governing the formation and maintenance of vascular cambium during this decade (Figure 8). However, in comparison with apical meristems, our knowledge of the vascular cambium and its regulation are still very poor.

In Arabidopsis apical meristems, the position of stem cells and their niche has been determined with molecular markers that are specifically expressed in the stem cells or their niche cells. On the contrary, the position of the stem cells and accompanying niche cells in the cambium is still unclear. The main obstacle in answering these questions has been the lack of reliable molecular makers, which will enable us to define the stem cells and associated niche cells. A genome-wide expression analysis combined with technologies that can isolate an individual cell will aid in establishing a reliable gene expression map of the vascular cambium. In addition, such an approach will provide the marker genes that can differentiate the collectively called 'daughter cells' into cell identities with distinctive functions. Furthermore, a clonal analysis of the secondary cambium can aid in generating the precious cell lineage map, which permits defining the location and identity of the stem cells and the niche cells.

Figure 8 Regulatory networks in the control of vascular stem cell activities.

In *Arabidopsis* roots, the radially symmetric cambium is formed from the bisymmetrically organized procambial cells, indicating that the bisymmetric pattern is broken and radial symmetric pattern is established during the transition phase. Based on the anatomical analyses, the pericycle cells adjacent to protoxylem pole also seem to be recruited into cambial

zone during transition phase, suggesting that the radially symmetric cambial tissue is derived from two distinct types of cells, procambial cells and pericycle cells. As described above, cytokinin is the key regulator of the initiation of the secondary vascular development. However, it is completely unknown how this symmetry conversion is regulated during the transition from primary to secondary development. A forward genetic screen with the appropriate gene marker will allow uncovering the molecular basis of this process.

Recent works started to reveal various transcription factors that regulate several aspects of vascular development including the cell proliferation and differentiation. However, little is known about how these transcription factors construct the regulatory networks during vascular development. The expression patterns of these transcription factors are tightly controlled; however, our knowledge about the upstream regulators of these transcription factors is limited. Recently, in an attempt to delineate the gene regulatory networks, comprehensive yeast one-hybrid and two-hybrid assays have been performed against a large number of transcription factors that are expressed in a tissue-specific manner in *Arabidopsis* stele (Brady *et al*, 2011). A similar approach will provide us with the vital information regarding the transcriptional networks in the secondary cambium.

Overall plant growth and development depends heavily on environmental factors. Different environmental and physiological conditions such as resource availability, temperature and stresses have a profound impact on the activities of vascular stem cells. Recently in *Pinus radiata*, it was reported that xylem transcriptome exhibits seasonal reorganizations in accordance with various physiological changes and tree age (Li *et al*, 2010). Vascular stem cell activity also shows plasticity in response to mechanical loads. In angiosperm trees, a local increase in cambial cell division in the leaning stems results in the formation of tension wood. Therefore, understanding how gene regulation and signaling in vascular stem cells is created and rewired in response to ever-changing environments is critical for predicting and maintaining plant growth and yields in the future.

Conflict of interest

The authors declare that they have no conflict of interest.

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