INVESTIGATION OF MOBILE TRANSCRIPTION FACTORS INVOLVED IN THE ROOT VASCULAR MORPHOGENESIS

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INVESTIGATION OF MOBILE TRANSCRIPTION FACTORS INVOLVED IN THE ROOT VASCULAR MORPHOGENESIS

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Vascular system plays an essential role as conductive and supporting machinery in the majority of land plants. Xylem and phloem, major tissues in the vascular system, are generated from vascular stem cells in a strictly organized fashion. Their patterning requires extensive cell-to-cell communications that have been remained largely unknown. In this dissertation, I present the discovery of two developmental programs that require the cell-to-cell movement of transcription factors.

One finding came out while screening the phenotype of knockout mutants of transcription factors that are expressed in the vascular cell type enriched manner. I found two closely related AT-hook family members AHL3/4 whose knockout mutants result in abnormal xylem patterns in the root. Interestingly, AHL4 proteins move from procambium cells to xylem precursors in the root. Our studies suggest that cell-to-cell mobility of AHL4 is required for defining the cell type boundaries. In addition, AHL4 forms protein complexes with its closest homolog AHL3. Together, I proposed that AHL4 and AHL3, moving from the procambium to xylem precursors, define the boundaries between the two tissues and restrict the expansion of a xylem domain to procambium.

The other finding involves the SHR regulation on the phloem patterning in two

distinct pathways, both of which require the cell-to-cell movement of SHR. The companion cell formation is mediated by the SHR-miR165/6 pathway that requires SHR to move to the endodermis. Meanwhile, the cell division for sieve element formation is mediated by the SHR moving into a phloem initial from procambium. By combining a cell sorting/microarray facilitated genome-wide expression profiling method with the high-resolution genome-wide root expression map, I found that a novel regulator NARS1, an NAC domain transcription factor, controls sieve element division as downstream of SHR.

Plant morphogenesis is a highly organized process that largely relies on positional information. Such positional information is established by cell-to-cell communication which turns on cell-type specific regulatory networks temporally and spatially. My study reveals novel cases where transcription factors that move between cells act as important informants during vascular tissue patterning. This further broadens our understanding of molecular mechanisms underlying a key developmental innovation in plant evolution.

BIOGRAPHICAL SKETCH

Jing Zhou grew up in Jinan the capital city of a coastal province Shandong, China. She started to study piano and drawing before entering junior high school and won awards in several piano competitions in Shandong. She attended high school affiliated to Shandong Teacher University and started to develop interests in science.

After admitted to Shandong University in Shandong, she studied biology and received her Bachelor's degree in 2003. Jing was involved in an environment analysis and improvement project as an analyst for a year, and in 2004 entered the National University of Singapore in Singapore as a graduate fellow. She studied flower development mechanisms and received her master's degree in 2006.

After graduation, Jing Zhou was admitted into the Ph.D. program at Department of Plant Biology, Cornell University. In 2007, she joined Dr. Ji-Young Lee's lab at Boyce Thompson Institute and studied the mechanisms regulating plant morphogenesis. She is married to Mr. Tengfang Huang, a Cornell alumnus.

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I also want to express my thanks to Cornell University for awarding me a Presidential fellowship for my first year's study and other funding agencies including National Science Foundation, Triad Foundation for providing grants to our research.

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Chapter 1 Introduction: Signaling and gene regulatory programs in plant vascular stem cells

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Abstract

A key question about the development of multicellular organisms is how they precisely control the complex pattern formation during their growth. For plants to grow for many years, a tight balance between pluripotent dividing cells and cells undergoing differentiation should be maintained within stem cell populations. In this process, cell-cell communication plays a central role by creating positional information for proper cell type patterning. Cell-type specific gene regulatory networks govern differentiation of cells into particular cell types. In this review, we will provide a comprehensive overview of emerging key signaling and regulatory programs in the stem cell population that direct morphogenesis of plant vascular tissues.

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Plant vascular system: Lifeline of plants

In multicellular organisms, molecules in various forms such as water, minerals, gases, nutrients, and signaling molecules are constantly exchanged between cells in short- and long distances. Such exchanges are mediated by vascular systems, transport conduits in plants and animals. Vascular systems make continuous networks that interconnect all the body parts. Though vascular systems in plants and animals are functionally similar to each other, there are significant differences between the two. First, transport in the plant vascular system is directional rather than circulatory (Figure 1.1A). Plants absorb water and minerals from the soil through roots, while synthesize carbohydrates by photosynthetic processes in the leaves. These molecules supplied from the opposite ends of a plant body are redistributed by two sub-functionalized compartments in the vascular system: xylem that conducts water and minerals from roots to shoots and phloem that transports the products of photosynthesis and other signaling molecules from leaves to the rest of the plant. Second, the plant vascular system is composed of stacks of cells, which allow for the transport of water and nutrients through intracellular highways as opposed to extracellular in animals. Third, different from animals, the plant vascular system has an additional function that is to protect and support a plant body. Xylem tissues that contain cells with thick secondary cell walls contribute to this role. In tree species, xylem tissues continuously grow from vascular stem cells via cell division and differentiation, generating wood, an important natural resource for construction, furniture, paper, and many other essentials (Figure 1.1B).

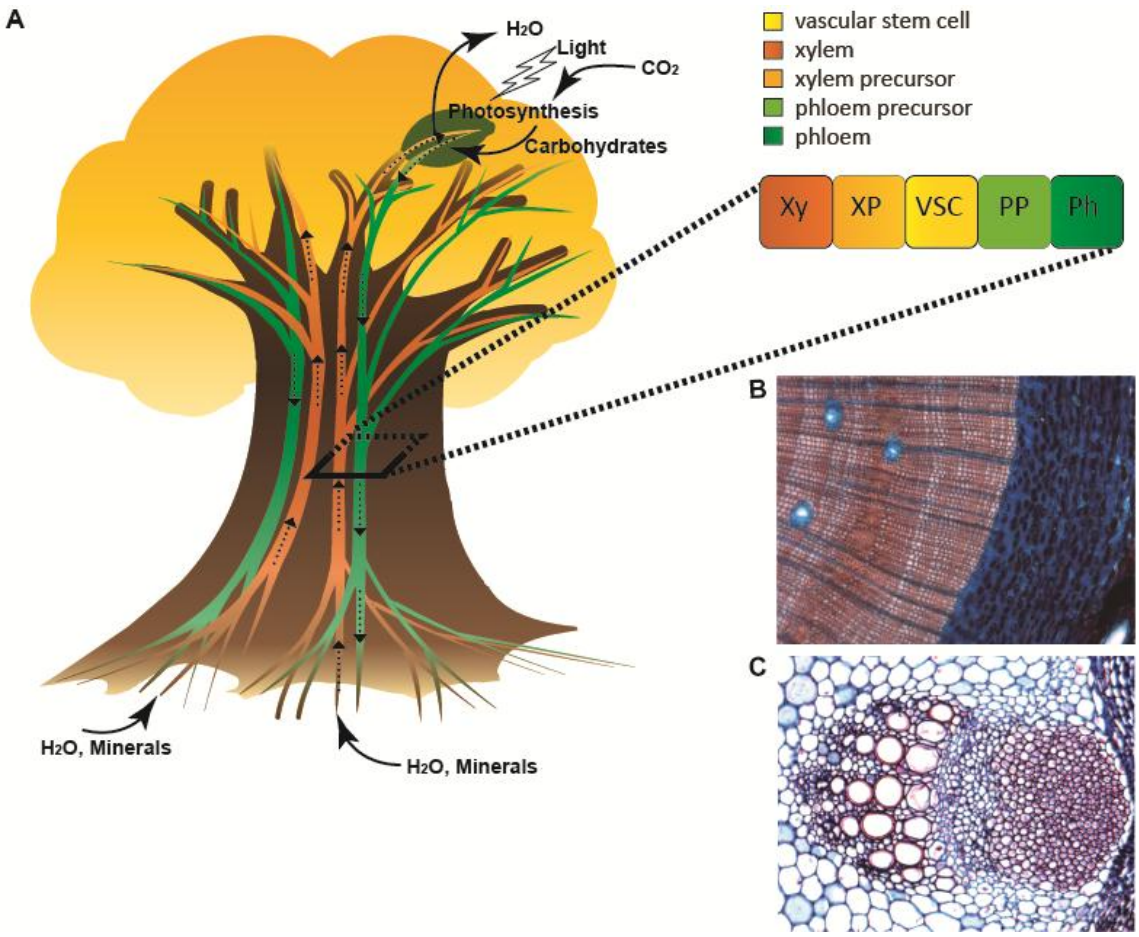

Figure 1.1. Vascular system in plants.

(A) Directional transport system conducts water and minerals from roots through xylem, and sugars from photosynthetic leaves through phloem. **(B)** Cross section of a woody stem in the secondary growth from an apple tree. **(C)** Cross section of a sunflower stem with vascular bundles (primary growth).

Evolution of vascular tissues solved the problem of water and food transport, which is the prerequisite for sessile plants to succeed in terrestrial environments. With the evolution of vascular tissues, plants successfully dominated and diversified their growth forms into trees, shrubs, and herbs (Niklas *et al.*, 1985). Global climate change, resulting from an increase in greenhouse gas emissions generated by combusting fossil fuels, is threatening the future of humanity and ecosystems. About

33% of CO_2 released by anthropogenic activities is converted to plant biomass. Trees in mature forests, store almost 45% of terrestrial carbons by converting CO_2 into biomass every year, significantly contributing to the carbon balancing (Bonan, 2008). Therefore, understanding mechanisms underlying vascular tissue development is closely linked to finding solutions for global warming.

Xylem and phloem are constituted of distinctive cell types. Xylem is composed of tracheary elements (TEs), xylem parenchyma, and fibers. Of these cell types, TEs are the main conducting cells for the transport of water and mineral nutrients (Wilson and White, 1986). TEs and fibers die as they mature and accumulate secondary cell walls with high levels of lignins, polymerized phenolic compounds (Mittler and Lam, 1995). Mature TEs, hollow and partially open-ended, stack on top of each other to make well-aligned pipelines. The rigidity of lignified cell walls of mature TEs is essential not only for strong mechanical support of plant bodies but also for enduring high turgor pressure created during water transport. Phloem is composed of sieve elements (SE), companion cells (CC), phloem parenchyma, and fibers (Esau, 1977; Oparka and Turgeon, 1999). SE and CC develop adjacently from the same precursor cells via asymmetric cell division. SEs transport photosynthetic products, signaling compounds, proteins and RNAs, which are loaded from CC.

The organization of xylem and phloem is tightly regulated by genetic programs. In young roots, the center is usually occupied by xylem, which forms outward ridge-like projections. Brackets between these ridges are occupied by phloem tissues and vascular stem cells, which form boundaries between the xylem and phloem. This arrangement results in one cylindrical form of vascular tissues in the root

center. In contrast to the root, xylem, phloem and vascular stem cells develop as multiple bundles in young stems (Figure 1.1C). In most of the higher plants, xylem grows towards the center of stem axis while phloem grows towards the periphery from vascular stem cells that are located between the two. Arabidopsis root, a widely used model system, has a relatively simple pattern with only two xylem ridges. Two types of TEs develop in the root, which are protoxylem and metaxylem. Protoxylem strands, characterized by the accumulation of lignified secondary cell walls in spiral forms, differentiate early in the periphery of xylem axis. In contrast, metaxylem strands, surrounded by dense lignified cell walls in reticulate patterns, differentiate later in the middle of the xylem axis. In Arabidopsis root, phloem develops at the two poles that are perpendicular to xylem axis. At each pole, two SEs are guarded by CCs.

Xylem and phloem develop from two meristematic tissues: procambium and cambium, collectively called vascular stem cells (Figure 1.1B and 1.1C). Procambium is the vascular stem cell population in young plant organs that grow in the apical direction (primary growth). In growing leaves and in young stems and roots, procambium cells establish and grow as residing organs grow, and then xylem and phloem cells differentiate from them. As plants mature, their stems and roots start growing in the lateral direction to increase the girth, undergoing a process called secondary growth. Secondary growth is promoted by cell division and differentiation of vascular tissues from the cambium, which is originally derived from the procambium and its neighboring cells. In most of higher vascular plants, daughter cells that are generated in the inner layers of the cambium (toward the center of the stem axis) by cell division are specified into xylem whereas those in the outer layers of

the cambium (toward the periphery of the stem axis) differentiate into phloem.

Vascular stem cells first appear during embryogenesis. However, in contrast to most of the animal embryos that form a miniature version of the adult body plans, plant embryos only establish major body axes and tissue layers with much less complexity than the mature plants (Goldberg *et al.*, 1994; Kaplan and Cooke, 1997). Complex pattern formation and organ differentiation that lead to the adult plant morphology take place during post-embryogenesis. To support such post-embryonic development, the genetic programs that establish vascular stem cells should operate continuously in the newly emerging organs during plant growth.

Recently a comprehensive understanding of regulatory networks in the initiation and specification of plant vascular stem cells started emerging (Figure 1.2A). Over the years, studies on plant vascular development have focused on the role of plant hormones (phytohormones). Phytohormones are small organic signaling molecules synthesized in plants. Among them, auxin, cytokinin and brassinosteriods have been shown as the major players in plant vascular stem cells. A number of studies in model systems such as *Arabidopsis*, *Populus*, and *Zinnia*, demonstrated indispensable roles of phytohormones. Recently, the involvement of signaling mediated by Leucine-Rich-Repeat (LRR) Receptor-Like Kinases (RLK) and CLAVATA3/ENDOSPERM SURROUNDING REGION (CLE) peptide in vascular development was identified. Advances in technologies of genome-wide expression profiling and perturbation of gene activities facilitated discoveries of transcription factors in gene regulatory networks, which are responsible for directing vascular tissue development. The importance of microRNAs in vascular development has been

recently re-discovered as mobile signals that regulate the activities of key xylem cell fate determinants.

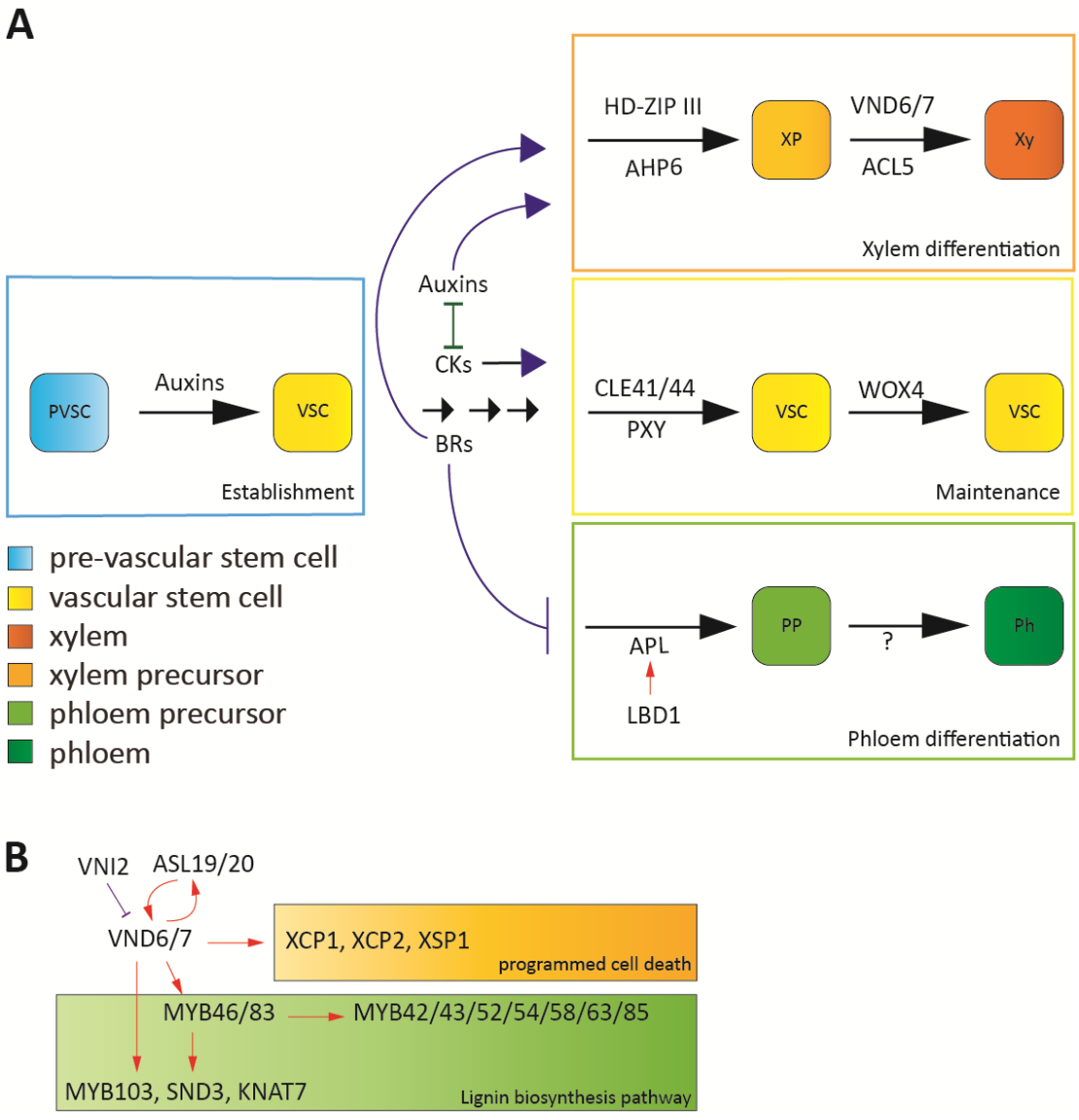

Figure 1.2. Gene regulatory networks in vascular development.

(A) An overview of the regulatory networks that differentially control cell fates in the vascular stem cells. **(B)** Detailed regulatory networks for xylem differentiation. Black arrows indicate the flow of vascular differentiation; Red arrows, transcriptional regulations; purple T-head, post-translational repression; green line, interaction between hormones; and blue lines, hormone outputs on the differentiation process.

In this review, we introduce and discuss the molecular mechanisms underlying the establishment and maintenance of stem cells, cell type specification, and cell type patterning during plant vascular tissue development. Patterning and differentiation of vascular tissues based on their structure/anatomy, evolution, and regulation by plant hormones have been recently reviewed elsewhere (Berleth and Mattsson, 2000; Baucher et al., 2007; Turner et al., 2007; Dettmer et al., 2009; Elo et al., 2009; Ohashi-Ito and Fukuda, 2010; Scarpella and Helariutta, 2010; Spicer and Groover, 2010).

Establishment of vascular stem cells: story of auxin

Auxin plays a central role in diverse aspects of plant growth, ranging from establishing embryo polarity to seed maturation (Muday and DeLong, 2001; Jenik and Barton, 2005; Teale et al., 2006; Sorefan et al., 2009; Stewart and Nemhauser, 2010). Vascular morphogenesis is no exception. As early as the 1950s, it was known that auxin can induce development of veins, which are networks of vascular tissues (Jacobs, 1952). First established during embryogenesis, auxin ensures continued formation of vascular tissues in the emerging organs throughout plant lifetime. The auxin flow-canalization hypothesis was proposed as a mechanism that explains the vein formation during organ growth (Sachs, 1981), and subsequent modeling and simulation approaches further supported this hypothesis (Mitchison, 1980, 1981; Feugier et al., 2005; Rolland-Lagan and Prusinkiewicz, 2005; Dimitrov and Zucker, 2006). According to this hypothesis, auxin flow/transport, facilitated by unknown positive feedback regulation, forms the basis for the specification of vascular stem

cells in narrow and continuous strands, predecessors of veins. A number of auxin signaling mutants isolated with severe defects in vein formation underscored the canalization hypothesis. Among them, *MONOPTEROS* (*MP*)/*AUXIN RESPONSE FACTOR5* (*ARF5*) encodes an auxin responsive factor and plays a vital role in vascular stem cell initiation (Figure 1.3). The *mp* knock-out mutant fails to establish vascular stem cells during embryogenesis (Hardtke and Berleth, 1998). In weak *mp* mutants, drastic reduction in vein formation occurred largely due to defective auxin signaling (Donner *et al.*, 2009; Schlereth *et al.*, 2010). Recently, it has been shown that MP commences vascular stem cell specification by directly activating expression of *ATHB8*, a Homeodomain Leucine-Zipper class III (HD-ZIP III) family transcription factor (Donner *et al.*, 2009).

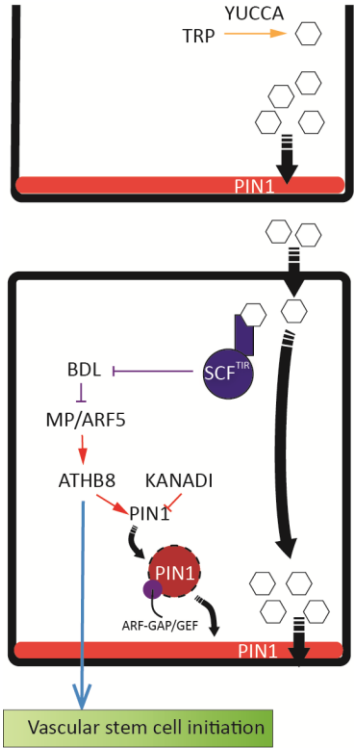

Figure 1.3. Auxin signaling pathway at the cellular level.

Auxin, synthesized from sources such as Tryptophan (Trp) via YUCCA, is transported between cells in a polar manner (PAT) by PINs. Inside the cell, auxin interacts with the TIR1, a subunit of the SCF^{TIR1} complex. This generates a signal that leads to ubiquitin dependent degradation of AUX/IAA transcriptional repressor (BDL), which in turn activates the expression of ARFs that modulate cellular auxin responses (MP/ARF5). Black squares indicate plasma membranes; thick arrow bars, molecule movement; red arrows, transcriptional regulation; purple T-heads, post-translational regulation; blue arrow, subsequent developmental processes; orange arrow, auxin biosynthesis. Auxin is depicted as hexagon, TIR receptor complex in dark blue, PIN1 in red, and ARF-GAP/GEF as a purple circle on a vesicle membrane that carries PIN1.

There are several pathways through which plants synthesize auxin *de novo*, primarily in a localized manner (Zhao, 2010). Among them, the YUCCA (YUC) pathway is a key route, mediated by the *YUC* family of flavin monooxygenase genes. In *Arabidopsis*, there are 11 *YUC* genes. Mutations in at least 4 members, *YUC1*, *YUC2*, *YUC4* and *YUC6*, caused abnormalities in vein initiation and development

(Cheng *et al.*, 2006). Together with an earlier observation that localized appliance of auxin induced new veins extending from the site of application, this finding signified the role of localized auxin as a key determinant in the establishment of vascular stem cells (Sachs, 1991). From the foci of synthesis, auxin is subsequently transported away by a process called polar auxin transport (PAT) (reviewed by (Blakeslee *et al.*, 2005; Grunewald and Friml, 2010)). PAT is central in establishing spatial auxin gradients (maxima and minima), which are required for vein growth (Benkova *et al.*, 2003; Grunewald and Friml, 2010). The first PAT associated mutant in *Arabidopsis* is the one that disrupts PIN-FORMED1 (AtPIN1), a major auxin efflux carrier protein. *Atpin1* displayed severe defects in vein formation, largely due to reduced PAT (Galweiler *et al.*, 1998). *AtPIN1* starts to be expressed in the cells of developing lateral organs before they turn into vascular stem cells (Scarpella *et al.*, 2006). *ATHB8* induced by incoming auxin via AtPIN1, further activates the expression of *AtPIN1* (Donner *et al.*, 2009). This positive feedback regulation facilitates the specification of vascular stem cells in narrow and continuous strands. Inhibition of PAT using inhibitors such as 1-N-naphthylphthalamic acid (NPA) resulted in anomalies similar to *Atpin1* in the formation of vascular tissues (Galweiler *et al.*, 1998; Mattsson *et al.*, 1999).

The importance of PAT in vein growth became more evident from studies of ARF-GAP/GEF (ADP Ribosylation Factor-GTPase Activating Protein/Guanine nucleotide Exchange Factor) family mutants. The ARF-GAP/GEF family proteins that mediate vesicle transport are essential for the intracellular polar transport/localization of PIN proteins to the plasma membrane. In the mutants of *GNOM/VAN7*, an auxin

signaling associated ARF-GEF gene, fragmented veins were formed mainly in the cotyledons and rosette leaves (Koizumi et al., 2000; Geldner et al., 2003; Fukuda, 2004). This was found to be from the failure in proper loading of PINs to the plasma membrane for PAT. Mutations in another ARF–GAP family gene, *VAN3/SCARFACE* also interrupted vein formation (Koizumi *et al.*, 2005; Sieburth *et al.*, 2006). VAN3 is part of the trans-Golgi network mediated vesicle transport system involved in PIN trafficking. *PINOID* (*PID*) encodes a serine/threonine protein kinase that phosphorylates PINs to recruit them in the direction opposite to where GNOM/VAN7 does to PINs (Christensen et al., 2000; Michniewicz et al., 2007; Kleine-Vehn et al., 2009). Therefore, balancing between these two PIN recruiting pathways is crucial for proper PAT. When such a balance was disrupted by either knocking out or ectopically expressing *PID*, the polar localization of PINs was again perturbed and discontinuous veins were formed (Christensen et al., 2000; Benjamins et al., 2001; Kleine-Vehn et al., 2009). Other known PAT defective mutants such as *lopped1* (*lop1)/tornado1* (*trn1*) and *trn2* also exhibited similar vein phenotypes (Carland and McHale, 1996; Cnops *et al.*, 2006).

Similar to PAT, defects in auxin perception also lead to abnormal vein formation. In the absence of auxin, activities of MP and other auxin responsive factors (ARFs) stay blocked by Aux/IAA repressors. In the presence of auxin, Aux/IAA repressors are degraded by the ubiquitin-mediated pathway. This protein degradation pathway is triggered by the interaction between auxin and TRANSPORT INHIBITOR RESPONSE 1 (TIR1), a part of protein degradation complex (more details reviewed in (Mockaitis and Estelle, 2008)), unleashing the transcriptional activity of ARFs. Some

mutants that are resistant to the auxin-dependent protein degradation exhibit highly reduced vein formation. These include *auxin resistant-6* (*axr6*), a mutant in a component of ubiquitin ligase complexes, and *bodenlos* (*bdl*), an IAA12 mutant resistant to the ubiquitin-mediated protein degradation, in *Arabidopsis* (Hamann et al., 1999; Hobbie et al., 2000; Hamann et al., 2002).

During secondary growth, auxin promotes proliferation activities in the cambium. It was shown that auxin maxima are established and genes associated with auxin signaling are up-regulated in the actively proliferating cambial cells (Moyle *et al.*, 2002). By contrast, general reduction in PAT and down-regulation of auxin signaling genes were observed during the phase of cambium dormancy (Schrader *et al.*, 2003). Consistently, disruption in auxin signaling/responsiveness led to detrimental effects on cambial activities, reducing secondary growth in hybrid aspen plants (Nilsson *et al.*, 2008). Studies on a mutant of *INTERFASCICULAR FIBERLESS1/REVOLUTA* (*IFL1*/*REV*), another HD-ZIP III member in *Arabidopsis*, also suggested a positive correlation between auxin and cambial activities. In the null mutant of *IFL1*, due to reduced PAT, there was no secondary xylem development in vascular bundles (Zhong and Ye, 1999, 2001).

Maintenance of vascular stem cells

Role of RLK-CLE signaling

A prevailing mechanism, which plants and animals employ for cell-cell communication, is a receptor-mediated signaling pathway (plant signaling pathways

were reviewed in (Tor et al., 2009; Fukuda and Higashiyama, 2011)). It was the signaling pathway mediated by CLAVATAs (CLVs) that was first reported maintaining stem cell populations in the shoot apical meristem (Clark *et al.*, 1993). Three key components were initially identified in this pathway: CLV1, a LRR-RLK; CLV2, a LRR-receptor-like protein (RLP); CLV3, a glucosylated dodecapeptide (Clark *et al.*, 1997; Fletcher *et al.*, 1999; Jeong *et al.*, 1999; Trotochaud *et al.*, 2000) (Figure 1.4A). Recently, CORYNE (CRN), another receptor kinase, was found to interact with CLV2 and form a receptor complex that perceives CLV3 (Bleckmann *et al.*, 2010; Replogle *et al.*, 2011). In the knock-out mutants of these signaling components, overgrowth of a stem cell population happens in the shoot apical meristem. Conversely, over-expression of *CLV3* reduces the stem cell population. These findings indicate the presence of a mechanism that balances proliferation and differentiation of stem cells (Brand *et al.*, 2000). Such a system was found to be achieved by a combination of positive and negative feedback regulations between the signaling pathways of CLVs and WUSCHEL (WUS), a homeobox-family transcription factor (Laux *et al.*, 1996; Mayer *et al.*, 1998; Schoof *et al.*, 2000). CLV3, secreted from stem cells to peripheral cells, binds to CLV1 and/or CLV2/CRN receptor complex in the plasma membrane. This interaction subsequently triggers a signal that represses the expression of *WUS* in the organizing center that maintains stem cells. *WUS* in turn activates *CLV3* expression, and thereby promotes stem cell proliferation. (Mayer et al., 1998; Brand et al., 2000; Schoof et al., 2000; Muller et al., 2008; Ogawa et al., 2008).

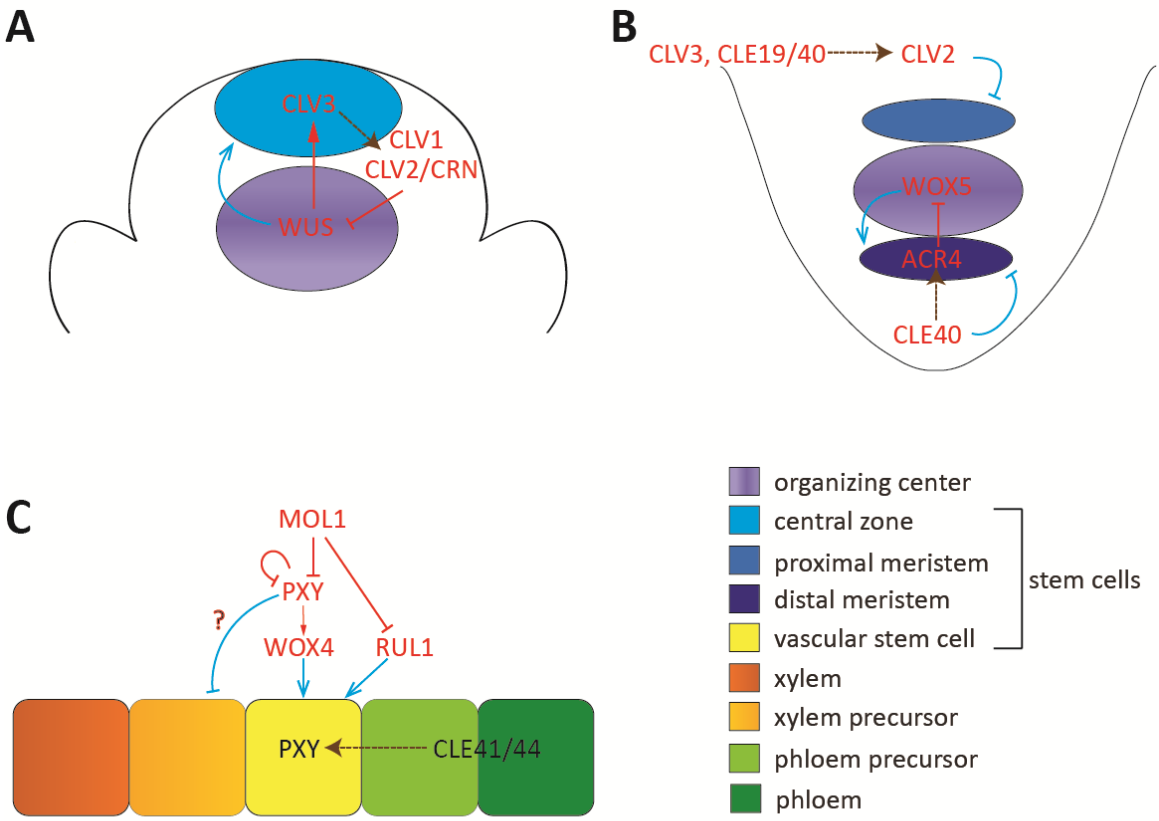

Figure 1.4. Intercellular regulatory network of CLE signaling pathways.

In the shoot **(A)**, root **(B)**, and vasculature **(C)** in *Arabidopsis*. Red arrows indicate transcriptional regulation; brown arrows, peptide intercellular movement and binding to the plasma membrane receptors; blue arrows, cell type specification.

A similar signaling pathway operates in the root apical meristem (Figure 1.4B). The feedback regulation between WOX5 (WUSCHEL-RELATED HOMEOBOX5) and CLE40 balances the distal stem cells and their differentiation (Stahl *et al.*, 2009). This regulation is distinctive from the pathway in the shoot system since CLE40 is secreted from differentiating cells to the stem cells. Subsequently, in stem cells, CLE40 is perceived by *ARABIDOPSIS CRINKLY4* (*ACR4*), a receptor-like kinase of the *CRINKLY4* family (De Smet *et al.*, 2008). CLE40-ACR4 defines the size and

location of stem cell population (Stahl *et al.*, 2009). Although CLV2 is not required to perceive CLE40 in maintaining distal stem cells, it seems to control the proximal stem cells in response to CLV3 and CLE19/40 (Fiers *et al.*, 2005; Stahl *et al.*, 2009).

In vascular stem cells, CLE41/44 (also known as TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF)) and CLV1-like receptor PXY (PHLOEM INTERCALATED WITH XYLEM) (also known as TDIF RECEPTOR (TDR)) are indispensable (Figure 1.4C) (Fisher and Turner, 2007; Hirakawa *et al.*, 2008). A high dose of CLE41/44 in *Arabidopsis* promotes the proliferation of vascular stem cells, whereas such an activity is inhibited in the loss-of–function mutant of *PXY*. Ligand-binding assay and photoaffinity labeling experiments suggested that CLE41/44 physically binds to PXY. Interestingly, it was found that *CLE41/44* is expressed in the phloem, while their proteins are also found in the adjacent vascular stem cells where *PXY* is expressed (Hirakawa *et al.*, 2008). Therefore, CLE41/44 peptides in the phloem seem to move to the adjacent vascular stem cells and then bind to the PXY in the plasma membrane. Subsequently, CLE41/44-PXY complexes activate the expression of *WOX4*, another homolog of *WUS*, and promote vascular stem cell proliferation (Hirakawa *et al.*, 2010). In the *wox4* null mutant, the size of vascular stem cell population is significantly reduced. This regulatory mechanism is in contrast to the negative regulation of CLE peptides on *WUS* and *WOX5* in shoot and root apical meristems. Furthermore, to ensure the balance between cell proliferation and differentiation activities of vascular stem cells, CLE signaling suppresses the expression of *PXY* as negative feedback regulation (Etchells and Turner, 2010).

A recent study using the genome-wide expression profiling in the cambium

cells identified two additional LRR-RLKs involved in the vascular stem cell activities (Agusti *et al.*, 2011). Mutants of these LRR-RLK genes, *MORE LATERAL GROWTH1* (*MOL1*) and *REDUCED IN LATERAL GROWTH1* (*RUL1*), respectively promotes and reduces the vascular stem cell proliferation. Genetic studies suggested that *MOL1* acts upstream of *PXY*. In the loss-of-function mutant of *MOL1*, both *PXY* and *WOX4* were up-regulated. In this study, an increase in the expression of *RUL1* was also detected in the *mol1*. Nevertheless, *MOL1* and *RUL1* are unlikely to function in a completely linear fashion, given that the null mutant of *RUL1* does not affect *WOX4* expression.

In addition to promoting vascular stem cell proliferation, CLE41/44-PXY pathway has also been found to control the differentiation of vascular stem cells in a polar manner (Fisher and Turner, 2007; Hirakawa et al., 2008; Etchells and Turner, 2010). As previously mentioned, under natural circumstances, xylem and phloem differentiate in opposite directions: xylem centripetally and phloem centrifugally. However, in the null mutant of *PXY*, such a vascular patterning is disturbed, exhibiting a phenotype that phloem and xylem are partially interspersed (Fisher and Turner, 2007). Ectopic expression studies and peptide feeding experiments indicated that CLE41/44-PXY complex formed on the phloem side promotes phloem differentiation and stem cell proliferation while represses xylem differentiation (Hirakawa *et al.*, 2008). The ectopic expression of CLE41/44 in the xylem domain disrupted such polar differentiation and led to the differentiation of phloem in the xylem pole (Etchells and Turner, 2010). However, *WOX4* does not seem to be involved in this pathway since *wox4* does not exhibit any disturbance in vascular patterning (Hirakawa *et al.*, 2010).

In summary, CLE-PXY signaling has multiple functions in the vascular development, which include defining polarity of cell differentiation and maintaining stem cell population (Etchells and Turner, 2010). CLE41/44, secreted from phloem, binds to PXY in the plasma membrane of vascular stem cells and then creates the boundary between the two cell types. In parallel, CLE-PXY signaling activates *WOX4* to maintain the characteristics of vascular stem cells. With the signal suppressing xylem differentiation in the vascular stem cells, the boundary between cambium and xylem is also established. However, the mechanism underlining the control of vascular polarity and xylem differentiation remains elusive.

Cytokinin

Cytokinins (CK) are adenine derivatives with an isopentenyl side chain (Werner et al., 2001; Miyawaki et al., 2006). They are produced in plants through the enzyme isopentenyltransferases (IPTs) (Figure 1.5) (Aloni et al., 2004; Aloni et al., 2005; Miyawaki et al., 2006). Plants have two classes of IPTs, ATP/ADP IPTs and tRNA IPTs. *Arabidopsis* has 9 *IPT* genes that encode either of these two classes. Based on mutant analyses, ATP/ADP IPTs seem to be the major source of cytokinins in *Arabidopsis*. In quadruple mutants of *IPT1*, *IPT3*, *IPT5* and *IPT7*, all encoding ATP/ADP IPTs, the endogenous cytokinin level is drastically reduced. Cytokinin signaling pathway has been well documented in *Arabidopsis* (Muller and Sheen, 2007a). It comprises of three main components: cytokinin receptors (AHKs), histidine phosphotransfer proteins (AHPs) and *Arabidopsis* response regulators (ARRs). AHKs

perceive cytokinins and initiate phosphorelay. Two types of ARRs positively (B-type ARR) or negatively (A-type ARR) regulate cytokinin signaling responses upon being phosphorylated by AHPs.

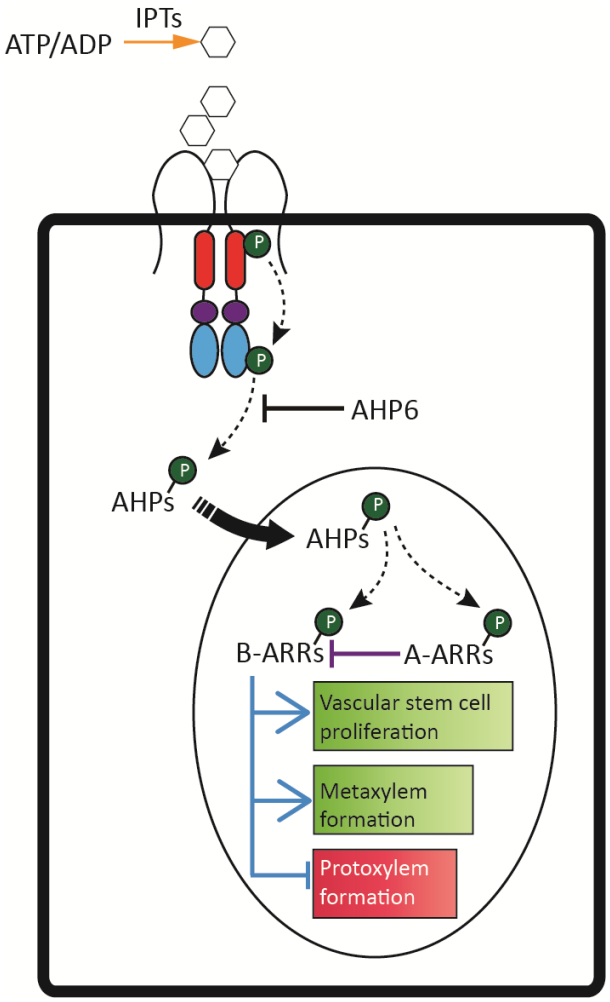

Figure 1.5. Cytokinin signaling pathway.

Cytokinin signaling commences with the binding of cytokinin to AHKs located on the plasma membrane. This triggers a phosphorelay cascade leading to the activation of ARRs that subsequently regulate cellular responses to cytokinin. Black square indicates plasma membrane; black circle, nuclear membrane; thick arrow bars, molecule movement; dotted black arrows, phosphorelay; purple T-heads, post-translational regulation; blue arrow, subsequent developmental processes; orange arrow, cytokinin biosynthesis. Cytokinin is shown as a hexagon.

It has been known for a while that a tight balance between cytokinin and auxin is required to induce xylem TE formation in the *in vitro* tissue culture system (Fosket and Torrey, 1969; Dalessan, 1973). However, its role in vascular development became more evident from the recent study of *WOODEN LEG* (*WOL,* also known as *ARABIDOPSIS HISTIDINE KINASE4* (*AHK4*)/ *CYTOKININ RESPONSE1* (*CRE1*)) mutant (Scheres et al., 1995; Mahonen et al., 2000). The *wol* mutation disrupts one of the three cytokinin receptors, *CRE1*/*AHK4*, in *Arabidopsis*. In the *wol* plants, all the vascular precursor cells in the root meristem differentiate into protoxylem and the vascular cell number is reduced drastically (Inoue *et al.,* 2001; Mahonen *et al.,* 2006). CRE1 possesses both kinase (in the presence of cytokinin) and phosphatase (in the absence of cytokinin) activities, similar to some of the bacterial histidine kinases. The *wol* mutation abolishes cytokinin binding ability of CRE1 and stabilizes its phosphatase activity. This results in a situation similar to when cytokinin signaling is absent. Depletion of cytokinin in the *Arabidopsis* root vascular stem cells by over-expressing *CYTOKININ OXIDASE2* resulted in a similar phenotype (Werner et al., 2001; Mahonen et al., 2006). Complete knock-out of all the three cytokinin receptor genes, *AHK2*, *AHK3* and *AHK4*, also gave similar conversion of vascular stem cells to the protoxylem (Higuchi et al., 2004; Nishimura et al., 2004; Muller and Sheen, 2007b). A similar phenotype resulted in the triple mutant of B type-ARRs, *ARR1*, *ARR10,* and *ARR12* (Argyros *et al.,* 2008; Ishida *et al.,* 2008). These results suggest that the default pathway of vascular stem cells in the absence of cytokinin is the differentiation into protoxylem and that the cytokinin signaling maintains a subset of vascular stem cells by inhibiting their differentiation.

The role of cytokinin in vascular development was further unveiled with *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6* (*AHP6*). Cytokinin signaling pathway shares analogy with two-component signaling systems in bacteria (West and Stock, 2001). Cytokinin receptors, upon binding to cytokinin, auto-phosphorylate and subsequently relay the phosphate to AHPs. AHPs subsequently migrate into nuclei and transfer their phosphates to ARRs. In *Arabidopsis*, there are five true phosphotransfer proteins (AHP1-5), which are positive regulators of cytokinin signaling, and one pseudo-phosphotransfer protein, AHP6, which acts as a negative regulator of cytokinin signaling (Mahonen *et al.*, 2006). Unlike other AHPs, AHP6 lacks a histidine residue essential for phosphotransfer. This characteristic makes AHP6 unable to transfer its phosphate group to ARRs upon being phosphorylated. Consistent with this feature, a double mutant of *WOL* and *AHP6* represses the phenotype of *wol* by recovering metaxylem formation.

In *ahp6*, protoxylem does not develop in the root, similar to what a high level of cytokinin does to the wild-type roots. Recently, certain CLE peptides, such as CLE10, were found to suppress protoxylem formation in the *Arabidopsis* root (Kondo *et al.*, 2011). It turned out that these peptides, sensed by CLV2, enhance the cytokinin sensitivity by suppressing the expression of *ARR5* and *ARR6*, A-type ARRs that negatively regulate cytokinin signaling. In addition to the interplay between the cytokinin signaling and CLE-RLK pathway, the mutually inhibitory regulation between cytokinin and auxin seems to balance between differentiated and un-differentiated states in the vascular stem cells (Bishopp *et al.*, 2011a; Bishopp *et al.*, 2011b). In the studies of vascular stem cells in the *Arabidopsis* root, high cytokinin in

the root procambium was found to promote the PAT towards xylem precursors by inducing the expression of *PIN3* and *7*. This results in the auxin maxima in the xylem precursors where auxin signaling promotes expression of *AHP6* and differentiation of protoxylem in the periphery of xylem axis. When a balance between cytokinin and auxin was broken with high cytokinin treatment in the roots, PIN3 and 7 domains expanded to xylem precursors, and thereby auxin maxima retracted. This resulted in the inhibition of *AHP6* expression and the differentiation of vascular stem cells into protoxylem. Consistently, when the PAT was blocked in knockout mutants of *PIN3* and *7*, auxin maxima expanded to vascular stem cells adjacent to xylem precursors, where extra protoxylem strands developed. These studies highlight a complex crosstalk among signaling processes that is the key driver of vascular stem cell regulation.

In *Populus*, experiments with ectopic expression of *CYTOKININ OXIDASE2* suggested a positive correlation between cytokinin levels and cell division activities in the vascular stem cells (Nieminen *et al.*, 2008). Transgenic trees with reduced endogenous cytokinin severely compromised vascular stem cell divisions and the secondary growth leading to the thinner stems. Using quadruple mutants of *IPT1*, *IPT3, IPT5* and *IPT7* in *Arabidopsis*, Matsumoto-Kitano *et al.* also demonstrated a dose-dependent effect of cytokinin on cell division activities in the vascular stem cells (Matsumoto-Kitano *et al.*, 2008). In *ipt1 ipt3 ipt5 ipt7*, plants showed reduced thickening of roots and stems, similar to what was observed in transgenic *Populus*. Further investigations showed that vascular cambium formation fails in stems and the onset of the secondary growth is delayed in the roots of *ipt1 ipt3 ipt5 ipt7*.

Interestingly, while not displaying any other obvious defects, *ipt3* single mutant plants exhibited reduced secondary growth (Matsumoto-Kitano *et al.*, 2008). Based on this observation, vascular stem cell divisions and secondary growth appear to be sensitive to alterations in the cytokinin level. Ectopic expression of *LONELY GUY* (LOG), a cytokinin activating enzyme, caused increased cell divisions in leaf vascular tissues (Kuroha *et al.*, 2009).

Brassinosteriods, gibberellin, and ethylene

Brassinosteriods (BRs) are steroid-based plant hormones vital for cell elongation and differentiation (Clouse, 1996; Nemhauser *et al.*, 2004). The role of BRs in vascular development was supported by phenotypes of BR signaling mutants in *Arabidopsis*, rice, and *Zinnia* (Mori et al., 2002; Cano-Delgado et al., 2010). The BR deficient mutant, *constitutive photomorphogenic dwarf* (*cpd*) was first identified to be defective in BR biosynthesis and has exhibited severe decline in xylem biogenesis (Szekeres *et al.*, 1996). The *dwarf7*, another BR biosynthesis mutant discovered in *Arabidopsis*, showed similar anomalies (Choe *et al.*, 1999). Consistently, chemical inhibition of BR signaling using brassinazole caused aberrant vascular patterning including reduction in xylem and an increase in phloem cells (Asami *et al.*, 2000). These findings suggested that BR signaling promotes xylem differentiation. Studies using uniconazole, another BR biosynthesis inhibitor, indicated that BRs are involved in specific stages of xylem TE development such as secondary cell wall formation and programmed cell death (PCD) (Yamamoto *et al.*, 1997). Yamamoto *et al.* reported a stage specific, dramatic

up-regulation in BR and related metabolites during TE differentiation, further augmenting the role of BR in xylem biogenesis (Yamamoto *et al.*, 2007).

BRs are perceived by binding to three plasma membrane-localized LRR-RLKs (BR receptors): BRASSINOSTEROID INSENSITIVE1 (BRI1), BRASSINOSTEROID INSENSITIVE LIKE1 (BRL1), and BRL3. Among them, a knockout mutant of *BRL1* altered vascular patterning with an increase in phloem and a decrease in xylem domains (Cano-Delgado *et al.*, 2004). In addition, severe vascular defects associated with *bri1 brl1 brl3* further supported the role BRs have in vascular morphogenesis. *BRL1* and its homolog *BRL3* are expressed specifically in vascular tissues. The *BRL2*/*VASCULAR HIGHWAY1*, another homolog of *BRI1*, seems more involved in the specification of vascular stem cells in the leaves (Clay and Nelson, 2002). Despite of its similarity to BRI1, BRL2 did not show binding activities to BRs (Cano-Delgado *et al.*, 2004). Based on these findings, it was proposed that BRs ensure the proper ratio between phloem and xylem by regulating specification of xylem cells from vascular stem cells (Cano-Delgado *et al.*, 2004). A recent approach based on mathematical modeling shows that a coordinated action of auxin and BR signaling guides vascular bundle patterning in *Arabidopsis* stems (Ibanes *et al.*, 2009). This model predicted that BRs influence vascular bundle number primarily through their control on proliferation of founding cells of vascular bundles in young stems. Various BR signaling mutants such as *bri1* and *cpd* generated fewer founding cells, therefore formed fewer vascular bundles than wild type plants.

Other phytohormones such as gibberellin (GA) and ethylene have also been implicated in the development of vascular tissues. GA's stimulatory role in vascular

stem cell proliferation came from studies on *Populus* (Bjorklund *et al.*, 2007; Dettmer *et al.*, 2009). Likely interaction between auxin and GA signaling pathways and their regulatory function in vascular stem cell activities were also proposed (Wang et al., 1997; Bjorklund et al., 2007). Results from *Populus* indicate that GA influences cambium function through its positive influence on auxin transport into vascular stem cells (Bjorklund *et al.*, 2007; Elo *et al.*, 2009). In recent studies of *Arabidopsis*, GA was found to act as a mobile signal that promotes hypocotyl xylem expansion (Ragni *et al.*, 2011). Ethylene's regulatory function in vascular stem cell activities was also reported (Telewski and Jaffe, 1986; Junghans et al., 2004). Exogenous treatment of ethylene enhanced cambium proliferation in the hybrid aspen (*Populus tremula* x *tremuloides*) (Love *et al.*, 2009). In these plants, xylem grew faster under high endogenous ethylene, likely due to an increased vascular stem cell activity.

Taken together, it appears that two or more of these hormonal signaling pathways coordinate with each other and precisely balance the vascular morphogenesis. In coming years, more detailed mechanisms of such hormonal interactions during vascular tissue development and pattern formation will be discovered.

Cell type specification in vascular stem cells

Mobile microRNAs as positional signals in xylem patterning

In plants, cell fate determination relies more on position than lineage (Benfey and Scheres, 2000). When wounded or stressed, plant cells can switch their identities in

response to new positional signals (Stewart and Dermen, 1975; Fukuda and Komamine, 1980). Such a mechanism allows plants to develop with remarkable plasticity. However, it also requires extensive communications between cells to obtain and provide precise positional information. Plasmodesmata are the major gates for cell-to-cell communication in plants. They aid in establishing positional information through exchange of signaling molecules, which are crucial for cell type specification and differentiation.

Various transcription factors, peptides and small non-coding RNAs act as signaling molecules in this process. Among them, small RNAs (ranging between 21-24 nucleotides in length) have drawn much interest in the last decade. In plants, the major species of small RNAs are the miRNAs and siRNAs (Bartel, 2004; Mallory and Vaucheret, 2006; Willmann and Poethig, 2007). siRNAs largely function in plant defense-related processes by targeting foreign RNA species. By contrast, miRNAs are primarily responsible for regulating endogenous genes, many of which encode regulatory proteins such as transcription factors. *MiRNA 165/6*, in particular, have been found to play crucial roles in the development of vascular tissues and lateral organs by regulating the levels and spatial distributions of HD-ZIP III transcription factors (Emery et al., 2003; Mallory et al., 2004; McHale and Koning, 2004; Kim et al., 2005). Recent studies further revealed that *miRNA 165/6* act as positional signals and contribute to the xylem cell type patterning in the root (Carlsbecker *et al.*, 2010; Miyashima *et al.*, 2011).

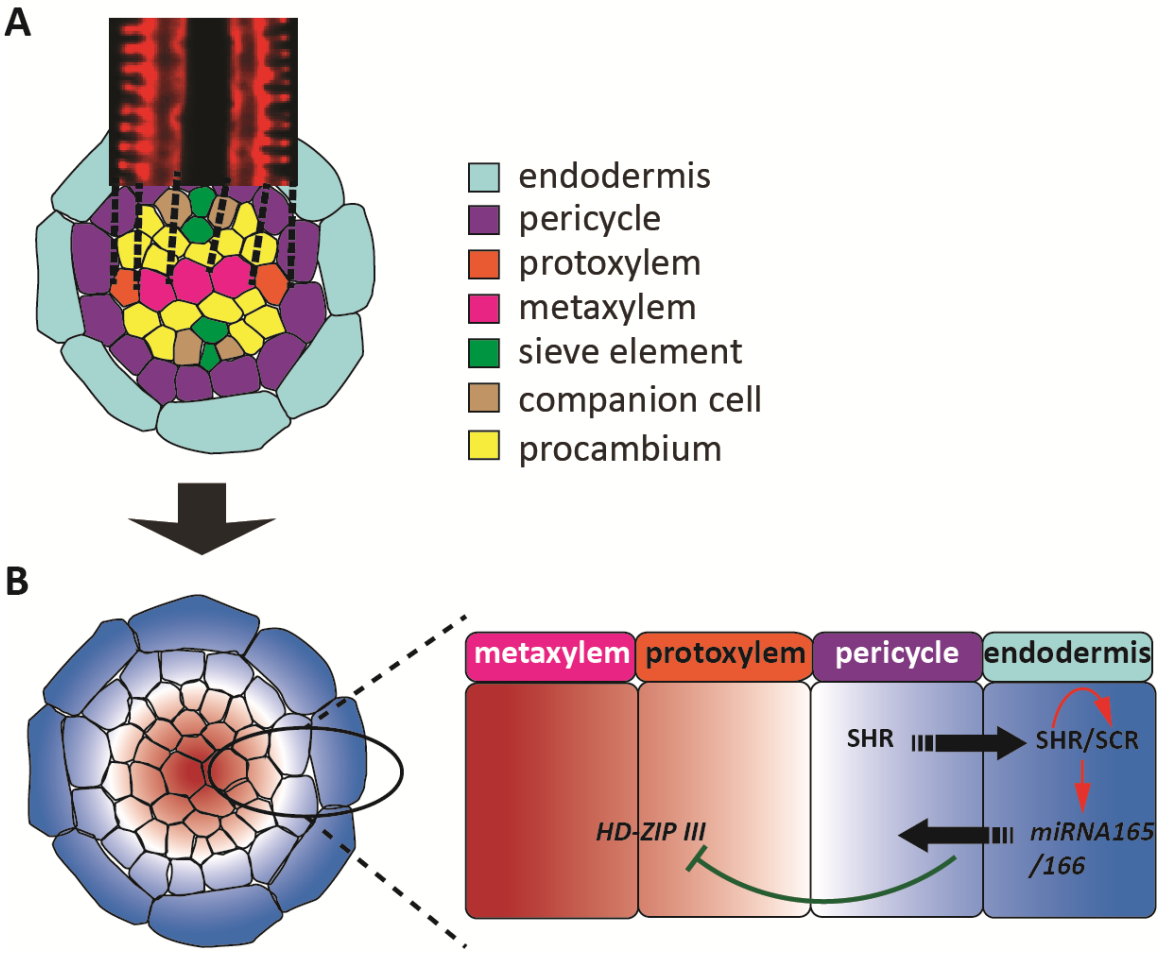

Figure 1.6. miRNA-HD-ZIP III pathway for xylem patterning.

(A) Schematic representation of an *Arabidopsis* root transverse section. Dashed lines link to a xylem image taken with a confocal microscope. **(B)** miRNA 165/6 generated by SHR/SCR in the endodermis moves to the stele. Color intensity indicates the distribution of miRNA 165/6 and HD-ZIP IIIs (dark blue for a high miRNA level; dark red for high HD-ZIP IIIs). Red arrows, transcriptional regulation; green arrow, post-transcriptional regulation; thick arrow bars, molecule movement.

In the root meristem, a cylindrical structure of vascular tissues is successively layered by pericycle, endodermis, cortex and epidermis from the center to the periphery (Figure 1.6A). As explained previously, two types of xylem TEs, metaxylem and protoxylem respectively develop in the center and periphery of vascular cylinder. It was found that HD-ZIP III transcription factors specify these two TE cell fates in a dosage-dependent manner: a high dose of HD-ZIP III transcription factors specifies metaxylem whereas a low dose specifies protoxylem. Therefore, for the proper xylem patterning, HD-ZIP III transcription factors should be distributed at a low level in the periphery of vascular cylinder and at a high level in the center. It turned out to be mobile miRNA165/6 from the endodermis that establishes such gradual distribution of HD-ZIP IIIs (Figure 1.6B) (Carlsbecker *et al.*, 2010). The same studies identified transcription factors that activate the expression of *miRNA165/6* in the endodermis. SHORT-ROOT (SHR), produced in the vascular cylinder, move to the initial cells of endodermis and cortex to activate the expression of *SCARECROW* (*SCR*) (Nakajima *et al.*, 2001). Subsequently, SHR and SCR together promote the expression of two genes that encode *miRNA165/6*. *MiRNA165/6*, generated in the endodermis, then diffuse into the vascular cylinder and direct xylem cell fate specification and patterning by aforementioned post-transcriptional regulation. When these regulatory processes were blocked in a miRNA resistant mutant (*phb-7d*) of *PHABULOSA* (*PHB*), one of the five *HD-ZIP III* genes, or a knock-out mutant of *SHR* and *SCR*, only metaxylem developed in place of protoxylem (Carlsbecker *et al.*, 2010).

Emerging transcriptional regulatory networks in vascular cell type specification

As been described, multiple signaling pathways participate in the establishment and maintenance of vascular stem cells. During plant growth, some of these stem cells are specified and then they differentiate into xylem and phloem. Extensive studies using genetic and genomic tools have revealed several transcriptional regulatory networks that operate during vascular cell type specification (Figure 1.2).

The first step of xylem development is to establish the organization of cell types. In the root, metaxylem is specified in the center of the vascular cylinder and protoxylem is at the periphery. In this process, HD-ZIP III transcription factors play a central role. In addition to the aforementioned *PHB*, *REV* and *ATHB8*, there are two more genes in this family, *PHAVOLUTA* (*PHV*) and *CORONA* (*CNA*). Even though none of the single mutants of these genes displayed abnormal xylem patterning, various triple mutants had metaxylem partially switched to ectopic protoxylem. This suggests that when the level of HD-ZIP III transcription factors is reduced to a certain threshold, the metaxylem identity starts being lost. Consistent with this idea, various quadruple mutants failed to form metaxylem and developed only protoxylem (Carlsbecker *et al.*, 2010). Strikingly, the quintuple mutant did not generate any xylem. In summary, HD-ZIP III transcription factors regulate de-novo xylem TE formation and specify TE types in a dosage-dependent manner. KANADI (KAN) family transcription factors regulate lateral organ formation antagonistically to HD-ZIP IIIs (Bowman *et al.*, 2002). A recent study suggests that KANs also antagonize HD-ZIP IIIs during vascular development. Ectopic expression of *KAN1* in vascular stem cells blocked vascular tissue formation in *Arabidopsis* (Ilegems *et al.*, 2010).

This was found to be due to negative effects of KAN1 on *PIN1* expression and its polar localization in the vascular stem cells. PAT led by PIN1 positively regulates *ATHB8* expression (Donner *et al.*, 2009) (Figure 1.3).

Studies on xylem differentiation were largely facilitated by the development of *in vitro* xylogenesis in *Zinnia elegans* (Ye and Varner, 1993; Hosokawa et al., 2001). In this system, cells isolated from leaves can trans-differentiate into xylem TEs with the external application of auxin and cytokinin. This *in vitro* xylogenesis system has provided a simple model for studying specification and differentiation of TEs (Fukuda and Komamine, 1980; Demura et al., 2002). The same approach was recently made using *Arabidopsis* leaf cells to profile gene expression dynamics during TE differentiation at a genome-wide level (Kubo *et al.*, 2005). In this study, two transcription factors, VASCULAR RELATED NAC-DOMAIN 6 (VND6) and VND7, were found to promote meta- and protoxylem formation, respectively (Figure 1.2B). Promoter analysis showed that *VND6* is expressed specifically in the metaxylem, while *VND7* in the immature protoxylem, suggesting their cell-autonomous regulation in TE specification. Furthermore, *VND6* and *VND7*, ectopically expressed in *Arabidopsis* and *Populus*, respectively promoted the formation of ectopic meta- and protoxylem (Kubo et al., 2005; Yamaguchi et al., 2010b). These suggest that VND6 and 7 are master regulators of xylem TE specification/differentiation.

VND6 and 7 up-regulate the expression of *AS2-LIKE19* (*ASL19*)/*LBD30* and *ASL20/LBD18* that encode two plant-specific, ASYMMETRIC LEAVES2 (AS2)/LATERAL ORGAN BOUNDARIES DOMAIN (LBD) transcription factors (Soyano *et al.*, 2008). Similar to *VND6* and *7*, the ectopic expression of *ASL19* and

ASL20 induced transdifferentiation of nonvascular cells into TEs. Interestingly, not only ASL20/LBD18 is up-regulated by VND7, but it also elevates *VND7* expression in return, creating a positive feedback loop to promote xylem differentiation.

Specification and differentiation of xylem are temporally regulated processes. A recent study by Yamaguchi *et al*. shows that VND-INTERACTING2 (VNI2), a novel NAC domain transcription factor, mediates such process by regulating activities of VND7 (Yamaguchi et al., 2010a). They found that VNI2 interacts with VND7, thereby negates the transcriptional activity of VND7. Unlike most of the NAC domain proteins that are transcriptional activators, VNI2 seems to be a transcriptional repressor. In *Arabidopsis* root, *VNI2* starts its expression in the procambial cells whereas *VND7* expression initiates later in the immature xylem TE. Such a temporal discrepancy in the expression of these two genes seems essential for proper TE differentiation. When *VNI2* expression domain was engineered to overlap with *VND7*'s by expressing it under the *VND7* promoter, xylem differentiation frequently failed. Therefore, in the xylem precursors, VNI2 binds to the VND7 activation domain and prevents the transcriptional activity of VND7, which otherwise might result in precocious differentiation of TEs.

Recently, additional downstream transcription factors that are responsible for the later stage of xylem development were identified. Among them, two closely related MYB family genes, *MYB46* and *MYB83*, are direct targets of VND6 and 7 (Zhong et al., 2007; McCarthy et al., 2009). Genetic evidence suggested that MYB46 and MYB83 are key regulators in the secondary cell wall biosynthesis during xylem differentiation. Over-expression of *MYB46* and *MYB83* induced a compendium of

transcription factors including *MYB42*, *MYB43*, *MYB52*, *MYB54*, *MYB58*, *MYB63*, *MYB85*, *MYB103*, *SND3* (*SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN3*) and *KNAT7* (*KNOTTED-LIKE HOMEOBOX OF ARABIDOPSIS THALIANA7*) and resulted in the ectopic deposition of secondary cell walls. Among them, MYB58, MYB63, and MYB85 were shown to activate the biosynthesis of lignin, a major component of TE secondary cell walls (Zhong *et al.*, 2008; Zhou *et al.*, 2009). Interestingly, *MYB103*, *SND3* and *KNAT7* are also direct targets of VND6/7, suggesting a feed-forward loop regulation that might speed-up responses to upstream master regulators.

As mentioned above, PCD is closely coupled with secondary cell wall synthesis during xylem differentiation. Pyo *et al.* found that a novel 11-bp *cis*-element TERE (tracheary-element-regulating *cis*-element) in the promoter of *Zinnia cysteine protease 4* (*ZCP4*) is sufficient for its TE-specific expression (Pyo *et al.*, 2007). Further analysis revealed that TERE is a common and essential promoter motif present in promoters of many genes involved in the secondary cell wall formation and PCD including *XCP1* (*XYLEM CYSTEINE PROTEASE1*), *XCP2*, and *XSP1* (*XYLEM SERINE PROTEASE1*). Interestingly, VND6 and 7 were found to up-regulate these TERE-containing TE-specific PCD genes, confirming the link between PCD and secondary cell wall synthesis during xylem development.

In contrast to xylem, our knowledge on phloem development is still limited. One of the best-characterized regulators in phloem development is the ALTERED PHLOEM DEVELOPMENT (APL), a coiled-coil MYB family transcription factor. *APL* starts to express in the phloem precursor cells and continues its expression in

mature sieve elements (Bonke *et al.*, 2003). The knock-out mutant of *APL* shows severe vascular patterning defects, which are due to failure in the formation of phloem sieve elements and companion cells via asymmetric cell division of phloem precursor cells. Furthermore, in *apl* mutants xylem cells frequently develop in place of phloem. Ectopic expression of *APL*, though did not switch xylem fate into phloem fate, inhibited xylem differentiation. Taken together, APL seems to have dual roles: promote phloem formation and repress xylem identity at the phloem pole. Recently, *APL* ortholog in *Populus* was shown to be up-regulated by LBD1, an AS2/LBD family transcription factor in the cambium (Yordanov *et al.*, 2010). Ectopic expression of *LBD1* in *Populus* enhanced secondary growth as a result of an increase in phloem formation (Figure 1.2A).

Other small molecules at work in vascular stem cells

The involvement of xylogen, a proteoglycan-like factor in xylem differentiation, was first discovered in the *Zinnia* xylogenesis system (Motose *et al.*, 2004). Xylogen exhibits properties of both arabinogalactan proteins and nonspecific lipid-transfer proteins and are reported to accumulate in vascular tissues as well as in meristem cells. Expression analysis suggested that xylogen might function as an intercellular signaling molecule, primarily involved in the differentiation of vascular stem cells to xylem (Motose et al., 2004; Fukuda et al., 2007). There are two xylogen genes (*XYLOGEN PROTEIN1* (*XYP1*) and *XYP2*) in *Arabidopsis*. A double knock-out mutant of *XYP1* and *XYP2* displayed discontinuous and thicker veins (Motose *et al.*, 2004; Sieburth

and Deyholos, 2006). However, it is unlikely that xylogen is essential for vascular morphogenesis since *xyp1xyp2* plants still forms vascular tissues.

Irregular vascular tissue formation, observed in the mutant of *ACAULIS5(ACL5)/THICKVEIN* in *Arabidopsis*, revealed the involvement of polyamines in vascular development (Hanzawa et al., 1997; Clay and Nelson, 2005) (Hanzawa *et al.*, 2000). Polyamines are small polycationic molecules found in most of the living organisms (Takahashi and Kakehi, 2010; Vera-Sirera *et al.*). These polyamines play essential roles in many cellular processes. *ACL5* which was originally described as a gene encoding a spermine synthase encodes a thermospermine synthase, which generates thermospermine from spermidine (Hanzawa et al., 2000; Knott et al., 2007; Kakehi et al., 2008). Muniz *et al.* found that ACL5 prevents premature PCD so that the cell can accomplish full expansion and cell wall lignifications (Muniz *et al.*, 2008). Without *ACL5*, xylem precursors undergo PCD precociously before they acquire xylem TE identity and function. Exogenous application of thermospermine or norspermine was able to rescue the *acl5* phenotype. Furthermore, thermospermine or norspermine upregulated *ATHB8, PHB*, and *SAC51*, a gene encoding a bHLH transcription factor found from the suppressor mutant screening of *acl5* (Imai *et al.*, 2006; Kakehi *et al.*, 2010). Based on these, thermospermine and norspermine seem to act as signaling factors for timing xylem TE differentiation program.

Defective vein joining in the *cotyledon vascular pattern1* (*cvp1*) mutant, resulted from the absence of STEROL METHYLTRANSFERASE2 (SMT2), suggests the action of sterols in leaf vein patterning (Carland *et al.*, 2002). Sterol(s) that affect

the vein growth are not BRs since vein defects in *cvp1* are not complemented by BR application, and *cvp1* and other *smt* mutants do not alter the BR profiles (Carland *et al.*, 2010). It is also noteworthy that HD-ZIP III transcription factors contain putative sterol binding domains though their sterol ligands remain elusive (Ponting and Aravind, 1999; Schrick *et al.*, 2004). Contribution of sterols to the maintenance of PAT was proposed based on the mutant phenotype of *STEROL METHYLTRANSFERASE1* (*SMT1/ORC*) (Scheres *et al.*, 1996; Willemsen *et al.*, 2003). Consistently, mutants for genes encoding sterol biosynthetic enzymes, including *hydra1* (*hyd1*), *fackel* (*fk*)*/hyd2*, and *cyclopropylsterol isomerase 1* (*cpi1*) caused mis-localization of PIN protein and ectopic *ATHB8* expression (Men *et al.*, 2008; Pullen *et al.*, 2010).

Other molecules such as inositol, glutathione, and glutathione disulfide are also known to regulate vascular tissue continuity in leaves (Henmi et al., 2001; Steynen and Schultz, 2003; Carland and Nelson, 2004; Carland and Nelson, 2009; Naramoto et al., 2009; Robles et al., 2010). Phosphoinositides, for example, have been shown to regulate vascular continuity by promoting VAN3 ARF-GAP activity and its trans-Golgi localization (Naramoto *et al.*, 2009). A double mutant of *CVP2* and *CVP2LIKE1* (*CVL1*), both of which encode inositol polyphosphate 5' phosphatases, phenocopies the *van3* mutant showing severe vascular discontinuity (Carland and Nelson, 2009; Naramoto *et al.*, 2009). A protein localization study showed that the targeting of VAN3 to trans-Golgi are lost in *cvp2 cvl1* (Naramoto *et al.*, 2009). Consequently, in these mutants, the recruitment of PIN1 to the membrane and the maintenance of *PIN1* expression were disrupted (Naramoto et al., 2009; Hou et al., 2010). It seems that

phosphoinositides affect the subcellular localization of VAN3 by directly binding to its PH domain, which is critical for its function. An amino acid change in the highly conserved residue of VAN3 PH domain resulted in strong vascular bundle defect phenotype (Carland and Nelson, 2009). Furthermore, VAN3 protein without PH domain lost its trans-Golgi localization mimicking VAN3 localization in the *cvp2* mutant (Naramoto *et al.*, 2009).

Future perspectives

In the last few decades, there has been considerable progress in our understanding of the biology behind plant vascular stem cells. Nevertheless, we are still far from having a holistic view. This is mainly due to the fragmented nature of information that is currently available. Various signaling pathways involved in vascular morphogenesis have been identified. However, it remains to be resolved how these are connected. Gene regulatory networks for specifying vascular cell types and positional signals responsible for positioning them need to be identified and connected. An issue such as the existence of a vascular stem cell specific organizing center, comparable to an organizing center in the shoot apical meristem or the quiescent center in the root apical meristem, remains to be answered.

New-generation genomic tools, such as cell-type specific transcriptome profiling, cell-type specific marker lines, high-throughput gene expression perturbation and genome sequencing, will facilitate constructing a comprehensive picture of plant vascular development and evolution. A better understanding of plant

vascular stem cell regulation has a great potential for advances in agriculture especially for enhancing biomass yields. This is particularly relevant in today's world, which faces considerable challenges of solving global warming and food and energy security.

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Chapter 2 Cell-to-cell communication mediated by AHL3/4 sets the boundaries between xylem and procambium in the early stage of vascular development

Jing Zhou and Ji-Young Lee[1]

Abstract

Local cell-to-cell communications play crucial roles during plant development. Mobile transcription factors are important signals that mediate intercellular communications. In this study, we report that AHL4 proteins, produced in the procambium, move to xylem precursors to serve as an intercellular signal regulating vascular patterning in the root. In *ahl4* mutant, boundaries between xylem and procambium are disturbed, resulting in the expansion of xylem domain into the procambium. When the mobility of AHL4 from procambium to xylem precursors was restricted by increasing the size of AHL4-tandemYFPs fusion proteins, the extra xylem formation in the *ahl4* mutant could not be suppressed successfully, suggesting that movement of AHL4 is crucial for setting up the boundaries. In addition, we found that AHL4 forms protein complexes with its closest homolog AHL3. Together, AHL4

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and AHL3 define the boundaries between xylem and procambium to limit the xylem axis to one-cell width.

Introduction

Morphogenesis in multicellular organisms is a tightly regulated process. Unlike animals growing from a body plan which is established during embryogenesis, plants grow by forming new organs throughout their life span. In this process, plants largely rely on positional information rather than lineage to control the pattern formation in a temporal and spatial manner (reviewed in (Scheres and Benfey, 1999; De Smet and Beeckman, 2011)). Since plant cells are confined by rigid cell walls, intercellular communications in a long or short distance play crucial roles in providing developmental cues. Communications between neighboring cells in particular contribute to specifying cell types and defining boundaries between them. Many forms of molecules including proteins, RNAs and small molecules serve as signals that mediate intercellular communications (reviewed in (Kurata et al., 2005a; Hirakawa et al., 2011; Van Norman et al., 2011)).

Vascular tissues serve as a major conductive and supporting system in vascular plants. Xylem and phloem, major tissues in the vascular system, are generated from procambium and cambium, stem cell populations specialized in the formation and growth of vascular tissues. Vascular system in the *Arabidopsis* root is organized in a simple manner, therefore serves as a good model for studying its developmental processes. In the Arabidopsis root, a single-cell wide xylem axis is composed of two xylem vessel types, protoxylem in the periphery and metaxylem in the center. Two phloem poles are localized perpendicular to the xylem axis, and procambium cells are between the xylem and phloem (Figure 2.1A).

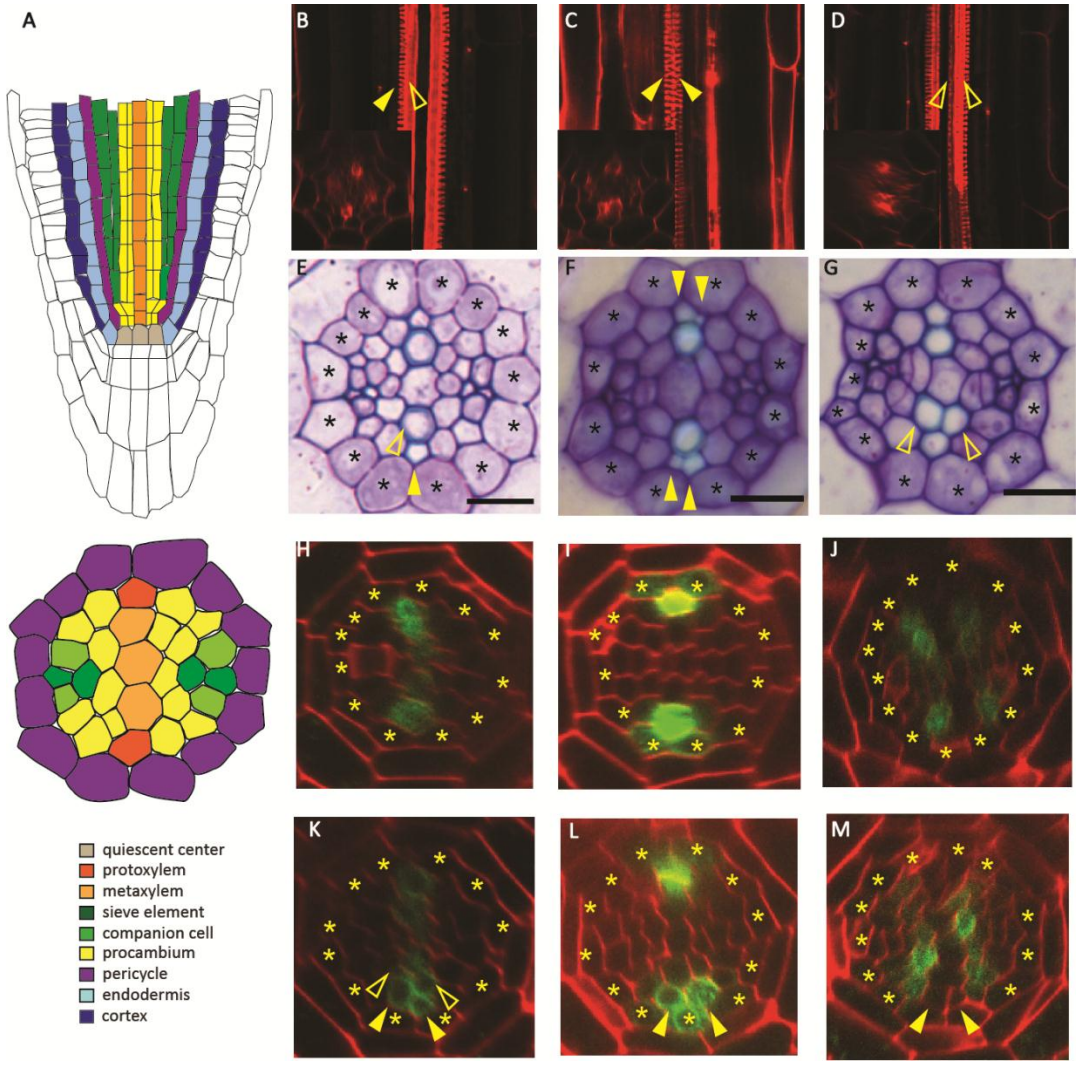


Figure 2.1. Vascular patterning in *ahl4-1*.

(A) Schematic representations of the *Arabidopsis* root meristem (longitudinal) and stele (transverse). Comparison of vascular pattern by fuchsin stained xylem **(B-D)** and toluidine blue stained root section **(E-G)** between wild type **(B, E)** and *ahl4-1* **(C, D, F, G)**. Marker analysis using *pTMO5::erGFP* **(H, K)**, *pAHP6::erGFP* **(I, L)**, and *pARR5::erGFP* **(J, M)** to compare the xylem precursor domain between wild type **(H-J)** and *ahl4-1* **(K-M)**. Scale bars, 10 µm; asterisks, pericycle position; arrow heads, protoxylem; hollow arrow heads, metaxylem.

During vascular development, the formation of xylem and phloem is controlled by cell-type specific developmental regulatory networks, while procambium/cambium remains undifferentiated. To regulate the polarity and boundaries between xylem and phloem, cell-to-cell communications are indispensible. PHLOEM INTERCALATED WITH XYLEM (PXY), CLV1-like Leucine-Rich-Repeat (LRR) Receptor-Like Kinases (RLK) and CLE41/44, CLAVATA3/ENDOSPERM SURROUNDING REGION (CLE) members are key regulators in this process (Fisher and Turner, 2007; Hirakawa et al., 2008). In this regulation, CLE41/44 peptides move from the phloem to the cambium cell where they bind to PXY (Hirakawa et al., 2008; Etchells and Turner, 2010; Hirakawa et al., 2010). The CLE/PXY complex in the cambium cells further activates as-yet unknown signals to maintain the boundary between cambium and xylem (Fisher and Turner, 2007; Hirakawa et al., 2008). Such a regulation by CLEs and PXY has been only observed in the mature root and hypocotyls but not in the root meristem.

Nevertheless, in the root meristem, the vascular pattern is established long before vascular cell types become morphologically distinctive. It has been reported that high cytokinin and high auxin are distributed in the root meristem in a mutually exclusive manner. Such a distribution pattern was suggested to contribute to delineating procambium and protoxylem domains (Bishopp et al., 2011a). A high level of auxin in the protoxylem precursor cells was found to promote the expression of *AHP6* (Bishopp et al., 2011a). AHP6, a cytokinin signaling inhibitor, in turn suppresses the cytokinin signal to establish protoxylem precursor cell domain in the root meristem (Mähönen et al., 2006). High cytokinin in the procambium facilitates

this process by regulating auxin transporters that channel auxin maxima to the protoxylem precursors and thereby maintains procambium cell identity. Breaking a balance between cytokinin and auxin resulted in increasing or reducing the formation of protoxylem strands (Bishopp et al., 2011a; Bishopp et al., 2011b). Collectively, cytokinin and auxin inhibit each other to draw the boundaries between procambium and protoxylem (Bishopp et al., 2011a; Bishopp et al., 2011b).

In this study, we present the discovery of two closely-related AT-hook family members, AHL3 and 4 that regulate boundaries between the procambium and xylem precursor cells in the Arabidopsis root meristem. Intriguingly, AHL3 and 4 proteins move between cells. Our investigation suggests that the cell-to-cell communication mediated by mobile AHL3 and 4 is critical for setting up the boundary between procambium and xylem axis.

Results

AHL4 regulates boundaries between the xylem and the procambium

In the root, xylem precursors are established in the meristem very close to the underlying quiescent center (QC) (Mähönen et al., 2000). In the wild-type Arabidopsis root, five or six xylem precursor cells form a single row of xylem axis. These cells in the periphery and center of the axis respectively differentiate into protoxylem and metaxylem vessels (Figure 2. 1B and 2.1E).

While analyzing T-DNA insertion lines of transcription factors that are enriched in the xylem precursors in the *Arabidopsis* root (Brady et al., 2007), we

noticed that a T-DNA insertion line (*SALK_124619*; *ahl4-1* hereafter) of an AT-hook family gene *AHL4* (*AT5g51590*) showed a defect in the xylem patterning. In the *ahl4-1* where a T-DNA is inserted into the third exon of *AHL4* (Figure 2.2B), additional strands of protoxylem (Figure 2.1C and 2.1F; filled arrow heads) or metaxylem (Figure 2.1D and 2.1G; hollow arrow heads) were observed. Real-time RT-PCR confirmed that the *AHL4* expression is reduced by 99% in the *ahl4-1* mutant compared to the wild-type, indicating that *ahl4-1* is likely a null mutant (Figure 2.2A). To find whether AHL4 has an additional function in the vascular patterning, we examined the phloem in the *ahl4-1*. However, our histological analysis did not indicate any obvious defect in phloem patterning (Figure 2.1E to 2.1G).

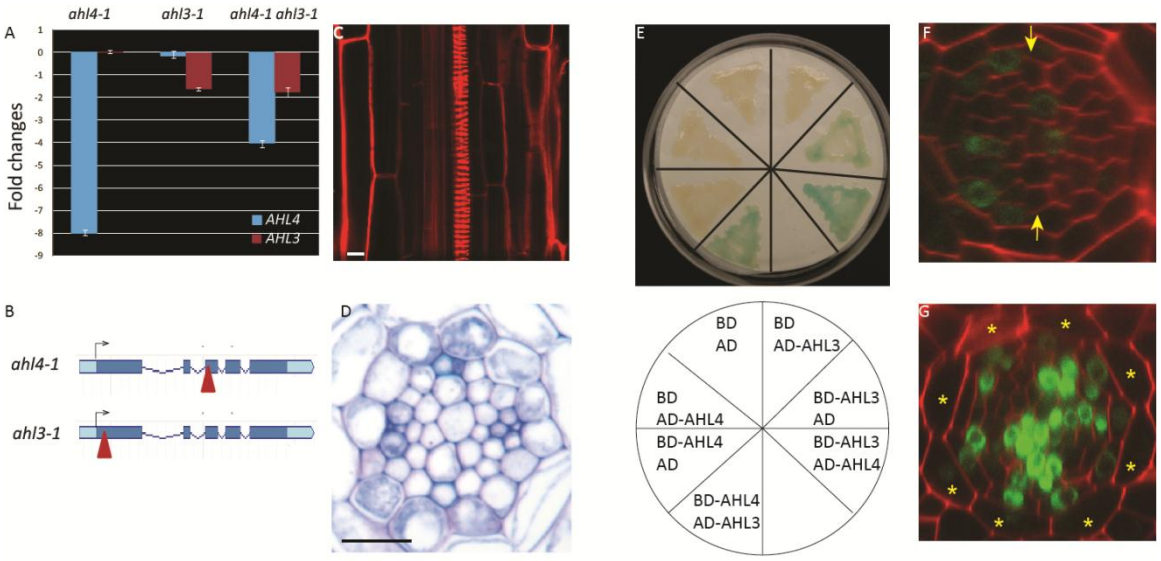
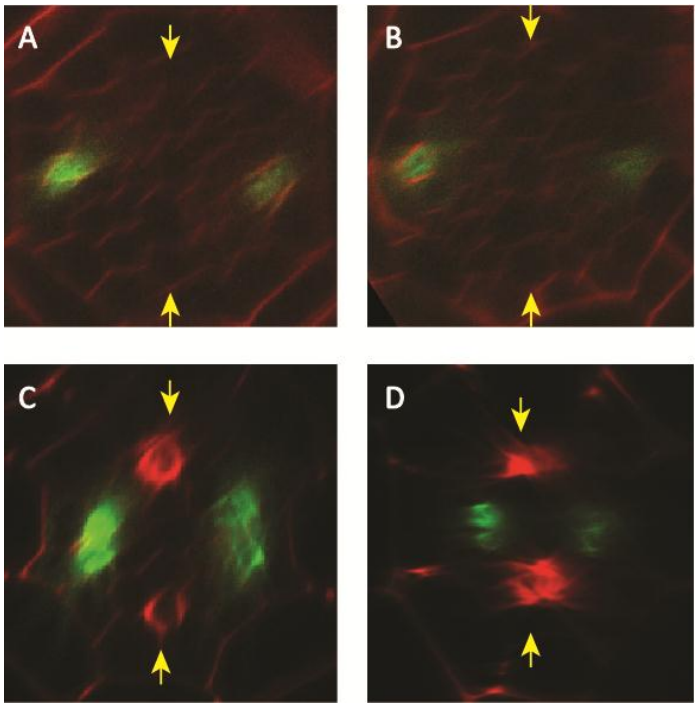


Figure 2.2. AHL3/4 proteins interact with each other and together influence the xylem patterning.

(A) qRT-PCR results showing Transcript levels of AHL3 and APN4 genes in single and double mutant background relative to wild type (Error bars represent sd). **(B)** Predicted intron/exon structure of *AHL4* (upper panel) and *AHL3* (lower panel). Exons are shown as dark blue boxes (Arrow, the start codon of *AHL4 and AHL3* transcripts; arrow head, positions of *ahl4-1* and *ahl3-1* T-DNA insertions). *ahl3-1 ahl4-1* forms ectopic extraxylem strands shown by confocal section **(C)** and toluidine blue staining **(D)**. **(E)** Yeast two-hybrid showing the physic interaction between AHL3/4 proteins. Confocal sections of *pAHL3::AHL3:GFP* **(F)** and *pCRE1::AHL3:GFP* **(G)**. Scale bars, 10 µm; asterisks, cortex position; yellow arrows, xylem axis.

Additional xylem vessels in the *ahl4-1* appear in the maturation zone of the root. However, cell-type specific root expression data indicated that *AHL4* is expressed and functions in the root meristem before vascular cells start differentiation. To identify its role in the root meristem, we introduced several vascular cell-type specific markers into *ahl4-1* mutant and compared their expression patterns in *ahl4-1* to those in the wild type. First, we investigated the influence of *AHL4* on the xylem domain by introducing *pTMO5::erGFP* (ER-localized GFP) into *ahl4-1*, which is

specifically expressed in the xylem precursor cells (Figure 2.1H) (Lee et al., 2006). By contrast to the wild type where *pTMO5::erGFP* expression is restricted to a single row of xylem precursors, the expression of *pTMO5::erGFP* in the *ahl4-1* expanded to the adjacent cell layer (Figure 2.1K). This expression pattern is consistent with the additional xylem strand formation in *ahl4-1*. *AHP6* is expressed in a protoxylem precursor and the two neighboring pericycle cells (Figure 2.1I) (Mähönen et al., 2006). Consistent with the expansion of *TMO5* domain in *ahl4-1*, the expression domain of *pAHP6::erGFP* also expanded in *ahl4-1*, being detected in the vascular cells in the procambium position of the wild type (Figure 2.1L). These xylem marker analyses suggested that the proper formation of boundaries between procambium and xylem might require *AHL4*. To further test this idea, we checked the status of procambium in the *ahl4-1* by introducing *pARR5::erGFP*, which precisely marks procambium domains that neighbor xylem precursors in the root meristem (Figure 2.1J) (Lee et al., 2006). GFP expression was absent in the cells adjacent to the xylem precursors, into which xylem markers expanded in the *ahl4-1* (Figure 2.1M). To examine phloem development in *ahl4-1*, we employed *pAPL::erGFP* which is expressed in the developing protophloem sieve element and then switches to the companion cells and metaphloem sieve element (Bonke et al., 2003). Consistently with our histological analysis, there was no obvious difference of phloem patterning between wild type and *ahl4-1* (Supplementary Figure 2.1). Based on both anatomical and marker based analyses, we conclude that AHL4 is involved in setting up the boundary between xylem and procambium by suppressing the ectopic xylem formation in the procambium.



Supplementary Figure 2.1. The phloem development is normal in *ahl4-1*.
Confocal section for the expression pattern of *pAPL::erGFP* in wild type **(A, C)** and *ahl4-1* **(B, D)**. **(A, B),** meristematic zone; **(C, D)**, maturation zone.

AHL4 moves between cells

Our investigation showed that *AHL4* in the root meristem suppresses the specification of procambial cells into xylem vessels to ensure the boundary between the two cell types. To further characterize the spatio-temporal regulation of xylem development by AHL4, we generated transcriptional and translational fusion lines of *AHL4*. For this, we developed constructs with *erGFP* or the coding region of AHL4 fused to free GFP (*AHL4-GFP*), expressed under the 2 kb-long upstream intergenic region of *AHL4*. When *pAHL4::AHL4:GFP* was expressed in the *ahl4-1* mutant, all the 6 independent

transgenic lines complemented the mutant phenotype, and did not generate ectopic xylem strands (Figure 2.3A and 2.3B and Table 2.1). Thus, it seems that the AHL4-GFP fusion protein is fully functional and the selected promoter is sufficient for the *AHL4* activity. We further analyzed the spatial domain where AHL4-GFP proteins are found. Consistent with its regulation of the boundaries between xylem and procambium in the root meristem, AHL4-GFP was enriched in the stele of a root meristem (Figure 2.3C). Interestingly, the expression domain of transcriptional GFP was not the same as the domain of translational GFP. Contrary to the ubiquitous distribution of AHL4-GFP throughout the stele cells of root meristem, the expression of transcriptional GFP was restricted to the procambium (Figure 2.3D).

Table 2.1. AHL4-GFP/AHL4-3xYFP localization in the xylem and its correlation to phenotype complementation.

Genotype	number of plants counted	Plants with GFP expression in the xylem	number of plants that complement phenotype		Genotype	number of plants counted	Plants with GFP expression in the xylem	number of plants that complement phenotype	phenotype/ movement correspondence
pAHL4::AHL4:GFP; ahl4-1 line#1	10	10 (100%)	10		*pAHL4::AHL4:3xYFP; ahl4-1 line#1*	10	10 (100%)	10	100%
pAHL4::AHL4:GFP; ahl4-1 line#2	10	10 (100%)	10		*pAHL4::AHL4:3xYFP; ahl4-1 line#2*	10	6 (60%)	6	100%
pAHL4::AHL4:GFP; ahl4-1 line#3	10	10 (100%)	10		*pAHL4::AHL4:3xYFP; ahl4-1 line#3*	10	2 (20%)	2	100%
pAHL4::AHL4:GFP; ahl4-1 line#4	10	10 (100%)	10		*pAHL4::AHL4:3xYFP; ahl4-1 line#4*	10	10 (100%)	10	100%
pAHL4::AHL4:GFP; ahl4-1 line#5	10	10 (100%)	10		*pAHL4::AHL4:3xYFP; ahl4-1 line#5*	10	9 (90%)	9	100%
pAHL4::AHL4:GFP; ahl4-1 line#6	10	10 (100%)	10		*pAHL4::AHL4:3xYFP; ahl4-1 line#6*	10	3 (30%)	3	100%

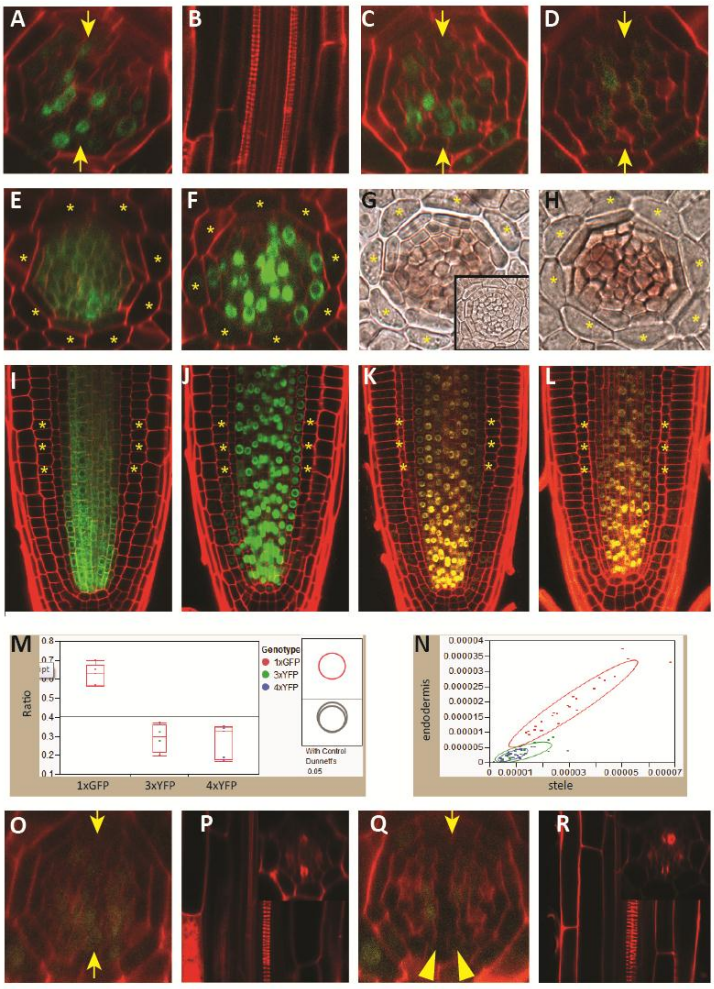


Figure 2.3. Movement of AHL4 is crucial for setting up the boundaries between xylem and procambium.

pAHL4::AHL4:GFP can be found both in procambium and xylem in and *ahl4-1* **(A)** and wild type **(C)**. *pAHL4::AHL4:GFP; ahl4-1* recovers the ectopic xylem phenotype in *ahl4-1* **(B).** *pAHL4::erGFP* is only expressed in the procambium. *pCRE1::erGFP* is expressed in the stele **(E, I)**, while *pCRE1::AHL4:GFP* is also found outside of stele in the endodermis **(F, J)**. *In situ* hybridization with an antisense *GFP* probe suggested similar mRNA expression pattern in *pCRE1::GFP* **(G)** and *pCRE1::AHL:GFP* **(H)** (inset in (**G)** showed a sense *GFP* probe). *pCRE1::AHL4-3xGFP* **(K)** and *pCRE1::AHL4-4xYFP* **(L)** showed reduce mobility of AHL4 and supported by quantification of the movement **(M, N)**. An independent line of *pAHL4::AHL4:3xYFP; ahl4-1* with AHL4 mobility **(O)** is capable of rescue xylem phenotype **(P)**, while another line of *pAHL4::AHL4:3xYFP; ahl4-1* with retarded AHL4 mobility **(Q)** cannot rescue xylem phenotype **(R)**. Asterisks, cortex position; arrow heads protoxylem position; arrows, xylem axis.

The spatial expansion of AHL4-GFP domain from the transcriptional GFP domain indicated that AHL4 proteins or RNAs might move between cells. To further explore this, we analyzed the spatial distribution of AHL4 proteins expressed under the stele-specific *CRE1* promoter (Figure 2.3E and 2.3I) (Mähönen et al., 2000). Consistent with observations made with transcriptional and translational fusion lines, AHL4-GFP signals expanded radially from the stele and reached out to the lateral root cap cells in the meristem region (Figure 2.3F and 2.3J). In the meristematic zone, we always detected AHL4-GFP in the endodermis where CRE1 is not normally expressed. Though the most of mobile transcription factors have been shown to move as proteins, there are cases when mRNAs move between cells (Lucas et al., 1995; Ruiz-Medrano et al., 1999). To find whether *AHL4:GFP* mRNAs are mobile between cells, we examined the mRNA domains of *AHL4:GFP* in *pCRE1::AHL4:GFP* transgenic plants by performing RNA *in situ* hybridization. An antisense *GFP* probe successfully detected *AHL4:GFP* mRNA, only in the stele cells (Figure 2.3H). This expression pattern mirrors *erGFP* expression driven by *CRE1* promoter (Figure 2.3G) (Mähönen et al., 2000). Based on our analysis of proteins and mRNAs of *AHL4:GFP* in the *pCRE1::AHL4:GFP* transgenic plants, we conclude it is the AHL4 proteins rather than its mRNAs that move between cells.

Movement of AHL4 is critical for the boundary between xylem and procambium

Numerous studies showed that transcription factors move from one cell to the other through plasmodesmata (PD) (Lucas et al., 1995; Zambryski and Crawford, 2000;

Nakajima et al., 2001; Kurata et al., 2005b; Schlereth et al., 2010; Tsukagoshi et al., 2010). The size of AHL4 protein is estimated to be around 45 kDa, thus the fusion protein AHL4-GFP becomes around 72 kDa (Molecular weight of 1xGFP is 27 kDa). Given that the size exclusion limit of PD between pericycle and endodermis in the root meristem is around 60 kDa, AHL4-GFP likely moves between cells in a targeted manner, in which AHL4 might dilate PD openings to increase its size exclusion limit (Crawford and Zambryski, 2001; Rim et al., 2011).

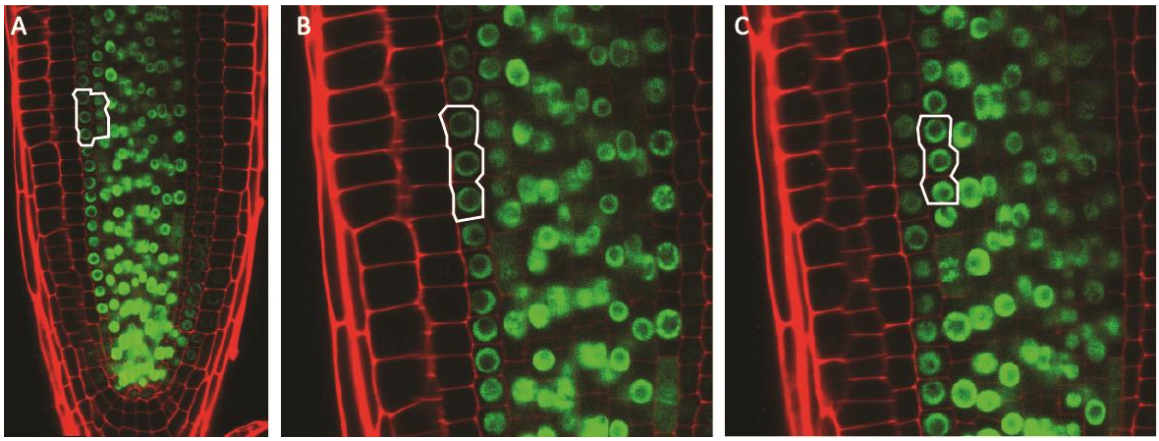
Table 2.2 Movement comparison between AHL4-GFP and AHL4-3x/4xYFP.

Genotype	%(N) of independent lines expressing GFP(YFP) in the endodermis	Total number of lines examined (N)	%(n) of individual plants expressing GFP(YFP) in the endodermis	%(n) of individual plants rescued the ectopic xylem phenotype	Total number of individual plants scored (N)
pCRE1::AHL4:GFP; wt	100%(5)	5	100%(25)	N/A	25
pCRE1::AHL4:3xYFP; ahl4-1	75%(3)	4	60%(12)	100% (20)	20
pCRE1::AHL4:4xYFP; ahl4-1	60%(3)	5	52%(13)	100% (25)	25

.

To gain a better understanding of AHL4 movement, the coding region of *AHL4* was fused with tandem *YFPs* (3x or 4x*YFP*) driven by *CRE*1 promoter and transformed into *ahl4-1* mutant. The extra xylem phenotype was complemented in all the lines of *pCRE1::AHL4:3x/4x-YFPs; ahl4-1* (9/9) (Table 2.3). This suggests that fusion proteins between AHL4 and tandem YFPs are also fully functional. We then

compared their cell-to-cell movement with *pCRE1::AHL4:GFP(1x)* lines. 3x and 4x YFP have large molecular weights (81 kDa and 108 kDa respectively) and therefore can significantly reduce the protein movement between cells (Crawford and Zambryski, 2001; Kurata et al., 2005b; Tsukagoshi et al., 2010). Our confocal analysis suggested that adding tandem YFPs significantly interferes with the movement of AHL4 but cannot abolish the movement completely (Figure 2.3K and 2.3L). We compared the percentage of individual lines that show protein movement to the endodermis between AHL4-GFP and AHL4-tandem-YFPs. Movement of AHL-GFP occurred 100% in all the 5 independent lines, however movement of AHL4-3x and 4xYFP occurred at the frequency of 75% and 60%, respectively (Table 2.3). We then quantified the movement of proteins in the same transgenic lines by measuring and comparing fluorescence signals in the endodermal cells and the neighboring pericycle cells (For further details about the imaging and calculation, see Supplementary Figure 2.2 and Materials and Methods). The average fluorescence intensity of AHL4-GFP in the endodermis was 62% (± 5.7% SD) of the one in the adjacent pericycle (Figure 2.3M). By contrast, 29 ± 7.4% (SD) and 27 ± 8.9% (SD) signal intensities were observed for AHL4-3xYFPs and AHL4-4xYFP, respectively. Based on our analysis of the signal ratio between pericycle and endodermis in transgenic lines, increasing the protein size of AHL4 seems to negatively impact cell-to-cell movement. However, even with a significant increase in the size of AHL4, AHL4 proteins were still capable of moving between cells, further supporting that AHL4 might move between cells in a targeted manner.



Supplementary Figure 2.2. Confocal sections for the quantification of AHL protein movement, shown Figure 2.3M and Figure 2.3N.

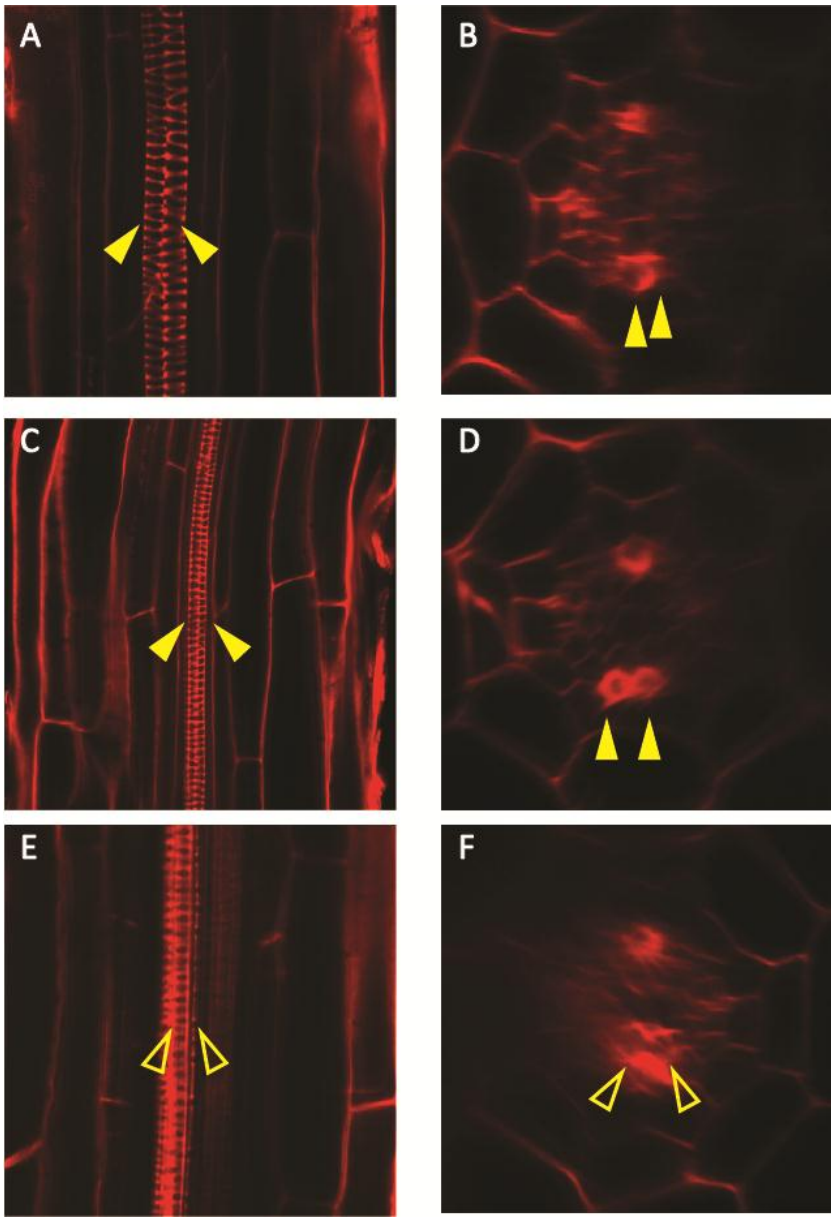
(A) Confocal section of root tip to localize the ground tissue where the adjacent endodermis and pericycle cell files were parallelly aligned for GFP signal intensity measurement (enclosed by white circle). Zoom-in images of ground tissue highlighted in **(A)** focusing first on endodermis **(B)** and then on adjacent pericycle **(C)**.

To investigate the connection between AHL4 protein movement and its developmental regulation, we expressed *AHL4* fused with 3xYFPs under *AHL4* promoter in *ahl4-1*. Similar to what was observed in *pCRE1::AHL4:3xYFP* plants, by fusing 3xYFP to AHL4 we could only partially restrict AHL4 movement (Table 2.1). We then counted protein localization in the xylem precursors and xylem phenotypes in 10 individuals from each of 6 independent lines to find whether the movement of AHL4 into the xylem precursors contributes to the complementation of xylem phenotype. Unlike *pAHL4::AHL4:GFP* that rescued xylem phenotype of *ahl4-1* at 100%, *pAHL4::AHL4:3xYFP* rescued xylem phenotype only at ~50% (Table 2.1). In individuals where AHL4-3xYFP proteins moved into the xylem precursors, we found the rescue of extra xylem phenotype (31/31) (Figure 2.3O and 2.3P). By contrast, extra xylem phenotype could not be rescued when there was no AHL4-3xYFP in the

xylem precursors (29/29) (Figure 2.3Q and 2.3R). Such a strong correlation between AHL mobility to the xylem precursors and xylem phenotype indicates that the movement of AHL4 from procambial cells to xylem precursors is required to set up and maintain the boundary between xylem and procambium.

AHL3 and 4 function together as protein complexes

A previous phylogenetic analysis suggested that *AHL3* (*AT4g25320*) is the most closely related homolog of *AHL4* in *Arabidopsis* (Fujimoto et al., 2004). Interestingly, the root expression data indicated that *AHL3/4* are expressed in a very similar pattern. To investigate the relationship between *AHL3* and *4* in the regulation of boundary between xylem and procambium, we obtained the line, *FLAG_445H04* (*ahl3-1* thereafter) with a T-DNA inserted into exon 1 of *AHL3* and verified by sequencing (Figure 2.2B). Real-time PCR showed 60% reduction of *AHL3* expression in this insertion line compared to the one in a wild type, suggesting that *ahl3-1* might be a knock down mutant of *AHL3* (Figure 2.2A). Interestingly, in *ahl3-1* extra xylem strands were observed in the same fashion as what was observed in the *ahl4-1* (Supplementary Figure 2.3A and 2.3B). Thus we asked whether AHL3 and 4 influence their expression each other. Expression analysis of AHL3 and 4 by real-time RT-PCR suggested it is unlikely the case. We did not find a change in the expression level of *AHL4* in the *ahl3-1* mutant or *vice versa* (Figure 2.2A).

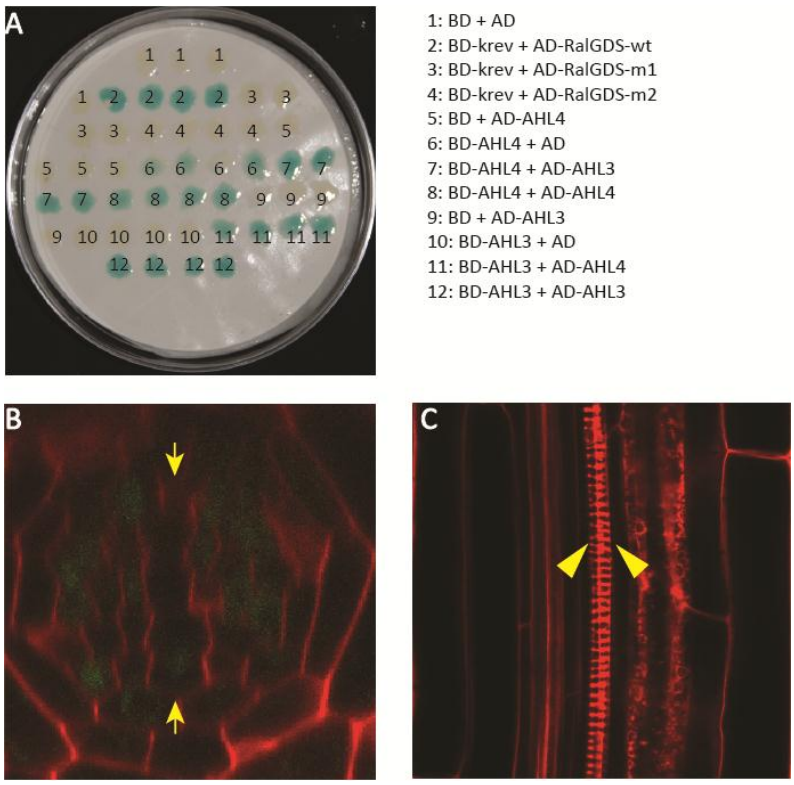


Supplementary Figure 2.3. Ectopic xylem formation in the *ahl3-1* and amiRNA lines.

Confocal sections of *ahl3-1* **(A, B)**, amiRNA line #1 **(C, D)** and amiRNA line #5 **(E, F)**. Arrow heads, protoxylem; hollow arrow heads, metaxylem.

Given that AHL3 and AHL4 share 69% amino acid similarity and that both showed the involvement in the xylem patterning, we asked whether they function in a

redundant manner. However, the *ahl3-1ahl4-1* double mutant did not show any additional xylem phenotype to what was observed in each of single mutants (Figure 2.2C and 2.2D). We also generated and analyzed artificial microRNA knock-down lines. These lines showed the similar phenotype to the single and the double mutants (Supplementary Figure 2.3C to 2.3F). These results suggested that AHL3 and 4 might function together in a complementary manner. We further asked whether this happens through the direct protein-protein interaction. Indeed, our *in vitro* study using the yeast-two-hybrid assay indicated that AHL3 and AHL4 physically interact to each other as well as to its own (Figure 2.2E and Supplementary Figure 2.4A). Thus, AHL3 and 4 likely function together as a tetrameric protein complex *in vivo*.



Supplementary Figure 2.4. Protein-protein interaction between AHL3 and AHL4 is required for the boundary between xylem and procambium.

(A) Yeast two-hybrid showing the self-interaction of AHL3 and AHL4. Expression of pAHL4::AHL4:GFP in the *ahl3-1* cannot be restricted **(B)**, yet the ectopic xylem formation cannot be rescued **(C)**.

We further characterized the involvement of AHL3 in the boundary between xylem and procambium. To this end, *pAHL3::AHL3:GFP* was transformed into the wild-type and the localization of AHL3-GFP was investigated. Very similar to *pAHL4::AHL4:GFP* lines, AHL3-GFP proteins were observed in the nuclei of both procambium and xylem precursors (Figure 2.2F). We asked whether such similar protein localization patterns are from the protein movement. Indeed, *pCRE1::AHL3:GFP* introduced into the wild-type background exhibited GFP signals

in the endodermis (Figure 2.2G). Cell-to-cell movement of some transcription factors has been shown to be interfered by the physical interaction with other transcription factors (Cui et al., 2007; Balkunde et al., 2011; Xu et al., 2011). To address whether the interaction between AHL3 and 4 influences the intercellular trafficking, we introduced *pAHL4::AHL4:GFP* into *ahl3-1*. In the absence of AHL3, the movement of AHL4 was not affected, suggesting that the interaction between AHL3 and 4 might not influence the cell-to-cell movement of AHL4 (Supplementary Figure 2.4B). The extra xylem phenotype observed in *ahl3-1* could not be rescued in these transgenic plants, either (Supplementary Figure 2.4C). Taken together, we conclude that AHL3 and AHL4 together regulate the boundaries between xylem and procambium by forming protein complexes.

Discussion

AHL4 movement provides positional information for vascular patterning

In the Arabidopsis root meristem, several transcription factors that regulate the formation of xylem and phloem have been reported. ALTERED PHLOEM DEVELOPMENT (APL), a MYB coiled-coil-type transcription factor, is required for the phloem formation (Bonke et al., 2003). Multiple transcription factors have been identified to regulate the xylem development (Yamaguchi et al., 2008; Carlsbecker et al., 2010; Yamaguchi et al., 2010b; Yamaguchi et al., 2010a). Among them, *HD-ZIP III* transcription factors act as dosage-dependent regulators and promote the de-novo formation of proto- and metaxylem under their low and high dosages, respectively.

Mobile *miR165/6* and SHORTROOT have been shown to spatially regulate the dosage of *HD-ZIP III* transcription factors (Carlsbecker et al., 2010). The organization of vascular tissues tends to be unique in each species. Vascular tissues in the Arabidopsis roots, for example, are always bisymmetrically organized. This suggests a presence of robust regulatory programs that define boundaries between vascular cell types.

So far, it has been poorly understood how the cell-cell signaling governs the boundaries between the cell types within vascular tissues. In this study, we find that mobile transcription factors, AHL4 mediate the cell-to-cell communication between xylem and procambium. Mutations in *AHL4* resulted in the formation of additional proto- and meta-xylem strands. The formation of extra xylem strands are most likely resulted from either of the following two reasons. First, it could be from the abnormal cell division that triggers the formation of extra xylem precursor cells. Second, it could be from the breakage of boundaries between cell types, which leads to the misspecification of cell fates. Our analysis favors the latter. We used cell markers *pTMO5::erGFP* and *pARR5::erGFP* which label the xylem precursors and procambium respectively in the root meristem. According to the cell markers, their cell identity establish in the very early stage, only a few cells away from the QC. In the *ahl4-1*, both *pTMO5::erGFP* and *pARR5::erGFP* expression patterns are affected by the expansion of xylem domain throughout the meristematic zone (Figure 2.1 K and 2.1M). If the ectopic xylem is a result of abnormal proliferation activity in the xylem precursor cells, it would suggest a mis-designated procambium activity, which we did not observe through *pARR5::erGFP* at xylem precursors in *ahl4-1*. Therefore,

we propose that AHL4 movement draws the boundaries between procambium and xylem for vascular patterning.

In terms of movement, our investigation suggests that AHL4 proteins move between cells very actively in a potentially targeted manner. When we increased the size of AHL4-YFP fusion proteins to 154 kDa by expressing *pCRE1::AHL4:4xYFP* in the *ahl4*, we still observed the movement of AHL4-YFP outside the stele domain. Studies indicated that the size exclusion limit in the meristem does not exceed more than 60 kDa in the root (Oparka et al., 1999; Kim et al., 2005; Rim et al., 2011). Therefore, AHL4 might actively increase the PD opening, similar to what has been found with CAPRICE (Kurata et al., 2005b). However, by contrast to CAPRICE and SHORT-ROOT that move in a directional manner, AHL4 does not seem to move directionally (Sena et al., 2004; Gallagher and Benfey, 2005; Kurata et al., 2005b; Gallagher and Benfey, 2009). Our study suggests that AHL4 moves not only between the vascular cell types but also outside the vascular cylinder.

In addition to the movement between cells, it appears that AHL4 also forms protein complex with its closest homolog AHL3 in the boundary definition. Reducing *AHL3* expression results in extra xylem phenotype similar to *ahl4-1*. Furthermore, the double mutant *ahl3-1 ahl4-1* phenocopied the single mutant phenotype indicating that AHL3 and 4 are involved in the same pathway. Our protein interaction study using the yeast two-hybrid assay further suggests that AHL3 and 4 form protein complexes. This is consistent with a recent study showing that *BARREN STALK FASTIGIATE1* (*BAF1*), a monocotyledon-specific AT-hook member in maize, forms both hetero- and homo-dimers with other putative AT-hook DNA binding proteins in yeast (Gallavotti

et al., 2011). Our study reveals that the protein interaction between AHL3 and AHL4 is functionally important *in planta* to define the boundaries between xylem and procambium.

AHL3/4 movement, a novel characteristic of the AT-hook transcription factors

The AT-hook is a small DNA-binding protein motif which is frequently associated with modulating chromatin architecture to co-regulate transcription. The extensively-studied AT-hook containing proteins in mammals, the high mobility group (HMG) non-histone chromosomal proteins (HMGA), revealed their important roles in a diverse range of biological processes (for reviews see (Reeves, 2001; Reeves and Beckerbauer, 2001)). The AT-hook motif, characterized by a highly conserved tripeptide of glycine-arginine-proline (GRP), exists as a single or multiple copies in a wide range of organisms (Aravind and Landsman, 1998). In plants, there is a unique group of AT-hook family proteins harboring both an AT-hook domain and an unknown plant and prokaryotes conserved (PCC) domain. PPC domain as its name indicated is also found in prokaryotic proteins. Yet in prokaryotes those proteins do not contain AT-hook motif (Fujimoto et al., 2004). These plant specific AT-hook members have been shown to be involved in diverse developmental processes such as hypocotyl elongation, flower development, gibberellins biosynthesis, leaf senescence, stem cell niche specification (Lim et al., 2007; Matsushita et al., 2007; Street et al., 2008; Ng et al., 2009; Gallavotti et al., 2011). There are total 29 members belonging to this AT-hook family in *Arabidopsis* (Fujimoto et al., 2004; Matsushita et al., 2007).

Homologs of AHL3/4 has been shown to act as promoter-binding proteins by yeast one-hybrid screening consistent with the proposed transcription factor activity (Matsushita et al., 2007)

In our analysis, AHL4-GFP fusion proteins are selectively localized to the nuclear periphery and excluded from nucleoli. Their nuclear localization pattern is consistent with the suggested association of AT-hook members to the matrix attachment regions in the nuclei (Morisawa et al., 2000; Fujimoto et al., 2004; Lim et al., 2007; Ng and Ito, 2010). We also showed that neither the nuclear-localization nor the intercellular movement of AHL4 is affected by the increment of protein size (Figure 2.3K, 2.3L, and Table 2.3). It suggests that both inter- and intra-cellular movements are in targeted manners. So far, only AHL3 and AHL4 in the AT-hook family are reported of moving. Further analysis on the domains of AHL3/4 proteins would improve our understanding on the mechanisms of protein movement in AT-hook family and whether or not the movement is shared based on the domain employed. The well-studied targeted mobile transcription factors suggested two main mechanisms. First, a PD-targeting signal might resemble an NLS as seen in KNOTTED1 (Lucas et al., 1995). Deletion of the NLS in KN1 abolished proteins from moving between cells. PCC domain has been reported to be important for the nuclear localization for AHL1, another AT-hook member in *Arabidopsis* (Fujimoto et al., 2004). It would be interesting to learn whether PCC domain also serves as intercellular signals, which in turn would open up the question that whether the movement is a shared characteristic in the family. Alternatively, mobile transcription factors such as SHORTROOT and CAPRICE have both shared and distinct domains

for inter- and intra-cellular movement (Kurata et al., 2005b; Gallagher and Benfey, 2009). In that case, the mobility might be unique to AHL3/4 and the domain(s) distinctive from the rest of the family members might be important for the intercellular mobility.

AHL3/4 and hormonal signaling in the vascular patterning

When the plant loses AHL3/4 function, we observed not only the formation of additional protoxylem, but also additional metaxylem. No regulators like AHL3/4 have been reported before as setting up boundaries for both protoxylem and metaxylem in the early stage of root development. Further investigation on the AHL3/4 targets has the potential to reveal novel mechanisms in the vascular patterning regulated by cell-cell communication. Also, it is not clear whether AHL3/4 set up the boundaries for xylem axis as a whole group or employ different mechanisms for protoxylem and metaxylem. Particularly, their regulation on the protoxylem domain might be connected to the cytokinin and auxin regulation. The formation of additional protoxylem strands is frequently reported in the mutants losing response to cytokinin signaling (Mähönen et al., 2000; Yokoyama et al., 2007; Bishopp et al., 2011a; Kondo et al., 2011). Our studies using *pAHP6::erGFP; ahl4-1* suggested that *AHP6* has an expanded expression when losing AHL4 function (Figure 2.1L). AHP6 is both an auxin responding gene and a repressor of cytokinin signaling in the auxin-cytokinin feedback loop (Mähönen et al., 2006). Similarly, ARR5, the cytokinin positive response regulator that also responds to auxin signals changed its expression

domains in the *ahl4-1* mutant (Figure 2.1M) (D'Agostino et al., 2000; Bishopp et al., 2011a). Taken together, when losing the AHL4, both the auxin and cytokinin domains in the vascular tissue were affected. It is likely that AHL3/4 acts upstream of the auxin-cytokinin feedback loop or at least two pathways merged in regulating the same group of hormone signaling components in the control of boundaries between procambium and protoxylem. In addition, when *ahl4-1* seedlings were treated with exogenous cytokinin, the protoxylem formation was abolished, showing the equal level of cytokinin sensitivity to the wild type plants treated with cytokinin (data not shown). This is consistent with the speculation that AHL4 pathway is either upstream of cytokinin pathway or acts parallel with it. Previous studies have linked other AT-hook genes in response to hormone signals and feedbacks (Matsushita et al., 2007; Vom Endt et al., 2007). Our results provide a new basis for connecting AHL3/4 to the crosstalk between cytokinin and auxin.

Materials and Methods

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used. Seeds were surface-sterilized, plated (0.5 × MS medium with 1% sucrose) and grown under a 16-h-light/8-h-dark cycle at 22-23 C in a plant growth chamber. *ahl4-1* (*SALK_124619*) was obtained from the Arabidopsis Biological Resources Center (ABRC) and *ahl3-1* (*FLAG_445H04*) was obtained from Versailles Genetics and Plant Breeding Laboratory Arabidopsis thaliana Resource Centre (INRA Versailles France,

http://dbsgap.versailles.inra.fr/vnat/). The following marker lines were characterized previously: *pTMO5::erGFP* (Lee et al., 2006), *pARR5::erGFP* (Lee et al., 2006); *pAHP6::erGFP* (Mähönen et al., 2006); *pCRE1::erGFP* (Mähönen et al., 2000). Primers used for genotyping were listed in Supplementary Table 2.1.

Supplementary Table 2.1 List of primers used in Chapter 2

Primers for genotyping		
	Primer name	Sequence
Genotyping *ahl4-1* wt allele	X9-1RP	CTGACCCGCTATAAAACTCCC
	X9-1LP	CTGTGTGTGTACATTGGCCTC
Genotyping *ahl4-1* mutant allele	X9-1RP	CTGACCCGCTATAAAACTCCC
	LBb1.3	ATTTTGCCGATTTCGGAAC
Genotyping *ahl3-1* wt allele	X17-1F	TTCACCTAACGCTATAATATCAACC
	X17-1R	GACGTGGCAAGTCTAATCGATGGC
Genotyping *ahl3-1* mutant allele	X17-1F	TTCACCTAACGCTATAATATCAACC
	Tag5	CTACAAATTGCCTTTTCTTATCGAC
Primers for cloning		
Cloning *AHL4* promoter into pDONR P4_P1R	pX9-2F	GGGGACAACTTTGTATAGAAAAGTTGC ATTTCGTGAAGTGTAGCTC
	pX9-2R	GGGGACTGCTTTTTTGTACAAACTTGC TGAAAAGATTCTGATTCCG
Cloning *AHL4* cDNA without stop coden into pDONR221	X9-2F	GGGGACAAGTTTGTACAAAAAAGCAG GCATGGAGGAGAGAGAAGGAAC
	X9-2R	GGGGACCACTTTGTACAAGAAAGCTG GGTATCAGCTTGGAACCTCGGT
Cloning *AHL3* promoter into pDONR P4_P1R	pX17-F	GGGGACAACTTTGTATAGAAAAGTTG GTGTTGTTGTATTCGGGTTTA
	pX17-R	GGGGACTGCTTTTTTGTACAAACTTGC CGGACATAAGATCAAGTGAT
Cloning *AHL3* cDNA without stop coden into pDONR221	cX17-F	GGGGACAAGTTTGTACAAAAAAGCAG GCTATGGAGGAGAGAGAAGGA
	cX17-R	GGGGACCACTTTGTACAAGAAAGCTG GGTGGCTCGGAATCTCGTTGT
Cloning *AHL4* cDNA with stop coden into pDONR221 for	cX9-F_N	GGGGACAAGTTTGTACAAAAAAGCAG GCTCCATGGAGGAGAGAGAAG
	cX9-R	GGGGACCACTTTGTACAAGAAATCAG CTGGGTGGCTTGGAACCTCGGTGT

Y2H		
Cloning *AHL3* cDNA with stop coden into pDONR221 for Y2H	cX9-F_N	GGGGACAAGTTTGTACAAAAAAGCAG GCTCCATGGAGGAGAGAGAAG
	cX17-R_N	GGGGACCACTTTGTACAAGAAAGCTG GGTCTAGCTCGGAATCTCGTTGT
Cloning *amiRNA-AHL3-4* into pDONR221	attB1-A-F	GGGGACAAGTTTGTACAAAAAAGCAG GCTCTGCAAGGCGATTAAGTTGGGTAA C
	attB2-B-R	GGGGACCACTTTGTACAAGAAAGCTG GGTGCGGATAACAATTTCACACAGGA AACAG
	miR-s	GATCATAAGTTAGAGTACCGCTGTCTC TCTTTTGTATTCC
	miR-a	GACAGCGGTACTCTAACTTATGATCAA AGAGAATCAATGA
	miR*s	GACAACGGTACTCTATCTTATGTTCAC AGGTCGTGATATG
	miR*a	GAACATAAGATAGAGTACCGTTGTCTA CATATATATTCCT
Primers for riboprobe amplification for *in situ*		
GFP-probN-SF		TAATACGACTCACTATAGGGAGGTGG AGTTGTCCCAATTCTTGTT
GFP-probN-SR		CCATGTGTAATCCCAGCAGCTGTT
GFP-probN-ASF		CTGTCAGTGGAGAGGGTGAAGGTG
GFP-probN-ASR		TAATACGACTCACTATAGGGAGGCCAT GCCATGTGTAATCCCAGC
Primers for real time qPCR		
qX9-F		TCACAGTAAATGCCGGTGAGGATG
qX9-R		ACGCGAGCCTTGTTGAGAGAATG
qX17-F		CGTGGCAAGTCTAATCGATGGC
qX17-R		ACCTGCCAAATTGGTATCAACAGG

Plasmid Construction

Gateway cloning technology (Invitrogen) was used for DNA manipulations. *AHL4* and *CRE1* promoters were amplified from *Arabidopsis* Col-0 genomic DNA and cloned into pDONR P4_P1r. *AHL3* and *AHL4* cDNA were cloned into pDONR221. *3xYFP* and *4xYFP* in pDONR P2R_P3 were previously described in (Tsukagoshi et al.,

2010). *pCRE1::AHL3/4:GFP*, *pAHL4::erGFP*, *pAHL4::AHL4:GFP*, were constructed into dpGreen-Bar by Multisite gateway LR recombination. *pCRE1::AHL4:3x/4xYFP* were constructed into dpGreen-BarT. A suitable target site for the microRNA targeting *AHL3/4* was identified and generated by following the instructions on (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) with the exception that oligoA was modified to clone *amiRNA-AHL3-4* into pENTR/D-TOPO vector. *pCRE1::amiRNA-AHL3-4* was constructed into dpGreen-Bar by Multisite gateway LR recombination. All the clones in the binary vector was transformed into Agrobacterium GV3101 with pSOUP and transformed into either wild-type or *ahl4-1*. *AHL3* and *AHL4* cDNA were introduced into pDEST22 and pDEST32 by gateway LR recombination to fuse AHL3 and AHL4 protein with GAL4 activation domain and GAL4 DNA-binding domain, respectively. Primers used for cloning were listed in Supplementary Table 2.1.

Histological Analysis

All seedling samples were collected at 6 days after germination. Confocal images were obtained using a Leica TCS SP5 Laser Scanning Confocal Microscope with the preset emission/excitation wavelength of 488 nm/505 to 530 nm for GFP, 510nm/ 525 to 560nm for YFP, and 561 nm/591 to 635 nm for propidium iodide (PI). For the visualization of the root structure, all the seedlings were stained in 2 μg/ml of PI. Root transverse sections and toluidine blue staining were done as described in (Scheres et al., 1995). Basic fuchsin staining was described in (Mähönen et al., 2000).

Confocal imaging for fluorescence intensity measurement

For quantitative analysis of AHL4 movement, GFP/YFP intensity was measured in both endodermis and its neighboring pericycle cells in *pCRE1::AHL4:GFP* and *pCRE1::AHL4:3x/4xYFP*. For each genetic background, 5 plants from each 4-5 independent lines were examined. Plant was first examined longitudinally under 63x objective to locate a focal plane where the adjacent endodermis and pericycle cell files were parallelly aligned (Supplementary Figure 2.2A). Then the image was zoomed in 2.3 times to center the aforementioned endodermis and pericycle cells (Supplementary Figure 2.2B and Supplementary Figure 2.2C). Two sequential images were taken with slightly different focal planes to respectively capture the fluorescence signals in the center of nuclei in the endodermis and pericycle. All the images were taken under the same setting. At least 3 pairs of nuclei parallelly aligned endodermis and pericycle cells were measured for fluorescence signal intensity by imageJ software and the integrated pixel intensity was recorded for further analysis.

In situ hybridization

Roots from 6-day old were fixed, embedded and sectioned for *in situ* hybridization as previously described in (Mähönen et al., 2000). 700-bp long GFP DNA templates including T7 promoter for either sense or antisense probes were amplified and probes were hydrolyzed to generate working probes of 150 bp long. Primers used for riboprobes were listed in Supplementary Table 2.1.

Quantitative Real-Time RT-PCR

2mm-long section of Root tips of from 6 day-old seedlings were harvested and total RNAs were isolated by RNasey mini kit (Qiagen). cDNA was synthesized using SuperScript III first strand synthesis system for RT-PCR (Invitrogen) as described in (Carlsbecker et al., 2010). Primers used for gene expression level measurement were listed in Supplementary Table 2.1.

Yeast 2 Hybrid

PreQuest two-hybrid system (Invitrogen) was used for yeast two hybrid analysis. All the procedures were done according to the manufacture's standard protocol.

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Chapter 3 Role of transcription factor SHORT-ROOT in the control of phloem patterning: every move counts

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Abstract

Plants largely rely on both long- and short-distance intercellular communications to control their morphogenesis in a temporal and spatial manner. Previous studies showed that GRAS family transcription factor SHORT-ROOT (SHR) regulates the patterning of root ground tissues and xylem vessels by moving from the stele to the endodermis. Here, we report that SHR also controls phloem patterning through two distinctive pathways, both of which require the cell-to-cell movement of SHR. One that regulates companion cell formation is mediated by the SHR-miR165/6 pathway that requires SHR to move from the stele to the endodermis. The other that regulates cell division for sieve element formation is mediated by the SHR moving into a phloem initial from procambium. In the latter pathway, we found NARS1, an

[1] **Author contributions** J.Z. and J.Y.L. designed experiments and performced microarray analysis, J.Z. performed all the histological analysis, sieve element characterization with immunolocalization and aniline blue staining, J.Z. generated all the constructs mentioned in the method description except for *pCRE1::PHBem-GFP* which was generated by J.S, J.Z. and J.S. generated microarray data, J.Z. and C.Y. performed genotyping analysis for T-DNA insertion line identification. J.Z. and J.Y.L. wrote the manuscript.

NAC domain transcription factor, controls the sieve element division process as downstream of SHR.

Introduction

The evolutionary success of multi-cellular organisms poses the key developmental question of how their complex morphogenesis is regulated. Positional information plays a critical role in the temporal and spatial regulation of tissue patterning during morphogenesis. Plant cells, in particular caged in the rigid cell wall, largely rely on the direct cell-to-cell communication through plasmodesmata to receive or send out positional information.

Studies have shown that SHORT-ROOT (SHR), a GRAS family transcription factor acts as both a key regulatory factor and an intercellular signal to control root morphogenesis. Knocking out *SHR* results in the perturbation in the xylem and ground tissue patterning as well as the root meristem activities (Benfey et al., 1993; Helariutta et al., 2000; Nakajima et al., 2001; Carlsbecker et al., 2010). SHR mRNA is transcribed in parts of the stele, the xylem precursors, procambial cells and pericycle cells that neighbor xylem and procambium. Subsequently, SHR proteins actively move into the phloem initial, the rest of pericycle cells, endodermis and the quiescent center (Helariutta et al., 2000; Nakajima et al., 2001; Sena et al., 2004).

SHR movement to the endodermis/cortex initial is crucial to trigger its asymmetric cell division to form the distinct endodermis and cortex layers (Nakajima et al., 2001). For this to happen, SHR in the endodermis activates and interacts with SCARECROW (SCR), another GRAS family transcription factor, and they together regulate downstream genes (Levesque et al., 2006; Cui et al., 2007). In addition to the asymmetric cell division, it was recently found that SHR and SCR in the endodermis

activate the expression of *microRNA165/*166 (*miR165/6*) to pattern xylem vessels (Carlsbecker et al., 2010). M*iR165/6* produced in the endodermis, move and target the mRNAs of HD-ZIP III family transcription factors in the stele periphery. This action forms the gradient of *HD-ZIP III* mRNAs and proteins in the stele, with the highest level in the center and lowest in the periphery (Carlsbecker et al., 2010). Differential levels of *HD-ZIP III* subsequently specify metaxylem vessel in the center and protoxylem in the periphery of the stele in a dosage-dependent manner (Carlsbecker et al., 2010; Miyashima et al., 2011). Taken together, SHR moving from the stele to the endodermis serves as positional information for the ground tissue and xylem patterning.

Phloem and xylem constitute the major conducting and supporting system in vascular plants. They are generated from the vascular stem cells, namely procambium and cambium. Phloem tissue is one of the fine structures evolved to facilitate nutrient transport from source to sink in a long distance. In angiosperms, sieve elements and companion cells form functional units for nutrient transport (reviewed in (Oparka and Turgeon, 1999; Van Bel, 2003)). Sieve elements (SEs), the enucleated live cells form tubular networks and serve as a conduit for nutrient fluids including carbohydrates, amino acids and minerals throughout the plant (Sjolund, 1997; Knoblauch and van Bel, 1998; Hayashi et al., 2000). Companion cells (CCs) develop next to SEs and unload macromolecules to SEs through plasmodesmata (Lohaus et al., 1995; Sjolund, 1997). However, due to its fragile and fine nature, our understanding of phloem development is very limited. Phloem structure in the Arabidopsis root is rather simple, such that each phloem pole is composed of a proto- and a metaphloem SE that are

guarded by two CCs (Figure 3.1A and 3.1B). Because of its structural simplicity, phloem in the Arabidopsis root is well suited for studying its developmental processes.

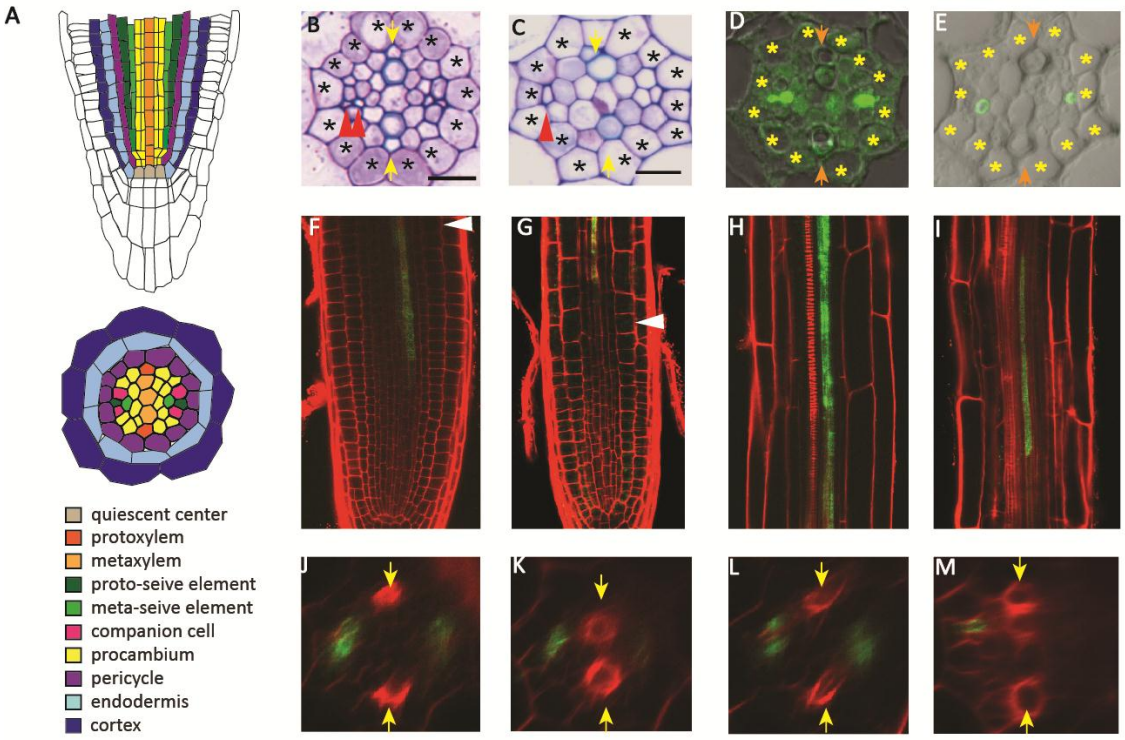


Figure 3.1. SHR regulates the phloem development in the Arabidopsis root.

(A) A schematic diagram of Arabidopsis root anatomy. Comparison of phloem development between wild type **(B, D, F, H, J, L)** and *shr-2* **(C, E, G, I, K, M)** roots. **(B, C)** Transverse sections through the maturation zones of wild type (**B**) and *shr-2* (**C**) roots, stained with toluidine blue. **(D, E)** Immunolocalization of the SE-ENOD in wild type (D) and *shr-2* (E) mature roots. Expression of *pAPL::GFP* in wild type **(F, J)** and *shr-2* **(G, K)**. Expression of *pSUC2::GFP* in wild type **(H, L)** and *shr-2* **(I, M)**. Scale bars, 10 μm; asterisks, pericycle position; arrows, xylem axis; red arrow heads, SEs; white arrow heads, the start of transition zone of the root.

In this study, we characterized and dissected the role of SHR in phloem patterning. Our investigation suggests that SHR moving into to a phloem initial triggers the cell division that forms two SEs. We identified SHR targets in the SE development using a cell type specific genome-wide expression profiling. Through further characterization of SHR targets, we discovered NARS1, an NAC domain transcription factor, as a regulator of the cell division for SE formation. Our findings add another layer to the complex roles of SHR in the root development and open up an understanding of phloem patterning, a previously under-explored field in plant development.

Results and Discussion

SHR controls phloem patterning

To determine whether SHR is involved in the phloem development, we carefully examined phloem morphology in *shr-2* roots. In addition to the xylem patterning deficiency reported previously, we found severe disruption in the phloem development. In the wild-type Arabidopsis root, xylem axis is a single row of cells made of protoxylem and metaxylem (Figure 3.1A). Perpendicular to the xylem axis, two poles of phloem tissues are formed. Different from the wild type where two SEs develop, in *shr-2* we observed only one SE-like cell in a phloem pole (Figure 3.1C). To examine SEs more accurately, we used immunohistochemistry to locate early SE nodulin-like proteins (SE-ENOD). These proteins specifically accumulate in differentiating and mature SE plasma membrane (Khan et al., 2007). In the wild type,

immunofluorescence detection of SE-ENOD under a confocal microscope allowed us to visualize both proto- and metaphloem SEs in each phloem pole (Figure 3.1D, Supplementary Figure 3.1A). In contrast, only one SE per phloem pole was detected in *shr-2* (Figure 3.1E).

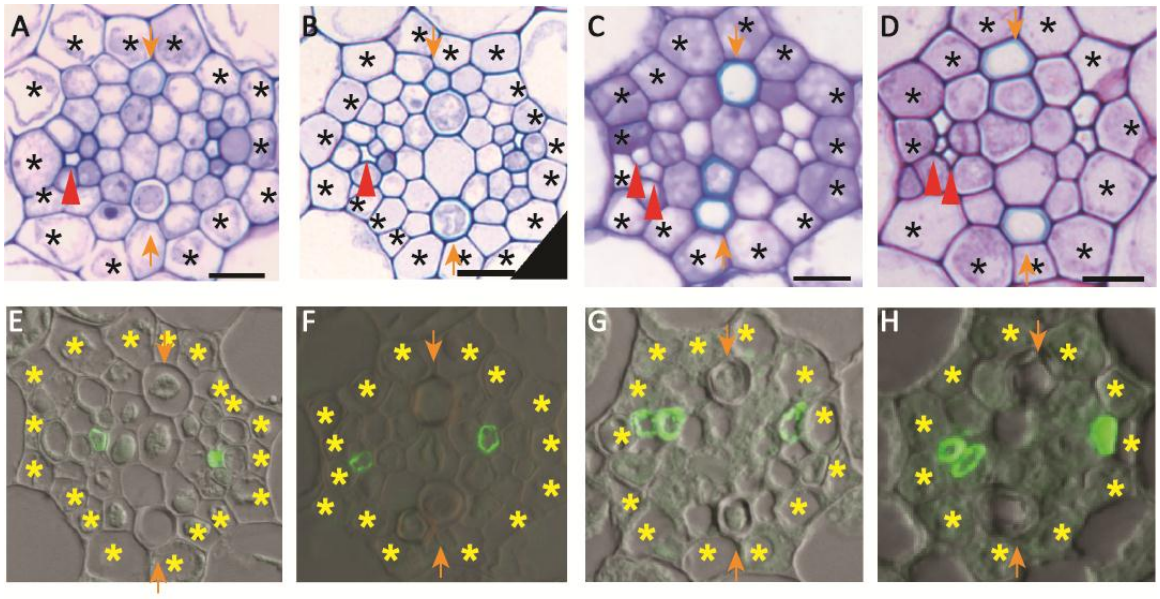
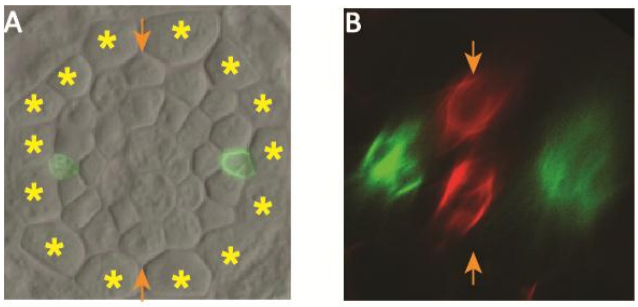


Figure 3.2. Stele cell proliferation and phloem sieve elements.

Toluidine blue stained transverse sections and immunolabled SE-ENOD of *pUAS*::*MIR165A; shr J0571* **(A, E)**, *shr-2 phb-6* **(B, F)**, *scr-4* **(C, G)** and *pCRE1::PHBem-GFP* **(D, H)**. Scale bars, 10 µm; asterisks, pericycle position; arrows, xylem axis; arrow heads, SEs.



Supplementary Figure 3.1. Phloem patterning in wild type and *shr-2 phb-6*.

(A) Immunolocalization of the SE-ENOD labels when the SEs start to differentiate in the wild type. **(B)** Expression of *pSUC2::erGFP* in *shr-2 phb-6*. Asterisks, pericycle position; arrows, xylem axis.

To further define cells that develop into phloem, we introduced phloem markers and examined their expression patterns in the wild type and *shr-2*. Previous lineage analysis on vascular cells in the *Arabidopsis* root showed that proto- and metaphloem SEs are generated from a periclinal division of a SE initial cell and each CC is generated by a tangential division of a procambium cell (Baum et al., 2002; Bonke et al., 2003). These events occur at 50 µm above the quiescent center. *pAPL::erGFP* (ER-localized GFP) expression starts in the developing protophloem SEs as soon as they emerge from the asymmetric division of a SE initial cell in the meristem (Figure 3.1F) and then switches to the developing CCs and metaphloem SEs when the protophloem SEs mature (Bonke et al., 2003) (Figure 3.1J). Expression of *pAPL::erGFP* in the *shr-2* started not in the meristem but in the transition zone between meristem and elongation, suggesting a delay in phloem SE initiation (Carlsbecker et al., 2010) (Figure 3.1G). In *shr-2*, GFP expression of *pAPL::erGFP* was first observed in a single SE-like cell and then the expression expanded asymmetrically to only one of its neighboring cells (Figure 3.1K). However, the

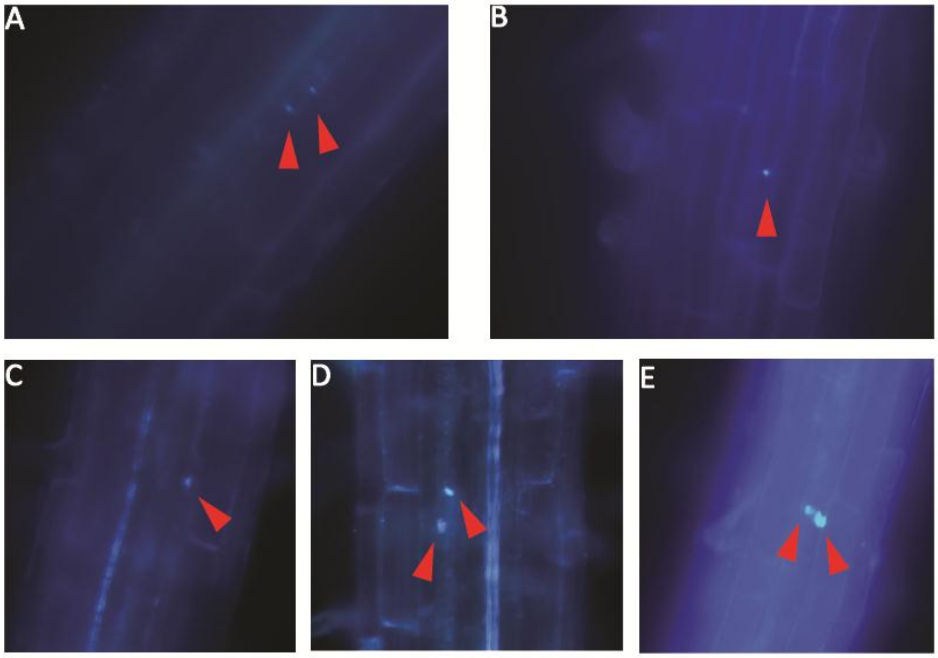
expression in the SE-like cell stayed throughout the root instead of switching to the metaphloem SE cell, consistent with our observation of a single SE cell in a phloem pole of *shr-2*. Furthermore, the cell expressing GFP in a later stage locates in the anticlinal side of the SE-like cell, which is very likely to be the CC. To further examine the status of CC, we analyzed the expression of *pSUC2::erGFP*, which is specifically expressed in the CCs in the mature part of a root, in the wild-type and *shr-2* roots (Stadler and Sauer, 1996) (Figure 3.1H and 3.1L). Unlike in the wild-type roots where *pSUC2::erGFP* was continuously expressed in the two CCs, in the *shr-2* we observed sporadic expression of GFP only in one cell next to the hypothetical SE (Figure 3.1I and 3.1M). Taken together, it appears that when SHR function is lost, cell division activities for the formation of both CCs and SEs are compromised.

SHR controls proliferation of phloem companion cells in a non-cell-autonomous manner

We previously reported that SHR together with SCR in the endodermis regulates xylem patterning in a non-cell-autonomous manner (Carlsbecker et al., 2010). SHR achieves this through the activation of *miR165/6* that moves into the stele and then posttranscriptionally represses *HD-ZIP III* members. We asked whether SHR-SCR-miR165/6 pathway also influences the phloem patterning. To this end, we examined the phloem patterning in the *pUAS*::*MIR165A; shr-2 J0571* line which drives *miR165* expression in the ground tissue (Carlsbecker et al., 2010). As we showed before, this transgenic line rescued xylem phenotype in the *shr-2*. Furthermore, we observed a recovery of cell numbers in the stele (Figure 3.2A). Among the five *HD-ZIP III*

members, PHABULOSA (PHB) was reported as a major downstream regulator of SHR in the control of xylem patterning (Carlsbecker et al., 2010). Consistently, *shr-2 phb-6* also restored the stele cell number, similar to the *UAS*::*MIR165A; shr-2 J0571* (Figure 3.2B).

Though the stele cell population recovered significantly in *pUAS*::*MIR165A shr-2 J0571* and *shr-2 phb-6*, the number of functional phloem SEs did not seem to increase in these lines. In the wild type, mature SEs deposit callose in boundaries between SEs and exhibit unique histological characteristics, which are small cell size, ribbed cell shape and relatively thick cell walls. Based on these morphological features, both *pUAS*::*MIR165A shr J0571* and *shr-2 phb-6* appeared to have only one SE, similar to *shr-2* (Figure 3.2 A and 3.2B arrow head and Supplementary Figure 3.2 for aniline blue staining). We then used SE-ENOD antibody to detect the SEs in these two genetic backgrounds. Consistent with callose analysis, only one SE per phloem pole was detected in *pUAS*::*MIR165A shr J0571* and *shr-2 phb-6* (Figure 3.2E and 3.2F). Since the cells recovered in the phloem pole of *shr-2 phb-6* are not SEs, we asked whether they are ectopic CCs. Indeed, more than two cells that surround a SE showed the expression of *pSUC2::GFP* in *shr-2 phb-6* (Supplementary Figure 3.1B).



Supplementary Figure 3.2. Aniline blue staining for SEs.

(A) wild type, **(B)** *shr-2*, **(C)** *shr-2 phb-6*, **(D)** *pCRE1::SHRΔNLELDV:nlsGFP; shr-2* and **(E)** *pS32::SHRΔNLELDV:nlsGFP; shr-2*. Arrow heads, SE.

SCR interacts with SHR in the endodermis to produce *miR165/6*. *scr-4*, the loss-of-function mutant displays similar disruption of cell proliferation activity and xylem patterning to *shr-2*. To further examine the influence of miR165/6 on the cell division for SE development, we examined the SEs in the *scr-4*. Although the cell number in the stele was reduced to the similar level to *shr*'s, *scr-4* constantly showed two SEs in at least one of the phloem poles (Figure 3.2C and 3.2G). A similar event was observed when we examined the SEs in the transgenic plants with *pCRE1::PHBem-GFP* that expresses a microRNA resistant version of *PHB* under the promoter of a stele specific gene *CRE1* (Mahonen et al., 2000) (Jose et al. unpublished data). This results in the over-expression of *PHB* throughout the stele, like in the *shr*

mutant. Similar to the *shr-2*, roots expressing *pCRE1::PHBem-GFP* exhibited significant reduction in the stele cell number (Figure 3.2D). However, the number of SEs did not decrease (Figure 3.2H). Based on these data, we conclude that stele cell proliferation, including CCs in the phloem pole, is under the control of bidirectional signaling that involves SHR, SCR, miR165/6 and PHB. However, our data indicate that the cell division for SE formation is regulated independently of the bidirectional signaling.

PHB is transcribed throughout the stele in the meristem of a wild-type Arabidopsis root, however its mRNAs and proteins are excluded from the stele periphery including the phloem pole (Carlsbecker et al., 2010). We asked whether ectopic PHB expressed in the phloem pole of the *shr* represses cell proliferation in the stele. To address this, we expressed microRNA resistant *PHB* (*PHB-M*) fused to GFP under the phloem specific promoter *pAT2G18380* (*S32*) in *shr-2 phb-6* (Lee et al., 2006). Indeed, *pS32::PHB-M:GFP* in *shr-2 phb-6* repressed cell proliferation in the stele. Therefore, for proper cell proliferation in the stele, *PHB* mRNAs in the phloem pole of the wild type root should be actively degraded via SHR-SCR-miR165/6 pathway.

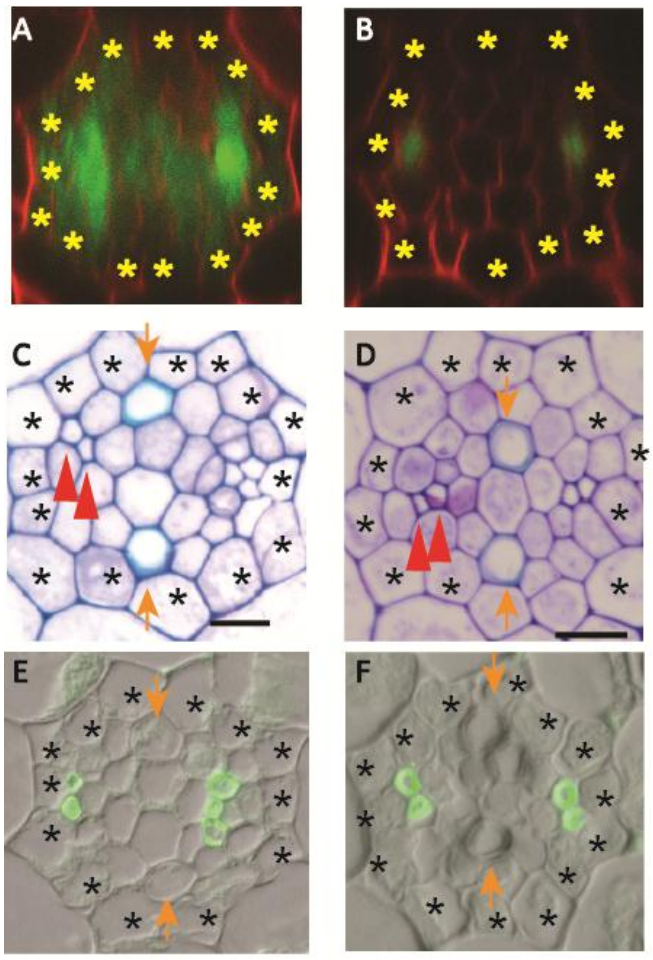


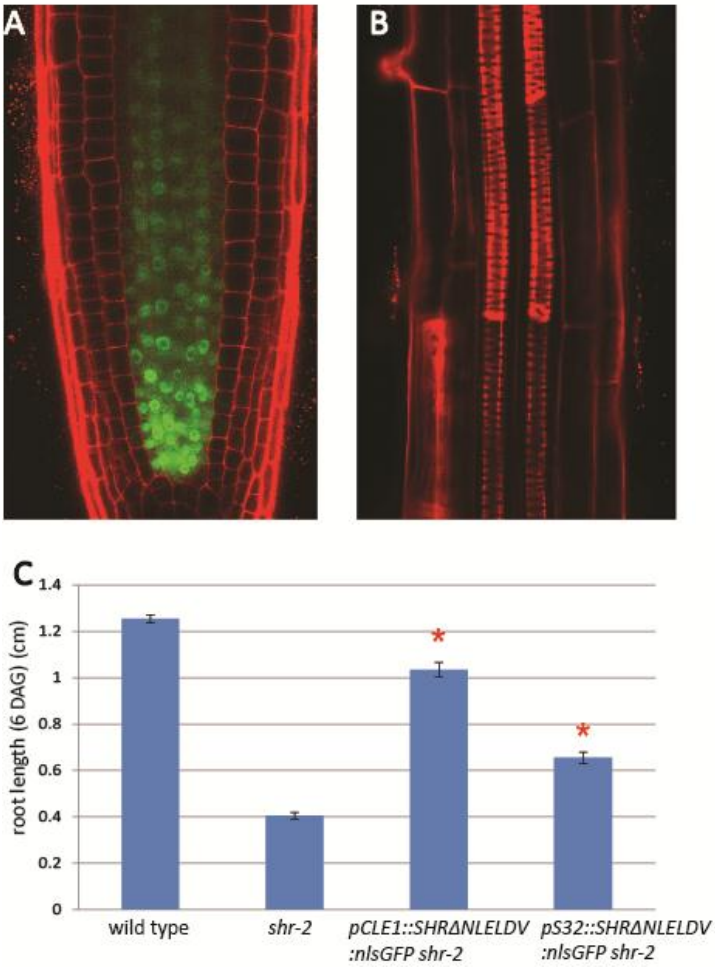
Figure 3.3. SHR expressed in the stele rescues SE cell division in *shr-2*.

Confocal cross sections of root meristematic zone of *pCRE1::SHRΔNLELDV:nlsGFP; shr-2* **(A)** and *pS32::SHRΔNLELDV:nlsGFP; shr-2* **(B)** show the GFP signal indicating the expression pattern of non-mobile SHR protein. Toluidine blue stained transverse sections and immunolocalization of the SE-ENOD of *pCRE1::SHRΔNLELDV:nlsGFP; shr-2* **(C, E)** and *pS32::SHRΔNLELDV:nlsGFP; shr-2* **(D, F)**. Scale bars, 10 μm; black asterisks, pericycle position; arrows, xylem axis; arrow heads, SEs.

SHR in the phloem initial promotes cell division for phloem sieve elements

SHR proteins are present in the stele, endodermis and quiescent center. Since the

bidirectional signaling does not regulate the formation of two SEs, we asked if SHR in the stele promotes this process. To address this, we expressed *SHR* exclusively in the stele in the *shr-2* background. We did this by expressing a non-mobile version of SHR, *SHRΔNLELDV* which was fused to GFP with a nuclear localization signal, under the *CRE1* promoter (Mahonen et al., 2000; Carlsbecker et al., 2010). *SHRΔNLELDV* does not have cell-to-cell mobility but still retains its biological function (Gallagher and Benfey, 2009). By using *pCRE1::SHRΔNLELDV:nlsGFP; shr-2*, we can analyze SHR function in the stele in the *shr* mutant background (Figure 3.3A, Supplementary Figure 3.4A). In *pCRE1::SHRΔNLELDV:nlsGFP; shr-2*, we observed a partial recovery of root growth and increase in phloem cell population (Figure 3.3C, Supplementary Figure 3.4C). Consistent with the previous finding (Carlsbecker et al., 2010), xylem patterning was not restored in this transgenic line (Supplementary Figure 3.4B). However, based on the histological analysis, the increase in the cell number on the phloem poles seemed to accompany the increase in SEs (Figure 3.3C). Immunolocalization of the SE-ENOD verified the increase in SEs in each phloem pole in *pCRE1::SHRΔNLELDV:nlsGFP shr-2* (Figure 3.3E). These data strongly support the idea that SHR in the stele promotes the cell division for SE formation.

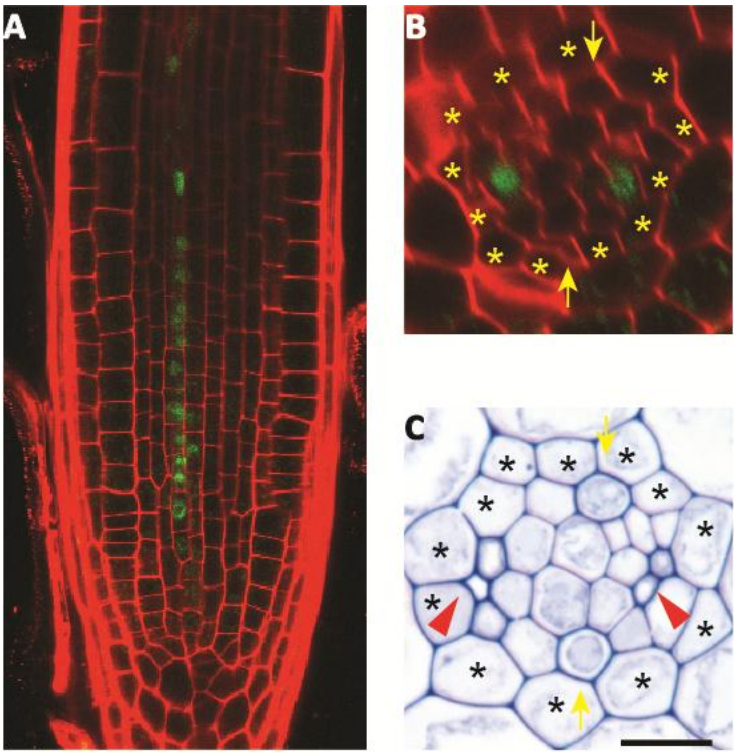


Supplementary figure 3.4. SHR expression in the stele affects root growth.

(A) *SHRΔNLELDV* driven by *pCRE1* is expressed in the stele of meristematic zone in the shr-2. **(B)** *pCRE1::SHRΔNLELDV:nlsGFP; shr-2* showed the same ectopic metaxylem phenotype as in *shr-2*. **(C)** Root length comparison among 6 DAG seedlings of wild type, *shr-2*, *pCRE1::SHRΔNLELDV:nlsGFP; shr-2* and *pS32::SHRΔNLELDV:nlsGFP; shr-2*. Error bars represent sd; red Asterisks indicate the difference is significant as compared with *shr-2*, $p<0.001$.

In the root meristem, *SHR* is natively expressed in the xylem precursors and procambium cells but not in the phloem poles. But, SHR protein moves into phloem poles. We asked whether mobility of SHR into phloem initials is required for their cell

division for SEs. To address this, we expressed *SHRΔNLELDV* in the phloem initial in the *shr-2* by driving gene expression under *S32* (Figure 3.3B). *pS32::SHRΔNLELDV:nlsGFP* did not recover the stele cell proliferation in *shr-2* at a significant level (Figure 3.3D). However, we found the formation two SEs in *pS32::SHRΔNLELDV:nlsGFP; shr-2* instead of one in the *shr-2* (Figure 3.3F). These suggest that SHR proteins moving into the phloem initial promote the cell division for the SE formation. Interestingly, the root length of *pS32::SHRΔNLELDV:nlsGFP; shr-2* was slightly longer than *shr-2*'s ($p<0.05$) (Supplementary Figure 3.4C).



Supplementary figure 3.4. PHB expression in the phloem suppresses the stele cell proliferation activity in *shr-2 phb-6*.

Longitudinal **(A)** and transverse **(B)** confocal sections of *pS32::PHB-M:GFP* in *shr-2 phb-6*. Toluidine blue stain indicates that there are fewer cells in the stele **(C)**. Scale bars, 10um; Asterisks, pericycle position; arrows, xylem axis; arrow heads, phloem SEs.

Previous histological studies suggested that SEs and CCs are generated from the asymmetric division of different initial cells instead of a series of asymmetric divisions from a single initial cell (Baum et al., 2002; Bonke et al., 2003). Consistent with these, our study suggests that cell division activities for the formation of SE and CC are controlled by two distinctive pathways. It is SHR moving into the phloem initial that triggers asymmetric division for the formation of two SEs. Meanwhile, SHR in the endodermis controls the asymmetric cell division of procambial cells for the CC formation in a non-cell autonomous manner through *miR165/6*. PHB seems to

inhibit this process.

Despite of the recovery of SEs in *pS32::SHRΔNLELDV:nlsGFP; shr-2*, we frequently observed that SEs in this transgenic line did not align perpendicular to the xylem axis as seen in the wild type (Figure 3.3H). Such misalignment of SEs was also observed in the *scr-4* and transgenic plants that express microRNA-resistant *PHB* throughout the stele (Figure 3.2C, 3.2D, 3.2G and 3.2H). These indicate that there might be unknown cell-cell interaction between SE and CC which allows them to coordinate with each other and generates a more precise pattern of the phloem cell types. It is also noteworthy that the cell division for SEs relies more on the presence of SHR in the phloem cells than on the proliferation of other stele cells.

SHR regulates the phloem specific gene expression

Our results strongly suggest that SHR moving into the phloem initial promotes cell division for SE formation in the root meristem. In this process, SHR likely turns on the expression of another transcriptional regulator(s) in the phloem initial that promotes the cell division. To further investigate the downstream pathway that controls the cell division for SE formation, we employed high-resolution cell-type specific gene expression data in *Arabidopsis* roots (Nawy et al., 2005; Lee et al., 2006; Levesque et al., 2006; Brady et al., 2007; Carlsbecker et al., 2010). First, we identified phloem-enriched genes using cell type specific data (at least 3 fold enriched in phloem cell types and corrected p-values < 0.001). 1089 genes fell into the given

criteria (Figure 3.4A).

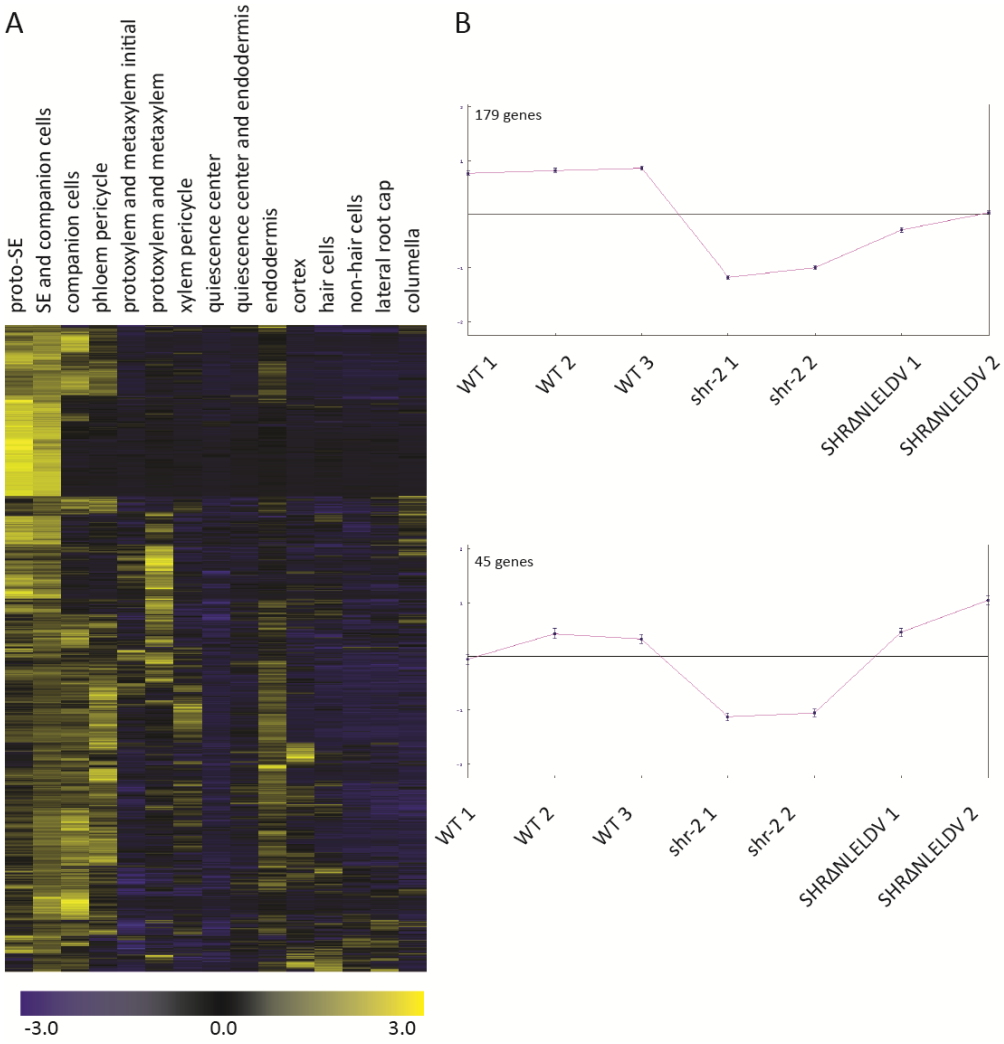


Figure 3.4. Expression analysis to identify downstream genes of SHR that control phloem development.

(A) Root expression of genes that are enriched in the phloem cell types. **(B)** Two QT clusters that identified phloem-enriched genes which are under the regulation of SHR in the stele.

To find out which of the genes enriched in the phloem might be involved in the cell division of a phloem initial mediated by SHR, we further dissected SHR function

and analyzed the influence of SHR in the stele by generating genome-wide gene expression data. First, *pCRE1::erGFP* was introduced into wild type and *shr-2* backgrounds to express GFP in the stele cells in the root meristem. Then, we collected GFP-expressing stele cells from the wild type, *shr-2* and *pCRE1::SHRΔNLELDV:nlsGFP; shr-2* through Fluorescence Activated Cell Sorter (FACS). RNAs were extracted from the sorted cells of each line and labeled probes prepared from these RNAs were hybridized onto GeneChip Arabidopsis Tiling 1.0R Array (Affymetrix).

To understand how SHR controls SE formation, we examined the influence of SHR on the aforementioned phloem enriched genes in the tiling array. We made this approach because SHR is a positive regulator of SE formation, thus it likely promotes the expression of phloem enriched genes. To find phloem-enriched genes that are up-regulated by SHR, we clustered expression of the 1089 phloem enriched genes in the wild type, *shr-2* and *pCRE1::SHRΔNLELDV:nlsGFP shr-2* (Heyer et al., 1999). In this analysis, we found two clusters made of 224 genes that are down-regulated in *shr-2* in comparison to the wild type and then restore expression in *pCRE1::SHRΔNLELDV:nlsGFP; shr-2* (Figure 3.4B; Supplementary Table 3.1). We found 24 transcription factors in this list. Based on the gene annotation and literature survey, we chose 6 transcription factors from these for further characterization (Table 3.1).

Table 3.1. Candidate TFs enriched in the phloem cells for further characterization in the SHR cell-autonomous pathway.

AGI no.	TF family	enrichment in the phloem (fold)	Annotation
AT1G79430	MYB (APL)	42.61072951	Control of phloem specification (Bonke et al., 2003)
AT4G28500	NAC (SND2)	30.80055263	Control of vascular cell differentiation in Arabidopsis and Populus (Grant et al., 2010)
AT1G71692	MADS (AGL12)	49.56715591	Involved in root cell differentiation (Burgeff et al., 2002)
AT3G45610	C2C2-Dof	14.5007208	Other family members displayed either phloem specific expression or control of phloem proliferation activity (Papi et al., 2002; Guo et al., 2009)
AT3G54390	Trihelix	4.780052215	
AT3G15510	NAC (NARS1)	3.916336659	

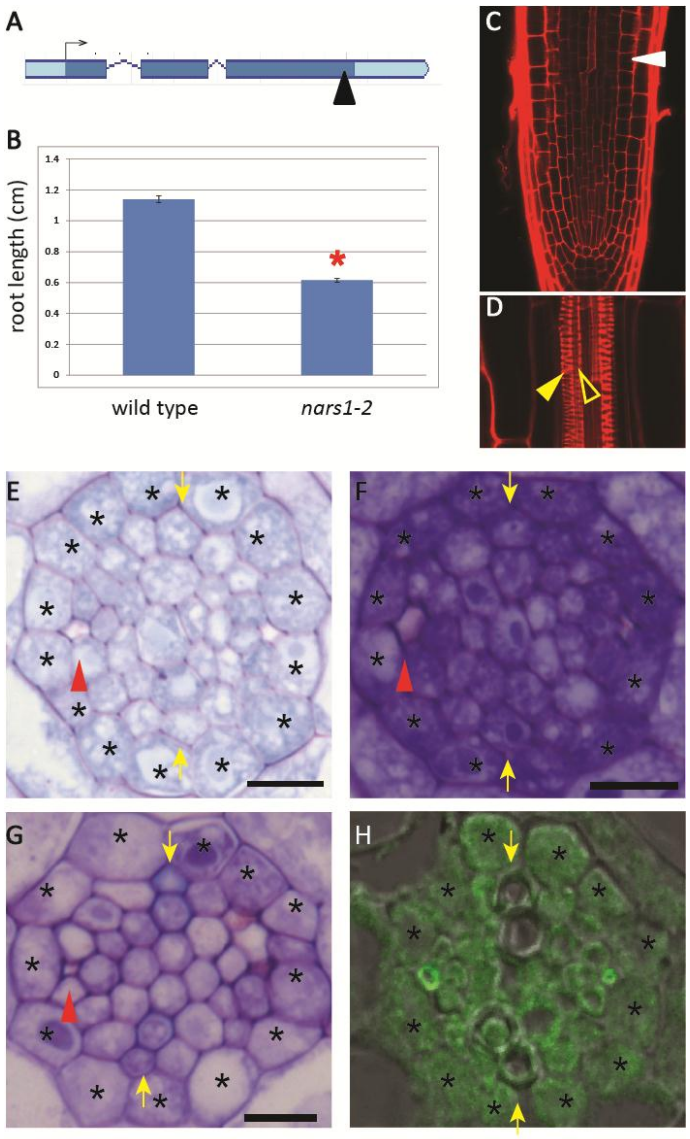


Figure 3.5. *NARS1* regulates SE cell division in the root.

(A) Predicted intron/exon structure of *AT3G15510*. Exons are shown as dark blue boxes (Arrow, the start codon of *AT3G15510* transcript; black arrow head, positions of *nars1-2* insertion). **(B)** Average root lengths in 6 day old seedlings of wild type and *nars1-2* (Error bars represent sd; red Asterisks indicate the difference is significant as compared with wild type, $p<0.001$). Longitudinal confocal section through the root tip **(C)** and mature xylem **(D)** of *nars1-2*. Comparison of steles in the meristems of wild type **(E)** and *nars1-2* **(F),** stained with toluidine blue. Toluidine blue stained transverse sections **(G)** and immunolocalization of the SE-ENOD **(H)** in the maturation zone of *nars1-2* roots. Scale bars, 10 µm; black asterisks, pericycle position; arrows, xylem axis; yellow arrow heads, protoxylem; hollow arrow heads, metaxylem; red arrow heads, SEs.

NARS1 regulates cell division for phloem SE formation as downstream of SHR

In the analysis of candidate genes, a T-DNA insertion line (*SALK_137131*; *nars1-2* hereafter) to *AT3G15510* displayed disturbed root growth phenotype (Figure 3.5A). NARS1 (NAC-REGULATED SEED MORPHOLOGY 1) is a NAC domain transcription factor. A previous study indicated that it regulates the development and degeneration of ovule integuments during embryogenesis (Kunieda et al., 2008). However, nothing was reported about its role in the root development. At 6 day after germination, seedlings of *nars1-2* had roots significantly shorter than the wild type (Figure 3.5B). Though the root length of *nars1-2* is longer than *shr-2*, the root length phenotype of *nars1-2* is consistent with previous studies that SHR controls root growth in a combinatorial manner from both in the stele and more importantly via *miR165/6* in the endodermis (Carlsbecker et al., 2010). Confocal analysis also suggested that the meristem size of *nars1-2* roots is much smaller than that of wild type roots (Figure 3.5C). Though the meristem size and root length were compromised in *nars1-2*, the stele cell number in *nars1-2* was not affected (Figure 3.5E and 3.5F). This is distinctive from the *shr*, *scr* and *wol* mutants, all of which display a significant reduction in cell division activities for both apical root growth and stele cells (Benfey et al., 1993; Di Laurenzio et al., 1996; Helariutta et al., 2000; Mahonen et al., 2000). Furthermore, unlike in the *shr-2* where xylem patterning is disturbed, xylem patterning remained normal in *nars1-2* (Figure 3.5D).

Histological analysis of the maturation zone in *nars1-2* roots indicated that CC patterning is normal, but SE formation is not (Figure 3.5G arrowhead). To further confirm SE phenotype, we applied immunostaining to detect the SEs in *nars1-2*.

Indeed, our analysis showed that only one SE is present in each phloem pole of *nars1-2*, mimicking the phenotype of the *shr-2* (Figure 3.5H). Taken together, we conclude that *NARS1* controls the cell division for SE formation as downstream of SHR.

Conclusion

In this study, we investigated an early developmental process of phloem and found that SHR is the key regulator of cell division for both SE and CC formation. We successfully dissected SHR function and found that SHR controls these two processes through distinct pathways. However, these pathways share a common feature in that both require the cell-to-cell trafficking of SHR. Our data suggest that the SE division is regulated by SHR that moves into the phloem initial. Using the microarray based approach, we identified *NARS1* as a potential downstream target of SHR that promotes cell division for SEs. A mutation in *NARS1* disrupts the SE division similar to the *shr-2* mutant phenotype. However, neither the xylem patterning nor the CC development is affected in *nars1*. These suggest that *NARS1* is specifically involved in the cell division for SEs. Though the radial growth of meristem seems normal in the *nars1-2*, the root growth activity is reduced (Figure 3.5B). Consistently, in the *pS32::SHRΔNLELDV:nlsGFP; shr-2*, where the only noticeable restoration of the root structural morphology is the SE differentiation, root length is significantly longer than in *shr-2* (Figure 3.3I). Taken together, fully functional SEs seems to have an impact on the root growth. It might be because the reduced SE files fail to translocate adequate carbohydrates to the roots, and thereby hinder the root growth (Freixes et al.,

2002; Ingram et al., 2011).

In summary, our studies expanded the knowledge on the diverse role of SHR during root development. The novel roles of SHR in the control of two aspects of phloem patterning add another layer of understanding on the sophisticated root development mediated by extensive cell-to-cell communications. So far, studies on the early phloem developmental processes are very limited. Further studies on NARS1 and other remaining potential targets of SHR in the SE pathway will allow us to decipher the phloem patterning and further the understanding of the phloem transport system.

Materials and Methods

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used. Seeds were surface sterilized, plated (0.5 × MS medium with 1% sucrose), grown under a 16-h-light/8-h-dark cycle at 22-23℃ in a plant growth chamber. *nars1-2* (*SALK_137131*) was obtained from the Arabidopsis Biological Resources Center (ABRC). The following marker lines were described previously: *pAPL::GFP* (Bonke et al., 2003); *pSUC2::GFP* (Stadler and Sauer, 1996). Primers used for genotyping were listed in Supplementary Table 3.1.

Supplementary Table 3.1 List of primers used in Chapter 3

Primers for genotyping		
	Primer name	Sequence
Genotyping *shr-2* wt allele	SHR-2876R	ATCAACCATCACCACACGTG
	SHR-1670F	GACACTGCACGTGCGCAACA
Genotyping *shr-2* mutant allele	SHR-2F	ATACGCTTTTGCAGGTTATATCAGC
	SHR-2R	GAACTCCCATAAGCCTAGCGAATTT
Genotyping *phb-6* wt allele	Phb-6F2	TCGAGATTGGCGTCTGAGATAAA
	Phb-6R2	TTGGAAACGCATTCAAAGACAAT
Genotyping *phb-6* mutant allele	Phb-6F2	TCGAGATTGGCGTCTGAGATAAA
	Ds 3'-1	GGTTCCCGTCCGATTTCGACT
Genotyping *nars-2* wt allele	P6-1LP	CGCCGAAGTCGATCTCTATAA
	P6-1RP	CTCAGTCCAAAAACATCAGTG
Genotyping *nars1-2* mutant allele	P6-1LP	CGCCGAAGTCGATCTCTATAA
	P6-1RP	CTCAGTCCAAAAACATCAGTG
Primers for cloning		
Cloning *WOL* promoter into pDONR P4_P1R	proWOL-2F	GGGGACAACTTTGTATAGAAAAGTTGTTCC TAGATTTTCTCACACACCA
	proWOL-1R	GGGGACTGCTTTTTTGTACAAACTTGA TCT GAGCTACAACAATAGAGAA
Cloning *SHRΔNLELDV* cDNA without stop coden into pDONR221	pSHR-2F	GGGGACAACTTTGTATAGAAAAGTTGGCG ATTCTGCTGAGTTATGTGT
	pSHR-2R	GGGGACTGCTTTTTTGTACAAACTTGTTTTT TTTTTTAATGAATAAGA
Cloning nlsGFP promoter into pDONR P2R_P3	nlsGFP-B2r	GGGACAGCTTTCTTGTATAGAAAAGTTGAT GGAGCAGAAGCTGATCC
	nlsGFP-B3	GGGGACAACTTTGTATAATAAAGTTGCTAC CCGGACTTGTACAGCTC
Generating PHB-m harboring a point mutation in the microRNA complementary site	PHB-G202G-F	GGATGAAGCCTGGACCGGATTCTATTGGC
	PHB-G202G-R	GCCAATAGAATCCGGTCCAGGCTTCATCC

Plasmid Construction

Gaterway cloning technology (Invitrogen) was used for DNA manipulations. *CRE1* promoter was amplified from *Arabidopsis* Col-0 genomic DNA and cloned into pDONR P4_P1R. Generation of *S32* promoter in pDNOR P4_P1R was described in (Lee et al., 2006). *PHB* cDNA was cloned into pDONR221 and mutagenized to *PHB-M* as previously described (Carlsbecker et al., 2010). *SHRΔNLELDV* was amplified from the plasmid containing *SHRΔNLELDV* and cloned into pDONR221 (Gallagher and Benfey, 2009). *GFP* and *nlsGFP* were cloned into pDONR P2R_P3 by BP recombination. *pS32::PHB-M:GFP* was constructed into dpGreen-Bar by Multisite gateway LR recombination. *pCRE1::SHRΔNLELDV:nlsGFP* and *pS32::SHRΔNLELDV:nlsGFP* were constructed into dpGreen-BarT. All the clones in the binary vector was transformed into *Agrobacterium* GV3101 with pSOUP and transformed into *shr-2* or *shr-2 phb-6*. Primers used for cloning were listed in Supplementary Table 3.1.

Histological Analysis

All seedling samples were collected at 6 days after germination. Confocal images were obtained using a Leica TCS SP5 Laser Scanning Confocal Microscope with the preset emission/excitation wavelength of 488 nm/505 to 530 nm for GFP and 561 nm/591 to 635 nm for propidium iodide (PI). For the visualization of the root structure, all the seedlings were stained in 2 μg/ml PI. Root transverse sections and toluidine blue staining were done as described in (Scheres et al., 1995).

Immunostaining

Seedlings were collected at 6 days after germination. Immunolocalization of Arabidopsis roots was done as previously described in (Paciorek et al., 2006). The primary antibody RS6 (Khan et al., 2007) was diluted in a ratio of 1:100 into blocking solution and incubate for 1.5 hours at 37 ℃. The second antibody Alexa Fluor® 488 F(ab')2 Fragment of Goat Anti-Mouse IgG, IgM (H+L) (Life Technologies) was diluted in a ratio of 1:200 in blocking solution and incubated for 1 hour at room temperature. The microscope slides were mounted with antifadent AF1 (Citifluor Ltd) and examined using Leica TCS SP5 Laser Scanning Confocal Microscope with the emission/excitation wavelength of 488 nm/505 to 530 nm.

Microarray Experiments

All the seedling samples were collected at 6 days after germination and the half-bottom parts of roots were cut and harvested. The protoplast preparation and FACS facilitated cell sorting were done as described in (Birnbaum et al., 2005). Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen). RNA integrity was determined on a Bioanalyzer (Agilent BioAnalyzer 2100). Probe preparation were carried out according to the manufacturer's instructions (GeneChip Whole Transcript Double-Stranded Target Assay Manual from Affymetrix Inc.), and then biotinylated double stranded DNA probes were hybridized to the Arabidopsis Tiling 1.0R arrays (Affymetrix Inc.). Two to three biological replicate were generated.

Microarray Analysis

In order to isolate genes that are involved in the phloem regulatory networks, we compared gene expression data among phloem SE (S32), SE and companion cells (APL), companion cells (SUC2), phloem pole pericycle (S17), protoxylem and metaxylem initials (S4), protoxylem and metaxylem (S18), xylem pericycle (J0121), quiescence center (AGL42), quiescence center and endodermis (scr5), endodermis (E30), cortex (CORTEX), hair cells (COBL9), non-hair cells (gl2), lateral root cap (LRC), columella (pet111) (Nawy et al., 2005; Lee et al., 2006; Levesque et al., 2006; Brady et al., 2007; Carlsbecker et al., 2010) using LIMMA package (Smyth, 2004). We identified 1089 genes that are enriched in S32 and/or APL cell file (fold enrichment >3; corrected p-value < 0.001).

Microarray data from Tiling 1.0R array CDF that contains gene-specific single-copy exonic probe sets were normalized using RMA algorithm in BIOCONDUCTOR (Irizarry et al., 2003; Naouar et al., 2009). High correlation coefficients were confirmed within biological replicate data. Then the aforementioned 1089 phloem enriched genes were examined about their expression levels in wild type, *shr-2*, and *pCRE1::SHRΔNLELDV:nlsGFP; shr-2*. Quality threshold (QT) clustering of differentially expressed genes was performed and visualized using MultiExperimental Viewer (Saeed et al., 2006).

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Chapter 4 Vignettes and Conclusive Remarks

The main goal of my dissertation projects was to understand molecular mechanisms underlying the morphogenesis of plant vascular tissues. To this end, I investigated transcription factors that are expressed in the early xylem or phloem precursor cells in a cell type enriched manner using *Arabidopsis* root as a model system (Appendix I). Plant morphogenesis is a highly organized process that largely relies on positional information. Such positional information is established by cell-to-cell communication which turns on cell-type specific regulatory networks that lead to specification and differentiation of cell types. The approach to investigate the developmental function of cell type enriched transcription factors in vascular tissues turned out to be so powerful that I was able to discover several developmental regulators from a small number of candidates. It is very interesting yet not surprising to find the extensiveness of cell-to-cell communications via movement of transcription factors during vascular tissue development. Among several regulators of vascular tissue development, I concentrated on investigating mobile transcription factors as presented in Chapter 2 and 3.

In chapter 2, we reported an AT-hook transcription factor AHL4 that moves from the procambium to the xylem to draw the cell boundaries between two distinctive cell populations (Figure 4.1). It is the first time that a transcription factor is reported to serve as an intercellular signal to maintain the vascular pattern in the root meristem. In Chapter 3, we also reported SHR function in the phloem pattern formation. Again, SHR serves as a mobile signal from procambium to the phloem initials where it

triggers the asymmetric cell division to generate proto- and metaphloem SEs. We also reported the new findings on *SHR/miRNA* pathway in which its regulation on *PHB* likely influences the formation of companion cells. Together with other results, our studies broaden the knowledge on the fundamental yet complicated mechanisms that a plant employs in the control of precise pattern establishment during development. In addition, it also improves our understandings on the vascular development that is of great interest for applications in bioengineering.

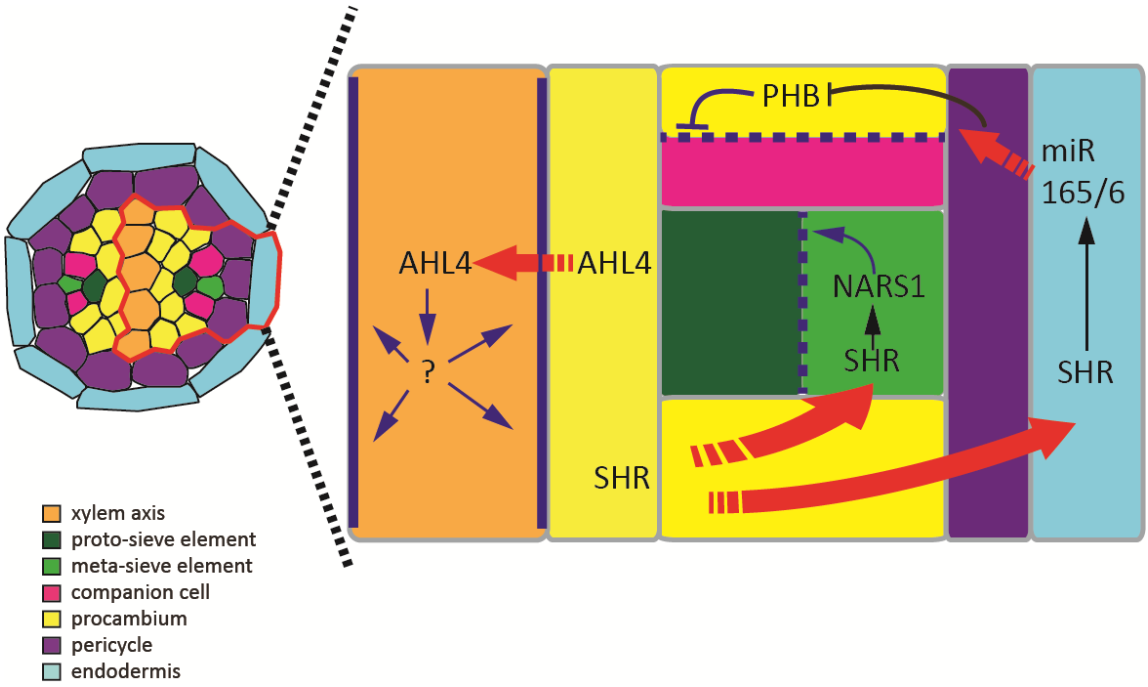

Figure 4.1. Proposed model for the pathways studied in the vascular patterning.

Both SHR and AHL4 proteins are expressed in the procambium. AHL4 proteins move into xylem axis mediating the cell-to-cell communication that defines the boundaries between xylem and procambium. SHR proteins move to both phloem and endodermis serving as intercellular signals for phloem patterning. SHR in the endodermis generates *miRNA 165/6* which then post-transcriptionally regulates *PHB* mRNA levels in the stele. In turn, the low level of PHB in the periphery of procambium promotes cell proliferation generating the companion cells. In the meantime, SHR moves to a phloem initial where it regulates cell division for SEs in a cell-autonomous manner. A transcription factor NARS1 has been identified to act the downstream of SHR in the control of cell division. Blue arrows, promoting the subsequent development processes; black arrows, transcriptional regulation; brown line, post-transcriptional regulation; thick arrow bars, molecule movement; blue dashed lines, cell division; blue solid lines, cell boundaries; question mark, unknown downstream networks.

Appendix I Characterization of transcription factors in the early vascular pattern formation

Jing Zhou, James Eaglesham, and Ji-Young Lee[1]

Transcription factors that affect xylem domains

In search of the transcription factors involved in the xylem patterning, we surveyed and selected transcription factors that are enriched in the xylem precursor cells. In this process, we used high resolution microarray-based gene expression data available in the most of cell types in Arabidopsis root (Brady et al., 2007). Transcription factors chosen for further characterization were at least 2-fold enriched in xylem precursor cell type with corrected p-values less than 0.001 in comparison to the other root cell types. 15 transcription factors were selected based on this query and their T-DNA insertion lines were screened for abnormal xylem phenotypes (Table I.1). Mutants of 4 out of 15 (27%) candidates showed abnormal xylem phenotype during the preliminary screen using confocal microscopy. This suggests that our approach to finding developmental regulators based on expression patterns is quite efficient. Interestingly, mutants of all the four transcription factors including two AT-hook

[1] **Author contributions** J.Z. and J.Y.L. designed experiments, J.Z. performed all the histological analysis and cloning, J.Z. and J.E. performed genotyping analysis for T-DNA insertion line identification listed in the xylem enriched transcription factor sceening. J.Z. and J.Y.L. wrote the manuscript.

family members reported in Chapter 2 share the similar extra xylem strand formation phenotype (Figures 2.1, I.1A and I.1B; Table I.1).

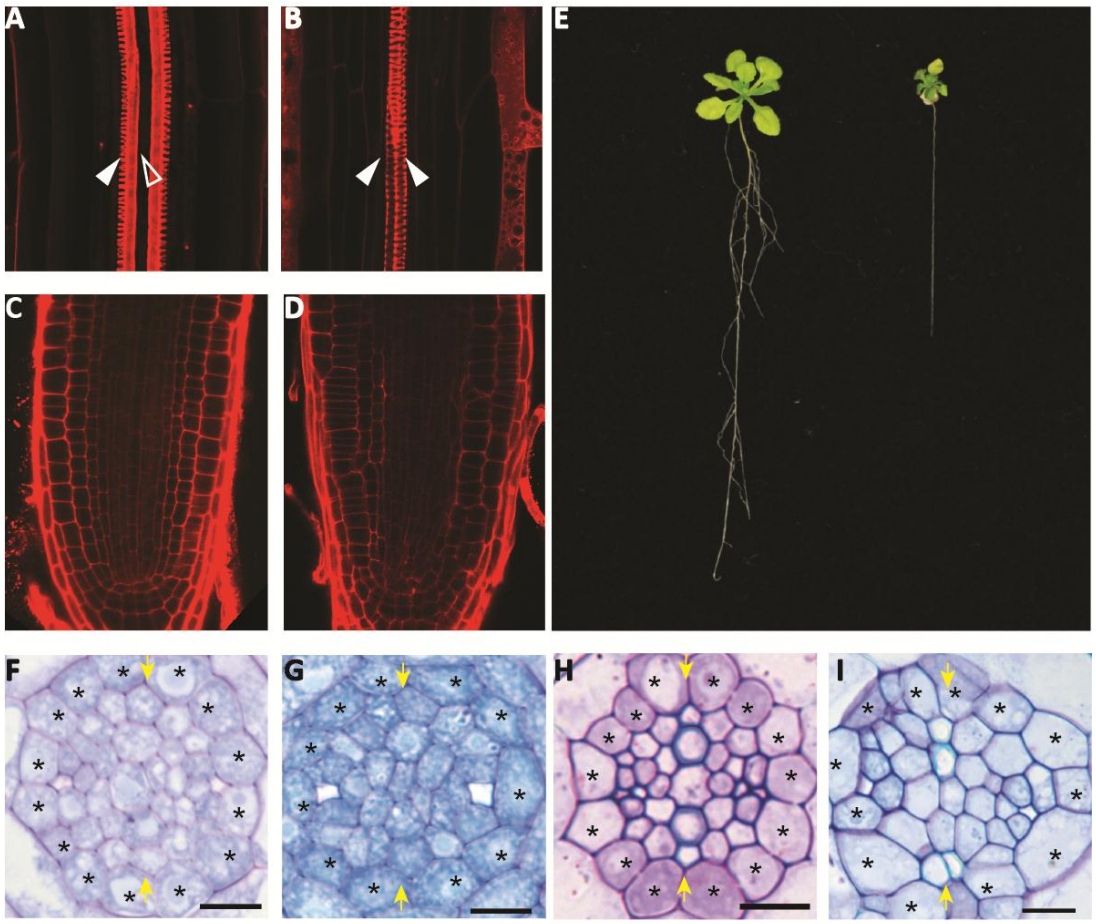

Figure I.1. Mutation of *AT1G29950* affects xylem patterning and root growth.

Confocal microscopy of xylem of wild type **(A)** and *SALK_147291* **(B),** stained with basic fuchsin**.** Longitudinal confocal section through the root tip of wild type **(C)** and *SALK_147291* **(D).** **(E)** Root growth comparison between wild type and *SALK_147291* of 20 day- old seedlings. Transverse sections of wild type **(F, H)** and *SALK_147291* **(G, I)** roots stained with Toluidine blue. (**F, G**) The meristematic zone. (**H, I**) The maturation zones. Scale bars, 10 μm; black asterisks, pericycle position; arrows, xylem axis; filled arrow head, protoxylem; hollow arrow head, metaxylem.

Table I.1. T-DNA insertion lines of xylem precursor enriched transcription factors and their preliminary phenotype description.

AGI no.	TF family	T-DNA insertion lines available	Phenotype of the insertion lines
AT5G51590	AT-hook	*SALK_124619c*	Extra protoxylem
		SALK_049264c	Extra protoxylem
AT4G25320	AT-hook	*FLAG_445H04*	Extra protoxylem
		FLAG_447A04	Extra protoxylem
AT4G12620	Alfin-like	*SALK_068008*	No phenotypes
		SALK_042536	No phenotypes
AT4G14700	Alfin-like	*SALK_104400*	No phenotypes
AT3G12270	C2H2(Zn)	*CS854084*	No phenotypes
AT1G44810	GeBP	*SALK_020363*	No phenotypes
AT1G09770	MYB	*CS348962*	No phenotypes
AT5G25475	ABI3/VP1	*SALK_086678*	No phenotypes
		SALK_015817C	No phenotypes
AT2G01940	C2H2(Zn)	*SALK_087765C*	No phenotypes
AT1G29950	BHLH	*SALK_147291*	Extra protoxylem; short root
AT3G22780	CPP	*SALK_074231*	No phenotypes
AT3G57150	NAC	*SALK_031065*	No phenotypes
AT5G64530	NAC	*SALK_023146*	Extra protoxylem
		SALK_023898	No phenotypes
AT1G52150	Homeobox	*CS879151*	No phenotypes
AT5G06710	Homeobox	*SALK_152121*	No phenotypes

However, root phenotypes of these mutants are not identical. For example, *SALK_147291* that harbors a T-DNA insertion in the first exon of *AT1G29950* displays a phenotype with short root and retarded meristem growth in addition to the disturbed xylem patterning (Figure I.1C to I.1E). Further histological analysis suggested that the vascular cell arrangement is also affected in the *SALK_147291* (Figure I.1F to I.1I). Using erGFP (endoplasmic reticulum localized GFP) driven by *AT1G29950* promoter, we observed expression of *AT1G29950* in the quiescent center, xylem cells, and endodermis (Figure I.2A). We also found GFP signal in the root meristem in the embryo (Figure I.2A inset). Cells expressing *AT1G29950* are consonant with the high level of auxin expression region (Sabatini et al., 1999; Schlereth et al., 2010; Bishopp et al., 2011). Previous studies also showed that AT1G29950 interacts with another bHLH protein TMO5-LIKE1, a putative target of auxin signaling regulator AUXIN RESPONSE FACTOR 5 (ARF5)/MONOTEROS (MP), in yeast (Ohashi-Ito and Bergmann, 2007; Schlereth et al., 2010). It is possible that *AT1G29950* acts in the auxin signaling pathway in the control of xylem patterning. Subsequently, losing the *AT1G29950* function may mimic the scenario that the auxin maxima in the protoxylem are somehow disturbed, thus results in the formation of extra xylem as reported before (Bishopp et al., 2011). In addition to *AT1G29950*, we presented in Chapter 2 that two AT-hook proteins AHL3/4 move from the procambium to the xylem to draw the boundaries between the two cell populations when the xylem is established. However, our preliminary investigation suggests that AHL3/4 might influence the boundary via an as-yet unknown pathway rather than a cytokinin/auxin pathway.

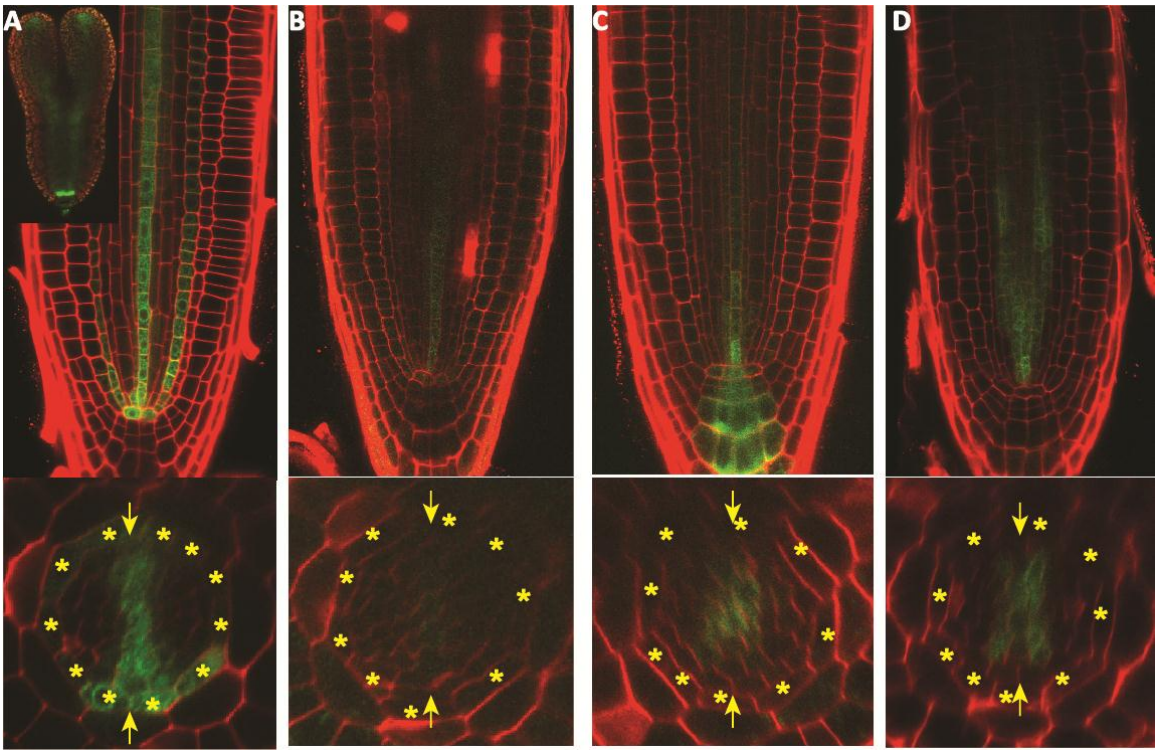

Figure I.2. Expression pattern of xylem enriched genes.

Confocal microscopic analysis of *pAT1G29950::erGFP* **(A)**, *pAT3G19300::erGFP* **(B)**, *pAT1G79630::erGFP* **(C)**, *pAT2G34060::erGFP* **(D)**. The longitudinal sections were shown in the upper panel and transverse sections in the lower panel. Asterisks, endodermis position; arrows, xylem axis.

Table I.2 Expression analysis of genes enriched in the xylem precursor cells

AGI no.	Annotation	Expression in the root	reference
AT4G32880	HD-ZIP III family transcription factor ATHB8	xylem cells of meristematic zone	(Carlsbecker et al., 2010)
AT2G34710	HD-ZIP III family transcription factor PHB	Strong in metaxylem cells and weak in procambium of meristematic zone	(Carlsbecker et al., 2010)
AT5G60690	HD-ZIP III family transcription factor REV	xylem cells and procambium of meristematic zone	(Carlsbecker et al., 2010)
AT1G80100	an cytokinin signaling inhibitory pseudophosphotransfer protein AHP6	Protoxylem cells and neighboring pericycle	(Mähönen et al., 2006)
AT1G29950	bHLH DNA-binding superfamily protein	Xylem cells and endodermis	Fig 4.2A
AT3G19300	Protein kinase superfamily protein	Xylem initials of meristematic zone	Fig 4.2B
AT1G79630	Protein phosphatase 2C family protein	Xylem initials and neighboring procambium of meristematic zone	Fig 4.2C
AT2G34060	Peroxidase superfamily protein	Strong in procambium next to the xylem and weak in the xylem cells	Fig 4.2D
AT3G57150	pseudouridine synthase	Everywhere in the root tip	N/A
AT5G08260	serine carboxypeptidase-like 35	Everywhere in the root tip	N/A
AT5G25830	GATA factor family of zinc finger transcription factor	Expression below detection	N/A

Genes that are enriched in the xylem could be used to mark specific stages of xylem development. Thus, we surveyed the aforementioned microarray database again looking for xylem marker genes (2-fold enriched in xylem precursor cell type with corrected p-values less than 0.001). 11 genes including 5 transcription factors were chosen for further analysis. Interestingly, 4 out of 11 candidate genes have been previous reported and shown to be expressed quite specific or enriched in the xylem in the root, illustrating the high efficiency and accuracy of our screening methods (Table I.2). In order to study the gene expression pattern in the root for the rest of the genes in the list, we generated DNA constructs of *erGFP* driven by each of the promoter regions of candidate genes and transformed them into the wild-type Arabidopsis. GFP signal was further analyzed with confocal microscopy. In addition to the *pAT1G29950::erGFP* discussed above that shows a very specific xylem expression in the vascular tissue, we also found two other genes (*AT3G19300* and *AT1G79630*) that are specifically expressed in the xylem of meristematic zone (Figure I.2B and I.2C). *pAT3G19300::erGFP* in particular was only observed in the first couple of xylem cells in the root tip indicating an particular phase of the xylem initials that can be marked by *AT3G19300* expression. Furthermore, we found *pAT2G34060::erGFP*, expressed strongly in the procambium cells that are next to the xylem, but only weakly in the xylem cells (Figure I.2D). Though *pAT2G34060::erGFP* cannot be used as a xylem marker, it certainly serves as a valuable procambium marker in the future studies. Overall, 7 out of 11 candidate genes showed specific or enriched xylem expression in the root and have a potential to serve as specific cell markers in future studies.

Table I.3. Candidate TFs with specific enrichment in the phloem precursor cells that belong to SHR cell-autonomous pathway for the complementation study.

AGI no.	TF family	Plant genotype for characterization	Preliminary phenotype
AT4G28500	NAC Family (SND2)	*pS32*::*AT4G28500:GFP* in *shr-2*	Partial restoration of root growth
AT3G45610	C2C2-Dof Family	*pS32*:: *AT3G45610:GFP* in *shr-2*	Missing expression in the meristem
AT1G79430	MYB family (APL)	*pS32*:: *AT1G79430:GFP* in *shr-2*	Reduced meristem size
AT1G71692	MADS Family	*pS32*:: *AT1G71692:GFP* in *shr-2*	No phenotype

Phloem enriched transcription factors that are downstream of SHORT-ROOT

In the study presented in Chapter 3, we used fluorescence activated cell sorting based gene expression profiling approach to identify transcription factors that control phloem cell division as downstream of SHR. We characterized T-DNA insertion lines of two candidate transcription factors. Among them, we found that NARS1 regulates cell division for phloem sieve elements (SEs), and described that finding in Chapter 3. In addition to these two candidates, the cDNAs of the other four candidate transcription factors listed in Table I.3 were fused with free GFP and expressed under a phloem specific promoter, *pAT2G18380* (*S32*) in *shr-2* background (Lee et al., 2006). We examined whether restoring the expression of these genes in the phloem initial can

recover the cell division for SEs. Preliminary analysis suggested that the expression of another NAC family transcription factor, *AT4G28500* (*SND2)*, in the phloem of *shr-2* partially restores root meristem size (Figure I.3A and I.3B arrowhead). NAC proteins constitute one of the largest plant specific families with more than 100 members in *Arabidopsis*. Studies implicated that NAC family members are involved in diverse processes. Given that SND2 and NARS1 only share 16% amino acid sequence identity, it is uncertain whether there are any connections between them in the control of meristem growth. Nevertheless, further investigation will improve our insight into NAC members in the control of root meristem activity. In addition, a previously reported phloem specific gene *APL* was also in our list of genes that are activated by SHR in the stele (Bonke et al., 2003) (Table I.3). A previous study suggested that APL regulates the phloem specification by being expressing in the differentiating SEs and companion cells (Bonke et al., 2003). Interestingly, when APL was expressed under the *S32* promoter in *shr-2*, the meristem size became even smaller than the *shr-2* without a transgene (Figure I.3C). In addition, the GFP signals suggested a potential movement of APL to other cells in addition to phloem pole. It seems that with the ectopic expression of APL in the early meristem, APL causes the precocious determination of procambial cells into phloem. Therefore, the meristem cell activity is retarded, resulting in a further reduction of the meristem size. Our findings on APL suggest that the SE cell division controlled by SHR likely happens before the phloem cell differentiation where *APL* starts being expressed.

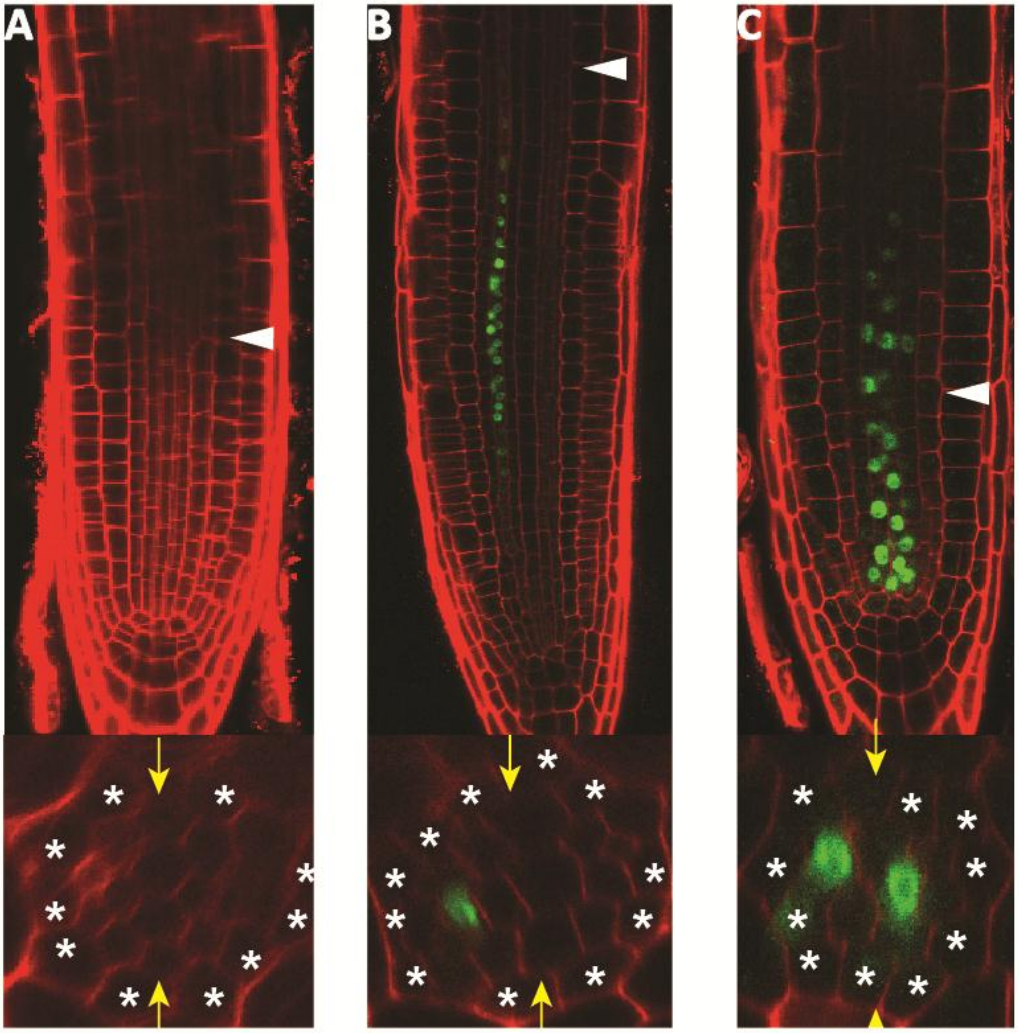

Figure I.3. Expression of SND2 and APL driven by *S32* in the *shr-2* influences the root growth.

Confocal section through root tips of *shr-2* **(A)**, *pS32::SND2:GFP; shr-2* **(B)**, and *pS32::APL:GFP ; shr-2* **(C)**. The longitudinal sections were shown in the upper panel and transverse sections in the lower panel. Asterisks, pericycle position; arrowheads, the start of transition zone of the root; arrows, xylem poles.

Summary

On the xylem side, using cell type specific root expression data we found four transcription factors that are involved in the xylem patterning process and three novel markers with enriched expression in the xylem. Though the mutants of these four transcription factors have similar xylem phenotype, they seem to regulate early xylem patterning processes through more than one pathway: one through the auxin-cytokinin signaling pathway and the other through the cell-to-cell movement of AHL3/4. Further studies on the candidate genes and markers have the potential to reveal the fine programs that a plant employs to precisely control an early stage of xylem patterning.

There is no question about how important phloem tissues are in the nutrient transportation. However, due to its naturally compact and fragile structure, studies on the phloem patterning have been very limited as compared to xylem formation. In my dissertation study, I investigated the early phloem pattering by focusing on the SHR function whose mutant causes the severe loss of phloem proliferation activity and normal pattern formation. Using histological and immunofluorescence methods, we pinned down that SHR moves into phloem from the procambium and triggers an asymmetric cell division for SEs. Our study also suggests that SHR in the endodermis controls the procambium activity in a non-cell-autonomous manner, which further influences the formation of companion cells. Identification of at least two NAC family transcription factors downstream of SHR in this dissertation provides the basis for further exploration of this delicately regulated developmental process.

Materials and Methods

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used. Seeds were surface-sterilized, plated (0.5 × MS medium with 1% sucrose) and grown under a 16-h-light/8-h-dark cycle at 22-23℃ in a plant growth chamber. All the T-DNA insertion lines described in Table I.1 were obtained from the Arabidopsis Biological Resources Center (ABRC) except for *FLAG_445H04* and *FLAG_447A04* which were obtained from the Versailles Genetics and Plant Breeding Laboratory Arabidopsis thaliana Resource Centre (INRA Versailles France, http://dbsgap.versailles.inra.fr/vnat/). Primers used for genotyping were listed in Supplementary Table I.1.

Supplementary Table I.1 List of primers used in Appendix I

Primers for genotyping		
	Primer name	Sequence
Genotyping *shr-2* wt allele	SHR-2876R	ATCAACCATCACCACACGTG
	SHR-1670F	GACACTGCACGTGCGCAACA
Genotyping *shr-2* mutant allele	SHR-2F	ATACGCTTTTGCAGGTTATATCAGC
	SHR-2R	GAACTCCCATAAGCCTAGCGAATTT
Genotyping *phb-6* wt allele	Phb-6F2	TCGAGATTGGCGTCTGAGATAAA
	Phb-6R2	TTGGAAACGCATTCAAAGACAAT
Genotyping *phb-6* mutant allele	Phb-6F2	TCGAGATTGGCGTCTGAGATAAA
	Ds 3'-1	GGTTCCCGTCCGATTTCGACT
Genotyping *AT4G12620* wt allele	X19-1LP	ACATGTCAGCCAATTTGTTC
	X19-1RP	CGAAATCAAGGCCTAAGACC
Genotyping *AT4G12620* mutant allele	LBb1.3	ATTTTGCCGATTTCGGAAC
	X19-1RP	CGAAATCAAGGCCTAAGACC
Genotyping *AT4G14700* wt allele	X20-1LP	GCATATTCAACTTGATGTTCTTGG
	X20-1RP	CGTCTTCTTCGTCTCAGATCG
Genotyping *AT4G14700* mutant allele	LBb1.3	ATTTTGCCGATTTCGGAAC
	X20-1RP	CGTCTTCTTCGTCTCAGATCG

Genotyping *AT3G12270* wt allele	X24-1LP	AGCCCAGTAGCCTCTAACGAG
	X24-1RP	TCTTTTTGCTGCTAAAGCTGG
Genotyping *AT3G12270*mutant allele	LBb1.3	ATTTTGCCGATTTCGGAAC
	X24-1RP	TCTTTTTGCTGCTAAAGCTGG
Genotyping *AT1G44810* wt allele	X32-1LP	ACCACCGTAAAGAGAAATGGG
	X32-1RP	CTGGATCTGAGACAAATTCCG
Genotyping *AT1G44810* mutant allele	LBb1.3	ATTTTGCCGATTTCGGAAC
	X32-1RP	CTGGATCTGAGACAAATTCCG
Genotyping *AT1G09770* wt allele	X38-F	GCTGACAAAATGATAAAGGAGGAG
	X38-R	ATATTGACCATCATACTCATTGC
Genotyping *AT1G09770* mutant allele	Gabi-Kat LB	ATATTGACCATCATACTCATTGC
	X38-R	ATATTGACCATCATACTCATTGC
Genotyping *AT5G25475* wt allele	X48-1LP	AGAGACTTCCAAAAGCAAGGC
	X48-1RP	CCTCTTGAATCCTGAAAACCC
Genotyping *AT5G25475* mutant allele	LBb1.3	ATTTTGCCGATTTCGGAAC
	X48-1RP	CCTCTTGAATCCTGAAAACCC
Genotyping *AT5G25475* wt allele	X48-2LP	AGAGACTTCCAAAAGCAAGGC
	X48-2RP	CCTCTTGAATCCTGAAAACCC
Genotyping *AT5G25475* mutant allele	LBb1.3	ATTTTGCCGATTTCGGAAC
	X48-2RP	CCTCTTGAATCCTGAAAACCC
Genotyping *AT2G01940* wt allele	X6-1LP	TTTGAAAAGCCTTTGTTGCAG
	X6-1RP	GGAGAAGACACGACCACAGTC
Genotyping *AT2G01940* mutant allele	LBb1.3	ATTTTGCCGATTTCGGAAC
	X6-1RP	GGAGAAGACACGACCACAGTC
Genotyping *AT1G29950* wt allele	X5-1LP	TTCAACGAAGGGGTATTCATG
	X5-1RP	AGCAAATTTTGTTGTTGCCAC
Genotyping *AT1G29950* mutant allele	LBb1.3	ATTTTGCCGATTTCGGAAC
	X5-1RP	AGCAAATTTTGTTGTTGCCAC
Genotyping *AT3G22780* wt allele	X4-1LP	CTCTTGCTGTTGGGAGAGATG
	X4-1RP	GCTCATACCCCCTAGCATCTC

Genotyping *AT3G22780* mutant allele	LBb1.3	ATTTTGCCGATTTCGGAAC
	X4-1RP	GCTCATACCCCCTAGCATCTC
Genotyping *AT3G57150* wt allele	X3-1LP	CTAACCCTCCTAAGCTCCTGC
	X3-1RP	ACATTTTGGGCACAACAAAAG
Genotyping *AT3G57150* mutant allele	LBb1.3	ATTTTGCCGATTTCGGAAC
	X3-1RP	ACATTTTGGGCACAACAAAAG
Genotyping *AT5G64530* wt allele	X7-1LP	ACAATTTGGAAGGGGAAAGTG
	X7-1RP	TGTATATACCGGGAAGGTCCC
Genotyping *AT5G64530* mutant allele	LBb1.3	ATTTTGCCGATTTCGGAAC
	X7-1RP	TGTATATACCGGGAAGGTCCC
Genotyping *AT1G52150* wt allele	S9-1LP	CATCAAATTGGAAGGTCTTGG
	S9-1RP	CTGTGGGAACTCAGAGCAAAG
Genotyping *AT1G52150* mutant allele	SAIL-LB1	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
	S9-1RP	CTGTGGGAACTCAGAGCAAAG
Genotyping *AT5G06710* wt allele	X13-1LP	AAACATGATTTTGCGGTTTTG
	X13-1RP	AGCGTAATTCTTGGTCACACG
Genotyping *AT5G06710*mutant allele	LBb1.3	ATTTTGCCGATTTCGGAAC
	X13-1RP	AGCGTAATTCTTGGTCACACG
Primers for cloning		
Cloning *AT1G29950* promoter into pDONR P4_P1R	pX5_w/o_utr-F	GGGGACAACTTTGTATAGAAAAGTTGCATCATGAGATTAACTATTTCGC
	pX5_w/o_utr-R	GGGGACTGCTTTTTTGTACAAACTTGAAAGAGAGAATGATGTGTGTGG
Cloning *AT3G19300* promoter into pDONR P4_P1R	pM7-2F	GGGGACAACTTTGTATAGAAAAGTTGACTTTGGGAAGAAGCAACTAC
	pM7-2R	GGGGACTGCTTTTTTGTACAAACTTGGGCCAAAGAACAGAGAGAGTC
Cloning *AT1G79630* promoter into pDONR P4_P1R	pM10-2F	GGGGACAACTTTGTATAGAAAAGTTGGATCGTAGACGTCTACCATG
	pM10-2R	GGGGACTGCTTTTTTGTACAAACTTGTGAAGCTTTCTTCTTCTTCT
Cloning *AT2G34060* promoter into pDONR P4_P1R	pM6-2F	GGGGACAACTTTGTATAGAAAAGTTGGAAGATATGCACAGTTCTCTGG
	pM6-2R	GGGGACTGCTTTTTTGTACAAACTTGGGTGGGGTTGTGACTTGTGA

Cloning *AT4G28500* cDNA without stop coden into pDONR221	cP1-F	GGGGACAAGTTTGTACAAAAAAGCAGGCT CAATGACTTGGTGCAATG
	cP1-R	GGGGACCACTTTGTACAAGAAAGCTGGGT AAGGGATAAAAGGTTGAG
Cloning *AT3G45610* cDNA without stop coden into pDONR221	cP3-F	GGGGACAAGTTTGTACAAAAAAGCAGGCT CAATGGATTATTCTTCGA
	cP3-R	GGGGACCACTTTGTACAAGAAAGCTGGGT ACAATAAAGCACCAGTAT
Cloning *AT1G79430* cDNA without stop coden into pDONR221	cAPL-F	GGGGACAAGTTTGTACAAAAAAGCAGGCT CAATGTTCCATGCTAAGA
	cAPL-R	GGGGACCACTTTGTACAAGAAAGCTGGGT ACCCAAATGGCGAGTTTC
Cloning *AT1G71692* cDNA without stop coden into pDONR221	cP2-F	GGGGACAAGTTTGTACAAAAAAGCAGGCT CAATGGCTCGTGGAAAGA
	cP2-R	GGGGACCACTTTGTACAAGAAAGCTGGGT AGAACTGAAATATTTCAC

Plasmid Construction

Gaterway cloning technology (Invitrogen) was used for DNA manipulations. For the purpose of xylem enriched gene expression study, *AT1G29950, AT3G19300, AT1G79630, AT2G34060, AT3G57150, AT5G08260,* and *AT5G25830* promoters were amplified from *Arabidopsis* Col-0 genomic DNA and cloned into pDONR P4_P1R. *pAT1G29950::erGFP*, *pAT3G19300::erGFP*, *pAT1G79630::erGFP*, *pAT2G34060::erGFP*, *pAT3G57150::erGFP, pAT5G08260::erGFP*, and *pAT5G25830::erGFP* were constructed into dpGreen-Bar by Multisite gateway LR recombination. All the clones in the binary vector was transformed into *Agrobacterium* GV3101 with pSOUP and transformed into wild type background.

For the complementary studies on the phloem candidate transcription factors listed in Table I.3, *SND2*, *AT3G45610*, *APL*, and *AT1G71692* cDNAs were cloned into pDONR221. *G*eneration of *S32* promoter in pDONR P4_P1R was described in (Lee et al., 2006). *nlsGFP* were cloned into pDONR P2R_P3 by BP recombination. *pS32::SND2:GFP*, *pS32:: AT3G45610:GFP*, *pS32::APL:GFP*, and *pS32::AT1G71692:GFP* were constructed into dpGreen-BarT by Multisite gateway LR recombination. All the clones in the binary vector was transformed into *Agrobacterium* GV3101 with pSOUP and transformed into *shr-2* heterozygous plants and *shr-2* homozygous plants were verified by genotyping. Primers used for genotyping and cloning were listed in Supplementary Table I.1.

Histological Analysis

All seedling samples were collected at 6 days after germination. Confocal images were obtained using a Leica TCS SP5 Laser Scanning Confocal Microscope with the preset emission/excitation wavelength of 488 nm/505 to 530 nm for GFP and 561 nm/591 to 635 nm for propidium iodide (PI). For the visualization of the root structure, all the seedlings were stained in 2 μg/ml PI. Root transverse sections and toluidine blue staining were done as described in (Scheres et al., 1995). Basic fuchsin staining was described in (Mähönen et al., 2000).

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Appendix II Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate

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Abstract

A key question in developmental biology is how cells exchange positional information for proper patterning during organ development. In plant roots the radial tissue organization is highly conserved with a central vascular cylinder in which two water conducting cell types, protoxylem and metaxylem, are patterned centripetally. We show that this patterning occurs through crosstalk between the vascular cylinder and

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A figure summarising the main result of this paper is also included as SI.
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the surrounding endodermis mediated by cell-to-cell movement of a transcription factor in one direction and microRNAs in the other. SHORT ROOT, produced in the vascular cylinder, moves into the endodermis to activate SCARECROW. Together these transcription factors activate MIR165a and 166b. Endodermally produced miR165/6 then acts to degrade its target mRNAs encoding class III homeodomain-leucine zipper transcription factors in the endodermis and stele periphery. The resulting differential distribution of target mRNA in the vascular cylinder determines xylem cell types in a dosage dependent manner.

Organ development involves extensive communication between cells to orchestrate tissue specification and differentiation. This communication is often mediated by mobile molecules such as hormones, mRNAs, proteins, and small RNAs [1]. In plants and animals, transcription factors (TFs) and/or mRNAs have been shown to move to neighbouring cells and transfer positional information [2-6]. Recently, small RNAs including microRNAs (miRNAs), small interfering RNAs (siRNAs), and transacting siRNAs (tasiRNAs) have emerged as potential mediators of cell-to-cell communication. Studies on the mobility of small RNAs have established that siRNAs and tasiRNAs are mobile but evidence for the mobility of miRNAs has remained elusive [2-7].

The plant root is well suited to study cell-to-cell communication in tissue patterning. Along the radial axis, epidermis, cortex, and endodermis form around the stele, in which the pericycle surrounds the vascular cylinder [8] (Fig. 1a). Here, xylem develops in the centre and forms symmetric arcs toward the pericycle. Phloem develops between these arcs, with procambium/cambium, the vascular stem cells separating xylem and phloem. Xylem precursors in the centre differentiate into metaxylem with pitted secondary cell walls while peripheral precursors differentiate as protoxylem with spiral walls. The strong evolutionary conservation of xylem patterning [9,10] suggests the presence of common molecular mechanisms that constrain its organization.

Here, we report a novel regulatory pathway (Supplementary Fig. 1) that involves bidirectional cell signalling mediated by miRNA165/6 and the TFs SHORT ROOT (SHR) and SCARECROW (SCR) controlling xylem patterning.

Endodermal SHR controls xylem patterning

SHR is produced in the stele and moves into the endodermis to activate *SCR* [11-15]. In mutants of *SHR* or *SCR*, the asymmetric cell division that forms endodermis and cortex fails to occur and the quiescent centre (QC) is not maintained, resulting in short roots with only one ground tissue layer [12,16,17]. Despite the endodermal-specific expression of *SCR* genome-wide mRNA profiling of wild type (wt), *shr*, and *scr* roots [14,15] (on-line methods) indicated that nearly half of the genes co-regulated by *SHR* and *SCR* were expressed at the highest level in the stele (Supplementary Fig. 2). Furthermore, in both *scr* and *shr* mutants, metaxylem differentiates ectopically in the place of protoxylem (Fig. 1e, f, g), suggesting that SHR and SCR affect stele development in a non-cell autonomous manner.

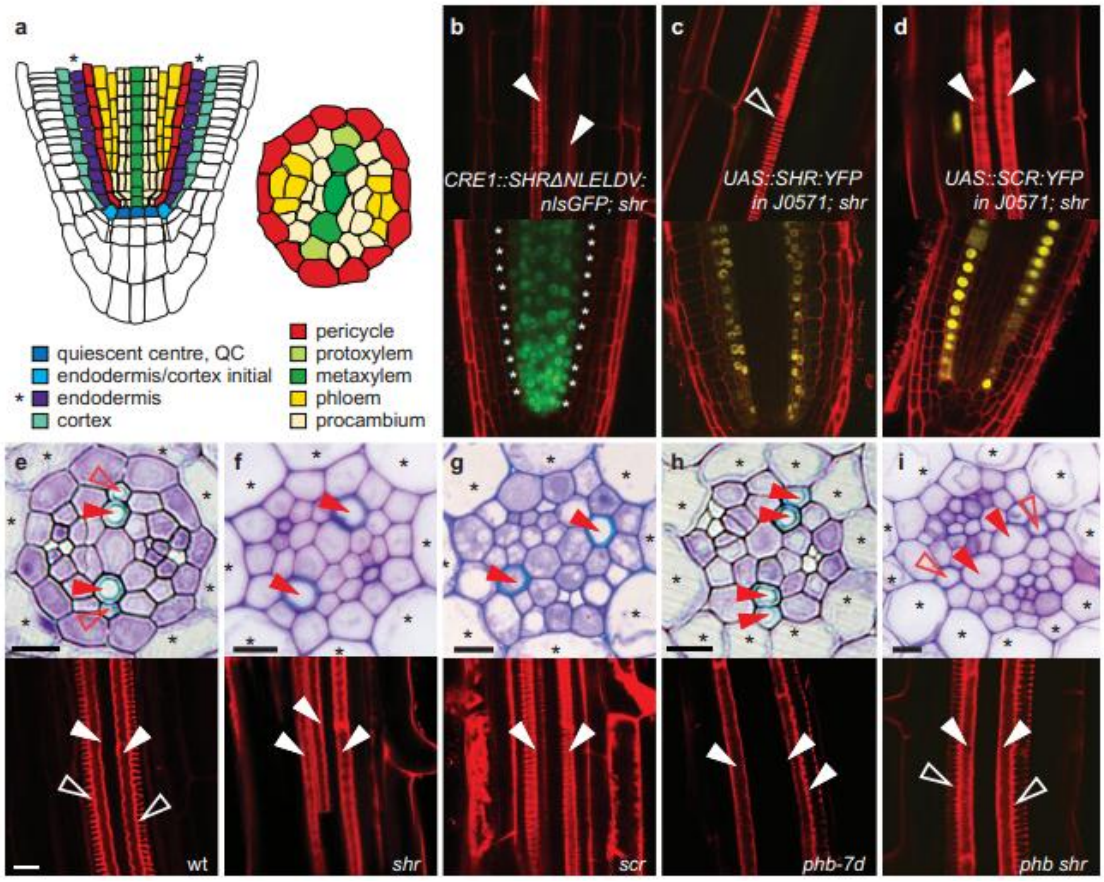

Figure 1. Endodermal SHR and SCR control xylem patterning via *PHB*. **a**, Schematic representation of the Arabidopsis root meristem and stele. Cells in the ground tissue layer adjacent to the stele are marked with asterisks. **b,** *CRE1:: SHRΔNLELDV:nlsGFP* in *shr-2*. **c,** *UAS::SHR:YFP* in *shr-2* harbouring *J0571*.**d**, *UAS::SCR:YFP* in *shr-2*, *J0571*. **e-i**, Toluidine blue stained cross sections and confocal laser scanning micrographs of basic fuchsin stained xylem of wt (**e**), *shr-2* (**f**), *scr-4* (**g**), *phb-7d* (**h**), and *phb-6 shr-2* (**i**). Filled arrowhead indicates metaxylem, and unfilled indicates protoxylem. Scale bar: 10μm.

Because SHR is normally present in both the stele and the endodermis, we determined where its activity is required for xylem patterning. We first expressed *SHR* strictly in the stele of *shr-2* by introducing a construct in which a non-mobile version of SHR [18] containing a nuclear localization signal was driven by the stele specific

promoter of *CRE1* [19]. Recovery of root meristem size (Student's t-test; $p<0.001$, $\alpha=0.05$) and root growth ($p<0.001$, $\alpha=0.05$) showed that this version of SHR was functional. Although we observed more immature phloem sieve cells than in *shr-2* (Supplementary Fig. 3), protoxylem formation was not rescued (Fig. 1b). We then expressed *SHR* strictly in the ground tissue by introducing *UAS::SHR:YFP* into *shr-2* harbouring *J0571*, an enhancer trap line that drives expression specifically in the ground tissue (http://www.plantsci.cam.ac.uk/Haseloff/; Fig. 1c; Supplementary Fig. 4). In addition to multiple ground tissue cell divisions, protoxylem formation was observed in 73% (24 of 33) of the lines (Fig. 1c), compared with 14% in *shr-2 J0571* alone. When *SCR* was expressed in the ground tissue in the absence of SHR, neither protoxylem nor endodermis was rescued (Fig. 1d). Therefore, xylem patterning requires both SHR and SCR to be present in the endodermis.

Xylem patterning requires *PHB* restriction

In a screen for altered vascular development (Supplementary Fig. 5) we identified a mutant with a short root and frequent differentiation of metaxylem in place of protoxylem (Fig. 1h). This mutant had a point mutation in the miR165/6 target site in the class III homeodomain leucine zipper (HD-ZIP III) gene *PHABULOSA* (*PHB*). Consistent with the phenotype of this new allele, *phb-7d*, the strong gain-of-function allele *phb-1d* [20] invariably formed ectopic metaxylem (Supplementary Fig. 5e).

Comparison of vascular development using cell and tissue markers reinforced the striking similarity of the *phb-7d* and *shr-2* phenotypes (Supplementary Fig. 6;

Supplementary Table 1). To determine if the *phb-7d* phenotype was caused by reduced SHR activity, we expressed *SHR*::*SHR*:GFP [13] in *phb-7d* (Supplementary Fig. 7) but found no deviation from wt expression, suggesting that this was unlikely.

As expected if the *phb-7d* mutation renders the *PHB* transcript resistant to miRNA-mediated degradation, its mRNA levels were elevated in the mutant (Supplementary Fig. 5d). RNA *in situ* hybridization in wt showed that *PHB* mRNA localized primarily to the metaxylem precursors and neighbouring procambial cells, and at a residual level in protoxylem precursors (Fig. 2a). In *phb-7d* the *PHB* mRNA domain expanded throughout as well as outside the stele (Fig. 2b), suggesting that miR165/6 normally acts to exclude *PHB* mRNA from the stele periphery and ground tissue.

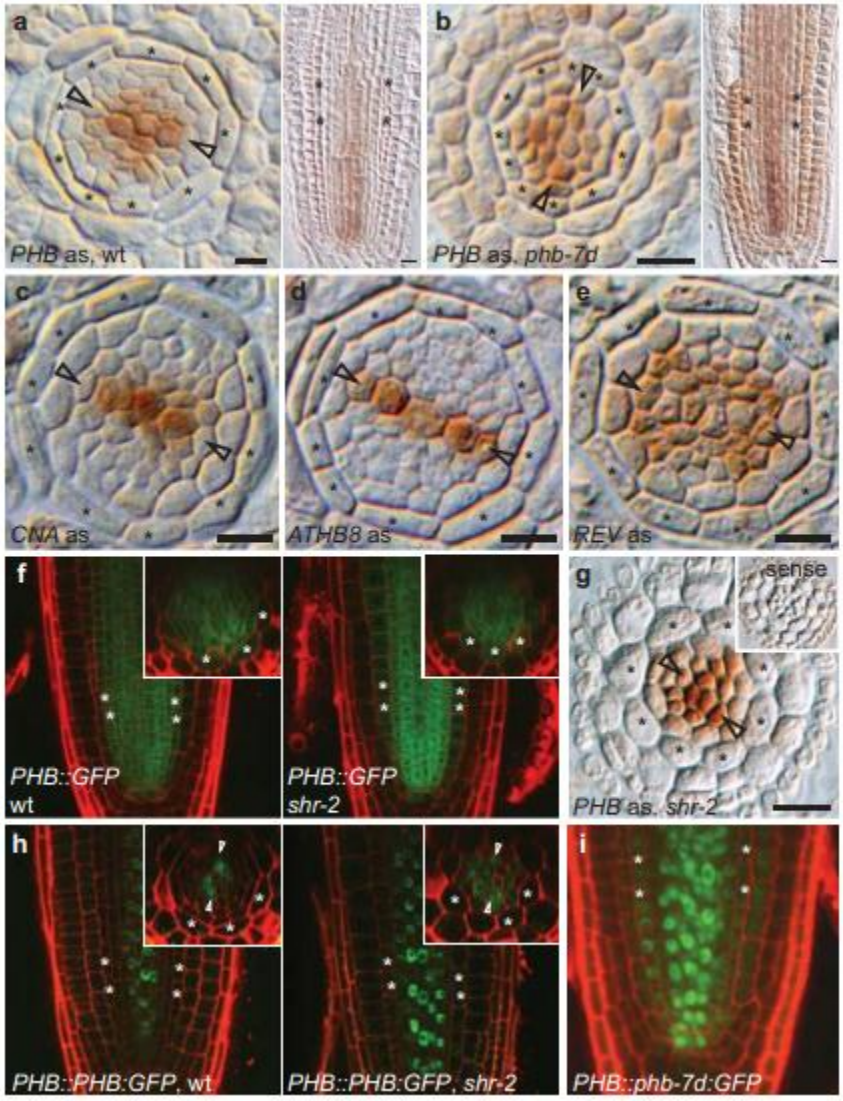

Figure 2. SHR post-transcriptionally represses *PHB*. **a**, **b**, *In situ* hybridization with a *PHB* mRNA specific probe on cross and longitudinal sections of wt (**a**) and *phb-7d* (**b**) roots. **c-e,** *In situ* hybridization with *CNA* (**c**), *ATHB8* (**d**) and *REV* (**e**) on cross sections of wt roots. **f**, Confocal laser scanning micrographs of transcriptional fusion of *PHB* to GFP in wt and *shr-2*. **g**, *PHB* mRNA *in situ* hybridization to cross section of *shr-2*. Inset is a section of *shr-2* hybridized with a *PHB* sense probe. **h**, Expression of translational fusion of *PHB* to GFP in wt and *shr-2*. **i**, Expression of translational fusion to GFP of *PHB* with the *phb-7d* mutation. Asterisks: endodermis position, arrowheads: protoxylem position, scale bar: 10μm.

The importance of miRNAs in xylem cell specification was further supported by the ectopic metaxylem phenotype of the mutant of HYL1, which specifically binds the miRNA during miRNA-mediated mRNA degradation [21,22] (Supplementary Fig. 8). We did not detect ectopic metaxylem in mutants of several well-characterized genes in the si- and tasiRNA-mediated RNA degradation pathways, indicating that xylem patterning is primarily mediated by miRNAs.

SHR post-transcriptionally represses PHB

Genome-wide expression profiling of *shr*, *scr*, and wt roots indicated that *PHB* and the other four *HD-ZIP III* genes targeted by miR165/6 [23-25] are up-regulated in the *shr* and *scr* mutant backgrounds (Supplementary Fig. 9). To further characterize the relationship between SHR and the HD-ZIP III TFs we crossed *shr-2* with loss-of-function mutants of three *HD-ZIP III* TFs, *PHB*, *PHAVOLUTA* (*PHV*), and *REVOLUTA* (*REV*), which are closely related and functionally redundant in leaf development [23]. The *phb-6 phv-5 rev-9* mutant did not show any major deviation in xylem patterning [26] from wt roots (Supplementary Fig. 10 & 19). However, in the *phb-6 phv-5 rev-9 shr-2* quadruple mutant, the xylem patterning defect of *shr* was completely rescued (Supplementary Fig. 10). This provides strong evidence that ectopic metaxylem formation in *shr* is the result of up-regulation of at least one of these *HD-ZIP III* TFs. This quadruple mutant also largely rescued the number of vascular cell files and root length (Supplementary Fig. 10). Analysis of the segregating double mutants showed that *phb shr* fully recovered protoxylem (in about 80%) and root growth, whereas *phv shr*, *rev shr*, and *phv rev shr* did not (Fig. 1i, Supplementary

Figs. 10 & 11). Neither *athb8-11* nor *corona-2* (*cna-2)* could restore the root growth of *shr-2* but in both double mutants stretches of protoxylem were infrequently observed (Supplementary Fig. 11). Combining mutation in *phb* with *scr* also fully recovered protoxylem (Supplementary Fig. 11). Hence, these genetic analyses suggest that *SHR/SCR* repression of *PHB* is the primary pathway for xylem patterning.

We asked if PHB was repressed by SHR at the transcriptional or posttranscriptional level. For this we analysed transcriptional (*PHB*::GFP) and translational GFP fusions (*PHB::PHB*:GFP) driven by the *PHB* promoter [27]. Patterns of *PHB*::GFP indicated that *PHB* is transcribed throughout the stele and endodermis (ground tissue in *shr*) in both wt and *shr* root meristems (Fig. 2f), whereas *PHB*::*PHB*:GFP was restricted to the central vascular cylinder in wt (Fig. 2h). When the *phb-7d* mutation was introduced into the cDNA of the translational fusion, the GFP domain became similar to the *PHB* transcriptional domain in wt (Fig. 2i). In *shr*, *PHB*::*PHB*:GFP and *PHB* mRNA expanded throughout the stele, similar to the pattern of *PHB*::GFP (Fig. 2g, h). These data suggest that SHR restricts PHB at the post-transcriptional level.

These results further implied that the meristematic zone is where PHB determines the root xylem cell types. Supporting this, we found that a miRNA-resistant version of *PHB* (with a silent mutation) expressed in the meristematic region under the stele-specific *CRE1* promoter was sufficient for ectopic metaxylem to form both in wt and in the *phb shr* double mutant (Supplementary Fig. 12).

In summary, our data suggest that miR165/6 post-transcriptionally restricts *PHB* within the root meristem to the stele centre for proper xylem patterning and that SHR regulates this process by promoting miR165/6 activity in the stele periphery and endodermis.

SHR activates miR165/6 in the endodermis

We compared levels of miR165/6 in root tips of *shr*, *scr*, and wt. In *shr*, miR165/6 levels were reduced 8-fold, and in *scr* 3-fold compared to wt (Supplementary Fig. 13; on-line methods). Comparison of gene expression profiles of components related to small RNA pathways in whole roots [14,15] showed no statistically significant expression changes in these mutant backgrounds (Supplementary Table 2), indicating that *SHR* and *SCR* primarily control miR165/6 activity by regulating *MIR165/6* expression.

To determine which of the *MIR165/*6 genes are controlled by *SHR* and *SCR*, expression patterns of transgenic promoter::GFP (ER-localized) lines with the complete intergenic region of eight of the *MIR165/6* genes (except *165b*) were analyzed in wt, *shr-2*, and *scr-1* (Fig. 3a, b; Supplementary Fig. 14). Only *MIR165a* and *166b* promoters drove detectable GFP in distinct patterns in wt roots. We note that another study [28] suggests that *MIR166a* is also expressed at low levels in roots. *pMIR165a::GFP* exhibited endodermis-specific expression throughout the root (Fig. 3a) while *pMIR166b::GFP* was expressed strongly in the endodermis and QC and weakly in cortex and epidermis of the meristems of embryonic, primary, and lateral roots (Fig. 3b; Supplementary Fig. 14). In *shr* roots, GFP was not detected from

pMIR165a::GFP in any tissue, and only low-level activity was observed in the ground tissue and epidermis from *pMIR166b::GFP* (Fig. 3b; Supplementary Fig. 14). The constructs behaved similarly in *scr*, exhibiting dramatic reduction in expression, with the exception that *pMIR165a::GFP* which was detected in the ground tissue from the late maturation zone (Fig. 3a; Supplementary Fig. 14). As in wt, GFP driven by any of the other six promoters could not be detected in *shr* roots. Consistent with the GFP data, real time RT-PCR analysis indicated significant reduction of pri-*MIR165a* and pri-*MIR166b* in both *shr* and *scr* roots (Supplementary Fig. 13).

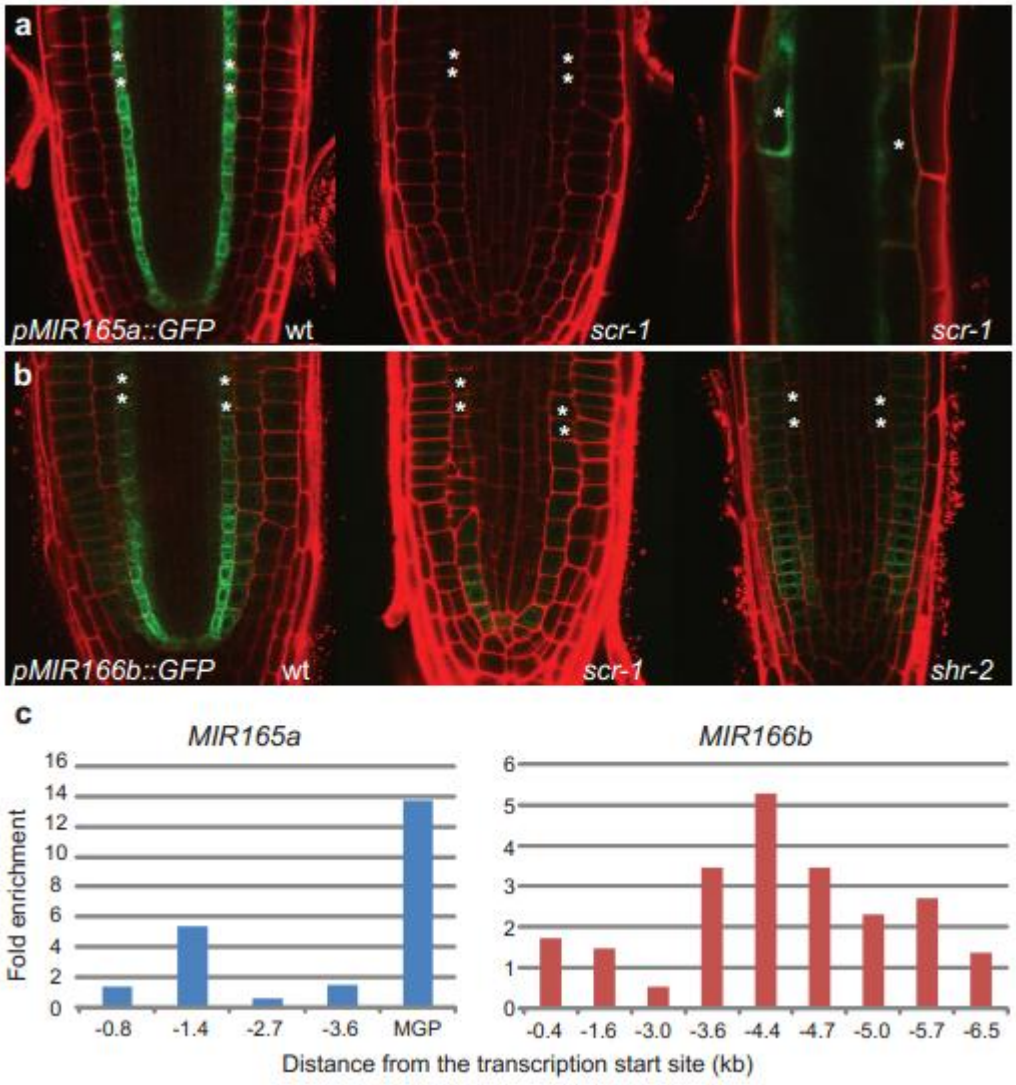

Figure 3. *miR165/6* activated by SHR in the endodermis is active in the stele. **a**, Expression of *pMIR165a::GFP* in wt and *scr-1* meristems and in maturation zone. **b**, Expression of *pMIR166b::GFP* in wt, *scr-1*, and *shr-2* meristems. **c**, Real-time PCR of ChIP on the upstream regulatory regions of *MIR165a* and *MIR166b* using anti-GFP antibodies and a transgenic plant expressing *pSHR::SHR:GFP*. The previously confirmed binding to the *MAGPIE* (*MGP*) promoter [21] was used as positive control. Asterisks: endodermis position.

To determine if SHR is a direct regulator of *MIR165/6* we performed Chromatin Immunoprecipitation (ChIP) followed by real-time qPCR (on-line methods). We

reproducibly found enrichment of fragments approximately 1 kb upstream of the transcription start site of *MIR165a*, 4.5 kb upstream of *MIR166b* (Fig. 3c), and 2.5 kb upstream of *MIR166a* (data not shown). Taken together, our data suggest that SHR directly activates transcription of *MIR165a* and *166b* in the endodermis.

miR165/6 acts non-cell-autonomously

We previously reported that the *HD-ZIP III* transcriptional domains are largely restricted to the vascular cylinder [27]. *PHB* is the only one whose transcriptional domain partially overlaps with the endodermal activity domain of *SHR* and *SCR*. Hence, for miR165/6 produced in the endodermis to encounter *HD-ZIP III* mRNA in the stele, there must be a mobile signal.

To understand the nature of this non-cell autonomous regulation, we first compared the spatial distribution of miR165/6 activity in wt and *shr-2* roots using a 'miRNA-sensor' [29] (Fig. 4a; on-line methods). In this system, high miR165/6 activity is reflected by low GFP expression. Without the miRNA recognition site the GFP level was uniform in the stele in both wt and *shr*. In wt roots the sensor GFP with the miRNA recognition site was significantly lower in the ground tissue and stele periphery than in other cell types, consistent with the observed reduction in the *PHB* mRNA and protein domains in wt. In *shr-2*, the sensor GFP expression level was uniform throughout the root.

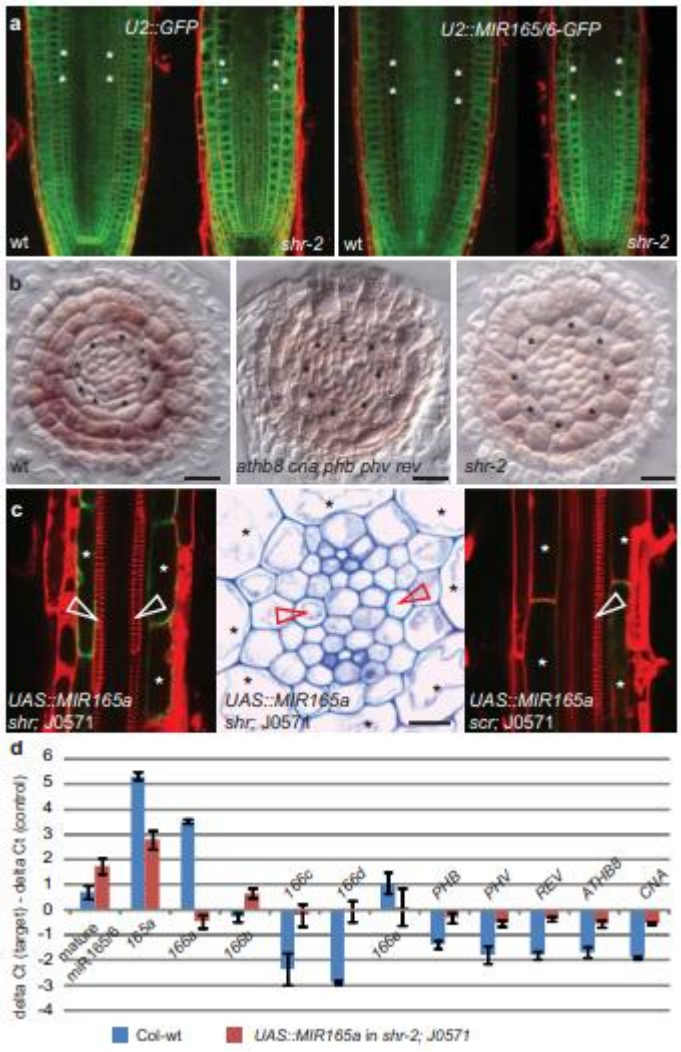

Figure 4. Non-cell-autonomous action of *MIR165a*. **a**, miR165/6 GFP sensor under the U2 promoter in wt and *shr-2*. **b**, *miR166*-specific LNA probe hybridization to sections proximal to the QC of wt, athb8-11 *cna-2 phb-13 phv-11 rev-6* and of *shr-2*. **c**, Protoxylem forms in *shr* and *scr* backgrounds when *UAS::MIR165a* is introduced into *shr-2* and *scr-4* harbouring *J0571*. **d**, Real-time RT PCR of pri-*MIRNA165/6* and *HD-ZIP III* in wt and a line with *UAS::MIR165a* in *shr-2, J0571*. n=4. Error bars indicate ±S.D. Asterisks: endodermis/ground tissue position, arrowheads: protoxylem position, scale bar: 10 µm.

Second, we determined the spatial distribution of mature miR165/6 in the root meristem of wt and *shr* using *in situ* hybridization with locked nucleic acid (LNA)

probes (Fig. 4b; Supplementary Fig. 15; on-line methods). In wt meristems, mature miR165/6 was detected at a low level in the QC and surrounding cells, but became progressively higher and ubiquitous throughout the root radius 30-40 m distal from the QC. In cells close to the QC, mature miR165/6 level was considerably higher in the cortical and epidermal cell layers than in the endodermis and stele cells. Hence, the pattern appeared complementary to the domain of *PHB* transcription. A complementary pattern of a mature miRNA and its target is consistent with previous observations [30], but rather contradictory to our results showing the highest promoter activity of *MIR165a* and *MIR166b* in the endodermis. We hypothesized that the hybridization signal may mainly reflect the distribution of free miR165/6. Consistent with this, we detected less of a differential distribution of miR165/6 in the *phb-13* mutant and in multiple loss-of-function *HD-ZIP III* mutants where target mRNA is very low or absent. In these backgrounds the miR165/6 signal became high and ubiquitous throughout the root radius even in tissues close to the QC (Fig. 4b; Supplementary Fig. 15). In *shr-2*, the miR165/6 pattern was similar to wt, but at a markedly lower level, likely reflecting the residual expression of *MIR166b* (Fig. 4b). Hence, the sensor and *in situ* hybridization results indicate that the mature miRNA165/6 moves radially both outward and inward from the endodermis.

Thirdly, we expressed *MIR165a* under a ground tissue specific promoter (*shr-2 J0571; UAS::MIR165a*) in *shr* and observed the resulting xylem pattern (Fig. 4c; Supplementary Fig. 4). Five independent segregating T2 lines showed a clear recovery of protoxylem (Fig. 4c) at frequencies ranging from 33% to 88%, accompanied by

suppression of mRNA levels of all the *HD-ZIP IIIs* (Fig. 4d) and restriction of *PHB* and *CNA* mRNA within the stele (Supplementary Fig. 16). Co-segregation of protoxylem formation with the activator was verified by back-crossing. Similarly, both *J0571>>MIR165a* (Fig. 4c) in *scr-4* and *MIR165a* driven under another ground-tissue specific promoter, *pSCR*, in the *scr-6* allele, rescued protoxylem formation (Supplementary Fig. 17).

Further support for this hypothesis is that protoxylem recovery observed when *SHR* was specifically expressed in the ground tissue of *shr* (Figure 1c) was accompanied by an increase in *MIR165a* and *166b levels*, and a decrease in mRNA levels of all five *HD-ZIP III* genes (Supplementary Fig. 18a), whereas SHR exclusively localized to the stele of the *shr* mutant did not affect either miR165/6 or *HD-ZIP III* mRNA levels (Fig. 1d; Supplementary Fig. 18b). Taken together, our results strongly support that miR165/6 in the endodermis mediates the non-cell autonomous action of *SHR* and *SCR* in xylem patterning, by moving into the stele to restrict the *HD-ZIP III* mRNA domains.

HD-ZIP III levels determine xylem type

Our results suggested that *PHB* was the primary determinant but other HD-ZIP IIIs may play a role in metaxylem specification. To further investigate the role of all five *HD-ZIP III* genes in root vascular patterning, we analysed their expression and assessed their loss-of-function phenotypes. Similar to *PHB, CNA* and *ATHB8* were expressed in xylem precursor cells (Fig. 2c, d): *CNA* in the metaxylem domain and

neighbouring procambial cells and *ATHB8* specifically in the xylem axis including the protoxylem precursors. *REV* was broadly expressed in the vascular tissue just above the QC (Fig. 2e) but disappeared from the metaxylem domain farther away from the QC. *PHV* mRNA was not detected. In summary, the *HD-ZIP III* mRNA levels in the root meristem appear highest in the centre of the xylem axis and become lower towards the stele periphery.

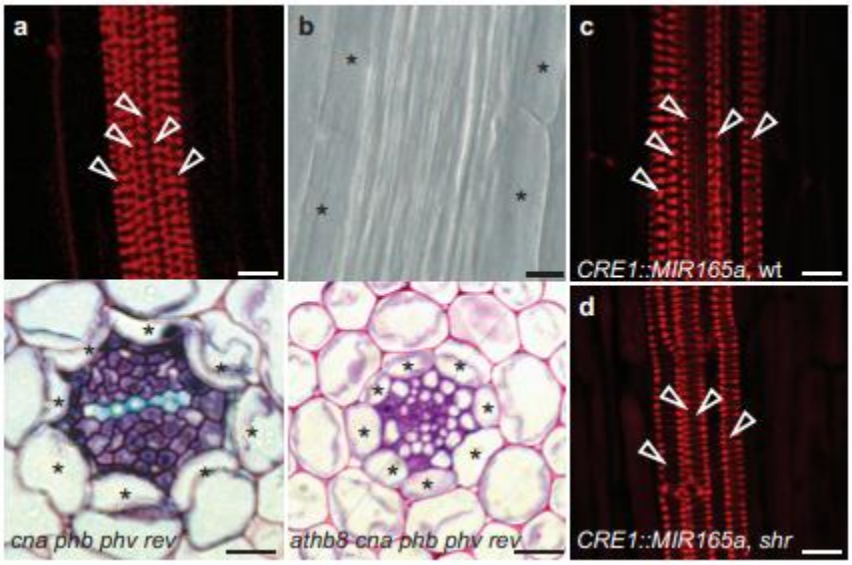

Figure 5. HD-ZIP III levels determine xylem type. **a**, Basic fuchsin stained xylem and cross section of *cna-2 phb-13 phv-11 rev-6.* **b,** cleared root and cross section of *athb8-11 cna-2 phb-13 phv-11 rev-6.* **c**, **d**, stained xylem of roots in which *MIR165a* is expressed from the *CRE1* promoter in wt and *shr-2*. Asterisks: endodermis position, arrowheads: protoxylem position, scale bar: 10 µm.

In contrast to ectopic *PHB* expression, which causes central metaxylem fate in the stele periphery, loss of multiple *HD-ZIP III* genes resulted in protoxylem differentiating in the central metaxylem positions or even abolished xylem

differentiation entirely (Fig. 5; Supplementary Fig. 19). This is consistent with previous studies showing that HD-ZIP III TFs may direct xylem development [23,25,31-33]. All single and most double mutants displayed normal xylem patterning. However the *athb8-11 phb-13* and various triple mutants had ectopic protoxylem partly replacing metaxylem. When four of the five genes were mutated no metaxylem was observed in any of the mutant combinations examined. Consistent with changes in xylem cell fates, low or no expression of the metaxylem marker gene *ACL5* [34] and ectopic expression in the centre of the xylem axis of the protoxylem marker *AHP6* [35] were observed in *athb8-11 cna-2 phb-13 phv-11* (Supplementary Fig. 20). This mutant also generated more vascular cells and, in contrast to the invariably diarch vascular arrangement in wt and often formed a tri- or tetrarch arrangement, suggesting that the *HD-ZIP III* genes redundantly restrict vascular cell proliferation (Supplementary Fig. 19). Surprisingly, loss of all five *HD-ZIP III* TFs failed to form any xylem (Fig. 5b). Partial failure in xylem differentiation was also detected in certain quadruple mutants (Supplementary Fig. 19). These results suggest that expression levels of *HD-ZIP III* TFs determine not only xylem cell types but also *de novo* formation of xylem.

Finally, we increased the level of miR165 throughout the stele in wt and *shr-2*. In both backgrounds an increased number of stele cells was observed (not shown) and all xylem precursors acquired peripheral fate differentiating exclusively as protoxylem (Fig. 5c, d), similar to the phenotypes of multiple loss-of-function *HD-ZIP III* mutants. Thus, xylem patterning requires suppression of *HD-ZIP III* mRNA in the stele periphery through the activity of miRNA165/6.

Discussion

Our study highlights a novel regulatory pathway that integrates transcriptional and post-transcriptional regulation and bidirectional cell-to-cell communication to drive tissue patterning in the *Arabidopsis* root (Supplementary Fig. 1). Formation of vascular tissue with a surrounding endodermal layer was a key milestone in the evolution of land plants [17,18]. Our study reveals that its underlying regulation involves evolutionarily conserved transcription factors, SHR/SCR and HD-ZIP III TFs, and miRNA165/6 [36,37], implying that this regulatory mechanism might underlie the evolutionary adaptation to terrestrial growth.

The mobility of miR165/6 in the shoot apical meristem has been suggested previously[2-6]. Our study indicates that miR165/6 is mobile in the root and its mobility over a short distance is critical for dosage-dependent regulation of *HD-ZIP III* TFs in xylem patterning. A recent modelling study indicates that a mobile small RNA can sharpen the boundary of the activity domain of its target [38]. Our study suggests that this may be the role for the endodermally produced miR165/6 as it moves into the vascular cylinder to encounter its target mRNA thereby communicating radial positional information between cells of the root meristem.

Methods Summary

Detailed methods can be found in Supplementary Online Methods

For anatomical and histological analyses primary roots of vertically grown 4-5 day old seedlings were used. Plastic sectioning, basic fuchsin staining and confocal imaging were performed as described [19].

For quantification of mRNA and miRNA, root tips from 6 to 7 day-old seedlings were harvested and total RNA including small RNAs was extracted with miRNeasy Mini Kit (Quiagen). To measure the expression level of pri-miRNA and other mRNAs, cDNA was synthesized using SuperScript III first strand synthesis system for RT-PCR (Invitrogen). For mature miRNA, a polyadenylation reaction was performed prior to the reverse transcription following the method of Shi and Chiang [39]. Differences in gene expression were measured by real time PCR using an ABI 7900HT (Applied Biosystems).

Sectioning, preparation of riboprobes, and *in situ* hybridization were performed as described [19]. LNA probes with complementary sequences to miR165 and miR166 were synthesized and 5' digoxigenin-labeled (Exiqon) then hybridized at 50 ℃.

Most transgenic constructs were generated using the modified multisite gateway system [27] and all were introduced into plants using the floral dip method [40].

To identify *in vivo* binding activities of SHR to the promoters of *MIR165/6*, plants expressing *SHR::SHR:GFP* in *shr-2* were used. Roots were harvested 6 days after germination and processed for ChIP [14]. SHR binding affinity was compared between the ChIP DNA treated with and without GFP antibody by measuring the differential enrichment of DNA fragments using real-time PCR.

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showed the non-cell autonomous action of non-mobile SHR. A.V. participated in positional cloning of *phb-7d* and establishment of the *J0571* lines to rescue *shr*. S.T. identified the *phb-7d* mutant. A.C. identified the *scr-6* allele. J.S. characterized *shr/scr* and *HD-ZIP III* double mutants and embryo expression patterns in GFP lines. J.L.B. shared informative non-published materials. Y.H. and P.N.B. participated in experimental design. A.C., J.Y.L., Y.H., and P.N.B. wrote the manuscript. All authors discussed the results and commented on the manuscript.

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Appendix III Function and Identification of Mobile Transcription Factors

*Ji-Young Lee[1,2] and Jing Zhou [1,2]

Abstract

Growth and development of multi-cellular organisms require mechanisms that allow for extensive cell-to-cell communication. In some incidences communication is established by signaling molecules that are directly transported from one cell to the other. These mobile signals in plants have been found in forms of proteins, RNAs, and small molecules. They are transmitted through the vascular system in a long distance (between organs), or through plasmodesmata in a short distance (between cell types). A growing number of studies show that transcription factors contribute as important mobile signals in plants. Transcription factors move in a short or long distance in forms of proteins and RNAs. Such transport activities are very important for patterning and growth of plant organs and tissues. In this chapter, we will comprehensively review transcription factors as mobile signals: factors that have been discovered, mechanisms of their mobility, new tools that will lead to the discovery of

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mobile transcription factors, and putative mobile transcription factors inferred from cell-type specific RNA profiling data.

Introduction

Coordinated growth and development of multi-cellular organisms require mechanisms that allow for extensive cell-to-cell communication. Sometimes these communications are mediated by signaling molecules that are directly transported from one cell to the other. Since cells in plants, in contrast to animal cells, cannot migrate, the direct transport of mobile molecules seems to play a more significant role in such communications than in animal system. Sessile nature of plant growth also necessitated the evolution of mechanisms that rapidly transmit signaling molecules in response to environmental changes or pathogen attacks. These mobile signals in plants have been found in forms of proteins, RNAs, and small molecules. They are transmitted through the vascular system in a long distance (between organs), or through plasmodesmata in a short distance (between cell types).

As mobile signals, transcription factors have been found to play crucial roles in a growing number of studies. For example, KNOTTED1 in maize is the first transcription factor that has been identified to move between cells. It regulates shoot apical meristem (SAM) activities by moving from the second cell layer (L2) of the meristem to the first cell layer (L1). SHORT ROOT (SHR) in Arabidopsis roots moves from the vascular cylinder to endodermis/cortex initials and then drives asymmetric cell division that generates endodermis and cortex. Recently cell-to-cell movement of SHR was also shown to be required for cell type patterning in the

vascular tissues. SHR does this in the endodermis by activating the expression of mobile microRNAs, which subsequently move out of the endodermis and affect their targets in the vascular stem cells. Cell-to-cell movement of these and several other transcription factors, which will be reviewed in this chapter, plays an indispensable role for providing positional information during specification and patterning of cell types in plants.

Transcription factors move in short distance mainly through plasmodesmata. However, only few mobile transcription factor proteins are smaller than the size exclusion limits of plasmodesmata, a maximum size of molecules that is allowed for free diffusion through openings of plasmodesmata. This suggests that many of transcription factors, which are mobile as proteins, might be actively recruited by components that increase the size exclusion limit and facilitate cell-to-cell movement. A lot of effort is invested to find the cellular components facilitating intercellular transport of proteins and RNAs (see also Chap. 1, Manfred Heinlein; Chap. 2, P. Zambrisky; Chap 4, D. Jackson, and Chap. 7, Ruiz-Medrano).

Transcription factors move not only between cell layers but also between organs. Movement of transcription factors in a long distance has been found to happen through phloem sieve cells in a form of RNA. For example, BEL5 mRNAs in potatoes move from shoot apices to stolons, which will develop into potato tubers. Messenger RNAs move in the form of protein-RNA complexes by binding to RNA binding proteins, which protect and facilitate the mRNA movement through phloem.

So far there are only a small number of transcription factors that have been found to be mobile and influence plant development. Recent progress in technology in genomics and proteomics and other biotechnologies will enable the discovery of much more mobile transcription factors in coming years.

In this chapter, we will comprehensively review mobile transcription factors: factors that have been discovered, mechanisms of their mobility, new tools that will lead to the discovery of mobile transcription factors, and putative mobile transcription factors inferred from cell-type specific RNA profiling data.

Mobile transcription factors in plants

Cell-to-cell movement of transcription factor proteins

KNOTTED1, the first mobile transcription factor discovered

KNOTTED1 (KN1), a homeobox family transcription factor, is the first transcription factor that has been discovered to move between cells (Lucas et al., 1995). Its gene was identified from a dominant mutant in maize, which develops knots on developing leaves (Vollbrecht et al., 1991). Expression analysis of KN1 showed the distinctive distribution of its RNAs from proteins' (Jackson et al., 1994). KN1 RNAs were detected in cells below the L1 layer of SAM, however its proteins were found throughout the meristem. Such difference in expression domains provoked the idea that KN1 proteins might move from inner cells to cells in the L1 layer of SAM. The localization of KN1 proteins was monitored after injecting the KN1 tagged with fluorescent molecules into mesophyll cells of tobacco and maize leaves. This

experiment showed that KN1 proteins move from one cell to the other (Lucas et al., 1995). Further domain analyses in the same study suggested that the homeodomain is critical for cell-cell trafficking of KN1. Interestingly, movement of KN1 proteins also facilitated its own mRNA movement. More detailed movement mechanisms of KN1 are described in Chap.4 (Dave Jackson).

As an important development regulator, cell-to-cell movement of KN1 is conserved during plant evolution (Kim et al., 2002; Kim et al., 2003). In *Arabidopsis* homeobox family, KNOTTED 1-like homeobox protein 1 / BREVIPEDICELLUS (KNAT1/BP) and SHOOTMERISTEMLESS (STM) are most closely related to KN1. STM and KN1 have very similar function in initiating and maintaining the SAM (Long et al., 1996). KNAT1/BP is involved in the regulation of inflorescence architecture (Venglat et al., 2002). Kim *et al* demonstrated that both KNAT1 and STM proteins are mobile from L1 to inner layers in the shoot apical meristem when they were expressed under a L1 specific promoter (Kim et al., 2003). In rice, three KN1-like homeobox class 1 transcription factors, Oskn1~3, were tested for their cell-to-cell trafficking by transiently expressing them in onion epidermis. Among these, Oskn1 (OSH1) showed extensive mobility between cells (Kuijt et al., 2004).

Mobile LAX PANICLE1 in the axillary meristem formation

In grasses, formation of axillary meristems affects the overall plant architecture. Among several mutants that affect axillary meristem formation, *lax panicle1* (*lax1*) phenotype (Komatsu et al., 2001) was found to be caused by the mutation in a bHLH

transcription factor. *LAX1* mRNA is expressed in an adaxial boundary between the axillary meristem and shoot apical meristem, however LAX1 function is required inside the axillary meristem. This indicated that LAX1 might function in a non-cell autonomous manner. Such non-cell autonomous behavior is explained by the mobility of LAX1 proteins. LAX1 proteins fused to a GFP monomer were distributed inside the axillary meristem as well as in the adaxial junction whereas its RNA was only restricted to the junction (Oikawa and Kyozuka, 2009). Since GFP:LAX1 fusion proteins only move toward the axillary meristem, the movement of LAX1 seems to be directional. When the size of LAX1 fusion proteins was increased by attaching 3xGFP, the mobility of 3xGFP:LAX1 decreased significantly, which might suggest that LAX1 does not influence the size exclusion limit of plasmodesmata.

Bidirectional communication of mobile transcription factors in the hair cell development

Epidermis in the root and leaf is a cell layer in direct contact with outer environments. Root hairs formed in the root epidermal layer are key cells that absorb water and minerals from soil. Above ground similar epidermis-derived cellular extensions are formed and named leaf trichomes. They function in protecting leaves from predators and pathogens, and preventing the overheating of leaf surface. Both, root and leaf hair cells, appear in a regular pattern, which is established by a similar set of transcription factors that act as signaling molecules between hair and neighboring non-hair cells. Detailed gene regulatory programs in epidermal cell patterning are reviewed

elsewhere (Ishida et al., 2008; Schiefelbein et al., 2009). Transcription factors that belong to R3 Myb and bHLH families contribute to the patterning of root hairs and trichomes by moving between cells.

CAPRICE (CPC), a small R3 Myb transcription factor, promotes root hair formation but inhibits trichome development (Wada et al., 1997; Wada et al., 2002; Kurata et al., 2005). CPC proteins, generated in the atrichoblasts (precursors of hairless cells), travel to the trichoblasts (precursors of hair cells) where CPC promotes the formation of root hairs. Kurata *et al.* (2005) demonstrated that two discrete regions in the N-terminus and Myb domain are required for CPC movement. A single amino acid substitution in W76 and M78 in the Myb domain of CPC drastically reduced its mobility. During its movement, CPC seems to increase the size exclusion limit of plasmodesmata. When *CPC* fused to multimerized GFP was expressed under *CPC* promoter, these proteins moved between cells whereas the same multimerized GFP without CPC did not. The inter-cellular movement of CPC also seems to be regulated in a tissue-specific manner. *CPC* was expressed under promoters that drive gene expression in the root stele (vascular tissues and pericycle) and trichoblasts, respectively. CPC proteins moved from trichoblasts to atrichoblasts, however they did not from the stele to an outer cell layer.

The mobility of two other R3 MYB family proteins, ENHANCER OF TRY AND CPC 3 (ETC3) and TRIPTYCHON (TRY), has been observed in the leaf epidermis (Digiuni et al., 2008; Wester et al., 2009). Wester *et al.* reported ETC3 transport between epidermal cells as well as between the epidermis and underlying

cells in the leaves (Wester et al., 2009). In the *etc3* null mutant, trichome density increased suggesting a negative role of ETC3 in the trichome identity.

CPC, ETC3, and TRY interact with GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3), bHLH transcription factors. In the root, these two transcription factors move from trichoblasts to atrichoblasts to define the non-hair cell fate (Bernhardt et al., 2005). Studies indicated that *GL3* and *EGL3* are preferentially up-regulated by CPC in the trichoblasts, while they repress their own expression after traveling to atrichoblasts. Therefore, CPC and GL3/EGL3 movement form a bi-directional regulatory circuit that mediates the communication between trichoblasts and atrichoblasts. Furthermore, an elevated level of GL3 proteins in the over-expression lines significantly reduced mobility of CPC, suggesting the protein-protein interaction interferes with the cell-to-cell movement of CPC (Wester et al., 2009).

SHORT ROOT movement in the root

Cell type patterning and growth in the root are governed by root apical meristem. Plant roots are composed of radial tissues that are centripetally organized: epidermis, cortex, endodermis, and pericycle. Inside the pericycle, vascular tissues are organized in multi-symmetry. Development of these root tissues are driven by activities of cell division and cell type specification in the stem cell niche, which is composed of undifferentiated stem cells and the quiescent center, a stem cell organizer (Bennett and Scheres, 2010; Sablowski, 2011).

Cell-to-cell movement of SHORT ROOT (SHR), a member of plant specific GRAS family transcription factors, plays a critical role in the patterning of root tissues (Helariutta et al., 2000). *SHR* is expressed in the stele, and then its proteins move into adjacent cell layers including quiescent center, cortex/endodermal initials, and endodermis. In these cell types, SHR directly activates the expression of *SCARECROW* (*SCR*), which encodes another GRAS family transcription factor (Helariutta et al., 2000; Nakajima et al., 2001). SHR and SCR in the endodermis/cortex initials subsequently trigger the asymmetric cell division that forms endodermis and cortex. Recently, SHR and SCR in the endodermis were also shown to be required for the cell type patterning in inner layers of the endodermis by activating the expression of microRNA 165/6 as a retrograde signal (Carlsbecker et al., 2010; Miyashima et al., 2011). Mobile microRNA 165/6 establishes the graded distribution of the mRNAs that encode class III Homeodomain Leucine-Zipper family (HD-ZIP III) transcription factors via post-transcriptional regulation, which is low in the stele periphery and high in the center.

Cell-to-cell movement of SHR is regulated in a tissue specific manner. When *SHR* was expressed in the cell types where it is not normally expressed, SHR proteins did not move between cells (Sena et al., 2004). Studies by Gallagher *et al* proposed a model that a balance between cytoplasmic and nuclear localization is required for SHR movement (Gallagher and Benfey, 2009). Similar to CPC, more than one region were found to be important for both mobility and function of SHR. However, a deletion of LNELDV in the second leucine heptad repeat region disrupted only mobility not functionality. It was also pointed out that signals for intercellular

movement are likely to be conserved within GRAS domain since a modified SCR protein without nuclear localization signal (NLS) showed a cell-to-cell mobility like SHR.

For proper patterning of endodermis and cortex, SHR has to be confined to the endodermis as ectopic SHR expression in the cortex causes supernumerary cell layers. Interestingly, a study demonstrated that SCR proteins, produced by transcriptional activation of SHR, interact with SHR and thereby restrict SHR proteins to the nucleus and inhibit their further movement (Cui et al., 2007). This is consistent with the finding that SHR needs to be in the cytoplasm to move to a neighboring cell layer (Gallagher et al., 2004).

Mobile bHLH transcription factors in the root initiation and growth

Three mobile transcription factors in the bHLH family have been found to play various roles in the root initiation and growth. One of them is *TARGET OF MP 7* (*TMO7*), a direct target of auxin-dependent transcription factor MONOPTEROS (MP) (Schlereth et al., 2010). MP, also known as *AUXIN RESPONSE FACTOR 5* (*ARF5*), regulates gene expression in response to auxin (Hardtke and Berleth, 1998). Its activity has been shown to be important for controlling hypophysis specification and embryonic root initiation. Schlereth *et al.* (2010) showed that MP directly activates the expression of *TMO7* in the embryo cells adjacent to hypophysis. TMO7 protein was then found to move into the hypophysis precursor cells. Such directional transport allows TMO7 to act as intercellular signal that mediates MP regulation on the initiation of root stem cells. Unlike CPC, the cell-cell movement of TMO7 seems to

be dependent on the size. At the same time, its movement seems to be directional. The addition of triple-GFP (84kD) to the 11kD TMO7 protein blocked movement beyond the transcriptional domain, and led to a mostly cytoplasmic localization. This TMO7:GFP fusion protein was detected in the hypophysis, but not in the apical half of the embryo.

Another mobile bHLH transcription factor recently found is UPBEAT1 (UPB1). UPB1 controls the transition from cell proliferation to differentiation in the root (Tsukagoshi et al., 2010). In its regulation, UPB1 proteins seem to move from the lateral root cap (LRC) to vascular tissues. In vascular tissues, UPB1 positively regulates expression of peroxidases that promote the production of reactive oxygen species (ROS). ROS subsequently promotes the transition of cell status from meristem to differentiation. Correlation between LRC and ROS activation zone indicates that LRC provides the positional information which is provided by the UPB1 moving horizontally and triggering the production of ROS in the right place. The movement of UPB1 was significantly reduced when it was fused to 3xYFP but not completely abolished.

Another bHLH transcription factor, named POPEYE, is very interesting because it seems to regulate root development and protein movement in response to abiotic stresses. In plants exposed to iron deficiency expression of *POPEYE* is induced specifically in the pericycle and root cap (Long et al., 2010). However, POPEYE protein moves to the neighboring root meristem and stele. Though not been emphasized in the paper by Long et al. (2010), POPEYE proteins seem to change their sub-cellular localization in response to iron deficiency. In the iron rich condition,

POPEYE is localized in the cytoplasms and cell boundaries. However, under iron deficiency, POPEYE is found mainly in the nuclei suggesting that nuclear localization might be required for the cell-to-cell mobility (Gallagher and Benfey, 2009).

Cell-to-cell movement of transcription factors in the flower development

There are several transcription factors documented to be mobile in studies on flower development. Comprehensive reviews for floral meristem formation and development are available elsewhere (Goto et al., 2001; Jack, 2004; Causier et al., 2009). LFY, an important floral meristem regulator, has been shown to move in a non-directional manner, which is different from the mode of cell-cell movement of most of aforementioned transcription factors (Sessions et al., 2000). When *LFY* was expressed under the promoter that drives gene expression in the L1 layer of developing flowers, the *lfy* mutant phenotype was fully rescued. In such transgenic plants, LFY proteins, but not LFY RNAs, were detected in all layers of the rescued flowers, indicating that LFY proteins move from the L1 into inner layers. Intercellular movement of LFY seems to be non-targeted. The mobility of LFY proteins into the inner cell layers of floral meristems was gradual and dependent on the size of protein, suggesting that LFY movement could be based on diffusion. Such type of intercellular trafficking might be related to the lack of particular domains required for targeted cell-to-cell movement, as indicated in the domain analysis of LFY movement (Wu et al., 2003).

Two MADS-box family transcription factors, DEFICIENS (DEF) and GLOBOSA (GLO), function in a non-cell-autonomous manner to control petal and

stamen organ identity in *Antirrhinum* (Perbal et al., 1996). In mutants of *DEF* or *GLO*, organ primordia in the second and third whirls in a flower, which respectively develop as petals and stamens in the wild type, turn into sepals and carpels, organs of the first and fourth whorls (Carpenter and Coen, 1990). When *DEF* expression was specifically restored in the L2 and L3 layers of floral meristem in the *def* mutant, petals developed in the second whirl and deposited pigments in the L1 layer. Such full recovery of petals in the L1 layer seems to be achieved by the regulation of DEF proteins that move into the L1 from L2 and L3 layers. However, the respective Arabidopsis orthologous B-type proteins APETALA3 (AP3) and PISTILLATA (PI) do not seem to have similar activities (Jenik and Irish, 2001). Another MADS-box family transcription factor, AGAMOUS (AG) has been shown to be able to move between cells in the L1 layer as well as into the inner cells from the L1 layer in the floral meristem. This cell-cell movement might suppress the *WUS* expression inside, thereby driving determinate formation of floral organs (Urbanus et al., 2010).

Transcription factors moving long distance

Mobile *LeT6* affects leaf architecture

In the wild-type tomato, leaves develop as unipinnate compound leaves with acutely pointed tips. Leaves in a dominant mutant *Mouse ears* (*Me*) develop round-shaped leaves with higher orders of compoundness. *Me* mutant was previously found to be caused by the fusion between *LeT6*, a gene encoding a KNOTTED-1–like homeobox (KNOX) transcription factor, and *PFP*, a gene for PYROPHOSPHATE-

DEPENDENT PHOSPHOFRUCTOKINASE, an enzyme in the glycolytic pathway (Chen et al., 1997). This gene fusion, which has 10 kb of native *PFP* upstream sequence and a part of *PFP* coding region, enhances *LeT6* expression in *Me* mutant, thereby increasing the complexity of compound leaf structure. A further study by Kim *et al.* (2001) suggested that *LeT6* RNAs are mobile through phloem (Kim et al., 2001). When wild type tomato plants were grafted onto *Me* stocks, newly developing leaves on the wild type scion turned into forms similar to *Me* leaves. Further expression analysis using *in situ* RT-PCR showed the presence of *PFP-LeT6* RNA in the shoot apices of wild-type scions.

Movement of *CmNACP* through phloem

Phloem sieve elements are thought to contribute to the transport of signaling molecules (see also Chap. 2, Ruiz-Medrano). To find whether those molecules include mRNA and their identity, mRNAs were isolated from phloem sap in the pumpkin (*Cucurbita maxima*) and cDNA library was constructed from them. Random sequencing of this library identified RNA species that encode several transcription factors that are highly similar to NAM (CmNACP), RING (CmRINGP), GAI (CmGAIP), WRKY (CmWRKYP), and STM (CmSTMP) (Ruiz-Medrano et al., 1999). The mobility of these RNAs was further confirmed by hetero-grafting experiments using a pumpkin as stock and a cucumber as scion. In these experiments, all of these transcription factor RNAs were detected in the phloem sap of cucumber stem only when it was grafted onto pumpkin stock. Further analyses using *in situ* RT-

PCR suggested that despite RNA movement through sieve cells in the stem happens widely, the movement of RNA into the meristem is more selective. Among five mobile transcription factors that showed the mobility along sieve cells in the stem, only *CmNACP* and *CmGAIP* mRNAs were detected in the apical region of cucumber scion after the hetero-grafting.

***St BEL5* regulates the development of potato tubers**

Tuber formation in potatoes is a complex developmental process that requires the interaction of environmental, biochemical, and genetic factors. In potato genotypes such as *S. tuberosum* ssp *andigena*, short-day (SD) photoperiods are strictly required for tuber formation, whereas long-day (LD) conditions inhibit tuberization. *St BEL5* is ubiquitously expressed in potato plants but accumulation of it RNA is enhanced in leaves and stolons by SD conditions (Chen et al., 2003). *St BEL5* RNA levels accumulated in an increasing concentration gradient from the shoot tip (low levels) through the stem to the stolon tip in response to SD conditions. Under LD conditions, the highest levels of RNA were observed in shoot tips and stolons with the lowest levels in the lower portion of the stem. Over-expression of *St BEL5* induced tuber formation even in non-inductive condition.

The grafting experiments suggested that *St BEL5* RNAs are mobile through phloem. This movement is induced by short day photoperiod and regulated by the 3′ untranslated region of *St BEL5*, indicating that 3′ untranslated region may contain a conserved motif which facilitates the binding of RNA-binding proteins (Banerjee et al., 2006; Banerjee et al., 2009; Hannapel, 2010)

Conserved mobility of GIBBERELLIC ACID-INSENSITIVE

Arabidopsis *GIBBERELLIC ACID-INSENSITIVE* (*GAI*) encodes a protein that belongs to the GRAS family, and functions as a negative regulator of gibberellic acid (GA) responses. Mutated GAI carrying deletions in the DELLA domain inhibits some components of the GA signaling pathway and causes dark green dwarf phenotype in a semi-dominant manner. The mobility of *GAI* RNA along sieve cells in the stem was initially found in the pumpkin (Ruiz-Medrano et al., 1999) and further characterized in detail in pumpkin, tomato, and Arabidopsis (Haywood et al., 2005). Hetero-grafting experiments indicated that RNAs of *GAI* from *Cucurbita maxima* (*CmGAIP*) are mobile from the pumpkin stock to the cucumber scion. The same phenomenon was observed when the wild-type scions of Arabidopsis and tomato were grafted onto the stocks that ectopically express *CmGAIP* and Arabidopsis Δ*DELLA-gai*. Δ*DELLA-gai* that moved from a stock to a scion in tomato affected the colors of leaves in a scion which emerged near the grafting junction. This suggests that mobile Δ*DELLA-gai* RNAs are translated to functional proteins. The mobility of *GAI* RNA was also observed in apples (Xu et al., 2010). However, unlike in the studies of Arabidopsis, pumpkin, and tomato which showed *GAI* RNAs only move from the stock to the scion, *GAI* RNAs were mobile in both directions.

This evolutionarily conserved RNA mobility of *GAI* seems to be mediated by the RNA secondary structure (Huang and Yu, 2009). Other closely related paralogues of *GAI* did not show the RNA mobility suggesting that mobility has specifically

evolved in *GAI*. Domain analyses of *GAI* RNA suggested that a part of coding region of RNA which starts ~1kb downstream of the translational start site and the 3' untranslated region are required for the RNA mobility. The random mutagenesis analysis of this region indicated that the secondary RNA structure involving a stem-and-loop structure might play a more important role than a specific nucleotide sequence.

Cellular contexts that influence cell-to-cell movement of transcription factors

As described in the previous section, a growing number of transcription factors have been found to move between cells in forms of proteins or RNAs. For many of them, their mobility plays an important role in plant functions. However, it remains an open question how many transcription factors move between cells. In a recent study, a comparison was made between cell type specific root expression data and expression patterns of transcriptional and translational GFPs driven by promoters of 24 cell type enriched transcription factors in the Arabidopsis root (Lee et al., 2006). Comparison of three data, which include transcriptional GFPs driven by endogenous promoters of individual transcription factors, translational GFPs which are fused to the coding regions of transcription factors and driven under promoters of corresponding transcription factors, and cell-type specific root expression data, indicated that nearly 25% of transcription factors (6 out of 24) might move between cells as proteins in the

Arabidopsis root. This frequency is much higher than anticipated based on the number of mobile transcription factors identified so far (Table III.1).

Table III.1. A list of transcription factors that move between cells as RNAs or proteins

Transcription factor	Gene family	Molecular weight*	What is moving?	Mechanism	Origin	Target	Organism	Literature
CAPRICE	R3-type MYB	11kD	protein	targeted	Atrichoblast	Trichoblast	Arabidopsis	(Wada et al., 2002)
KNAT1	KNOX I Homeobox	46kD	protein	targeted	L1 of shoot meristem	L2/3 of shoot meristem	Arabidopsis	(Kim et al., 2003)
KNOTTED1	KNOX I Homeobox	40kD	RNA/ protein	targeted	Below L1 of SAM	L1 of SAM	maize	(Lucas et al., 1995)
STM	KNOX I Homeobox	43kD	protein	targeted	L1 of shoot meristem	L2/3 of shoot meristem	Arabidopsis	(Kim et al., 2003)
LEAFY	LFY	47 kD	protein	non-targeted	L1 of shoot meristem	L2/3 of shoot meristem	Arabidopsis	(Sessions et al., 2000)
LAX PANICLE1 (LAX1)	bHLH	23 kD	protein	targeted	Adaxial boundary between axillary meristem and shoot apical meristem	Rest of axillary meristem	rice	(Oikawa and Kyozuka, 2009)
SHORT ROOT (SHR)	GRAS	59kD	protein	targeted	Stele in the root	Endodermis and QC	Arabidopsis	(Nakajima et al., 2001)
TARGET OF MP 7 (TMO7)	bHLH	11kD	protein	non-targeted	Outside the hypophysis	Hypophysis and vascular tissues	Arabidopsis	(Schlereth et al., 2010)
AT4G00940	C2C2-Dof	34kD	protein		Pericycle in the root	Endodermis	Arabidopsis	(Lee et al., 2006)
AT4G27410/	NAC	33kD	protein		Pericycle and	Cortex and	Arabidopsis	(Lee et al.,

RD26				endodermis in the root	epidermis		2006)
AT3G61850/ DAG1	C2C2-Dof	33kD	protein	Stele in the root	Endodermis	Arabidopsis	(Lee et al., 2006)
AT2G22850	bZIP	26kD	protein	Phloem pole pericycle	Endodermis and stele	Arabidopsis	(Lee et al., 2006)
AT4G37940/ AGL21	MADS	26kD	protein	Procambium and columella root cap	Stele, endodermis, columella, and epidermis	Arabidopsis	(Lee et al., 2006)
ETC3	R3-type MYB	9kD	protein	Trichome	Neighboring epidermal cells around a trichome	Arabidopsis	(Wester et al., 2009)
TRIPTYCHON (TRY)	MYB	13kD	protein			Arabidopsis	(Digiuni et al., 2008)
GL3	bHLH	70.5kD	protein	Hair cell	Non-hair cell	Arabidopsis	(Bernhardt et al., 2005)
UPBEAT1	bHLH	12kD	protein	Lateral root cap	Root elongation zone	Arabidopsis	(Tsukagoshi et al., 2010)
DEFICIENS	MADS	26kD	protein	L2 or L3 of floral organ primordia	L1	Antirrhinum	(Perbal et al., 1996)
GLOBOSA	MADS	25kD	protein	L2 or L3 of floral organ primordia	L1	Antirrhinum	(Perbal et al., 1996)
AGAMOUS	MADS	29kD	protein	L1 of floral meristem	L2 and L3	Arabidopsis	(Urbanus et al., 2010)
POPEYE	bHLH	27kD	protein	Root cap	Root	Arabidopsis	(Long et al.,

				meristem		2010)
St BEL5	homeobox	RNA	Shoot	Stolon	potato	(Banerjee et al., 2006)
GAI	GRAS	RNA	Stock	Scion	pumpkin, tomato, Arabidopsis	(Haywood et al., 2005; Xu et al., 2010)
LeT6	KNOX I Homeobox	RNA	Stock	Scion	tomato	(Kim et al., 2001)
CmNACP	NAC	RNA	Stock	Scion	pumpkin	(Ruiz-Medrano et al., 1999)
CmRINGP	RING	RNA	Stock	Scion	pumpkin	(Ruiz-Medrano et al., 1999)
CmWRKYP	WRKY	RNA	Stock	Scion	pumpkin	(Ruiz-Medrano et al., 1999)
CmSTMP	Homeobox	RNA	Stock	Scion	pumpkin	(Ruiz-Medrano et al., 1999)

* The molecular weights of mobile proteins and not of mobile RNAs are shown.

Now it is well established that proteins and RNAs move between cells through plasmodesmata. Cell-to-cell movement of transcription factors as proteins involves the alteration of size exclusion limit of plasmodesmata and the detailed mechanism of proteins and RNA movement through plasmodesmata is described in other chapters of the book (Chap. 1, Manfred Heinlein; Chap. 2, P. Zambrysky; and Chap. 7, Ruiz-Medrano et al.). In recent studies, size exclusion limits have been estimated using free GFP (Crawford and Zambryski, 2000; Kim et al., 2005b; Kim et al., 2005a). Monitoring the movement of GFP proteins at various sizes suggests that size exclusion limits are regulated dynamically. In *Nicotiana* leaf epidermis, 27kD free GFPs were observed to move between cells at a relatively high frequency (23%). When the size of GFP was doubled (54kD), GFP movement decreased dramatically (Crawford and Zambryski, 2000). By contrast, a similar size of GFP fusion proteins that include P30 in the Tobbaco Mosaic Virus (57kD) moved between cells at a frequency 30 times higher than 2xGFP. In addition to the mobility, free GFP and P30-GFP showed a difference in the sub-cellular localization. Whereas free GFP proteins were localized throughout the cell including the nucleus in a diffusive manner, P30-GFP proteins were located to punctae on the cell walls, which co-localize to plasmodesmata. Based the distinctive nature of these proteins (GFP vs. P30-GFP), Crawford and Zambryski categorized cell-to-cell protein movement into two types: targeted and non-targeted.

Non-targeted movement is more like diffusion, which is affected by the concentration and size of proteins. By contrast, targeted movement involves the interaction between mobile proteins and plasmodesmata components and thereby affects the size exclusion limit. Consistent with the idea that targeted movement

involves the active regulation of size exclusion limit of plasmodesmata, targeted movement was not affected much by changes in environments and developmental stages (Crawford and Zambryski, 2001). However, non-targeted movement was dramatically reduced by these factors.

As shown in Table III.1, protein sizes of mobile transcription factors are distributed at a broad range. Some seem to be small enough to move to neighboring cells simply by diffusion. However, studies indicate that the protein size does not predict the mode of cell-cell movement. For example, CPC is a small 11kD protein however moves between cells in a targeted manner (Kurata et al., 2005). When the protein size of CPC was dramatically increased by fusing it to 3xYFP, CPC:3xYFP still moved between cells. Furthermore, CPC proteins move only between epidermal cells not to the inner cell layers, suggesting that specific factors in the epidermis might direct cell-type specific movement. Contradictory to CPC, 47kD LFY fused to GFP moves from the L1 to inner layers in a rather diffusive manner (Wu et al., 2003). These suggest that the size of transcription factor proteins is not a good indicator for predicting the mode of cell-cell trafficking.

Although the mode of protein movement between cells was categorized into two, complex behaviors of mobile proteins suggest there might be multiple regulatory mechanisms that govern protein mobility. One important factor that affects protein movement is protein-protein interaction. A number of studies indicate that the protein-protein interaction can interfere with the cell-cell mobility. SHR proteins do not move any further once they reach in the endodermis where they interact with SCR proteins. SHR-SCR protein complexes are localized in the nuclei, and thereby cannot

move to the next cell layer (Cui et al., 2007). In the absence of SCR, SHR proteins are localized to both cytoplasms and nuclei, and frequently to the next cell layer (Sena et al., 2004; Cui et al., 2007). Similarly, the cell-cell movement of CPC was proposed to be interfered by the interaction to GL3 (Wester et al., 2009).

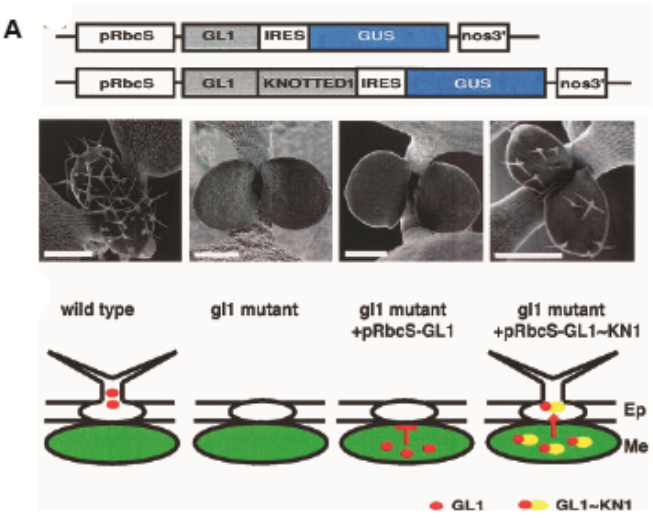
The second factor that was shown to affect protein movement is the sub-cellular localization. For SHR to move, it seems to require the localization to both nuclei and cytoplasms (Gallagher et al., 2004; Gallagher and Benfey, 2009). When SHR was fused to the GFP with a nuclear localization signal (NLS), SHR movement was significantly reduced suggesting that cytoplasmic localization of SHR is necessary for cell-to-cell trafficking. However, when domains that are crucial for the SHR movement are deleted, SHR proteins were localized to the cytoplasm. Interestingly, as modified non-mobile SHR proteins were retargeted to the nuclei by fusing to NLS-GFP, these proteins were able to move between cells again. These studies propose the presence of unknown mechanisms that require the delicate sub-cellular localization in the process of cell-to-cell trafficking.

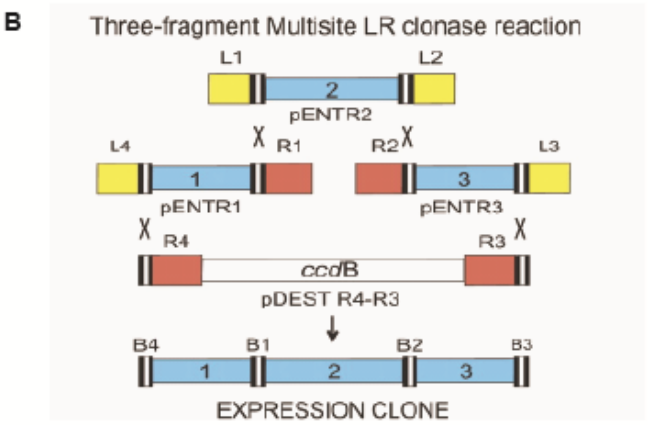
The third factor that affects protein mobility can be found in cell type specific mechanisms that affect cell-cell trafficking in a either positive or negative manner. Unlike free GFP and P30-GFP that move radially from the foci of their expression, movement of many mobile transcription factors seems directional. SHR expressed in the phloem sieve cells or in the epidermis did not move (Sena et al., 2004). CPC expressed in the stele did not, either (Kurata et al., 2005). These observations suggest the presence of cell type specific factors that specifically facilitate or repress the movement of SHR and CPC. Although diffusive behaviors of LAX1 and TMO7

proteins suggest their movement might be non-targeted, LAX1 and TMO7 move in a directional manner (Oikawa and Kyozuka, 2009; Schlereth et al., 2010). LAX1 proteins fused to a GFP monomer are distributed inside the axillary meristem as well as in the adaxial junction, but not to the opposite direction. TMO7 moves more actively in the shoot-ward direction. In the future it will be interesting to find whether these preferences in the direction of cell-to-cell trafficking is caused by the cell-type specific size exclusion limit of plasmodesmata or by other cell factors that actively regulate directional mobility.

Genome-wide approaches to identifying mobile transcription factors

Recent advances in technologies for profiling proteins and RNA at a global scale, isolating cell types, and imaging are speeding up the discovery of transcription factors mobile as proteins and RNAs. Here, several existing approaches that can enable the efficient discovery of mobile transcription factors at a global level are introduced. Furthermore, though not listed in original papers, transcription factors that might move as RNA in the phloem sieve cells are predicted from the cell type specific expression data.


A
pRbcS
GL1
IRES
GUS
nos3'
pRbcS
GL1
KNOTTED1
IRES
GUS
nos3'
wild type
gl1 mutant
gl1 mutant
+pRbcS-GL1
gl1 mutant
+pRbcS-GL1~KN1
Ep
Me
GL1
GL1~KN1


B
Three-fragment Multisite LR clonase reaction
L1
L2
2
pENTR2
L4
R1
R2
L3
1
3
pENTR1
pENTR3
R4
R3
ccdB
pDEST R4-R3
B4
B1
B2
B3
1
2
3
EXPRESSION CLONE

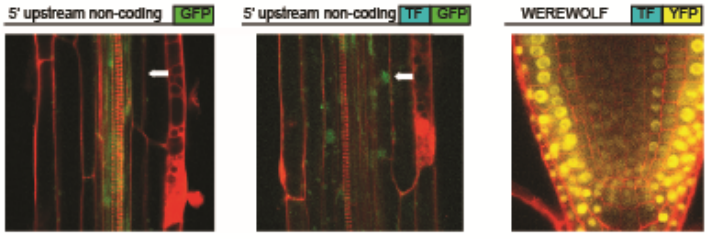

C
5' upstream non-coding
GFP
5' upstream non-coding
TF
GFP
WEREWOLF
TF
YFP

Figure III.1. Genetic and molecular tool kits for identifying mobile transcription factors.

A: Functional protein trafficking assay using trichome rescue. Upper panel; GL1 fusion constructs used for trichome rescue assay. Constructs include the mesophyll-specific RbcS promoter (pRbcS), GL1 or GL1~KN1 fusions, an internal ribosome entry sequence (IRES) upstream of the GUS coding sequence, and the nopaline synthase (nos) 3′ terminator. Middle and bottom panels show images of leaf surfaces in different genetic backgrounds and cartoons that explain the phenotypes. Trichome formation is promoted by GL1~KN1 that moves from the mesophyll cells into the epidermis. Image modified from Figure 1 by Kim *et al.* (2005c) with copyright permission from Cold Spring Harbor Press. **B:** Versatile cloning of multiple components using multisite Gateway recombination system (Invitrogen). Image modified from Figure 1 by Karimi *et al.* (2007) with copyright permission from American Society of Plant Biologists. **C:** Identification of a mobile transcription factor (DAG1) in the Arabidopsis roots with transgenes generated with the multisite Gateway recombination and the laser scanning confocal microscopy (Lee et al., 2006). Left panel; transcriptional GFP (endoplasmic-reticulum localized) expressed under the DAG1 promoter. Middle panel; translational free GFP fused to the coding region of DAG1 expressed under its own promoter. Right panel; YFP fused to DAG1 expressed by the *WEREWOLF* promoter. *WEREWOLF* promoter drives expression in the lateral root cap and epidermis. Arrows indicate the endodermis layer whether DAG1 proteins move from the stele.

Trichome-assay based approach

Using trichome assay system (Kim et al., 2005c), domains required for cell-cell movement in KN1 were identified (Figure III.1A) (see also Chap. 4, Dave Jackson). In this approach, GLABRA1 (GL1), a transcription factor that promotes trichome formation on leaf epidermis, was expressed under rbcS promoter, which drives gene expression in the mesophyll cell, in gl1 mutant background. In gl1 mutant, trichome does not develop on leaf epidermis. Since GL1 acts in a cell-autonomous manner, GL1 expressed in the mesophyll cells cannot complement gl1 mutant. However, when KN1 was translationally fused to GL1 in this construct and expressed in gl1, trichomes developed because KN1:GL1 proteins moved from the mesophyll cells to epidermal cells. This system can be applied in a cost-efficient manner to identify cellular factors altering the capacity of transcription factors to move between cells (Winter et al. 2007) or to identify novel transcription factors that actively move between cells.

Analysis of transcription factor movement using cell type specific promoters

The mobility of transcription factors can be assessed by expressing transcription factors, which are translationally fused to the visual markers, under a cell type specific promoter. This approach was used in the studies on the cell-to-cell movement of SHR in the root (Sena et al., 2004). SHR proteins, generated in the stele, move to the endodermis layer in the Arabidopsis root. SHR was expressed in the phloem companion cells in the root or in the epidermis. In either cell types, SHR proteins did

not move to other cells. However, when SHR was expressed in the epidermis in the scr mutant background, SHR proteins moved into the cortex layer suggesting that components regulated by SCR are involved in controlling the cell-to-cell movement of SHR.

A similar approach was made to investigate the movement of KN1 (Kim et al., 2002; Kim et al., 2005c). GFP fused to KN1 was expressed under WUSCHEL promoter, which drives gene expression specifically in the L3 layer of shoot apical meristem, and under SCR, which drives gene expression in the L1 layer of shoot apical meristem. Under both promoters, expansion of proteins was observed beyond where they are produced, suggesting KN1 can move in either direction in the shoot apical meristem.

Again, L1 specific promoter of ATML1 was used to assess the movement of LFY and AG (Wu et al., 2003; Urbanus et al., 2010). Both transcription factors moved to inner cells of floral meristems and partially complemented their mutant phenotypes.

There are rich sources of cell type specific promoters in Arabidopsis that can be used for systematic screening of potentially mobile transcription factors. However, the assessment of transcription factor mobility should be made carefully because studies show the cell-to-cell movement is significantly dependent upon cellular contexts. For example, a random analysis of selected 23 transcription factors by expressing their GFP fusion under WEREWOLF promoter, which drives gene expression in the lateral root cap and atrichoblasts, more than 40% (10 transcription factors) expanded their GFP domains (Lee, unpublished results; an example is shown

in Figure III.1C). This is a much higher frequency than when they are expressed in their endogenous expression domains.

Analysis of transcription factor movement using a versatile cloning technology

Technologies for isolating gene promoters and coding regions became very efficient with development of DNA polymerase with a high proof reading function and recombination based cloning technology (Figure III.1B). Several binary vectors that can integrate multiple DNA fragments in frame using gateway cloning system have been developed (see the reviews (Chung et al., 2005; Karimi et al., 2005; Karimi et al., 2007)). ~1600 transcription factors in Arabidopsis have been cloned into a gateway vector (Ou et al., 2011). Using this highly efficient cloning system, individual transcription factors translationally fused to GFP were expressed under their own promoters in transgenic Arabidopsis. GFP expression patterns of these lines (translational fusion) were compared with the ER localized GFP expressed under the same promoters (transcriptional fusion). In this experiment (Lee et al., 2006), transcription factors that are expressed in a cell type enriched manner were selected from the genome-wide expression data generated from several root cell type specific GFP expression lines. Distinctive expression domains of transcriptional GFPs made it feasible to identify translational GFPs that had different expression patterns. Among 61 transcription factors whose GFP fusion lines generated, 24 yielded lines with GFP patterns that clearly recapitulated cell type specific root expression data. Six of them (25%) were found to have translational fusion GFP with expression domains broader

than the corresponding transcriptional GFP, suggesting that their proteins might move between cells (Figure III.1C).

Profiling RNAs transported by RNA binding phloem proteins

RNA-binding proteins (RBPs) form ribonucleoprotein (RNP) complexes and thereby play crucial roles in RNA stability, processing, targeted delivery, and novel modes of mRNA protection (Shyu et al., 2008). Recent studies have established that in plants some RNP complexes can act non-cell-autonomously (Gómez et al., 2005; Ham et al., 2009). These RNP complexes help a unique population of mRNA species to move through the phloem sieve cells. Profiling RNA species bound to RBPs can help to find new RNA species that are transported through the phloem.

Recently, 50-kD pumpkin phloem RNA-binding protein (RBP50), a polypyrimidine tract binding protein, has been characterized as a non-cell-autonomous RNA-binding protein (Ham et al., 2009). This phloem mobile protein constitutes RBP50-based RNP complexes with multiple phloem proteins (Lin et al., 2009). To identify RNA that binds to RBP50, RBP50 RNP complexes were co-immunoprecipitated, from which RNAs were extracted, reversed transcribed and then amplified. From these, mRNAs of *PP16-1*, *GIBBERELLIC ACID-INSENSITIVE PHLOEM PROTEIN*, *SHOOT MERISTEMLESS*, *SCARECROW-LIKE 14* and *MYELOBLASTOSIS* family transcription factor were identified. To further investigate the degree to which RBP50 exhibits specificity in its binding to these identified phloem transcripts, co-immunoprecipitation experiments were performed using PP2, a phloem protein previously shown to bind RNA in a non-sequence-specific manner

(Gómez et al., 2005). RNAs isolated from these PP2 complexes were broader than those from RBP50; in contrast with the six mRNA species identified from the RBP50 RNP complexes, 75 different transcripts were cloned from the PP2 complexes. Some of these transcripts encode transcription factors. The heterogeneous nature and high number of the transcripts identified from the PP2 co-immunoprecipitation contrast markedly from the six transcripts identified from the RBP50 experiments. This suggests that RBP50 binds to a specific set of phloem-mobile mRNA species. All the six transcripts bound by RBP50 contain canonical polypyrimidine tract binding motifs. Further tests of RBP50 binding to these sequences confirmed that polypyrimidine tract binding motifs are required for the interaction.

Identification of potential mobile transcription factor RNAs from cell type specific RNA profiling

Most of mobile transcription factors as RNAs or proteins so far have been identified from studies of individual transcription factors. Thanks to the enhanced efficiency of molecular cloning and in vivo imaging, the number of mobile transcription factors is growing fast. Recent advances of cell type specific profiling of RNA, however, will further facilitate finding more mobile transcription factors. Here, recent data generated in the phloem sieve cells and companion cells using various techniques are described and potential mobile transcription factor RNAs are introduced.

Phloem expression profiling

To find mRNAs that are mobile through phloem, Deeken et al. generated a combination of datasets using various approaches (Deeken et al., 2008). The first data set was based on microarray experiments on mRNAs found in the whole phloem tissue harvested by Laser Microdissection Pressure Catapulting (LMPC) from the Arabidopsis inflorescence stems. The second microarray data were generated from mRNA extracted from the stem exudates. The last set of data was 444 ESTs generated from RNAs, which were isolated from companion cells (Ivashikina et al., 2003). Having confirmed the quality of the microarray data, only genes with hybridization signals that met the significance criterion of a *P*-value ≤ 0.01 on all three replicate microarray data were referred to as present in the phloem sap or LMPC-derived phloem tissue. According to these criteria, 2417 transcripts were detected by microarrays in the phloem sap and 1291 in LMPC-derived phloem tissue of Arabidopsis. From these lists, a total of 828 genes were found in both phloem sap and phloem tissue and 13 of them were transcription factors (Table III.2). Among 444 ESTs from companion cells, 144 ESTs were also found in both, exudates transcripts and LMPC-based phloem transcripts, suggesting that these mRNAs from companion cells might be unloaded into the phloem sieve cells.

Table III.2. Potential mobile transcription factor RNAs inferred from cell-type specific RNA profiling

TAIR number	Transcription factor gene family	Reference
AT3G60490	AP2-EREBP	(Mustroph et al., 2009)*
AT4G11140	AP2-EREBP	(Brady et al., 2007)**
AT1G17880	basic transcription factor	(Deeken et al., 2008)***

AT4G14410	bHLH	(Deeken et al., 2008)
AT1G59640	bHLH	(Brady et al., 2007)
AT1G61660	bHLH	(Mustroph et al., 2009)
AT3G23210	bHLH	(Mustroph et al., 2009)
AT5G04150	bHLH	(Mustroph et al., 2009)
AT5G67110	bHLH	(Mustroph et al., 2009)
AT1G75390	bZIP	(Mustroph et al., 2009)
AT1G66140	C2H2	(Mustroph et al., 2009)
AT2G36930	C2H2	(Deeken et al., 2008)
AT5G16470	C2H2	(Deeken et al., 2008)
AT1G07640	Dof-type zinc finger	(Brady et al., 2007; Mustroph et al., 2009)
AT2G28510	Dof-type zinc finger	(Mustroph et al., 2009)
AT2G28810	Dof-type zinc finger	(Mustroph et al., 2009)
AT2G37590	Dof-type zinc finger	(Brady et al., 2007)
AT3G45610	Dof-type zinc finger	(Brady et al., 2007)
AT3G55370	Dof-type zinc finger	(Mustroph et al., 2009)
AT3G61850	Dof-type zinc finger	(Mustroph et al., 2009)
AT1G49560	G2-like	(Mustroph et al., 2009)
AT1G69580	G2-like	(Mustroph et al., 2009)
AT1G79430	G2-like	(Brady et al., 2007; Mustroph et al., 2009)
AT3G04030	G2-like	(Mustroph et al., 2009)
AT4G18020	G2-like	(Mustroph et al., 2009)
AT4G37180	G2-like	(Mustroph et al., 2009)
AT5G18240	G2-like	(Mustroph et al., 2009)
AT5G29000	G2-like	(Mustroph et al., 2009)
AT2G18380	GATA	(Brady et al., 2007)
AT4G36620	GATA	(Brady et al., 2007)
AT2G04890	GRAS	(Brady et al., 2007)
AT5G52510	GRAS	(Mustroph et al., 2009)
AT1G04880	HMG (high mobility group)	(Brady et al., 2007)
AT1G69580	Homeodomain	(Brady et al., 2007)
AT2G03500	Homeodomain	(Brady et al., 2007; Mustroph et al., 2009)
AT3G04030	Homeodomain	(Brady et al., 2007)
AT3G12730	Homeodomain	(Brady et al., 2007; Mustroph et al., 2009)
AT1G19000	Homeodomain	(Deeken et al., 2008)
AT2G33310	IAA13	(Deeken et al., 2008)
AT1G72450	JASMONATE-ZIM-DOMAIN PROTEIN 6 (JAZ6)	(Deeken et al., 2008)

AT2G22540	MADS	(Deeken et al., 2008)
AT1G71692	MADS	(Mustroph et al., 2009)
AT2G42830	MADS	(Brady et al., 2007)
AT5G05790	MYB	(Mustroph et al., 2009)
AT1G54330	NAC	(Brady et al., 2007)
AT1G60350	NAC	(Brady et al., 2007)
AT1G65910	NAC	(Brady et al., 2007)
AT2G27300	NAC	(Brady et al., 2007)
AT3G03200	NAC	(Brady et al., 2007)
AT5G17260	NAC	(Brady et al., 2007)
AT1G73230	NAC	(Deeken et al., 2008)
AT4G29080	phytochrome-associated protein 2 (PAP2)	(Deeken et al., 2008)
AT1G43000	PLATZ transcription factor family	(Brady et al., 2007)
AT1G20823	RING/U-box superfamily protein	(Deeken et al., 2008)
AT1G33480	RING/U-box superfamily protein	(Brady et al., 2007)
AT1G06040	salt tolerance protein (STO)	(Deeken et al., 2008)
AT2G46020	SWI/SNF	(Deeken et al., 2008)
AT1G28520	VOZ-9	(Mustroph et al., 2009)
AT2G04880	WRKY	(Mustroph et al., 2009)
AT4G26640	WRKY	(Mustroph et al., 2009)

* Enriched in the companion cells. Selection criteria for significantly enriched RNAs for each pairwise comparison: Signal-log-ratio >1; False discovery rate <0.01.
** RNAs enriched in both, S32 and APL, expression domains (>1.2 fold and False discovery rate <0.001).
*** Expression was considered ‘present’ if the significance call P value equals or is below 0.01.

Cell-type specific expression map in the Arabidopsis root

The root expression map generated by cell sorting/microarray technology can serve as a great resource for identifying putative transcription factors that are mobile in form of RNA (Birnbaum et al., 2003; Nawy et al., 2005; Lee et al., 2006; Levesque et al.,

2006; Brady et al., 2007). Cell sorting combined with microarray technology uses transgenic plants that express fluorescent markers in cells of interest. Fluorescent cells among protoplasts are selectively isolated through the Fluorescence Activated Cell Sorter and their RNAs are purified and processed for expression profiling using microarray or next-generation sequencing technology (Birnbaum et al., 2005).

A recently combined root expression map includes expression profiles from 19 cell type specific lines, which cover 14 non-overlapping cell types in the root (Brady et al., 2007). In this expression map, data generated using three phloem lines are available, S32, APL, and SUC2. S32, APL, and SUC2 data respectively cover mRNA profiles from protophloem sieve cells starting from initials, companion cells starting from the region of root meristem, and companion cells starting from root maturation zone (Figure III.2). Using these and expression data from other cell types, phloem-enriched mRNA species that are mobile from companion cells into sieve cells can be predicted. For example, in the study by Brady et al. (2007), genes that are highly enriched in S32, APL, and SUC2 were predicted from the pair-wide comparison of gene expression values in each of phloem data against those in non-phloem cell types. 544 and 317 transcripts are respectively enriched in cell types labeled by S32 and APL. A surprisingly large number of genes (266) are expressed in both cell types in a highly enriched manner. Among these, 23 genes encode transcription factors (Table III.2). GFP expression domains in S32 and APL lines slightly overlap in the early stage of phloem. Therefore, some of these overlapping genes could be from the shared expression domain. In contrast, GFP expressed by the SUC2 promoter is expressed specifically in companion cells. Expression of 224 genes is predicted to be

highly enriched in the SUC2 domains. Among these, 97 genes are also enriched in the S32 domain. Taken together, comparison of enriched genes in phloem cell types suggests that a high proportion but not all of mRNA species expressed in companion cells might be loaded into sieve tubes. Such conclusion is also consistent with what was found by Deekens et al. (2008) (see above).

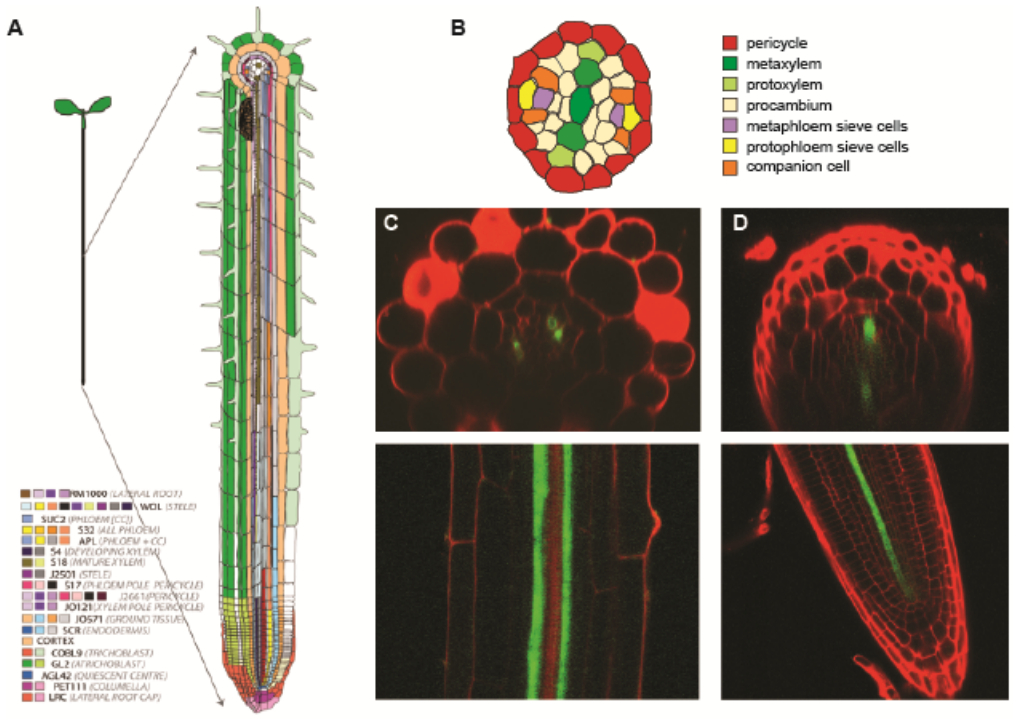


Figure III.2. Cell-type specific mRNA profiling in the Arabidopsis root for predicting potentially mobile transcription factor RNAs through phloem.

A: Microarray expression profiles of 19 fluorescently sorted GFP-marked lines were analyzed by Brady *et al* (2007). The colors associated with each marker line reflect the cell types sampled. Image modified from Figure 1 by Brady *et al.* (2007) with copyright permission from AAAS. **B**: Diagram of cell types inside the Arabidopsis stele. **C:** Cross (upper panel) and longitudinal (lower panel) sections of Arabidopsis roots that show GFP expression driven by *APL* promoter. Images were taken with a laser scanning confocal microscope. **D:** Cross (upper panel) and longitudinal (lower panel) GFP expression driven by *S32* (AT2G18380) promoter in the Arabidopsis root.

Cell-type specific translatome data

Cell-type specific root expression map generated using the cell-sorting/microarray technique provides a list of transcription factor transcripts that might be worthwhile to be tested for their capacity to allocate into phloem sieve tube cells. A major concern

in this approach, however, is the contamination of protoplasts by neighboring cells, which could potentially results in false positives.

Recently, mRNAs associated with ribosomes from specific cell populations were used for profiling on microarrays to find cell type specific changes of translatomes under hypoxia (Mustroph et al., 2009). In this study, FLAG-tagged ribosomal protein L18 (RPL18), a component of ribosome complexes, was expressed under the cell type specific promoters in Arabidopsis, and then mRNA-ribosome complexes were purified by immunoprecipitation using FLAG antibodies.

These translatomes obtained from phloem companion cell populations can help to predict mobile transcription factor RNAs. In a study by Mustroph et al. (2009), two promoters were used to profile translatome in the companion cells. *pSUC2:GFP-RPL18* was expressed in companion cells of the entire root whereas *pSULTR2;2:GFP-RPL18* was limited to companion cells present in the root elongation and maturation region. Consistent with the regional distinctions in expression of these promoters, the *pSUC2* and *pSULTR2;2* root translatomes were highly overlapping but not identical. The 270 co-enriched transcripts included a number of phloem companion cell markers such as *SUC2*, Sucrose-H^+ symporter (At1g22710); *AHA3*, plasma membrane H^+ ATPase (At5g57350); *APL*, G2-type transcription factor associated with phloem development (At1g79430); two phloem-specific lectins (At4g19840 and At2g02230), supporting the conclusion that mRNAs were effectively isolated from the targeted cell type. The comparison of the shoot and root *pSUC2* translatomes with mRNAs obtained from *pSUC2:GFP* protoplasts of seedling roots (Brady et al., 2007) identified 214 enriched mRNAs present in all 3 samples. Furthermore, 78 of the reported phloem

sap mRNAs were markedly enriched in the shoot *pSUC2* translatome (Deeken et al., 2008). In Table III.2, transcription factors that are enriched in all the companion cell data are listed. Some of these overlap with enriched transcription factors from a cell-sorting based approach. Considering that these lists were generated using different data set and different search criteria, it is very encouraging to find several overlapping transcription factors that might be mobile.

Bioinformatics approach to predict genes that are expressed in the vascular tissues

Studies using various approaches suggest that a large number of RNAs and proteins that move through the phloem sieve elements might be unloaded from the neighboring companion cells. Therefore, genes that are highly expressed in the cells associated with phloem sieve cells are good candidates as mobile proteins or mRNA. Gene expression patterns can be predicted if a small set of transcription factors drive the gene expression in particular cell types. A recent study suggests that it might be the case for phloem-enriched genes (Ruiz-Medrano et al., 2011).

Previously EST sequence database was generated for more than 1,200 transcripts from pumpkin phloem sap. This dataset was compared against Arabidopsis genes to pinpoint the putative orthologous genes. In this analysis, 150 Arabidopsis genes encoding putative transcription factors, protein kinases, protein phosphatases, cell cycle regulators, and hormone response factors were identified. Enriched motifs in 150 genes were searched using several motif search algorithms. In this search, motifs with CT and GA rich repeats were identified. Promoter analysis of a subset of

150 genes showed that genes that are expressed specifically in vascular tissues have these repeats at higher rates than those that are expressed in vascular tissues as well as other tissues. Furthermore, each of CT and GA motifs was able to drive vascular expression when they were attached to the 35S minimal promoter, confirming the importance of transcription factors that bind to these motifs in driving gene expression in vascular tissues.

Conclusions and Perspectives

The sessile nature of plants and different functions of plant tissues requires a versatile communication system between cells and organs in response to changes inside and outside the plant body. Studies that uncovered and investigated mobile transcription factors clearly indicate the functional significance of their cell-to-cell movement activity. An unbiased localization study of cell-type specific transcription factors suggests that nearly a quarter of transcription factors might move between cells. The underlying mechanisms of cell-to-cell movement of transcription factors have started to emerge, however, it is still an open question what cellular factors determine the cell-to-cell movement activity of proteins and mRNAs.

Recent advances in technologies enabling us to profile RNA and proteins at a high resolution will propel this research field at an unprecedented level. Simple comparison of cell type specific RNA profiling data already suggests that a high proportion of RNA species generated in the phloem companion cells might be unloaded into the sieve cells. With more systematic data analyses and validation

experiments, a large number of transcription factors as mobile signals will be unveiled.

In addition to the discovery of mobile transcription factors, unraveling a biological significance behind their mobility remained as one of the big challenges. Furthermore, so far only few transcription factors have been reported to move between cells in evolutionarily divergent species. Also very little progress has been made in understanding the meaning of cell-to-cell movement of transcription factors in the context of plant evolution. However, deep-sequencing and automatized mass-screening techniques combined with the complete genome information and molecular toolkits that have become available in various plant lineages, a deeper understanding of the cell-cell communications system based on mobile transcription factors might soon emerge.

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