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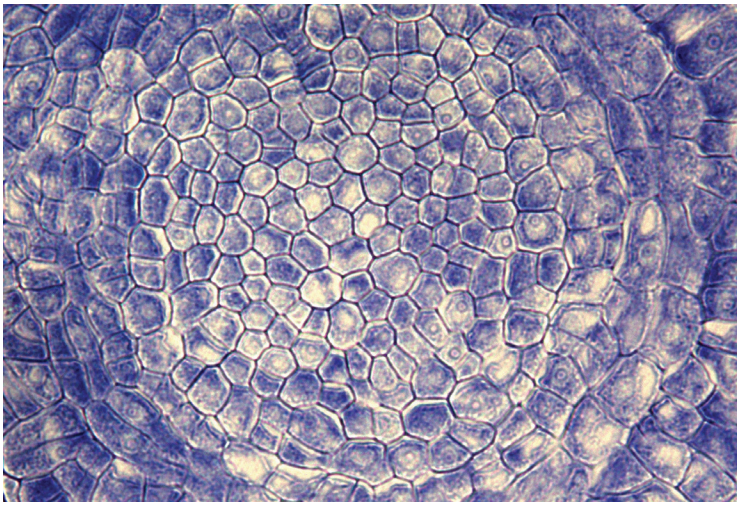
IRIS SEVILEM

The Integration of Developmental Signals during Root Procambial Patterning in *Arabidopsis thaliana*



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Doctoral Thesis

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ACADEMIC DISSERTATION

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In Loving Memory of my Grandfather,
Professor Osmo Liiri

"Understanding is the heartwood of well-spoken words"
The Buddha

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List of original publications

I S. Miyashima[1], P. Roszak[1], **I. Sevilem[1]**, K. Toyokura, B. Blob, J. O. Heo, N. Mellor, H. Help-Rinta-Rahko, S. Otero, W. Smet, M. Boekschoten, G. Hooiveld, K. Hashimoto, O. Smetana, R. Siligato, E. S. Wallner, A. P. Mahonen, Y. Kondo, C. W. Melnyk, T. Greb, K. Nakajima, R. Sozzani, A. Bishopp, B. De Rybel & Y. Helariutta (2019) Mobile PEAR transcription factors integrate positional cues to prime cambial growth. *Nature*, 565, 490-494.

II W. Smet[1], **I. Sevilem[1]**, M. A. de Luis Balaguer, B. Wybouw, E. Mor, S. Miyashima, B. Blob, P. Roszak, T. B. Jacobs, M. Boekschoten, G. Hooiveld, R. Sozzani, Y. Helariutta & B. De Rybel (2019) DOF2.1 Controls Cytokinin-Dependent Vascular Cell Proliferation Downstream of TMO5/LHW. *Current Biology*, 29, 520-529 e6.

III E. S. Wallner, V. Lopez-Salmeron, I. Belevich, G. Poschet, I. Jung, K. Grunwald, **I. Sevilem**, E. Jokitalo, R. Hell, Y. Helariutta, J. Agusti, I. Lebovka & T. Greb (2017) Strigolactone- and Karrikin-Independent SMXL Proteins Are Central Regulators of Phloem Formation. *Current Biology*, 27, 1241-1247.

IV **I. Sevilem**, S. R. Yadav & Y. Helariutta (2015) Plasmodesmata: channels for intercellular signaling during plant growth and development. *Methods in Molecular Biology*, 1217, 3-24.

V **I. Sevilem**, S. Miyashima & Y. Helariutta (2013) Cell-to-cell communication via plasmodesmata in vascular plants. *Cell Adhesion and Migration*, 7, 27-32.

[1]These authors contributed equally to this work

Author's contribution

I I.S. with input from S.M. identified phloem-specific DOF genes that promote periclinal cell divisions by cloning and analysing reporter and overexpression lines for the majority of DOF family members. I.S. identified downstream targets of PEAR1 and PEAR2 with input from co-authors. I.S. identified phloem/procambium-expressed target genes by performing *in silico* analysis and by generating and analysing overexpression constructs and reporter lines for selected targets. I.S. identified *SMXL3* as a putative direct target of PEAR2 that is sufficient to induce ectopic periclinal cell division. I.S. assisted in writing the manuscript and was responsible for writing the results and methods of the downstream analyses.

II I.S. identified *DOF2.1* as a gene that is sufficient for inducing periclinal divisions and determined its expression pattern and protein localization. I.S. generated the *pRPS5A::DOF2.1* and *pRPS5A::DOF2.1-GR* overexpression lines and the *pDOF2.1::GFP/GUS* transcriptional reporter line. I.S. commented on the manuscript.

III I.S. prepared samples for serial block-face scanning electron microscopy (SBEM) performed by I.B. and, as a phloem specialist, was responsible for the experimental design and interpreting the results.

IV I.S. wrote the manuscript together with S.R.Y. and Y.H.; I.S. was the primary author.

V I.S. wrote the manuscript together with S.M. and Y.H. I.S.; I.S. was the primary author.

Abbreviations

AB	aniline blue
CC	companion cell
DEX	dexamethasone
DNA	deoxyribonucleic acid
DOF	DNA-BINDING WITH ONE FINGER
ER	endoplasmic reticulum
GFP	green fluorescent protein
GR	glucocorticoid receptor
GUS	β-glucuronidase
HD-ZIP	HOMEODOMAIN-LEUCINE ZIPPER
IAA	indole-3-acetic acid
IPC	internal procambial cell
llt	lower lower tier
LOG	LONELY GUY
lt	lower tier
miRNA/miR	micro RNA
MP	MONOPTEROS
mRNA	messenger RNA
MSE	metaphloem sieve element
MX	metaxylem
NPA	1-naphthylphthalamic acid
OPC	outer procambial cell
QC	quiescent center
PC	procambium
PCR	polymerase chain reaction
PEAR	PHLOEM EARLY DOF
PM	plasma membrane
PSE	protophloem sieve element
PSE-IN	protophloem internal neighbor
PSE-LN	protophloem lateral neighbor
PX	protoxylem
RAM	root apical meristem
RNA	ribonucleic acid
RR	response regulator
SAM	shoot apical meristem
SBEM	serial block-face scanning electron microscopy
SE	sieve element
SMXL	SUPPRESSOR OF MAX2 1-LIKE
TE	tracheary element
TMO	TARGET OF MONOPTEROS
ult	upper lower tier
ut	upper tier
YFP	yellow fluorescent protein

Abstract

The vascular system of plants functions as a transportation route for water, nutrients and signaling molecules while also forming a support structure and generating most of the radial growth by increasing the number of cell files through periclinal cell divisions. These features have transformed life on Earth by enabling plants to colonize land and grow larger. In mature plants, the conductive tissues xylem and phloem are produced from stem cells in the vascular cambium, which develops from the procambium formed during early development. The vascular cylinder of the *Arabidopsis* root comprises a central xylem axis with a peripheral phloem pole on either side and procambial cells located between the xylem and phloem. Formation of the vascular pattern requires high auxin and cytokinin signaling domains in the xylem and phloem/procambium positions, respectively. However, the gene regulatory network acting downstream of these hormonal cues has remained unknown.

I investigated procambium patterning in the *Arabidopsis* root. Our research group discovered that radial growth is activated in the peripheral phloem domain by six mobile DOF transcription factors that we named PHLOEM EARLY DOF (PEAR) proteins, consisting of PEAR1, PEAR2, and their four homologues. PEAR proteins form an inverse concentration gradient to the HD-ZIP III transcription factors, which inhibit periclinal cell divisions in the central domain partially by inhibiting the movement of PEAR proteins. HD-ZIP III expression is promoted by auxin in the xylem axis and inhibited by endodermis-derived mobile microRNA165/166 in the periphery. The PEAR and HD-ZIP III genes form a feedback loop in which the PEAR proteins promote HD-ZIP III transcription while the HD-ZIP IIIs inhibit *PEAR* transcription and protein movement. The PEAR-HD-ZIP III regulatory module decodes hormonal and microRNA signals to result in the formation of a highly active peripheral zone and a more quiescent central zone during procambium development. We also determined that a member of the DOF family, DOF2.1, acts downstream of TARGET OF MONOPTEROS 5/LONESOME HIGHWAY-dependent cytokinin biosynthesis to regulate periclinal cell divisions in the outer procambial cells in contact with the xylem axis. Together, PEAR and DOF2.1 proteins control all of the periclinal divisions in the procambium through their activity in partially distinct domains.

We also identified *SUPPRESSOR OF MAX2 1-LIKE 3* (*SMXL3*), a member of SMXL subclade 2 which is expressed in the early phloem and procambium cells, as a putative direct target of PEAR2 that is sufficient to promote periclinal divisions. Characterization of SMXL subclade 2 identified *SMXL3, 4* and *5* as essential regulators of phloem formation that act very early in development and thus are required for all aspects of phloem development. Phloem specification requires periclinal divisions in the procambium. *SMXL3, 4* and *5* act in both the periclinal divisions and phloem specification in a partially redundant manner. Furthermore, analysis of regulators downstream of the PEARs revealed that they not only promote cell proliferation but also specify the identity of the surrounding cells non-cell autonomously, including procambial and phloem pole pericycle identity.

Our work highlights the importance of cell-to-cell communication in plant development. The interaction of mobile hormones, transcription factors and microRNAs originating from different tissues is required to coordinate developmental processes in the vascular cylinder. We have assembled the most complete understanding to date of the regulatory network coordinating procambial development and have identified the protophloem sieve elements as the organizers of radial growth during the early stages of vascular development in the *Arabidopsis* root. These findings can potentially be used to increase yields in forestry and agriculture.

Tiivistelmä

Kasvien johtosolukko kuljettaa vettä, ravinteita ja viestimolekyylejä, sekä toimii tukirakenteena, joka tuottaa suurimman osan kasvin paksuuskasvusta solun pitkittäisen akselin suuntaistesti tapahtuvien ns. periklinaalisten solunjakautumisten avulla. Näiden ominaisuuksien ansiosta kasvit pystyivät siirtymään maalle ja kasvamaan kooltaan suuremmiksi, minkä seurauksena elämä maanpäällä muuttui täysin. Täysikasvuisilla kasveilla johtosolukon puu- ja nilasolut kehittyvät kantasoluja sisältävästä jällestä, jota edeltää varhaisen kasvuvaiheen esijälsi. *Arabidopsis thalianan* eli lituruohon keskuslieriössä puu- eli ksyleemisolut muodostavat akselin keskelle, nilasolut ovat molempien puolien reunoilla ja esijällen solut sijaitsevat näiden välissä. Korkea auksiinipitoisuus ksyleemissä ja korkea sytokiniinipitoisuus nilassa ja esijällessä tarvitaan johtosolukon normaaliin kehitykseen. Näiden hormonaalisten viestien ohjaama geeninsäätelyverkosto on ollut kuitenkin tuntematon.

Tutkin esijällen muodostumista lituruohon juuressa. Tutkimusryhmämme havaitsi paksuuskasvun aktivoituvan keskuslieriön reunoilla olevissa varhaisen vaiheen nilan siiviläsoluissa ja tietyissä niitä ympäröivissä soluissa. Määritimme että näiden solujen jakautumisesta on vastuussa kuuden liikkuvan DOF-transkriptiotekijän perhe, jolle me annoimme nimeksi PHLOEM EARLY DOF (PEAR). Nämä koostuvat PEAR1 ja PEAR2 proteiineista ja niiden neljästä homologista. PEAR proteiinit muodostavat vastakkaisen pitoisuusgradientin ennestään tunnettujen HZ-ZIP III transkriptiotekijöiden kanssa. HD-ZIP III proteiinit estävät periklinaalisia solunjakautumisia keskuslieriön keskiosassa osittain estämällä PEAR proteiinien liikkumista. Auksiini edistää HD-ZIP III:n ilmenemistä keskuslieriön keskellä sijaitsevissa ksyleemisoluissa, kun taas keskuslieriön ulkopuolelta liikkuva mikroRNA165/166 heikentää HD-ZIP III:n ilmenemistä sen reunoilla. PEAR ja HD-ZIP III muodostavat takaisinkytkentämekanismin: PEAR lisää HD-ZIP III:n ilmenemistä, kun taas HD-ZIP III estää *PEAR* geenien ilmenemistä ja PEAR proteiinin liikkumista. PEAR-HD-ZIP III säätelymoduuli tulkitsee hormonaalisia ja mikroRNA signaaleja minkä seurauksena esijälteen muodostuvat aktiivisesti jakautuva reuna-alue ja hiljainen keskialue. Osoitimme myös, että TARGET OF MONOPTEROS 5/LONESOME HIGHWAY transkriptiotekijäparin aktivoima sytokiniinin biosynteesi aktivoi DOF perheeseen kuuluvan *DOF2.1* geenin ilmenemistä. DOF2.1 säätelee periklinaalisia solujakautumisia ksyleemin viereisissä reuna-alueen esijälsisoluissa. Osittain eri alueilla toimivat PEAR ja DOF2.1 proteiinit säätelevät kaikkia esijällen periklinaalisia solunjakautumisia.

Tutkimuksessamme havaitsimme, että PEAR2 aktivoi SUPPRESSOR OF MAX2 1-LIKE (SMXL) proteiinien toiseen alaluokkaan kuuluvan *SMXL3* geenin ilmenemistä. SMXL3 toimii varhaisen vaiheen nilan ja esijällen soluissa ja on riittävä aktivoimaan periklinaalisia solunjakautumisia. SMXL proteiinien toisen alaluokan jäsenet SMXL3, 4 ja 5 säätelevät lisäksi nilan kehitystä. Ne toimivat jo kehityksen varhaisessa vaiheessa ja täten ovat tarpeellisia kaikkiin nilan kehitysvaiheisiin. Esijällen periklinaaliset solunjakautumiset ovat tärkeä osa nilan kehitystä. Tutkimuksemme osoittaa, että SMXL3, 4 ja 5 toimivat sekä solunjakautumisten että erilaistumisen säätelyssä osittain päällekkäisesti. PEAR proteiinien

säätelykohteiden analyysi myös paljasti, että PEAR proteiinit toimivat sekä solunjakautumisten säätelyssä että ympäröivien solujen, kuten esijällen ja nilan viereisten perisyklisolujen, identiteetin määrittämisessä liikkumalla solusta toiseen.

Tuloksemme korostavat solujenvälisen viestinnän tärkeyttä: eri solukoista peräisin olevien liikkuvien kasvihormonien, säätelytekijöiden ja mikroRNA-molekyylien vuorovaikutus tarvitaan ohjaamaan kehitystä. Työmme luo tähän mennessä kattavimman ymmärtämyksen säätelyverkostoista, jotka ohjaavat esijällen kehitystä ja osoittaa varhaisen vaiheen nilan siiviläsolujen järjestävän paksuuskasvun aktivoinnin lituruohon juuren johtosolukon varhaisessa kehityksessä. Näitä löydöksiä voidaan mahdollisesti hyödyntää maa- ja metsätaloudessa lisäämään tuotantoa.

1. Introduction

Multicellularity evolved independently in the plant and animal kingdoms and consequently, plants and animals have acquired fundamentally different mechanisms for adapting to their environments. As sessile organisms, plants adapt by dynamic iterative growth which continues throughout their life, while animals, apart from some exceptions such as hydras, are able to move in response to environmental challenges and complete their organogenesis during embryogenesis and have limited regeneration potential. During development, animal cells are able to migrate, whereas plant cells are fixed in place due to their surrounding cell walls. Therefore, the development of three-dimensional structures in plants relies solely on tightly coordinated cell division, cell expansion, and cell differentiation, which sometimes involves cell death. The status of these three cellular processes in each cell defines its developmental identity. The fixed position of plant cells also implies that positional information is crucial in determining their identity (Tanaka and Reddien 2011, Basile, Fambrini and Pugliesi 2017).

During plant embryogenesis, only a rudimentary body plan is established, and this lays the foundation for postembryonic growth and patterning. Indeterminate growth is possible through the activity of meristems, which contain pluripotent stem cells that self-renew while their daughter cells are recruited for organ formation. The primary meristems are the shoot and root apical meristems, which form during embryogenesis and are responsible for the apical and basal growth of the primary axis. Secondary meristems, on the other hand, are established during postembryonic growth and are responsible for branching and radial growth (Weigel and Jürgens 2002, Stahl and Simon 2010, Aichinger et al. 2012, Basile et al. 2017, Ragni and Greb 2018, Wang and Jiao 2018). Cambium is the vascular meristem, which gives rise to the vascular tissues, xylem and phloem, in mature plants and produces most of the radial growth. The potential for radial growth is already laid down during primary development with the establishment of the procambium, which undergoes a set of oriented cell divisions. These longitudinal (or periclinal) divisions are also essential for primary phloem formation. In this thesis, I investigated the regulatory networks that orchestrate procambium and phloem development during the early stages of vascular development in the *Arabidopsis* root.

1.1 The vascular system

The formation of the vascular system was one of the most important events in the evolutionary history of land plants. It enabled plants to become terrestrial and increase in size in their competition for light. The vascular system serves three main functions: 1) to transport water and nutrients between organs, 2) to provide mechanical support and 3) to convey information by transporting long-distance signals that coordinate development or modulate the plant's activity. The vascular system consists of the conductive tissues, xylem and phloem, and the intervening procambium/cambium. In angiosperms, xylem vessels are composed of tracheary elements, which are dead cells that transport water and

minerals from the soil to all of the parts of the plant. In contrast, phloem sieve tubes consist of highly specialized living cells, known as sieve elements, that translocate sucrose from photosynthesizing organs, such as leaves, to sink tissues for growth or storage (Esau 1965, Furuta et al. 2014, Lacombe and Achard 2016, Fukuda and Ohashi-Ito 2019).

During embryogenesis, as well as primary and secondary development, xylem and phloem tissues are established by cell division and specification in the procambium/cambium, which consists of undifferentiated vascular stem cells organized in continuous strands. The vascular tissues are arranged in vascular bundles that vary in their organization in different organs and developmental stages but nevertheless serve to connect all of the parts of the plant (De Rybel et al. 2014a, Furuta et al. 2014, De Rybel et al. 2016, Hellmann et al. 2018). A diarch pattern of vascular tissue is characteristic of the *Arabidopsis* primary root, whereas the primary stem consists of multiple vascular bundles with peripheral phloem, internal xylem and intervening procambial cells. In leaves, the vascular bundles consist of adaxial (upper) xylem and abaxial (lower) phloem separated by procambium. Secondary development is initiated when a subset of the procambial cells starts to divide, leading to the formation of a cylindrical vascular cambium responsible for generating secondary phloem outwards (towards the epidermis) and secondary xylem inwards. Though this radial growth is most obvious in tree species, it also occurs in *Arabidopsis*, which can thus serve as a useful model to study the process.

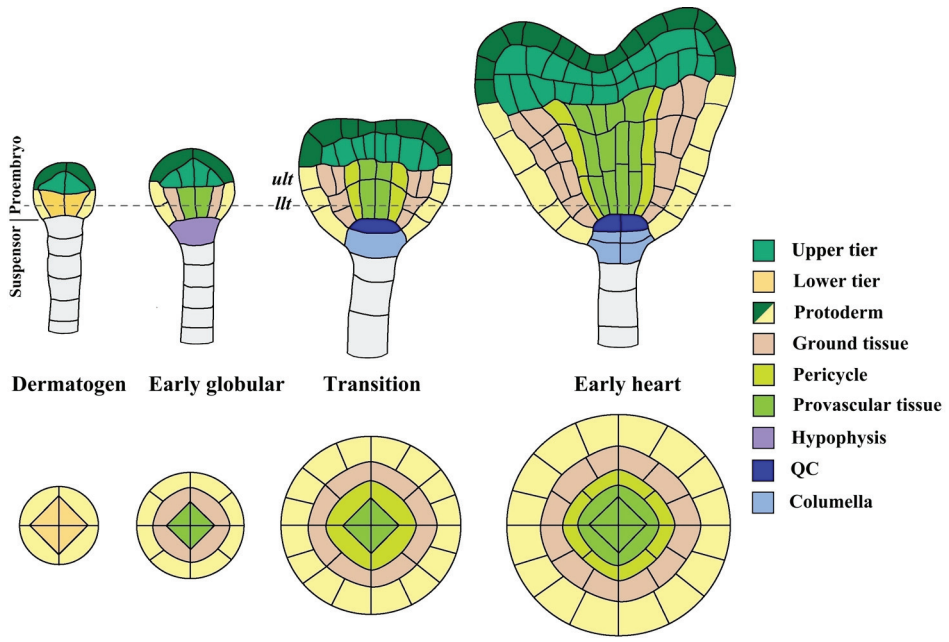

Figure 1. Schematic representations of longitudinal and transverse views of *Arabidopsis* embryos at different stages. *ult*, upper lower tier; *llt*, lower lower tier. Modified from Furuta et al. 2014.

1.2 The *Arabidopsis* root

The *Arabidopsis* root has a relatively simple structure and a highly predictable division pattern, which makes it an excellent model system for studying vascular patterning. The primary root, along with the primary vascular tissues, is established during embryogenesis. The first asymmetric division of the zygote forms a smaller apical cell and a larger basal cell, establishing the longitudinal growth axis on which the shoot apical meristem (SAM) and the root apical meristem (RAM) will form at the apical and basal ends, respectively. Divisions of the apical cell produce the proembryo (Figure 1), whereas the divisions of the basal cell result in the formation of the suspensor. The topmost cell of the suspensor is specified as the hypophysis, which will become the RAM and columella, whereas the remaining embryonic tissues, including the SAM and vascular cells, are derived from the proembryo. The mature embryo consists of two cotyledons, the hypocotyl (embryonic stem) and the embryonic root. Together, the hypocotyl and root constitute the embryo axis (Dolan et al. 1993, Jürgens 2001).

Cells in the meristems can divide in either a symmetric or asymmetric manner. Symmetric divisions, which constitute the majority of divisions, result in cell proliferation without the acquisition of a new fate—in other words, the daughter cells have the same identity as the parent cell—whereas asymmetric divisions produce new cell types by forming two daughter cells with distinct fates. The direction of growth is determined by the orientation of the cell division planes and the direction of cell expansion (Abrash and Bergmann 2009, Pillitteri, Guo and Dong 2016). In the RAM, new cells form in the stem cell niche, where slowly-dividing organizer cells known as the quiescent center (QC) maintain the identity of the surrounding stem cells (also called initials). Anticlinal asymmetric division of an initial produces a daughter cell adjacent to the QC which maintains its identity as an initial and a distal daughter cell which undergoes several rounds of cell division in the transit amplifying zone before elongating and finally differentiating. Thus, a cell file is formed along the longitudinal axis where cells mature as they get further from the QC (Benfey and Scheres 2000). Organized divisions occur simultaneously along the radial axis, resulting in the formation of a three-dimensional structure with concentric tissue layers. These periclinal cell divisions occur primarily in the vascular tissue located at the center of the root (Figure 2).

The vasculature of the primary root consists of a xylem axis through the middle, with central metaxylem (MX) and peripheral protoxylem (PX), and a phloem pole on either side consisting of a metaphloem sieve element (MSE), a protophloem sieve element (PSE) and two companion cells (CC). Procambial cells (PC) are located between the xylem and phloem. The vascular tissues are surrounded by the pericycle, which can be divided into xylem- and phloem-associated pericycle cells. The xylem-pole pericycle cells are involved in lateral root initiation and xylem loading, while the phloem-pole pericycle cells have been shown to function in phloem unloading. Pericycle cells are also involved in the activation of secondary growth (Beeckman and De Smet 2014, Ross-Elliott et al. 2017, Smetana et al.

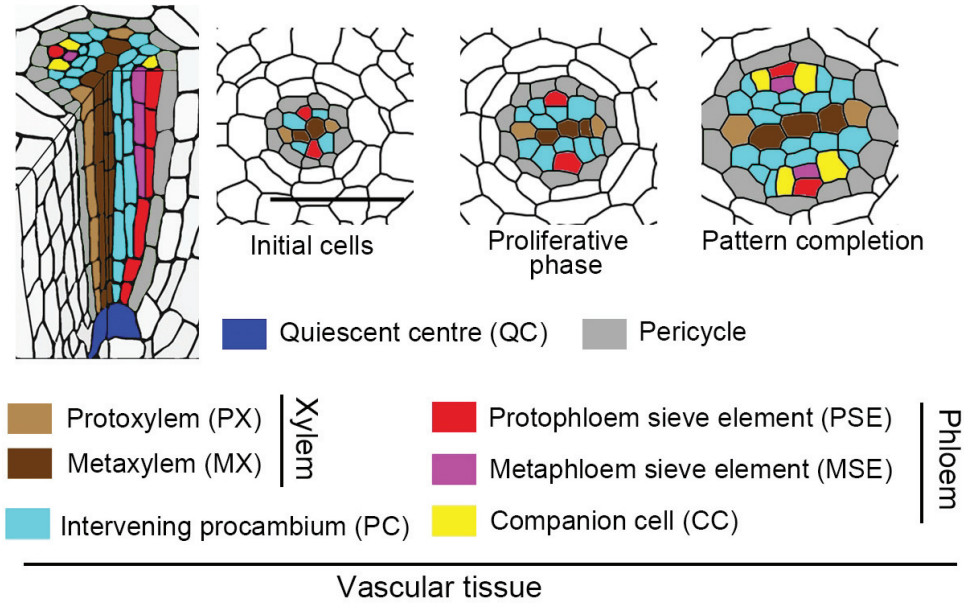

Figure 2. Schematic representation of the *Arabidopsis* root vascular tissue from the initials to the fully formed pattern. Modified from Miyashima et al. 2019.

2019). The pericycle is surrounded by the ground tissue layers, the endodermis and cortex, and finally the epidermis.

1.3 Positional information defines cell identity

Although the extremely regular pattern of the *Arabidopsis* root and the continuity of cell files starting from the initials would suggest that cell lineage is an important factor during plant development, laser ablation studies have demonstrated that the fate of plant cells depends mainly on positional cues from neighboring cells rather than their lineage (van den Berg et al. 1995, van den Berg et al. 1997, Scheres 2001). Consequently, cell-to-cell communication plays a crucial role in orchestrating plant development and serves as a basis for the highly flexible growth observed in plants despite the immobility of their cells. Various mobile regulatory molecules function as positional cues. For example, movement of hormones, transcription factors, peptides and small RNAs has been reported to play important roles during vascular development (Nakajima et al. 2001, Hirakawa et al. 2008, Carlsbecker et al. 2010, Bishopp et al. 2011b).

Plants have evolved several different routes through which to exchange information. Ligand-receptor signaling occurs via apoplastic transport. Molecules diffuse into the apoplast, the extracellular space along with the cell wall, and are perceived by their receptors at the plasma membrane (Jürgens and Geldner 2002). In transcellular transport, molecules that move through the apoplast are actively transported across the plasma membrane and

perceived inside the cell. This is the case for the plant hormone auxin, which is directionally transported from cell to cell over long distances by influx and efflux carriers (Naramoto 2017).

Plants have also evolved membrane-lined channels called plasmodesmata that serve as crucial connections in development and other contexts. Plasmodesmata provide cytoplasmic continuity by traversing the walls of adjacent cells, thus enabling the movement of molecules directly between cells (Lucas and Lee 2004, Yadav et al. 2014, Sevilem, Yadav and Helariutta 2015, Sager and Lee 2018). Dynamic deposition and degradation of callose, a β-1,3 glucan polymer, at plasmodesmata has been shown to be a key mechanism regulating their permeability (Bucher et al. 2001, Vaten et al. 2011, Zavaliev et al. 2011). By using a version of the *CALLOSE SYNTHASE 3* (*CALS3*) gene mutated to increase the deposition of callose, plasmodesmatal permeability can be modulated in a spatially and temporally specific manner, facilitating the investigation of the role of cell-to-cell communication in development (Vaten et al. 2011, Sevilem, Miyashima and Helariutta 2013). Both xylem and phloem function in the transport of signaling molecules over long distances (Lucas et al. 2013).

1.4 Auxin and cytokinin

The plant hormones auxin and cytokinin play a fundamental role in vascular development. Auxin and cytokinin often function in an antagonistic and complementary fashion, mutually inhibiting one another but also requiring each other in order to drive developmental processes. Auxin and cytokinin interact at many levels, affecting each other's biosynthesis and degradation, activation and inactivation, and transport and perception (Dello Ioio et al. 2008, Bishopp et al. 2011a, Marhavy et al. 2014, Schaller, Bishopp and Kieber 2015).

Auxin

Auxin is ubiquitously involved in plant development, influencing embryogenesis, postembryonic organ formation, root growth, tropic responses, and vascular development (Thimann and Schneider 1939, Vanneste and Friml 2009, Zhao 2010). Auxin biosynthesis is spatially and temporally regulated and occurs in the meristematic tissues of both the shoot and the root (Cheng, Dai and Zhao 2007, Stepanova et al. 2008, Tao et al. 2008, Petersson et al. 2009). The most abundant auxin, indole-3-acetic acid (IAA), is produced via a tryptophan-dependent and a tryptophan-independent pathway. The tryptophan-dependent pathway is thought to be the more important. In it, TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) family of aminotransferases converts tryptophan to indole-3-pyruvate, which is subsequently converted to IAA by the YUCCA flavin monooxygenases. Though the tryptophan-independent pathway is probably also important, it is less well understood (Tivendale, Ross and Cohen 2014, Zhao 2014).

Auxin perception involves the AUXIN RESPONSE FACTOR (ARF) transcription factors and their transcriptional repressors, the AUXIN/INDOLE ACETIC ACID (Aux/IAA) proteins. The binding of auxin to its receptors, the TRANSPORT INHIBITOR

RESISTANT1/AUXIN SIGNALING F-BOX (TIR1/AFB) F-box proteins, in the nucleus leads to the degradation of Aux/IAA by the ubiquitin-proteasome system. This releases the ARF proteins from inhibition by the Aux/IAAs, freeing them to interact with AUXIN RESPONSE ELEMENT (ARE) in the promoters of downstream genes. When auxin levels are low, ARF function is blocked by the Aux/IAAs and the transcriptional repressor TOPLESS. The differential expression of Aux/IAA and ARF genes plays an important role in patterning tissue-specific auxin responses (Hayashi 2012, Chandler and Werr 2015, Salehin, Bagchi and Estelle 2015, Li et al. 2016, Weijers and Wagner 2016).

Auxin is transported over short and long distances via the activity of transporter proteins in the plasma membrane, including influx carriers of the AUX1/LIKE AUX1 (AUX1/LAX) family, efflux carriers of the PIN–FORMED (PIN) family and B-type ATP-binding cassette transporters (Friml 2010, Zazimalova et al. 2010, Hayashi 2012). Auxin can enter cells by diffusion or via transport through the AUX1/LAX influx carriers (Swarup and Peret 2012), whereas it can only leave cells through the action of PIN efflux carriers, which pump it into the apoplast (Adamowski and Friml 2015). Patterned localization of the PIN transporters can thus generate directional movement of the hormone from cell to cell, known as polar auxin transport. Directional movement of auxin can spontaneously organize because auxin controls the expression and subcellular localization of PINs to direct its own movement (Benkova et al. 2003, Sauer et al. 2006, Dello Ioio et al. 2008, Vanneste and Friml 2009, Chen et al. 2015).

The self-organizing nature of auxin transport is essential during organogenesis and pattern formation. For example, during root development, the formation of the RAM and the positioning of lateral root primordia depend on the formation of local auxin response maxima (Sabatini et al. 1999, Benkova et al. 2003). Shoot-derived auxin as well as local biosynthesis are together responsible for auxin accumulation in the root tip, which is essential for RAM formation. Auxin circulation in the root meristem by the PINs (PIN1, PIN2, PIN3, PIN4 and PIN7) is required to maintain cell division in the root meristem. The *pin1 pin2* mutant, as well as higher order *pin* mutants with *pin2*, has a reduced meristem size which can be restored to normal by exogenous auxin application.

PINs affect both cell division and cell elongation, which both contribute to meristem size and root length (Blilou et al. 2005). The accumulation of auxin in the stem cells promotes the transcription of the *PLETHORA* (*PLT*) transcription factors, which form a longitudinal gradient as a result of cell-to-cell movement and protein stability. This gradient is required to maintain stem cell identity and to define RAM zonation by controlling the rate of cell division in a dose-dependent manner (Galinha et al. 2007, Mähönen et al. 2014). The auxin gradient also has a role in RAM zonation during gravitropic response; this response is independent of the PLT gradient, which changes more slowly and is required for stable long-term root growth (Mähönen et al. 2014).

A key ARF protein during root development and vascular pattering is MONOPTEROS (MP/ARF5), which functions as a master regulator that activates various developmental programs during embryogenesis and postembryonic growth. MP is also critical in the self-organization of auxin transport. MP directly controls the expression of several PINs (*PIN1,*

PIN3 and *PIN7*), which is necessary for RAM and SAM function, as well as for lateral root initiation, and thus is crucial for both shoot and root organ formation (Krogan et al. 2016). The importance of MP is illustrated by the pleiotropic phenotype of the *mp* mutant, which lacks an embryonic axis and root structures and has defective vasculature and often fused cotyledons (Przemeck et al. 1996, Hardtke and Berleth 1998).

Cytokinin

Cytokinins play various roles during plant development, regulating processes such as cell division and differentiation, vascular development, apical dominance and senescence (Werner and Schmulling 2009, Wybouw and De Rybel 2019). Cytokinins are derived from adenine. They contain an adenine ring with isoprenoid side chains attached to its N6 position and are synthesized by members of the ISOPENTENYL TRANSFERASE (IPT) and LONELY GUY (LOG) enzyme families. Zeatin, which was first identified in maize (Miller 1961, Letham 1963), is the most abundant naturally occurring cytokinin. Active cytokinins are free bases, but cytokinins are also found in a less active form bound to ribose sugars.

During cytokinin biosynthesis, IPT catalyzes the addition of an isoprene side group to the N6 position of ADP or ATP (Sakakibara 2006). The resulting isopentenyl-adenine-type cytokinin is converted to a zeatin-type cytokinin by the cytochrome P450 enzyme CYP735A1/2 (Takei, Yamaya and Sakakibara 2004). Subsequently, LOG enzymes synthesize free-base cytokinin from cytokinin ribotides to produce active cytokinin (Kuroha et al. 2009). Active cytokinin levels can be reduced by reversible conjugation to a sugar or irreversible cleavage by cytokinin oxidase (CKX) (Werner et al. 2006). Cytokinin biosynthesis and degradation is regulated locally, with tissue-specific expression of the IPT, LOG and CKX genes.

In *Arabidopsis*, cytokinins are perceived by the ER- and PM-localized ARABIDOPSIS HISTIDINE KINASE (AHK2, AHK3, and CRE1/WOL/AHK4) receptors (Mähönen et al. 2000, Hwang and Sheen 2001, Inoue et al. 2001, Yamada et al. 2001). The binding of cytokinin to its receptors initiates a His-Asp phosphorelay cascade mediated by five histidine-containing phosphotransfer proteins, ARABIDOPSIS HISTIDINE PROTEIN 1-5 (AHP1-5) (Hwang and Sheen 2001, Hutchison et al. 2006). This leads to the phosphorylation and thus activation of B-type response regulators (RRs), which are transcription factors that induce cytokinin responsive genes (Feng et al. 2013). On the other hand, A-type RRs, which are transcriptionally activated by B-type RRs, lack a DNA binding domain and function as negative feedback regulators of cytokinin signaling, probably by competing with B-type RRs for phosphorylation by AHPs and for phosphorylation-dependent binding of target proteins (To et al. 2007). Another negative regulator of cytokinin signaling is AHP6, an atypical AHP family member (Mähönen et al. 2006). AHP6 lacks the histidine residue required for the phosphorelay found in the other AHP proteins. Since it competes with other AHPs but cannot activate response regulators, it inhibits cytokinin signaling. In the

root, AHP6 expression marks the protoxylem position and is necessary for protoxylem differentiation.

Cytokinins are synthesized in different parts of the plant and transported both from the root to the shoot and vice versa. However, the mechanisms of cytokinin transport are not well understood. Trans-zeatin-type cytokinin has been shown to move via the xylem from roots to shoots, where it regulates shoot development and is involved in the loading of cytokinin to xylem sap by a G-type ABC transporter, ABCG14 (Ko et al. 2014, Zhang et al. 2014). Isopentenyl-adenine-type cytokinin, on the other hand, can move from shoot to root in phloem sap, and though this movement has been shown to play a role in maintaining vascular pattern of the root, the transporters involved are not known (Bishopp et al. 2011b). Cytokinin response was recently shown to be modulated by PUP14, a protein located at the plasma membrane that imports cytokinin from the apoplast to the cytosol and thus downregulates cytokinin response by decreasing the available cytokinin (Zurcher et al. 2016).

During apical growth of the root, cytokinin acts antagonistically to auxin in controlling meristem size by promoting differentiation of the meristematic cells in the transition zone. Cytokinin biosynthetic mutants (*ipt3 ipt5 ipt7*) and the cytokinin receptor mutant *ahk3* have larger meristems, whereas cytokinin application reduces meristem size. The *arr1 arr12* double mutant also has a large meristem, similar to the *ahk3* mutant, suggesting that ARR1 and ARR12 function downstream of *AHK3* in controlling the rate of differentiation. An auxin signaling inhibitor that is degraded by increased auxin levels, *AUX/IAA SHORT HYPOCOTYL2 (SHY2)*, is transcriptionally activated by cytokinin via ARR1 and ARR12 (Dello Ioio et al. 2007, Dello Ioio et al. 2008, Moubayidin et al. 2010). SHY2 inhibits *IPT5* expression, thus forming a negative feedback loop between cytokinin and auxin. In the following sections, I will discuss vascular patterning during embryogenesis and postembryonic root growth, and the importance of auxin and cytokinin and their interactions during these processes.

1.5 RAM specification

Specification of the RAM occurs at the basal pole of the embryo axis and requires apical to basal transport of auxin. Modeling suggests that local auxin biosynthesis in the central upper tier cells of the proembryo is required for polar localization of PIN1 (Wabnik et al. 2013). It has also been shown that the apical-basal axis formation is defective in mutants without functional tryptophan aminotransferases, TAA1 and TAR1/2 (Robert et al. 2013). The hypophysis, which is the uppermost cell of the suspensor, gives rise to QC, the source of initials, as well as the columella (Figure 1). Hypophysis specification occurs in the 32-cell globular embryo when the auxin efflux carrier PIN1 starts to be localized basally in provascular initials, directing auxin flow to the hypophysis position. This is marked by strong expression of *DR5*, a synthetic reporter of auxin signaling (Ulmasov et al. 1997). Interfering with this apical-basal auxin flow results in embryos lacking root and hypocotyl vascular tissue (Weijers and Jürgens 2005).

Auxin promotes RAM formation via MP and BODENLOS/IAA12 (BDL). In the proembryo, auxin triggers the release of MP from its inhibitor, the auxin response regulator BDL, and targets BDL for degradation by the ubiquitin proteasome pathway (Hamann et al. 2002, Weijers et al. 2006). MP then promotes expression of *PIN1* in the proembryo, resulting in directional auxin flow basally to the hypophysis precursor cell (Weijers et al., 2006). Both *mp* loss-of-function mutants and *bdl* gain-of-function mutants lack an embryonic root (Hamann et al., 2002). The *bdl* mutant phenotype can be rescued by *MP* overexpression (Hardtke et al. 2004). However, auxin transport alone is not sufficient for hypophysis specification, which also requires the movement of TARGET OF MONOPTEROS 7 (TMO7). MP activates the transcription of *TMO7* in the proembryo, and the TMO7 protein moves basally to the hypophysis precursor cell where it induces an asymmetric cell division. This results in an apical and basal daughter cell, which give rise to the QC and columella, respectively. Thus, initiation of the RAM is controlled by the auxin-MP/BDL-TMO7 pathway, which involves cell-cell signaling and the transport of both auxin and TMO7 from the proembryo to the extra-embryonic hypophysis precursor to establish the RAM (Schlereth et al. 2010).

1.6 Vascular tissue specification

Vascular tissue specification is initiated in the cotyledons and progresses downwards to the embryo axis (Bauby et al. 2007). The transverse divisions of the 2-cell stage embryo to form an 8-cell stage embryo lead to the formation of smaller upper tier cells (*ut*) and larger lower tier cells (*lt*) which have different fate. The vascular tissues of above-ground organs, such as the stem and leaves, originate from the SAM, which is specified in the *ut* cells (Figure 1). During the early globular stage, periclinal division of the inner *lt* cells (procambium precursors) produces the outer ground tissue and four inner provascular initials. The vascular tissues of the embryo axis originate from these four provascular initial cells (De Rybel et al. 2013, Yoshida et al. 2014).The provascular initials first produce xylem and procambial cells. Subsequently, a subset of the procambial cells undergoes asymmetric periclinal cell divisions that give rise to phloem tissue. A high auxin domain forms at the site of the cotyledon primordia, and basal flow of this auxin leads to two of the four provascular initials below the cotyledon primordia receiving more of the hormone. The increased auxin levels in these two provascular initials determines them to form the xylem axis. In this way, the xylem axis is aligned with the cotyledons. The other two provascular cells, which have less auxin, give rise to the phloem and procambium (Bishopp et al. 2011a). Thus, the bisymmetry of the cotyledon primordia is translated into the bisymmetry of the vascular tissue via basal auxin flow.

Auxin response plays an important role in asymmetric cell divisions, which are necessary for the formation of new cells types. In these divisions, the plane of division is not determined by cell geometry to ensure that the new wall spans the shortest distance between existing walls (Yoshida et al. 2014). The auxin response factor *MP* is required not only for hypophysis specification and root formation but also for the specification of

provascular cells. *MP* is already expressed in the first apical cell of the embryo and becomes specific to the vasculature later. In addition to lacking an embryonic root, *mp* mutants have defective vascular tissue due to the absence of the provascular initial cells in the embryo (Hardtke and Berleth 1998, Hamann et al. 2002). Analysis of cell division patterns in *mp* and *bdl* embryos revealed that the regulated periclinal division of the provascular initials is reduced. Instead, cells divide according to the shortest wall-to-wall distance, which results in defective vasculature (De Rybel et al. 2013, Yoshida et al. 2014). MP was found to activate the expression of its direct target, the basic helix–loop–helix (bHLH) transcription factor *TARGET OF MONOPTEROS 5* (*TMO5*), which is involved in vascular specification. *TMO5* expression is first observed in the four provascular initials and later specifically in the xylem precursor cells (Schlereth et al. 2010, De Rybel et al. 2013, De Rybel, Breda and Weijers 2014b). The embryonic vascular phenotype of *mp* can be suppressed by expressing *TMO5* under the *MP* promoter, indicating that MP mediates vascular initiation by activating transcription of *TMO5*. However, the single *tmo5* mutant does not display any obvious phenotype due to redundancy. *TMO5* has three close homologs, *TMO5-LIKE1-3* (*T5L1-3*) (Ohashi-Ito and Bergmann 2007, De Rybel et al. 2013). When the *tmo5* mutant was combined with *t5l1*, which is the closest homolog, reduced periclinal division of the provascular cells and their progenitors was observed in the embryos of the double mutant. Furthermore, division planes were found to be distorted, resulting in abnormally narrow vascular tissue with fewer cell files. TMO5 and its homologs were found to form heterodimers with the more distantly related bHLH transcription factor LONESOME HIGHWAY (LHW) and its closest homologs. Like the *tmo5 t5l1*double mutant, the *lhw ll1* double mutant also has fewer periclinal divisions of the vascular initials.

1.7 Root vascular patterning

The vascular tissue in the *Arabidopsis* root is organized in a bisymmetric pattern which is established during embryogenesis. Unlike the xylem, which is specified early, the phloem/procambium undergoes periclinal cell divisions during its patterning, which simultaneously increase the number of vascular cell files (Mähönen et al. 2000). Experiments and modeling have established that an auxin response maximum in the xylem axis and two domains of high cytokinin signaling in the flanking phloem/procambium are required for normal vascular patterning of the *Arabidopsis* root, which involves mutually inhibitory interaction of the two hormones (Mähönen et al. 2006, Bishopp et al. 2011a, De Rybel et al. 2014a, Muraro et al. 2014, el-Showk et al. 2015, Mellor et al. 2017). Cytokinin is required for radial cell proliferation in the phloem/procambium and to inhibit protoxylem differentiation. The *wooden leg (wol)* mutant, which has a defective *AHK4*, and the cytokinin receptor triple mutant *cre1 ahk2 ahk3* have a severely reduced number of vascular cell files, and all of their vascular cells differentiate into protoxylem (Mähönen et al. 2000, Bishopp, Mähönen and Helariutta 2006, Mähönen et al. 2006). In addition to activating *TMO7* and *TMO5* transcription, MP directly promotes the expression of *AHP6* in the protoxylem position (Mähönen et al. 2006, Ohashi-Ito et al. 2014). *AHP6* was identified in a *wol* suppressor

screen since the *wol ahp6* double mutant has more vascular cell files than the *wol* mutant. AHP6 enables protoxylem differentiation by inhibiting cytokinin signaling. Mutation of *AHP6* inhibits protoxylem identity in a manner similar to treatment with cytokinin. In the all-protoxylem *wol* mutant, all of the vascular cells express *AHP6* (Mähönen et al. 2006). By contrast, the auxin resistant *axr3* mutant with a gain-of-function mutation in *IAA17* lacks xylem altogether. Protoxylem development can also be inhibited by treatment with 1-naphthylphthalamic acid (NPA), which inhibits the transport of auxin to the correct position (Bishopp et al. 2011a).

The protoxylem position is thus marked by high auxin and low cytokinin signaling. Establishment of an auxin maximum in the xylem axis requires the active transport of auxin. The auxin maximum is unstable in the *pin3 pin7* double mutant, leading to variable *AHP6* expression and altered protoxylem development. High auxin signaling in xylem cells causes the PINs to be basally localized, leading to rootward transport of auxin. In contrast, high cytokinin signaling in the procambial cells promotes the expression and lateral localization of PINs, directing auxin toward the xylem axis. Modeling predicts that cytokinin transport through the phloem is required to provide the root meristem with enough cytokinin but does not function as a positional cue for vascular patterning (Muraro et al. 2014).

Indeterminate root growth is possible because of the continuous division of initials in the stem cell niche. The initiation and maintenance of the QC and the initials are therefore essential to ensure continuous growth. Indeed, regulators required for QC identity, such as the GRAS family members *SHORT ROOT* (*SHR*) and *SCARECROW* (*SCR*), as well as the *PLT* genes, are also required for indeterminacy (Benfey et al. 1993, Sabatini et al. 2003, Aida et al. 2004). The indeterminacy of the vascular tissues is also tied to the number of vascular cell files, as factors required for the periclinal division of vascular cells have been shown to also be essential for vascular indeterminacy (De Rybel et al. 2013). In the xylem axis, auxin signaling acts via MP to promote *TMO5* expression, which is required for periclinal divisions not only in the embryo but also during postembryonic vascular development. Roots of *tmo5 t5l1* double mutants are significantly narrower than wild type roots, and the vascular tissue does not form a diarch pattern but has only a single protoxylem and phloem pole. The *tmo5 t5l1 t5l3* triple mutants display a further reduction in vascular cell file number, and in *tmo5 t5l1 t5l2 t5l3* quadruple mutants the differentiated vascular tissue is completely absent. Root growth is also severely affected in higher order mutants in proportion to the number of genes silenced (De Rybel et al. 2013), indicating that the genes in the *TMO5* clade function redundantly.

Roots of *lhw* single mutants phenocopy the *tmo5 t5l1* double mutant root in having a monopole organization, whereas the *lhw ll1* double mutant also has a further reduction in vascular cell number (Ohashi-Ito and Bergmann 2007). All higher order *tmo5* and *lhw* mutants display determinate vascular growth. In wild type plants, periclinal cell divisions occur in the lower part of the meristem, doubling the number of meristem cells and leading to gradual radial growth of the vascular tissue between five and eight days after germination. These periclinal cell divisions and radial growth do not occur in higher

order mutants containing *tmo5*, and in the quadruple mutant the vascular tissues are completely consumed after a week of growth. These results indicate that TMO5/LHW promote periclinal cell divisions in the lower part of the meristem, and this maintains the indeterminacy of the root vascular tissue. This conclusion is further supported by the observation that TMO5 and LHW colocalize most abundantly in the lower half of meristematic cells. When overexpressed, both *TMO5* and *LHW* significantly increase the number of vascular cell files. Moreover, the effect is dramatically increased when the two genes are overexpressed simultaneously, which is in line with the observation that TMO5 and LHW act as a heterodimer. The effect is specific to the induction of periclinal cell divisions. Anticlinal divisions are not significantly affected, as demonstrated by the unaltered number of cortical cells in the meristem compared with wild type. Analysis of embryos showed that the severity of the embryonic phenotype does not predict whether postembryonic tissue growth will become determinate, which suggests that TMO5/LHW promote indeterminacy postembryonically rather than as an indirect consequence of embryonic defects (De Rybel et al. 2013).

TMO5/LHW promote periclinal cell divisions in a narrow domain of early xylem precursors. However, root xylem cells do not themselves divide periclinally, and therefore periclinal divisions and indeterminacy are promoted non-cell-autonomously in the surrounding procambium cells. The TMO5/LHW dimer was found to promote cytokinin biosynthesis by directly activating the transcription of *LOG4* and its closest homolog *LOG3* (De Rybel et al. 2014a, Ohashi-Ito et al. 2014). Like *TMO5*, *LOG4* is first expressed in the four embryonic provascular initials and later becomes restricted to the xylem axis, but it is also expressed in xylem-pole pericycle and xylem-pole endodermal cells in the postembryonic root. After TMO5/LHW expression, cytokinin levels go up and the expression of the cytokinin response marker *pTCSn-GFP* is induced ectopically, while *AHP6* expression and protoxylem differentiation are compromised, suggesting that TMO5/LHW activates cytokinin biosynthesis by promoting expression of *LOG4*. The *log4* single mutant does not display any obvious phenotype, whereas the *log347* triple mutant shows a reduced vascular cell number, and the number is further reduced in the *log1234578* septuple mutant, where it reaches the level observed in the *wol* mutant. Ectopic periclinal divisions caused by TMO5/LHW overexpression are partially suppressed in the *log347* mutant, whereas in the *wol* mutant they are completely absent. On the other hand, cytokinin treatment is able to increase periclinal divisions and restore the diarch pattern in the *tmo5 t5l1* double and *lhw* single mutants. Thus, a prominent role of TMO5/LHW in vascular development is the activation of cytokinin biosynthesis. However, how cytokinin activates periclinal cell division is unknown and is one of the key questions of my thesis.

Feedback mechanisms are essential to regulate cell division and differentiation rates in meristematic tissues. Auxin modulates periclinal divisions not only by activating *TMO5* expression but also via another MP target, *ACAULIS5* (*ACL5*), an inhibitor of periclinal division which encodes an enzyme catalyzing thermospermine biosynthesis (Knott, Romer and Sumper 2007, Schlereth et al. 2010). Thermospermine promotes the translation of the bHLH transcription factor SUPPRESSOR OF ACAULIS 51 (SAC51) and its homologs

SACL1-3 by interacting with their conserved upstream open reading frame (Imai et al. 2006, Takano, Kakehi and Takahashi 2012, Vera-Sirera et al. 2015). Mutants of *acl5* display an increased number of cell files. This phenotype requires the activity of LHW, since *acl5 lhw* double mutants have a reduced cell file number, similar to the *lhw* single mutant. SACL members can bind to LHW, and this competes with TMO5/LHW dimerization, thus negatively regulating TMO5/LHW function. Consistent with this, the expression of *LOG3/4* in *acl5* mutants is upregulated in an LHW-dependent manner. TMO5/LHW activates the expression of *ACL5* and *SACL3*, forming a negative feedback mechanism which inhibits excessive proliferation of vascular cells. Thus, auxin in the xylem axis both triggers cytokinin biosynthesis and periclinal divisions via the MP-TMO5/LHW-LOG4 pathway and inhibits them via the MP-ACL5-SACL pathway.

In addition to promoting vascular cell proliferation, TMO5/LHW members promote xylem fate cell autonomously (De Rybel et al. 2013, Ohashi-Ito, Matsukawa and Fukuda 2013, Ohashi-Ito et al. 2014). The quadruple *tmo5 t5l1 t5l2 t5l3* mutant completely lacks xylem vessels, whereas overexpression of *LHW* and *T5L1* causes ectopic protoxylem formation in roots and cotyledons (Katayama et al. 2015). Since *acl5* mutants also exhibit ectopic protoxylem and this phenotype is suppressed by *lhw*, *ACL5* likely inhibits protoxylem differentiation downstream of LHW/T5L1.

External signals affect root vascular patterning

The transcription factors *AT-HOOK MOTIF NUCLEAR LOCALIZED PROTEIN 3* (*AHL3*) and *AHL4* are involved in root vascular tissue boundary formation (Zhou et al. 2013). In *ahl3* and *ahl4* mutants, xylem cells appear in the procambium domain, and the boundary between xylem and procambium is altered, along with the auxin and cytokinin signaling domains. The expression of both *TMO5* and *AHP6* extends to the procambium, while the *ARR5* expression domain is reduced. *AHL4* is transcribed in the procambium, and the protein moves to all of the stele cells. *AHL3*, however, is transcribed in the endodermis of the maturation zone and moves to the xylem and procambium in the meristematic zone. Interestingly, the AHL3 and AHL4 proteins form a heteromeric complex, and this interaction may promote their cell-to-cell movement from the procambium. A non-mobile version of the AHL4 protein cannot complement the *ahl4* mutant phenotype. Furthermore, additional periclinal divisions of the xylem precursors are not observed in the mutants, suggesting that it is the tissue boundaries that are disorganized, not the periclinal divisions of the xylem initial. AHL3/4 movement from the procambium to the xylem is therefore necessary to restrict the xylem domain to the axis and thus define the boundary between xylem and the adjacent procambial cells. It appears that the AHL3-AHL4 pathway is a signaling system that connects the more mature part of the root to the meristem to coordinate the formation of tissue boundaries. This pathway was suggested to be a mechanism that could control xylem vessel formation in the root based on changing environmental signals (Zhou et al. 2013).

The GRAS family transcription factor SHORT ROOT (SHR) moves from the stele to the endodermis to specify the ground tissue layers (Helariutta et al. 2000, Nakajima et al. 2001), but it also initiates a pathway involving bidirectional movement from the stele to the endodermis and back again to regulate vascular patterning. Correct patterning of protoxylem and metaxylem requires the localization of SHR and another GRAS transcription factor, SCARECROW (SCR), in the endodermis, where they promote the transcription of the *microRNA165* and *microRNA166* (*miR165/166*) genes (Nakajima et al. 2001, Levesque et al. 2006, Cui et al. 2007, Gallagher and Benfey 2009). The miRNAs diffuse from the endodermis to the stele, forming a concentration gradient with its peak in the stele periphery and its lowest level in the central domain. miR165/166 degrade the mRNAs of members of the Class III HOMEODOMAIN LEUCINE ZIPPER (HD-ZIP III) transcription factor family, narrowing their posttranscriptional expression domain to the central stele, which allows protoxylem to develop at the periphery (Carlsbecker et al. 2010, Miyashima et al. 2011). HD-ZIP III transcription factors are well-known regulators of vascular development that act downstream of auxin (Baima et al. 1995, Prigge et al. 2005, Donner, Sherr and Scarpella 2009, Ramachandran, Carlsbecker and Etchells 2017). They consist of *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *INTERFASICULAR FIBRELESS1/REVOLUTA* (*IFL1/REV*), *ATHB8* and *ATHB15/CORONA* (*CNA*). All five members are expressed in a stele-specific manner during postembryonic root development. The HD-ZIP III genes regulate xylem fate in a dose-dependent manner. High HD-ZIP III levels promote metaxylem formation by inhibiting *AHP6* expression, whereas low levels of HD-ZIP III in the stele periphery due to miR165/6 regulation allow *AHP6* expression and protoxylem development. In the *shr* and *scr* mutants, miRNA regulation is absent, and as a result ectopic metaxylem is observed in the protoxylem domain. Ectopic metaxylem is also observed in *phb-d* gain of function mutants with elevated PHB levels. On the other hand, quadruple loss-of-function mutants of the HD-ZIP III genes (*phb phv cna athb8*), as well as miRNA overexpression lines, display a protoxylem-only phenotype (Carlsbecker et al. 2010). HD-ZIP III expression was shown to be controlled by local tryptophan-dependent auxin biosynthesis, and this is required for normal metaxylem development, as *trp2* mutants have defective metaxylem development due to reduced auxin levels (Ursache et al. 2014).

Research suggests that miRNA interaction with its target leads not only to the degradation of the mRNA but also to the elimination of the miRNA, and this mechanism is thought to be important in establishing sharp boundaries of target gene expression (Levine, McHale and Levine 2007). Consistent with this, mathematical modeling suggests that the interaction of PHB mRNA with miR165/6 leads to the degradation of both transcripts. In the peripheral stele, where PHB mRNA levels are the lowest, the miRNA levels are high enough to remove PHB. In the central stele, however, high levels of PHB mRNA are able to consume the miRNAs, forming a sharp boundary between cells with and without PHB. This sharp boundary is necessary to allow *AHP6* expression in the protoxylem position (Muraro et al. 2014).

In the phloem/procambium, high cytokinin signaling promotes the expression and lateral localization of PINs, thus channeling auxin flow radially to the xylem axis (Bishopp

et al. 2011a). Modeling showed that this lateral localization of PINs is sufficient for the formation of an auxin maximum at the xylem axis (Muraro et al. 2014). Furthermore, the bisymmetric pattern of PIN expression requires the SHR-miR165/6 pathway. In *shr* mutants, the PINs are expressed in a radially symmetric manner around the metaxylem. If the miRNAs are absent from the protoxylem position, *PHB* expression spreads there and inhibits *AHP6* expression. Low levels of AHP6 allow cytokinin signaling to occur, which leads to PIN expression and lateral localization in a radial manner. While *AHP6* inhibits cytokinin signaling at protoxylem, the identity of the cytokinin signaling in the metaxylem position is still unknown (Muraro et al. 2014).

In summary, miR165/6 is a positional cue from the outer endodermis that defines the boundary between central and peripheral stele, which are marked by high and low HD-ZIP III levels, respectively. In the xylem axis, this distinction defines the domains of central metaxylem and peripheral protoxylem. Therefore, the SHR/SCR-miRNA pathway also contributes to the bisymmetric hormonal pattern by promoting the formation of a diarch xylem axis in which PIN expression is inhibited at the protoxylem position. The HD-ZIP III genes also inhibit periclinal cell divisions in the vasculature, as shown by the supernumerary vascular files in the quadruple loss-of-function mutant and the reduced number of vascular cell files in the *phb-d* gain of function mutant (Carlsbecker et al. 2010). However, the mechanism behind this phenomenon is still unclear.

1.8 Xylem tracheary element differentiation

The xylem network enables water and minerals absorbed by roots to be transported to all of the tissues of the plant. The cohesion-tension theory proposes that upwards movement of water in xylem tracheary elements (TEs) occurs due to tension caused by evaporation from leaves coupled with the cohesive and adhesive nature of water molecules that cause them to stick to each other and to cell walls (Kim, Park and Hwang 2014). In order for the vascular tissue to become functional, xylem and phloem cells must undergo differentiation, which involves dramatic changes in their cell morphology. Although establishment of the vascular pattern occurs early, differentiation of the cells is only completed during postembryonic growth. During xylem differentiation, TEs undergo two simultaneous processes to be transformed into transport tissue: cell wall thickening and programmed cell death. Metaxylem and protoxylem cells are characterized by pitted and spiral secondary cell walls, respectively. The NAC family members VASCULAR-RELATED NAC-DOMAIN 6 (VND6) and VND7 are key transcription factors regulating TE differentiation. Either of these genes can induce ectopic xylem vessel differentiation. VND6 controls metaxylem differentiation and VND7 protoxylem differentiation (Kubo et al. 2005, Yamaguchi et al. 2010, Yamaguchi et al. 2011, Oda and Fukuda 2012). VND6/7 promote the transcription of genes which control secondary cell wall thickening, including the MYB family transcription factors MYB46/83, as well as genes involved in programmed cell death (Ohashi-Ito, Oda and Fukuda 2010, Yamaguchi et al. 2011, Zhong and Ye 2012). VND7 was found to interact with another NAC gene, VND INTERACTING 2, a repressor of TE differentiation (Yamaguchi

et al. 2010). Ectopic differentiation of phloem SEs and xylem TEs can be induced in culture with the VISUAL (Vascular cell Induction culture System Using Arabidopsis Leaves) system, which uses bikinin, a chemical that inhibits GLYCOGEN SYNTHASE KINASE 3 (GSK3) function (De Rybel et al. 2009, Kondo et al. 2014, Kondo et al. 2016). Using VISUAL, additional VNDs, VND1-3, were identified as potential upstream regulators of VND6/7 in the establishment of xylem fate (Endo et al., 2015; Kondo et al., 2016; Saito et al., 2018; Tan et al., 2018). LOB DOMAIN-CONTAINING PROTEIN 15 was also identified as positive regulator of TE formation upstream of VND7 (Ohashi-Ito, Iwamoto and Fukuda 2018).

CLE41 and *CLE44* are expressed in the phloem and encode the peptide TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF), which inhibits TE differentiation (Ito et al. 2006). The TDIF peptides are secreted from the phloem and perceived at the plasma membrane of procambial cells by their receptor, the Class XI leucine-rich repeat receptor-like kinase PHLOEM INTERCALATED WITH XYLEM (PXY)/TDIF RECEPTOR (TDR) (Hirakawa et al. 2008, Morita et al. 2016, Zhang et al. 2016). TDR-TDIF activates GSK3 family members which suppress TE differentiation in procambial cells by inhibiting the transcription of *BRI1-EMS-SUPPRESSOR 1* (*BES1*), a positive regulator of TE differentiation (Kondo et al. 2014). BES1 functions redundantly with its closest homolog BRASSINAZOLE RESISTANT 1 (BZR1) (Saito, Kondo and Fukuda 2018). GSK3 is negatively regulated by brassinosteroids, while BES1 and BZR1 activate the brassinosteroid pathway (Sun et al. 2010).

1.9 Phloem development

Phloem transport begins around two days after germination, when differentiation of the first phloem cells is complete (Bauby et al. 2007). Differentiated phloem consists of the conductive sieve elements (SEs), which form sieve tubes, and the adjoining companion cells (CCs). SEs are essential in plant growth and development since they carry photoassimilates and signaling molecules from photosynthesizing leaves to heterotrophic sink tissues, such as roots. Long distance transport of sucrose and other solutes is thought to occur by osmotic pressure-driven bulk flow (Knoblauch et al. 2016).

The narrow and elongated SEs are connected to each other by porous sieve plates that allow phloem sap to flow within the sieve tube. Sieve plates contain sieve pores with enlarged, callose-rich, plasmodesmata-like structures. Sieve areas develop on the lateral walls of SEs to enable lateral transport between them. Unlike TEs, SEs remain alive as they differentiate and thus provide symplastic connectivity throughout the plant. Nevertheless, drastic changes occur during SE differentiation. As SEs elongate, their cell walls thicken to provide support. During differentiation, many cellular components, including the nucleus, are lost via selective autolysis, while other organelles, such as plastids, mitochondria and ER, are reorganized to facilitate phloem flow. The enucleation makes SEs dependent on the adjacent CCs, which retain their nucleus and have high cytoplasmic density. This arrangement is facilitated by branched pore-plasmodesmata that connect SEs and CCs to

mediate the transport of proteins and other molecules between the two cell types (Lucas et al. 2013, Knoblauch et al. 2016, Heo, Blob and Helariutta 2017, Otero and Helariutta 2017). It has been recently discovered that the protophloem sieve element (PSE) also has a special connection with the phloem-pole pericycle cells (PPPs). These two cell types are connected via a special type of funnel plasmodesmata that specifically function in the unloading of macromolecules, such as proteins, from the PSE, which occurs in discrete pulses, unlike solute unloading. This unloading can be blocked using a PPP-specific *icals3m* line, *pCALS8::icals3m* (Ross-Elliott et al. 2017).

In the RAM, the SE/procambium initial cell adjacent to the QC undergoes an anticlinal division to produce the SE/procambium precursor, which divides periclinally to produce a procambial cell file and a SE precursor cell (Figure 2). Periclinal division of the SE precursor then results in the formation of PSE and metaphloem sieve element (MSE) cell files. Periclinal division of the procambium cells on either side of the PSE results in a procambium cell file and a CC cell file, demonstrating that in *Arabidopsis* the SEs and CCs are derived from different vascular stem cells. After further anticlinal divisions, the SE cells start expansion and differentiation. PSE differentiation, which is usually completed 20-25 cell lengths from the QC, occurs earlier than the differentiation of other root cell types, likely to ensure nutrient flow to the tip (Mähönen et al. 2000, Furuta et al. 2014, Rodriguez-Villalon et al. 2014). Phloem development is orchestrated by various regulators which are activated at different stages of phloem specification and differentiation. A key regulator is *ALTERED PHLOEM DEVELOPMENT* (*APL*), a MYB transcription factor which promotes phloem differentiation while inhibiting xylem differentiation (Bonke et al. 2003). *apl* mutants lack SEs and CCs, developing xylem-like cells at the phloem position instead. Consistent with this, xylem development can be inhibited by ectopic expression of *APL*. The early stages of phloem differentiation, including cell elongation and cell wall thickening, occur normally in *apl*. Downstream of *APL*, the NAC-type transcription factors *NAC45* and *NAC86* regulate SE differentiation by directing the degradation and reorganization processes (Furuta et al. 2014). In the *nac45 nac86* double mutant, differentiated PSEs are absent and phloem transport is impaired. Although cell wall thickening occurs in the *nac45 nac86* double mutant, enucleation and cytosol degradation do not. Downstream of NAC45 and NAC86, the *NAC45/86-DEPENDENT EXONUCLEASE-DOMAIN PROTEIN* (*NEN*) genes function to complete enucleation. Experiments using the VISUAL culture system suggest that *NAC20*, which is expressed in early SEs, might be an inhibitor of *APL* (Kondo et al. 2016).

Sieve plates are essential for connectivity between SEs. *CHOLINE TRANSPORTER-LIKE 1* (*CHER1*) involved in choline transport is required for normal sieve plate formation and phloem function, indicating that choline plays a central role in phloem development (Dettmer et al. 2014). Normal phloem differentiation also requires genes involved in callose accumulation. Sieve pores and sieve areas form from plasmodesmata during phloem differentiation. Mutation of the phloem-expressed *CALLOSE SYNTHASE7 (CALS7)* gene leads to reduced callose levels in the plasmodesmata of SEs and consequently the

development of fewer sieve plate pores (Xie et al. 2011). On the other hand, gain-of-function mutants of the *CALS3* gene decrease phloem transport due to increased callose levels (Vaten et al. 2011).

During phloem specification, the early SE-localized membrane-associated protein OCTOPUS (OPS) is required for the establishment of continuous SE strands. In *ops* mutants, some PSE cells do not differentiate, which is evinced as a lack of enucleation and cell wall thickening and the failure of sieve plate formation. These so-called gap cells introduce discontinuities to PSE strands, leading to defective phloem transport and reduced root growth (Truernit et al. 2012). Mutations in another early SE-expressed membrane-associated protein, which is also nuclear-localized, BREVIS RADIX (BRX), result in a very similar phenotype to *ops* (Scacchi et al. 2009, Scacchi et al. 2010). *BRX* is a brassinosteroid biosynthesis gene that is a direct target of MP and is strongly upregulated by auxin and repressed by brassinolide, thus forming a feedback loop between the two hormones (Mouchel, Osmont and Hardtke 2006).

Brassinosteroids are required for PSE differentiation, as the triple brassinosteroid receptor mutant *bri1 brl1 brl3* has gap cells in PSEs, but the phenotype is milder than in *ops* or *brx* mutants (Kang, Breda and Hardtke 2017). Furthermore, using VISUAL, *BES1* and *BZR1* were shown to induce not only TE differentiation but also SE differentiation (Kondo et al. 2016). *BRASSINOSTEROID INSENSITIVE2* (*BIN2*) is a member of the GSK3 family that acts as a negative regulator of the brassinosteroid pathway. BIN2 negatively regulates PSE fate by repressing *BES1* and *BZR1* (Yin et al. 2005). OPS was found to positively regulate brassinosteroid signaling by binding BIN2 to the plasma membrane, which lifts BIN2-mediated repression and thus promotes PSE fate. The phloem phenotype of *ops* can be partially rescued by treatment with bikinin, which inhibits GSK3s, or by dominant *bes1-d* and *bzr1-d* mutations (Anne et al., 2015). However, the OPS-BIN2 module seems to not be required for OPS function (Breda, Hazak and Hardtke 2017). Since the *ops brx* double mutant has a more pronounced phenotype, it is possible that OPS and BRX act in parallel pathways (Breda et al. 2017).

The maintenance of a strict balance in phosphatidylinositol-4,5-biphosphate levels by phosphoinositide 5-phosphatases COTELYDON VASCULAR PATTERN 2 (CVP2) and CVP2-LIKE 1 (CVL1) is essential for normal PSE differentiation. The *cvp2 cvl1* double mutant displays gap cells in PSEs similar to *ops* and *brx* mutants. Elevated phosphatidylinositol-4,5-biphosphate levels promote xylem differentiation, therefore affecting xylem and phloem differentiation in opposite manner (Rodriguez-Villalon et al. 2015, Gujas et al. 2017). In contrast, *CLAVATA 3/EMBRYO SURROUNDING REGION 45* (*CLE45*) and its receptor the receptor-like kinase *BARELY ANY MERISTEM 3* (*BAM3*) function to inhibit PSE differentiation. Treatment with the CLE45 peptide leads to the formation of gap cells in the SE. The *brx* and *ops* mutant phenotypes can be suppressed by *bam3* (Depuydt et al. 2013). Moreover, PSE differentiation is not suppressed when *ops* gain-of-function mutants are treated with the CLE45 peptide, and this resistance to CLE45 is dependent on BRX function. Thus, the commitment to PSE fate seems to be promoted by BRX-OPS and suppressed by the CLE45-BAM3 pathway (Rodriguez-Villalon et al.

2014). The function of CLE45 as inhibitor of SE differentiation is analogous to the role of CLE41/44 in inhibiting TE differentiation.

Recently, auxin was shown to play an important role in phloem differentiation via BRX (Marhava et al. 2018). In the root, PSE cells start elongation and differentiation before other cell types, and this is accompanied by increased auxin activity compared with surrounding cells. This involves the interaction of BRX with another membrane associated protein, PROTEIN KINASE ASSOCIATED WITH BRX (PAX), an AGC-family kinase that promotes auxin efflux by activating the PINs. Colocalization of BRX with PAX at the rootward plasma membrane of PSEs represses the PAX-mediated auxin efflux when auxin levels are low. This results in increased auxin levels. BRX is removed from the plasma membrane by auxin action, which activates PAX and leads to greater auxin efflux. Since auxin increases BRX transcription, this regulatory loop dynamically stabilizes auxin levels in the PSE cell file. The defects in PSE differentiation in *pax* mutants are identical to those in *brx* mutants, and expression of *MP* in PSEs under the *CVP2* promoter promotes PSE differentiation, indicating that auxin response in PSEs affects their differentiation state (Marhava et al. 2018). These results demonstrate that a complex network of interacting regulators coordinate SE differentiation. However, the very early steps in phloem specification have remained unknown.

2 Aims of the study

In order to investigate the role of multidirectional cell-to-cell communication during vascular development, we inhibited symplastic signaling using the *icals3m* system under various promoters. After expressing *icals3m* in early SE cells, we observed a dramatic reduction in the number of vascular cell files. This led to the hypothesis that early SE-derived mobile signals could play a major role in organizing vascular cell proliferation and patterning. Moreover, we hypothesized that this signaling could occur downstream of cytokinin due to its central role in vascular cell proliferation.

The specific aims of this study were:

1. Identification of the regulators of vascular cell proliferation originating from the early phloem

2. Identification of factors downstream of the TMO5/LHW-cytokinin pathway that control vascular proliferation

3. Characterization of the role of the genes *SMXL3, 4* and *5*, which are expressed in the early phloem/procambium

3 Materials and methods

The materials and methods used are described in publications I, II and III. The publication in which they appear are summarized in Table 1.

Table 1. Methods used in this study. Those in brackets were performed by co-authors in the respective publications. Asterisks indicate methods used for data included in the thesis results section.

Method	Publication
Agrobacterium-mediated transformation of *Arabidopsis*	I, II, (III)
Anatomical analyses	I, II, III
Aniline blue staining	(I), (III)
Confocal Laser Scanning Microscopy (CLSM)	I*, II, (III)
CRISPR/CAS9 mutant generation	(I), (II)
Differential interference contrast (DIC) microscopy	(II)
DNA extraction	I, II, (III)
Genetic crossing of *Arabidopsis*	(I), (II), (III)
Grafting	(III)
Histological sectioning of plastic embedded samples	(I), (II), (III)
Hormone / induction assays	I, II, (III)
In silico gene expression analysis	I
In situ RNA hybridization	(I)
Light microscopy	I, II, (III)
Mapping of the position of periclinal cell division	(I)
Mathematical modeling	(I)
Microarray hybridization	(I), (II)
mPS-PI staining	(II), (III)
Network inference analysis	(II)
Phloem transport assay	(I), (III)
Plasmid construction	I, II, (III)
Polymerase chain reaction (PCR) analysis	I, II, (III)
Quantification of periclinal cell divisions	I, II, (III)
Quantitative real-time PCR analysis	(I), (II)
Raster image correlation spectroscopy (RICS)	(I)
Reporter analysis	I, II, (III)
RNA extraction	I, (II)
Sequence analysis	I, II, (III)
Serial block-face scanning electron microscopy	(III)
Statistical analysis	(I), (II), (III)
Sugar measurements by ion chromatography	(III)
Vascular Cell Induction Culture System Using *Arabidopsis* Leaves (VISUAL)	(III)

4 Results and discussion

4.1 Periclinal cell divisions in the root vasculature are concentrated on PSEs

Cell-to-cell communication is known to play a critical role during plant development. In order to investigate the role of symplastic communication during vascular patterning, we inhibited symplastic transport using the *icals3m* system, which enhances callose deposition at plasmodesmata in an inducible manner (Vaten et al. 2011). When we expressed *icals3m* under an early PSE-specific promoter (Lee et al. 2006) (*pPEAR1[XVE]::icals3m*), we observed enhanced callose deposition in early PSE cells after induction. This was accompanied by a dramatic reduction in the number of vascular cell files. Furthermore, reduced expression of the CC markers *SISTER OF APL* (*pSAPL::GFP-GUS*) and *ATPASE 3* (*pAHA3::RFPer*) indicated that CC identity was compromised in the *pPEAR1[XVE]::icals3m* background, suggesting that the formative divisions in the procambium were affected (I, Extended Data Fig. 2A-E). In order to determine the exact location of periclinal cell divisions in the root vasculature of wild type plants, we mapped the position of each periclinal division using confocal microscopy cross section images from every developmental stage. We established new names for the procambium/phloem cells to reflect their relative position and calculated how many periclinal cell divisions occurred in each cell type (Figure 3). Cells touching the PSE as well as the pericycle were named PSE-lateral neighbors (PSE-LN); cells touching only the pericycle were named outer procambial cells (OPC); and internal cells touching only the PSE were named PSE-internal neighbors (PSE-IN). Our analysis revealed that PSE and PSE-LN cells divide actively, whereas OPC, protoxylem and PSE-IN cells are more quiescent. Furthermore, no periclinal cell divisions were observed in the metaxylem (MX)

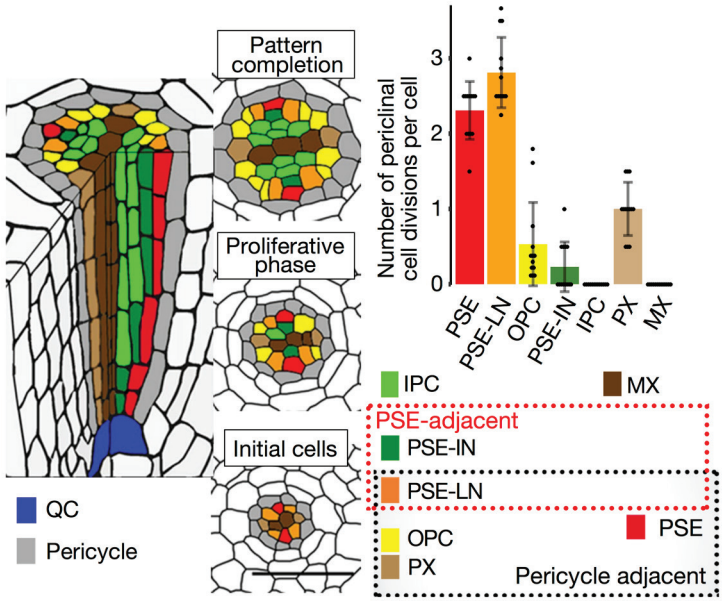

Figure 3. Periclinal cell divisions occur primarily in the PSE and its lateral neighbors, PSE-LN cells, during procambial development. IPC, internal procambial cell; OPC, outer procambial cell; MX, metaxylem; PX, protoxylem; QC, quiescent center. Modified from Miyashima et al., 2019

or internal procambial cells (IPC). These observations indicate that only cells touching the pericycle have the ability to divide in the radial dimension and highlight the PSE and PSE-LN cells as the domain of high periclinal cell division activity during vascular development in the *Arabidopsis* root. In the *pPEAR1[XVE]::icals3m* line, divisions of both PSE and PSE-LN cells were altered (I, Extended Data Fig. 2A-E). Together, these results indicate that periclinal cell divisions in the vasculature occur mainly in PSE and PSE-LN cells. The disruption of these divisions by increased callose accumulation suggests that they require symplastic signaling between PSEs and the surrounding cells.

4.2 PEARs are PSE abundant DOF transcription factors that are mobile

Since transcription factors are known to move via plasmodesmata (Vaten et al. 2011), we hypothesized that a mobile transcription factor expressed in PSE cells moves to the surrounding cells to direct cell division. We performed *in silico* analysis to find transcription factors expressed in the early PSE using the AREX database (Brady et al. 2007). We identified the DNA-BINDING WITH ONE FINGER (DOF) family members *DOF2.4* and *DOF5.1* and named them *PHLOEM-EARLY-DOF 1* (*PEAR1*) and *PEAR2*, respectively. DOFs are plant-specific transcription factors that were first identified in maize, and there are 36 DOF members in the Arabidopsis genome (Yanagisawa and Izui 1993, Yanagisawa 2002). *DOF5.1/PEAR2* was previously reported to regulate adaxial/abaxial patterning of leaves (Kim et al. 2010). Our analysis of *PEAR1* and *PEAR2* transcriptional reporter constructs and *in situ* hybridization confirmed that both are transcribed specifically in the early PSE (I, Fig. 1C, Extended Data Fig. 3D).

Many DOFs have been reported to be expressed in the vascular tissue and to be mobile (Le Hir and Bellini 2013). To test the mobility of PEAR1 and PEAR2, GFP-tagged proteins were expressed under their own promoters. We observed that both protein fusions, PEAR1-GFP and PEAR2-GFP, displayed a broader GFP distribution than their respective transcriptional reporters (I, Fig. 1C, D, Extended Data Fig. 3D). During the initial stage, PEAR1-GFP was observed in the PSE and neighboring procambial cells but not in the xylem axis. PEAR1-GFP was also detected in phloem-pole pericycle (PPP) cells during early development (I, Extended Data Fig. 4A-D). During the proliferative phase, PEAR1-GFP was detected in a gradient through the SE and procambial cells, with the strongest signal observed in the SE. PEAR1-GFP was also absent from xylem cells during the proliferative phase. The distribution of the PEAR1-GFP protein gradually became restricted to the SE and its neighboring cells during pattern formation. To examine how the inhibition of symplastic connections affects PEAR1-GFP movement, we introduced *pPEAR1::PEAR1-GFP* into a line inducibly expressing *icals3m* in the stele (*pCRE1[XVE]::icals3m*). SHR movement was shown to be abolished in this line (Vaten et al. 2011). After induction, the PEAR1-GFP signal became restricted to PSE cells, indicating that the protein moves via plasmodesmata (I, Extended Data Fig. 2F, G). Together, these observations show that

PEAR1 and PEAR2 move from the early PSE cells to the surrounding cell layer and are thus localized in cells with high periclinal cell division activity.

4.3 PEARs are necessary and sufficient for periclinal cell division

To investigate whether the PEARs influence periclinal cell division, we overexpressed *PEAR1* and *PEAR2* in the stele under the control of the inducible *CRE1[XVE]* promoter. We observed that both genes promoted periclinal divisions specifically, suggesting that they play a central role in regulating this process (I, Extended Data Fig. 3C). To determine whether *PEAR1* and *2* are required for the divisions, we analyzed the phenotypes of *pear1* and *pear2* single and *pear1 pear2* double mutants. We did not detect an obvious reduction in cell file number, which led us to hypothesize that other DOF family members might act redundantly in this process.

To identify early phloem-abundant DOFs that can promote periclinal cell divisions redundantly to *PEAR1* and *2*, we performed a systematic overexpression analysis as well as *in silico* and reporter analysis of the DOF family members. Our analysis revealed additional PSE-expressed DOFs with broader protein localization, including *DOF1.1/OBP2* (Skirycz et al. 2006), *DOF3.2/DOF6* (Rueda-Romero et al. 2012), *DOF5.6/HCA2* (Guo et al. 2009), and *DOF5.3/TMO6* (Schlereth et al. 2010). All of these genes could induce periclinal cell division (I, Extended Data Fig. 3A-C). Moreover, PEAR1-GR and PEAR2-GR could induce periclinal cell divisions ectopically in ground tissue using a *J0571 GAL4* driver line (Figure 4), demonstrating that PEARs have the capacity to induce periclinal divisions in a way

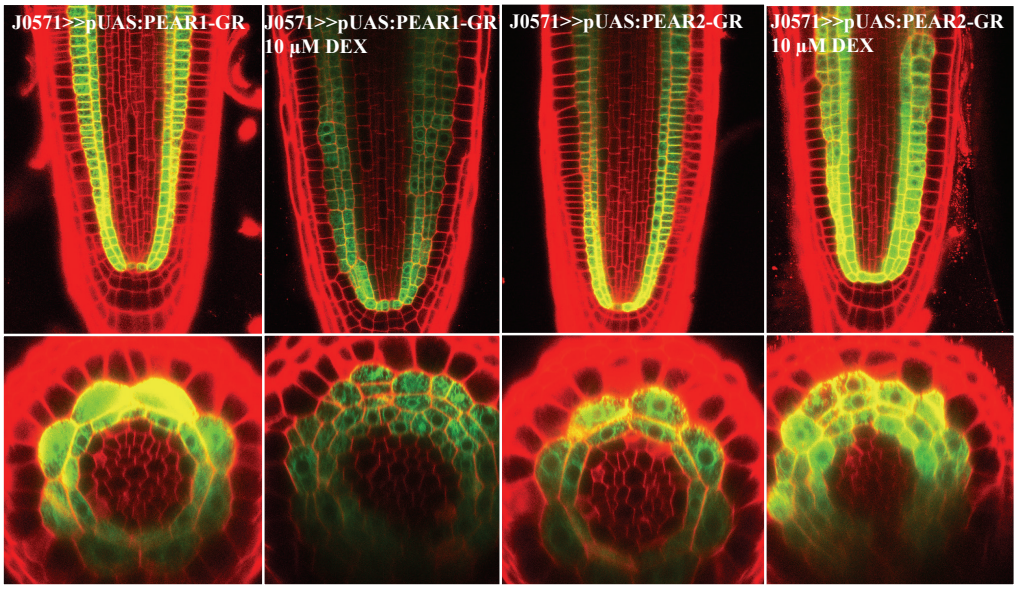

Figure 4. PEAR1-GR and PEAR2-GR can induce ectopic periclinal cell divisions when expressed in the ground tissue using the *J0571 GAL4* driver line. Dex, dexamethasone.

similar to TMO5 (De Rybel et al. 2013). Altogether, our observations suggest that PEARs are likely to play a major role in regulating vascular cell file number in a redundant manner. We performed a qRT-PCR analysis to determine whether the expression of the other PEARs was elevated in the *pear1 pear2* double mutant, potentially compensating for the mutations. The results revealed that HCA2, DOF6 and TMO6 transcripts were increased in the double mutant, suggesting that there could be a compensatory genetic regulation mechanism (I, Extended Data Fig. 3E). We also did not observe phenotypes in other *pear* double mutant combinations, so we generated higher order mutants. Since a *T-DNA* insertion line for *TMO6* was not available, we generated a knock-out for this gene using the CRISPR-CAS9 system (Fauser, Schiml and Puchta 2014). In the resulting *pear1 pear2 tmo6* triple mutant we observed a strong but variable phenotype, with significantly fewer vascular cell files (I, Fig. 2A-F). A strong and uniform phenotype was achieved by introducing a *dof6* mutation into the triple mutant to create the *pear1 pear2 tmo6 dof6* quadruple mutant, which phenocopied the *pPEAR1[XVE]::icals3m* line. Thus, we conclude that these four *PEAR* genes play a major role in regulating periclinal divisions. We also were able to define a minor role for *HCA2* and *OBP2* through analysis of the phenotypically variable *pear1 pear2 dof6 obp2 hca2* quintuple mutant: 30% of the population showed a significantly reduced cell file number in short-rooted plants. However, adding *hca2* and *obp2* to the quadruple mutant to form *pear1 pear2 tmo6 dof6 obp2 hca2* sextuple mutant did not increase the severity of the phenotype (I, Fig. 2B, E, F).

In addition to cell division defects, some *pear* combinatorial mutants had undifferentiated PSE cell files. To evaluate the functionality of the phloem in these mutants, we loaded the fluorescent dye 5(6)-carboxyfluorescein diacetate (CFDA) to one of the seedling cotyledons and monitored its transport and unloading at the root tip (Oparka et al. 1994). In the *pear* sextuple mutant, transport was defective, whereas transport was normal in the narrow roots of the *pear1 pear2 dof6 obp2 hca2* quintuple mutant (I, Extended Data Fig. 5E). Thus, the reduction in cell number does not correlate with a lack of phloem functionality. This suggests that, in contrast to *HCA2* and *OBP2*, *PEAR1*, *PEAR2*, *TMO6* and *DOF6* have a role in phloem differentiation. To further evaluate the relationship between cell number and phloem functionality, we analyzed the cell number of *pear* sextuple mutant roots 1.5 days after germination, before phloem has differentiated. There were fewer cell files at this early stage (I, Extended Data Fig. 5F). This indicates that the reduced cell number is not a consequence of a defective phloem flow but instead PEARs affect periclinal cell division and phloem differentiation separately.

Our results indicate that the *PEARs* are expressed abundantly in the PSE and the proteins move to the surrounding cells to promote radial growth. In addition, the *PEARs* act redundantly, with *PEAR1*, *PEAR2*, *TMO6* and *DOF6* being the central regulators, whereas *HCA2* and *OBP2* have a more minor role.

4.4 PEARs activate targets non-cell autonomously

In order to further investigate the non-cell autonomous function of the DOFs, we performed microarray experiments to identify transcriptional targets of PEAR1 and PEAR2 using dexamethasone (DEX) inducible overexpression lines with PEAR1 and PEAR2 proteins fused to the glucocorticoid receptor (GR) tag (*pRPS5A::PEAR1-GR* and *pRPS5A::PEAR2-GR*). The *RPS5A* promoter is expressed broadly in the root meristem, including in the xylem (II, Fig. 2J) (Weijers et al. 2001). After two hours of induction, we identified 212 and 435 genes upregulated by PEAR1 and PEAR2, respectively. Of these, 106 were shared between PEAR1 and PEAR2. Furthermore, by using cycloheximide treatment to inhibit protein synthesis, 20 and 84 putative direct targets were found for PEAR1 and PEAR2, respectively (I, Extended Data Fig. 6A, SI Table 2). We performed *in silico* analysis using the AREX database to predict the expression patterns of the targets. Intriguingly, approximately half of them were found to be expressed in the procambium. The targets also included a cluster of PPP-expressed genes (Figure 5). We cloned promoter reporter lines for selected putatively procambium-expressed genes using GFP-GUS or YFPer markers; most of the reporters were for PEAR2 direct targets since PEAR2 upregulated more genes in our microarray. We found that *AT1G09460*, *AT3G54780*, *SUPPRESSOR OF MAX2 1-LIKE3* (*SMXL3*), and *ROPGEF5* were expressed broadly in the phloem and procambium, but *AT1G09460* and *AT3G54780* were not expressed in the PSE (I, Fig. 2G, Extended Data Fig. 6B, C, D, I). In addition, *AT1G15080* and *AT4G00950* were identified as genes expressed in the PSE and surrounding cells (I, Fig. 2H, Extended Data Fig. 6F, H), and *AT1G49230* and *AT3G16330* as genes expressed only in PSE-surrounding cells (I, Extended Data Fig E, G). We also analyzed the expression of these targets in the *pRPS5A::PEAR2-GR* and *pear* sextuple mutant backgrounds. All of the genes were ectopically activated after PEAR2-GR induction (I, Fig. 2G, H, Extended Data Fig. 6B-I). However, activation was often not observed in the xylem position, despite abundant expression of the *RPS5A* promoter in that position. In the *pear* sextuple mutant background, we observed a strong reduction in expression of five of the targets (*AT3G54780*, *AT1G49230*, *ROPGEF5*, *AT4G00950* and *AT3G16330*) (I, Extended Data Fig. 6D, E, G, H, I). These results confirm that PEAR2 activates genes expressed in the phloem/procambium domain. This activation occurs also in non-cell autonomous manner, since *PEAR2* is expressed in the PSE and the protein moves to the surrounding cell layer, thus confirming the importance of this movement. Some of the targets also require PEAR activity for their expression. Furthermore, an unidentified inhibitor appears to block the transcription of many of the targets in the xylem position.

To understand more about the function of the target genes, we first analyzed which gene ontology terms were overrepresented among the PEAR1 and PEAR2 direct targets. However, this did not yield significant results. Furthermore, previously identified regulators of cell divisions were not found among the target genes. We therefore overexpressed approximately 50 targets genes under the inducible *CRE1[XVE]* promoter and analyzed theses lines for a cell division phenotype using confocal microscopy. This revealed that *SMXL3*, a putative direct target of *PEAR2*, induces periclinal cell divisions in the vasculature

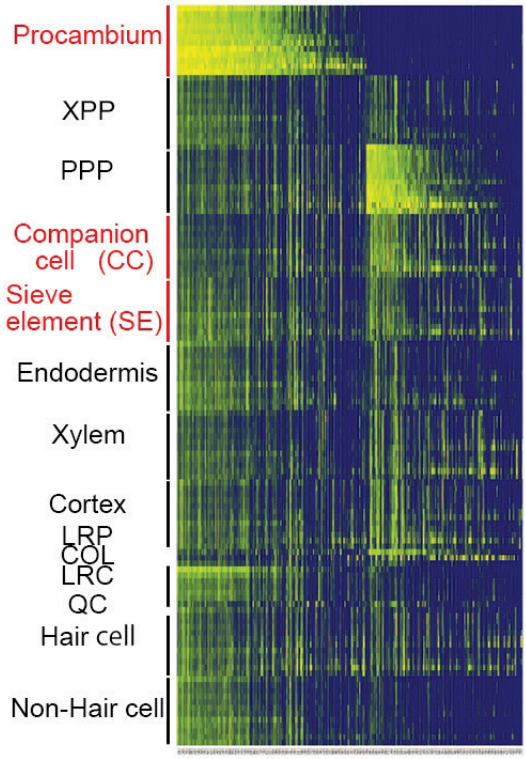

Figure 5. Spatiotemporal expression patterns of genes induced by PEAR1/PEAR2 reveals clusters of procambium and phloem-pole pericycle (PPP) expressed genes among the targets. The expression level in each cell type is shown in rows running from early (top) to late (bottom) stage cells. XPP, xylem-pole pericycle; LRP, lateral root primordium; COL, columella; LRC, lateral root cap; QC, quiescent center. Modified from Miyashima et al., 2019.

(I, Extended Data Fig. 6J). In summary, these results demonstrate that the *PEAR* genes activate their targets non-cell autonomously, mostly in the procambium but also in the PPP, which indicates that the mobility of the PEARs is central for their function. Furthermore, PEAR2 was found to activate, possibly directly, *SMXL3*, which also regulates periclinal cell divisions.

4.5 Cytokinin is required for PEAR expression

Cytokinin is required for periclinal cell divisions in the vascular tissue; however, the underlying molecular mechanism has remained a mystery. In order to evaluate whether the PEARs function downstream of cytokinin, we first analyzed cytokinin and auxin responses simultaneously using plants carrying a double marker for auxin and cytokinin response (*pIAA2::GUS-GFP* for auxin and *pARR5::erRFP* for cytokinin response). Auxin response was observed in the xylem axis throughout development, whereas high cytokinin response was first observed in the phloem and procambium during the initial and early proliferative phases but was restricted to procambium cells adjacent to the xylem during later stages (I, Fig. 3A). Thus, during early stages the cytokinin response domain is similar to the PEAR1-GFP expression domain. Next, we analyzed plants carrying a double marker for cytokinin response and *PEAR1* transcription (*pARR5::erRFP* x *pPEAR1::GFP-GUS*). We observed that *ARR5* and *PEAR1* transcription overlapped during the initial and proliferative phase of vasculature development. This suggests that *PEAR1* might be activated by cytokinin (I,

Fig. 3A). We therefore analyzed *PEAR1* promoter activity in the cytokinin receptor mutant *wol*, which can be rescued by the induction of *CRE1* expression (*pPEAR1::GFP-GUS/ pCRE1[XVE]::CRE1/wol*). Prior to induction, *PEAR1* transcription was severely attenuated in *pCRE1[XVE]::CRE1/wol* roots, but transcription was completely restored after induction of *CRE1* reestablished cytokinin signaling (I, Fig. 3B, C). Similarly, we analyzed *TMO6* transcription in the *pCRE1[XVE]::CRE1/wol* background and observed severely reduced levels in the vascular tissue accompanied by an increased signal in the pericycle before induction, but the pattern was restored after *CRE1* induction (I,Extended Data Fig. 7D). Since the effect of *ARR22* overexpression on cytokinin signaling is similar to *wol* (Kiba et al. 2004), we analyzed the expression of *PEAR1* after *ARR22* induction and found it to be downregulated (I, Fig. 3D, E). To further investigate the relationship between the PEARs and cytokinin, we measured *PEAR* transcript levels after cytokinin treatment using qRT-PCR. The results revealed that a short cytokinin treatment led to significant induction of *PEAR2*, *DOF6*, and *TMO6*, while induction of *PEAR1*, *HCA2* and *OBP2* was observed only after sustained incubation with cytokinin for 1-4 days (I, Extended Data Fig. 7B, C). These results suggest that while all of the *PEAR* genes are transcriptionally activated by cytokinin, *PEAR2*, *DOF6* and *TMO6* are activated more directly than *PEAR1*, *HCA2* and *OBP2*.

As vascular tissue patterning begins during the early stages of embryogenesis, we analyzed auxin and cytokinin responses (*pIAA2::GFP-GUS* and *pARR5::RFPer*, respectively) and *pPEAR1::GFP-GUS* expression patterns during embryogenesis in wild type and *wol* mutant plants. During the late globular stage, the provascular initials in the *lt* divide anticlinally, producing an upper and lower layer (named *ult* and *llt*, respectively). In wild type, *pIAA2* was observed in the provascular cells during the globular stage before this division, whereas initiation of *pARR5* expression occurred later, in *ult* cells during the early heart stage. During the mid-heart stage, a bisymmetric pattern was observed, with *pARR5* expressed in cells below the SAM and *pIAA2* expressed in the cells closest to the cotyledons. During the torpedo stage, we observed expansion of *pARR5* expression downward to *llt* cells (I, Extended Data Fig. 7E-H). However, in the *wol* mutant, *pIAA2* expression remained radially symmetric and *pARR5* expression was not activated (I, Extended Data Fig. 7I-L). These results suggest that the activation of cytokinin signaling is required to establish a bisymmetric auxin response during embryogenesis. *pPEAR1* expression began in the provascular cells of early globular embryos, and enhanced *pPEAR1* expression was observed in *ult* cells during the early heart stage, which is exactly where a *pARR5* expression maximum was detected (I, Extended Data Fig. 7M-O). By contrast, the enhancement of *pPEAR1* expression in *ult* cells was not observed in the *wol* mutant, although *pPEAR1* expression was initiated normally at the globular stage. Consequently, *pPEAR1* expression was severely downregulated during the heart stage (I, Extended Data Fig. 7P-R). These results indicate that the initiation of *pPEAR1* expression in early embryogenesis is independent of cytokinin signaling, but once the bisymmetric auxin-cytokinin pattern in the vascular tissue is formed during the heart stage, cytokinin re-activates *pPEAR1* expression and is required to maintain it in PSE cells postembryonically, indicating that PEARs function downstream of cytokinin.

4.6 *PEAR* and HD-ZIP III genes have antagonistic roles in regulating periclinal divisions

During procambial development, periclinal cell divisions occur only in the peripheral cells touching the pericycle. Since the PEAR1 protein and cytokinin response are both localized in the PSE-IN, which very rarely divides, an inhibitory mechanism is likely preventing division of the inner cells. As the HD-ZIP III genes inhibit periclinal cell divisions in the vasculature (Carlsbecker et al. 2010), we hypothesized that they might also inhibit PEAR function in the PSE-IN. To investigate this, we analyzed the localization of four HD-ZIP III proteins (PHB, REV, CNA and ATHB8) in detail in the root. PHB-GFP, REV-GFP and CNA-GFP were strongly localized to the central metaxylem as well as the internal procambial cells and PSE-IN, which virtually do not divide periclinally. On the other hand, they were absent from PSE and PSE-LN cells, which show high periclinal division activity. ATHB8-GFP was specific to the xylem axis (I, Extended Data Fig. 8F-K). The HD-ZIP III proteins are excluded from the periphery by the presence of miR165/6. Thus, HD-ZIP III localization correlates with low periclinal cell division activity. We analyzed the cell division pattern of the hd-zip III quadruple mutant *phb phv cna athb8* more closely and found periclinal division of cells that were not adjacent to the pericycle, including PSE-IN cells (I, Extended Data Fig. 8A-E). Furthermore, overexpression of *PEAR1* in heterozygous *phb1-d* mutants, which have a miR165/6-resistant *phb* allele and thus an expanded PHB expression domain and fewer vascular cell files (Carlsbecker et al. 2010, Miyashima et al. 2011), did not increase periclinal cell divisions as effectively as in the wild type background (I, Extended Data Fig. 8L-O). These results indicate that the *PEAR* and HD-ZIP III genes have antagonistic roles in regulating periclinal cell divisions, and their expression domains need to be kept in check in order to maintain a normal cell division pattern.

Mutual regulation between PEAR and HD-ZIP III genes

HD-ZIP III expression is promoted by auxin in the xylem axis, whereas the factors promoting their expression in procambial cells remain unknown. PEAR1 has been previously shown to bind to HD-ZIP III promoters (Gaudinier et al. 2011, O'Malley et al. 2016), raising the possibility that PEAR1 could regulate HD-ZIP III transcription. To determine whether PEAR1 controls HD-ZIP III transcription, we analyzed the transcriptional pattern of *CNA* (*pCNA::GV>UAS::GFPer*), *PHB* (*pPHB::GV>UAS::GFPer*), *ATHB8* (*pATHB8::HTA6-YFP*) and *REV* (*pREV::RFPer*) after PEAR1 overexpression. The transcriptional domain of both *CNA*, which is expressed mainly in procambial tissue, and *PHB*, which is normally maximally expressed in the xylem cells, were expanded following PEAR1 overexpression. We also observed a broadening of the *ATHB8* transcriptional domain, which in wild type is specific to xylem axis. Unlike the other HD-ZIP III genes, *REV* expression peaks in the phloem and is reduced towards the xylem, but its transcription was also enhanced after *PEAR1* overexpression (I, Extended Data Fig. 9A-H). We also analyzed *CNA* (*pCNA::GV>UAS::GFPer*) transcription in the *pear* quintuple mutant and

observed a significant reduction in PSE-IN cells (I, Fig. 4H-J). These results indicate that PEAR1 promotes HD-ZIP III expression.

We then investigated whether HD-ZIP III genes control *PEAR1* transcription. First, we analyzed the expression of *PEAR1-GFP* in the *phb-1d* mutant and observed that *PEAR1-GFP* was expressed only in a single phloem pole in the majority of *phb-1d* heterozygotes and was even completely absent in some cases. Furthermore, *PEAR1* transcription was absent in the *shr-2* mutant, in which *miR165/6* levels are dramatically reduced (I, Extended Data Fig. 8P-T). This indicates that HD-ZIP III genes inhibit *PEAR1* expression.

Together, these results suggest that PEAR1 maintains HD-ZIP III expression in PSE-IN cells, while further expansion of the HD-ZIP III domain is inhibited by miRNA regulation. On the other hand, in the peripheral PSE cell, where both cytokinin signaling and miRNAs are present, *PEAR1* expression can occur, while HD-ZIP III genes inhibit *PEAR1* expression in the internal domain. Furthermore, HD-ZIP III genes likely antagonize PEAR function in the PSE-IN cell, which could explain why this cell rarely divides. These results strongly suggest that complementary localization of HD-ZIP III and PEAR1 with an overlap at the PSE-IN is required to form two zones, one with actively dividing and the other with more quiescent cells.

HD-ZIP III genes control PEAR1 movement

Based on these findings, we hypothesized that the ectopic divisions observed in the hd-zip III quadruple mutant could be due to increased *PEAR* expression. However, we did not observe an expansion of the *PEAR1* transcriptional domain in the hd-zip III quadruple mutant, where *PEAR1* transcription remained limited to the PSE (I, Extended Data Fig. 8U-W). Therefore, we next analyzed PEAR1-GFP protein localization in the hd-zip III quadruple mutant. This revealed that PEAR1-GFP expanded to the central vascular tissues in the mutant (I, Fig. 4K-L, Extended Data Fig. 4A-I). In the hd-zip III quadruple mutant, the PEAR1-GFP signal was broadly detected in the vascular tissue without a clear gradient during the initial stage, and PEAR1-GFP was maintained in the internal domain during the proliferative phase. In wild type plants, PEAR1-GFP localization showed a steeper gradient, and we detected it primarily in the PSE and its neighboring cells. These localization patterns show that the protein moves further in hd-zip III quadruple mutants, suggesting that HD-ZIP III genes control the mobility of PEAR1 and restrict its localization to the PSE and neighboring cells.

To further test this hypothesis, we calculated the diffusion coefficient of PEAR1-GFP in wild type and hd-zip III quadruple mutants using Raster Image Correlation Spectroscopy (Clark et al. 2016). We found a higher diffusion coefficient in the hd-zip III quadruple mutants than in wild type, indicating increased mobility of the protein (I, Fig. 4M). To further analyze the relationship between HD-ZIP III and PEAR, we generated *pear1 pear2 dof6 obp2 hca2 phb phv cna athb8* nonuple and *pear1 pear2 dof6 tmo6 obp2 hca2 phb phv cna athb8* decuple mutants. Analysis of these mutants showed that in both cases the number of vascular cell files was reduced compared to the hd-zip III quadruple mutant, although to a

lesser extent than in the *pear* sextuple mutant. The reduction in vascular cell files was also observed in internal cells, which are outside the normal PEAR localization domain (I, Fig. 4A-G). This indicates that the increased cell number in the hd-zip III quadruple mutant is partially dependent on PEARs. Thus, HD-ZIP III genes inhibit periclinal cell divisions partially by inhibiting PEAR movement but also via a PEAR-independent pathway. However, additional DOF genes that promote periclinal divisions in the vasculature may await discovery.

Modeling HD-ZIP III and PEAR interaction

The interaction between the PEAR and HD-ZIP III families has unique properties, as the HD-ZIP III genes inhibit both the transcription and the movement of the PEARs. We used mathematical modeling to better understand the significance of these interactions for the spatial distribution of PEAR and HD-ZIP III in the phloem/procambium (I, Supplementary Modeling Information). For simplicity, the various PEARs and HD-ZIP IIIs were treated as two generic PEAR and HD-ZIP III proteins. We used a one-dimension model containing 3, 4 or 5 cells from the central metaxylem to the outer PSE, thus including the metaxylem (MX), internal procambial cell (IPC), PSE-IN and PSE cells. Auxin, miR165/6 and cytokinin are known to regulate either *PEAR* or HD-ZIP III genes, and their spatial patterns are well established, so they were included in the model. An input of auxin was imposed at the metaxylem position and of miR165/6 at the PSE position, and the cytokinin distribution was derived from the auxin distribution. This model was used to predict the spatial distribution of PEAR and HD-ZIP III (I, Extended Data Fig. 10D) based on three types of interaction: 1) HD-ZIP III inhibits PEAR diffusion, 2) HD-ZIP III inhibits *PEAR*

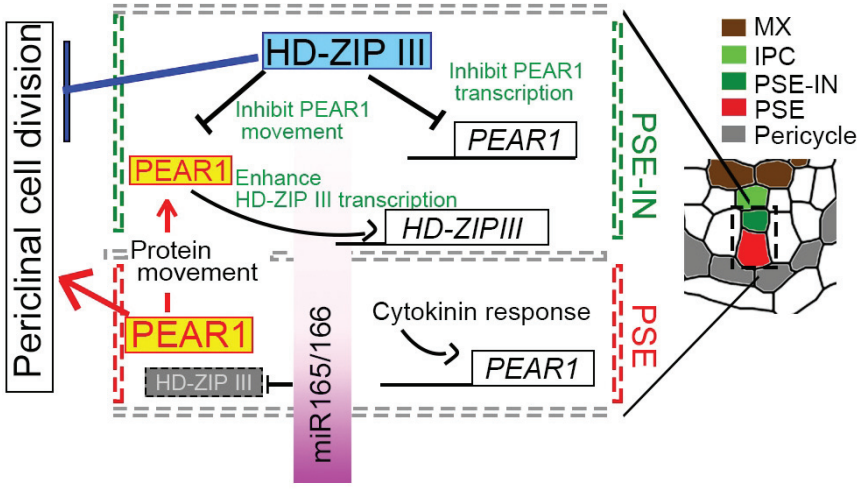

Figure 6. A regulatory network establishing the boundary between the dividing PSE and the non-dividing PSE-IN during procambial development. MX, metaxylem; IPC, internal procambial cell. Modified from Miyashima et al., 2019.

transcription and 3) PEAR promotes HD-ZIP III transcription (I, Extended Data Fig. 10C). The simulations demonstrated that all three interactions are required to form sharp and opposing concentration gradients of PEAR and HD-ZIP III, with PEAR concentration high in the PSE and PSE-IN cells but absent from inner procambial cell, whereas the HD-ZIP III level is high in the inner procambial cell and PSE-IN cells but not in PSE cells (I, Extended Data Fig. 10F-H) (Figure 6).

4.7 DOF2.1 acts downstream of TMO5/LHW and cytokinin

The results reported in the previous section demonstrate that the majority of periclinal divisions in the root vasculature occur in PSE and PSE-LN cells and are induced by *PEAR* genes downstream of cytokinin. However, divisions that occur in the outer procambial (OPC) cells cannot be explained by PEAR function alone since they occur mainly outside the PEAR expression domain, and periclinal divisions are not completely abolished in the *pear* sextuple mutant. The TMO5/LHW transcription factor complex located in early xylem cells promotes procambial periclinal cell divisions non-cell autonomously in the embryo and the root (De Rybel et al. 2013, De Rybel et al. 2014a). The activation of cell division by TMO5/LHW is thought to occur at least partly through increased cytokinin biosynthesis by the direct activation of *LOG3/4* expression. To discover how the TMO5/LHW-cytokinin pathway activates cell proliferation, we performed a microarray analysis using a DEX-inducible double overexpression line with both the TMO5 and LHW proteins fused to GR tag leading to simultaneous overexpression upon DEX treatment (*pRPS5A::TMO5:GR* x *pRPS5A::LHW:GR*; *double GR* or *dGR* in short). The *dGR* line exhibits a uniform and strong phenotype of cell proliferation compared to overexpression of either gene alone (II, Fig. 1A-I). After induction, the first ectopic periclinal cell divisions were seen in the epidermis after just four hours, suggesting that the gene activation preceding these divisions had occurred before this time point.

Based on these results, we performed microarray profiling at multiple time points (0.5, 1, 2, 3, 4, 5, and 6 hours). We identified 237 genes that were activated during the time course (II, Table S1). The data were validated by qRT-PCR, confirming the activation of 25 targets, including genes previously known to be activated by TMO5/LHW (II, Table S2). Gene activation occurred in several waves, the first occurring at 0.5-1 hours and including direct target genes such as *LOG3*, *LOG4* and *SACL3*. The next wave occurred after 1-3 hours and included A-type RRs (II, Fig. 1J, Table S2). These results are consistent with previous observations that TMO5/LHW directly activates cytokinin biosynthesis genes like *LOG*, which subsequently activate cytokinin responses, with A-type RRs serving as a typical readout. Furthermore, we analyzed cytokinin biosynthesis (*pLOG4::n3GFP*) and signaling (*pTCSn::3GFP*) in the *dGR* background using confocal microscopy. *LOG4* is normally expressed in the xylem axis, xylem-pole pericycle (XPP), and XPP-associated endodermis, while *TCSn* is expressed in procambium, columella, epidermis and root cap (II, Fig. 1K, M). Both genes were ectopically activated in the *dGR* background, indicating a pronounced increase in cytokinin production and signaling in the *dGR* line (II, Fig. 1L, N). Our profiling

experiment thus enabled us to identify the temporal pattern of gene activation downstream of TMO5/LHW.

We used network inference analysis to map transcriptional hubs that are activated downstream of TMO5/LHW using the GENIST regulatory network inference algorithm (de Luis Balaguer et al. 2017). This revealed six distinct networks, where, intriguingly, *DOF2.1* was a central hub that was activated downstream of TMO5/LHW 3 hours after *dGR* induction (II, Fig. S2, Data S2). The analysis did not place *DOF2.1* directly downstream of TMO5, but instead downstream of the cytokinin response regulator ARR12 (II, Data S2), suggesting that *DOF2.1* is activated by cytokinin signaling instead of directly by TMO5/LHW. Due to the role of *PEARs* in regulating periclinal divisions, we decided to investigate the function of *DOF2.1* further.

We began by analyzing the transcriptional pattern of *DOF2.1* (*pDOF2.1::GUS-GFP*) in embryos and seedlings. *DOF2.1* transcription begins in the *ult* cells during the early heart stage and is observed in the embryonic root during the torpedo stage (II, Fig. 2A-C). In the postembryonic root tip, strong expression was observed in the XPP, in OPC cells next to XPP, and in endodermal cells next to the XPP (II, Fig. 2D-E). The DOF2.1 protein localization pattern (*pDOF2.1::DOF2.1-YFP*) was identical to the transcriptional pattern, indicating that DOF2.1 is probably not mobile, unlike the PEARs (II, Fig. 2F-G). Furthermore, our expression analysis suggested that DOF2.1 and the PEARs have no overlap in expression.

To confirm that DOF2.1 functions downstream of TMO5/LHW, we analyzed the *DOF2.1* transcriptional pattern in the *dGR* line and the *tmo5 t5l1* double mutant. In the *dGR* line, *pDOF2.1* is ectopically expressed outside of the vasculature, whereas expression was reduced in the *tmo5 t5l1* double mutant, suggesting that *DOF2.1* transcription is activated by TMO5/LHW (II, Fig. 2H, I, M, N). However, the ectopic expression in the *dGR* line did not extend to the vascular tissue, including the phloem, inner procambial cells and metaxylem, despite the *RPS5A* promoter and cytokinin signaling (*pTCSn::ntdTomato*) being active in these cells after *dGR* induction (II, Fig. 2J). Thus, there is likely an inhibitory mechanism excluding *DOF2.1* expression from these cells, which could be mediated by HD-ZIP III.

To test whether *DOF2.1* acts downstream of cytokinin as well as TMO5/LHW, we performed qRT-PCR after cytokinin treatment. Cytokinin rapidly induced *DOF2.1* transcription in the roots of wild type seedlings, whereas this induction was abolished in the *wol* mutant (II, Fig. 2O-P). This is consistent with earlier reports that the B-type cytokinin RRs *ARR1*, *ARR10* and *ARR12* directly bind to the *DOF2.1* promoter (Xie et al. 2018). Furthermore, *DOF2.1* expression was previously shown to be upregulated upon *ARR10* induction and after cytokinin treatment in seedlings (Bhargava et al. 2013, Zubo et al. 2017). Together with our findings, this strongly suggests that *DOF2.1* expression is at least partly regulated by cytokinin.

4.8 DOF2.1 regulates periclinal division of the outer procambial cells

To investigate whether DOF2.1 regulates periclinal cell division, we first analyzed the phenotype of the *dof2.1* single mutant. *dof2.1* does not exhibit any obvious reduction in the number of vascular cell files (II, Fig. 3A-C). We suspected that the reduction of *DOF2.1* might be compensated for by enhanced expression of two PEARs, *DOF6* and *TMO6*, which are the closest homologs of *DOF2.1* (II, Fig. S3E). We therefore measured their expression level in the *dof2.1* mutant and found *DOF6* to be significantly elevated compared with wild type (II, Fig. 3D). This suggests that compensation could be responsible for the lack of a phenotype in *dof2.1*, although we did not analyze the expression patterns of these genes.

To overcome the potential compensation and the lack of complete silencing in the *dof2.1* T-DNA insertion line, we utilized the CRISPR/Cas9 genome editing technology to generate a *dof2.1-2 tmo6-1 dof6-2* triple mutant. We then quantified the vascular cell number in the triple mutant and found significantly fewer cell files than in wild type (II, Fig. 3C, E). To further dissect the effect of each gene, we quantified the total procambial cell number as well as OPC and IPC cell number in single, double and triple mutants. In *dof2.1-1*, we observed a significantly reduced number of OPC cells, while the IPC cell number was unaffected (II, Fig. 3M, Fig. S3J). However, there was no reduction in either OPC or IPC cell numbers in another allele, *dof2.1-2*, although *dof2.1-2* enhanced the phenotype of the *tmo6-1 dof6-2* double mutant. These observations suggest that both of the *dof2.1* alleles are likely to be true loss-of-function mutants. On the other hand, multiple *dof6 tmo6* double mutant combinations had specifically reduced IPC cell numbers (II, Fig. S3J). Furthermore, we observed fewer cells in the embryonic root of the *dof2.1-2 tmo6-1 dof6-2* triple mutant compared with wild type (II, Fig. S3H).

To investigate whether *DOF2.1* is sufficient to induce periclinal cell division, we overexpressed *DOF2.1* in an inducible manner (*pRPS5A::DOF2.1-GR*) and observed ectopic periclinal divisions in this line after DEX treatment (II, Fig. 4E-G, S3I). Whereas *TMO5/LHW* overexpression led to phenotypes resembling those of mutants with elevated cytokinin levels, including protoxylem defects, inducible overexpression of *DOF2.1* had no effect on protoxylem development, which supports the notion that *DOF2.1* specifically regulates cell proliferation downstream of cytokinin (II, Fig. 4H-J, S3C-D). Additional protoxylem files were observed in plants constitutively overexpressing *DOF2.1* (*pRPS5A::DOF2.1*), likely due to overproliferation of early xylem cells. Furthermore, *DOF2.1-GR* overexpression suppressed the cell division phenotypes of the *tmo5 tmo5-like1* and *lhw* mutants (II, Fig. 4K).

Taken together, these results suggest that *DOF2.1* acts downstream of TMO5/LHW-dependent cytokinin biosynthesis to control periclinal division of the OPC cells, which is the domain where *DOF2.1* is expressed in the vasculature and from which PEAR expression is largely absent. Thus, DOF2.1 and the PEARs seem to control procambial division in separate domains, and together they are responsible for activating all procambial divisions, although DOF2.1 functions in a smaller domain than the PEARs.

4.9 SMXL subclade 2 members are expressed in early phloem and procambium

In original publication I, we identified *SMXL3* as a putative direct target of PEAR2 that is able to induce periclinal cell division. The SMXL family is known to mediate strigolactone and karrikin signaling, and it consists of eight members that are distantly related to the heat responsive gene *HSP101/HOT1* (Stanga et al. 2013). Strigolactones are endogenous phytohormones involved in several developmental processes, including shoot and root branching (Borghi et al. 2016). Karrikins, on the other hand, are exogenous compounds found in smoke that promote seed germination (Waters et al. 2014). The response to these hormones is mediated by the F-box protein MORE AXILLARY GROWTH 2 (MAX2), which is part of the SKP1-CUL1-F-BOX (SCF) protein ubiquitin ligase complex (Nelson et al. 2011). Strigolactones and karrikins are perceived by the D14 and KAI2 receptors, respectively, leading to the degradation of different target repressors of the SMXL family in a MAX2-SCF dependent manner (Soundappan et al. 2015).

SMXL3 belongs to SMXL subclade 2, which consists of *SMXL3*, *4* and *5* (III, Fig. 1A). In order to characterize SMXL subclade 2, we analyzed the transcriptional patterns of the three members in detail. *SMXL3* (*pSMXL3:YFPer*) expression was most abundant in the root vasculature, with only weak expression in the cotyledon veins. *SMXL4* and *SMXL5* (*pSMXL4:YFPer* and *pSMXL5:YFPer*) showed strong expression in the root vasculature and also in the veins of the cotyledons and leaf primordia (III, Fig. S1F-N). In the RAM, all three gene were expressed strongly in the phloem as early as the initial stage (III, Fig. G-H). *SMXL3* was broadly expressed in the phloem and procambium and was also present in the pericycle. *SMXL5* was expressed broadly in phloem and procambium in a pattern similar to *SMXL3* but excluding the pericycle. *SMXL4* expression was more specific, limited to the SE and its surrounding cells. For comparison, we analyzed the transcriptional pattern of another SMXL gene, *SMAX1* (*pSMAX1:YFPer*), which was observed in the vascular tissue and columella cells. However, the signal was very weak in the phloem/procambium of the root tip (III, Fig. 1G-H). Thus, *SMXL3, 4* and *5* are all highly active in the root vasculature due to abundant transcription in early phloem/procambium cells, unlike *SMAX1*. The overlapping expression patterns suggest that *SMXL3, 4* and *5* could act redundantly.

4.10 *SMXL3, 4* and *5* are essential for phloem formation

To unravel the function of *SMXL3*, *4* and *5*, we examined their loss-of-function phenotypes. The single mutants of all three loci did not show any apparent phenotype, whereas all of the double mutants (*smxl3 smxl4*, *smxl3 smxl5* and *smxl4 smxl5*) had a shorter root than wild type. Notably, the *smxl3 smxl4 smxl5* triple mutant was seedling lethal, although in early development it mirrored the phenotype of the double mutants. In contrast, no root growth phenotype was observed in mutants of other *SMXL* subclades (*smax1 smxl2* double or *smxl6 smxl7 smxl8* triple mutants) (III, Fig. 1B-F). These results indicate that *SMXL3, 4* and *5* redundantly control root growth in a dose-dependent manner. In order to determine

the effect on RAM size, we counted the number of meristematic cortex cells in 2-10 day old *smxl4 smxl5* double mutants. This revealed that the meristem was the same size as in wild type in 2-day old plants but was decreased to half and one-fifth the wild type size after 5 and 10 days of growth, respectively (III, Fig. 2A-G).

Since *SMXL3*, *4* and *5* are already abundantly expressed in the phloem during the initial stage, we hypothesized that they could be involved in regulating phloem fate. Furthermore, decreased RAM size has previously been associated with phloem defects (Depuydt et al. 2013, Rodriguez-Villalon et al. 2015). To investigate whether phloem development is affected in the *smxl4 smxl5* double mutant, we used confocal and serial block face scanning electron microscopy (SBEM) (Denk and Horstmann 2004). In wild type roots, the first periclinal division of the phloem/procambium precursor cell results in one procambium and one PSE precursor cell, whereas the second periclinal division results in the formation of the PSE and PSE-IN cell files. Confocal images of root cross sections from 2 days old seedlings showed that although the number of cells in the stele was unaltered, the second periclinal division was often delayed in the mutant compared with wild type. In addition, the characteristic cell wall thickening of differentiated PSE cells, which is marked by intensified propidium iodide staining, did not occur in the mutant (III, Fig. 2H-K, S1O-Q). With SBEM, it is possible to reconstruct a highly detailed 3D image from ultrathin sections. These images revealed that SE enucleation does not occur in *smxl4 smxl5* double mutants and the cytosolic density remains high, unlike in wild type (III, Fig. 3A-E). We also analyzed the expression pattern of *pCALS7:H2B-YFP*, which marks the nuclei of PSE cells just before enucleation (Kondo et al. 2015, 2016). In wild type, H2B-YFP is localized to the nucleus until the PSE undergoes enucleation, at which point the YFP signal is scattered and eventually disappears. However, in *smxl4 smxl5* double mutants, *pCALS7:H2B-YFP* is not expressed in PSE (III, Fig. 3F-G). Our observations show that phloem formation is severely compromised in *smxl4 smxl5* roots, indicating a major role for *SMXL4* and *5* in phloem development.

To investigate the role of *SMXL4* and *5* in phloem development in a context-independent manner, we employed the VISUAL system (Kondo et al. 2016) to assess phloem trans-differentiation in the *smxl5*, *smxl4 smxl5,* and *smxl3 smxl4 smxl5* mutants. Before VISUAL induction, the phloem strands in the cotyledons of the *smxl5* single and *smxl4 smxl5* double mutants were indistinguishable from wild type when stained with aniline blue (AB) to mark the sieve plates. However, after a 6-day VISUAL induction, the level of AB staining was significantly lower in the mutants compared with wild type, indicating less trans-differentiation in the mutants (III, Fig. 3 H-M). Furthermore, the transcription of genes promoting phloem formation was induced significantly less than in wild type cotyledons (III, Fig. 3N). AB staining was completely absent in 36% of 10-day old *smxl3 smxl4*/+ *smxl5* plants and 82% of *smxl3 smxl4 smxl5* plants (III, Fig. 3R-T). In contrast, differences in TE differentiation were not observed, based on autofluorescence observation and measurement of the induction of xylem-related genes (III, Fig. 3N, S2A-F). We therefore conclude that *SMXL3*, *4* and *5* redundantly regulate the early stages of phloem development by promoting the formative periclinal division of the PSE precursor into PSE

and PSE-IN cells, resulting in the formation of PSE and MSE cell files and promoting SE identity. The reduced root growth and meristem size are likely due to defects in phloem development, especially as the shrinkage of the RAM in the mutants starts after 2 days of growth, which is when root growth becomes dependent on functional phloem transport.

Phloem flow is compromised in the smxl4 smxl5 double mutant

In order to investigate the systemic effects of compromised root phloem development, we grafted wild type shoots onto *smxl4 smxl5* double mutant roots and vice versa. As a control, shoots were grafted onto roots of the same genotype. Root growth of the *smxl4 smxl5* rootstock was not rescued by grafting with a wild type scion (wt/*smxl4 smxl5*), as these roots grew similarly to mutant rootstocks grafted with mutant scions (*smxl4 smxl5*/*smxl4 smxl5*). Likewise, grafting a mutant scion onto wild type rootstock (*smxl4 smxl5*/wt) resulted in a growth rate similar to wild type self-grafts (wt/wt) (III, Fig. S2G-H). However, grafts onto mutant rootstocks (wt/*smxl4 smxl5* and *smxl4 smxl5*/*smxl4 smxl5*) had smaller leaves and higher sucrose levels than grafts onto wild type rootstocks (wt/wt and *smxl4 smxl5*/wt) (III, Fig. S2I-J). Measurement of sugar concentrations in phloem exudates from wild type, *smxl5* and *smxl4 smxl5* showed that sugar export from mature leaves was not reduced in either mutant compared with wild type (III, Fig. S2K). We then assessed the state of phloem flow by grafting 5-day old wild type scions expressing *GFP* under the CC-specific *SUCROSE TRANSPORTER 2* promoter (*pSUC2:GFP*) (Vaten et al. 2011) onto wild type or *smxl4 smxl5* rootstock. As expected, GFP unloading to the root tip was impaired when wild type scions were grafted onto *smxl4 smxl5* rootstocks compared with grafts onto wild type rootstocks (III, Fig. S2L-M). The GFP signal was weak but present in the tips of lateral roots, which were longer than the primary root, whereas it was completely absent in the shorter primary root (III, Fig. SN-O). Taken together, these results show that the *smxl4 smxl5* double mutant has defects in phloem formation in the RAM which result in impaired phloem unloading to the root tip, reducing RAM size and root growth. The observed leaf phenotypes are a result of these defects in root phloem formation, as the leaf phenotype is dependent on the root but not the shoot genotype.

SMXL4 and SMXL5 function independently of strigolactone/karrikin signaling

To determine whether *SMXL3*, *4* and *5* mediate strigolactone/karrikin signaling, similar to other SMXLs, we examined whether *MAX2* is required for *SMXL3*, *4* and *5* signaling. To this end, we analyzed root length, RAM size, SE precursor division and SE formation in *max2* and in the *smxl4 smxl5 max2* triple mutant and compared them to the wild type and *smxl4 smxl5* double mutant, respectively. No differences were observed, suggesting that *SMXL4* and *SMXL5* promote phloem development independently of strigolactone/karrikin signaling mediated by *MAX2* (III Fig. S3).

SMAX1 was identified as a suppressor of multiple *max2* phenotypes (Soundappan et al. 2015). To investigate whether *SMAX1* can functionally replace *SMXL5*, we expressed

pSMXL5:SMXL5-YFP and *pSMXL5:SMAX1-YFP* in the *smxl4 smxl5* double mutant background. We observed that either *SMXL5* and *SMAX1* could suppress the double mutant phenotype (III, Fig. 4A-B). Rac-GR24, a synthetic strigolactone that induces strigolactone signaling as well as karrikin signaling, enhances the interaction of MAX2 with its target suppressors, including SMAX1, leading to their degradation (Li et al. 2016). We observed that the suppression of the *smxl4 smxl5* double mutant phenotype by *SMAX1* expression was reversed by growing the mutant on medium supplemented with rac-GR24. By contrast, rac-GR24 supplementation did not affect the suppression of the *smxl4 smxl5* double mutant phenotype by *SMXL5* expression (III, Fig. 4A-B). Finally, we assessed the protein stability of SMXL5-YFP, SMAX1-YFP, SMXL4-YFP and SMXL3-YFP after rac-GR24 supplementation and observed that only SMAX1-YFP was degraded, while SMXL5-YFP, SMXL3-YFP and SMXL4-YFP levels were unaltered. Together, these results indicate that unlike *SMAX1*, *SMXL3*, *4* and *5* likely do not mediate MAX2-dependent strigolactone/karrikin signaling and thus regulate phloem development independent of the MAX2-dependent strigolactone/karrikin pathway.

5 Concluding remarks

Previous studies have established that the interplay of auxin and cytokinin is fundamental for correct vascular patterning in the *Arabidopsis* root. However, the gene regulatory network downstream of the hormones regulating procambial development has remained unknown. Our results demonstrate that the periclinal cell division activity in the root vasculature is not a simple reflection of the hormone domains, with high cytokinin and high auxin signaling domains resulting in high and low cell division activity, respectively. Instead, procambial cells are a heterogeneous population in which the PSE and PSE-LN cells located next to pericycle contribute most of the divisions while the central metaxylem and inner procambial cells remain quiescent. The observed pattern of cell division depends crucially on an additional positional cue, the mobile miRNA165/166 that moves from the ground tissue to the stele. The high miRNA concentration together with high cytokinin response defines the location of high periclinal cell division activity at the PSE and the PSE-LN position.

We further show that the regulators downstream of these positional cues consist of two sets of interacting transcription factors; the well-known HD-ZIP IIIs, which act downstream of auxin, interact with the newly-identified mobile PEAR transcription factors, which act downstream of cytokinin. The HD-ZIP III and PEAR proteins form opposing gradients in the vascular tissue that overlap at the PSE-IN position. HD-ZIP III expression is not only activated by auxin in the xylem axis but also by the PEARs in the PSE-IN, while HD-ZIP III mRNAs are eliminated from the PSE by miRNA regulation. In a complementary fashion, *PEAR* transcription is promoted by cytokinin signaling at the PSE, and the proteins move to the surrounding cells (PSE-LN and PSE-IN), while HD-ZIP IIIs inhibit *PEAR* transcription at the PSE-IN and further movement of PEAR proteins to inner procambial cells. These interactions result in opposing gradients of HD-ZIP III and PEAR activity which establish a sharp boundary between dividing and quiescent cells within the procambium, a conclusion supported by modeling experiments. How exactly the HD-ZIP IIIs inhibit PEAR movement remains an interesting question for future studies.

We also found that the cytokinin-induced gene *DOF2.1* activates divisions in the outer procambial cells, though these make only a minor contribution to the total procambial cell file number. It therefore seems that DOFs, activated by cytokinin, are the major switches turning on all procambial periclinal divisions. The identification of *DOF2.1* offered further insight into the heterogeneity of the procambial cell population, where different DOFs contribute to specific divisions. The *DOF2.1* expression pattern and its cell-autonomous activity distinguish it from the *PEARs*. Our analyses suggest that the expression of *DOF2.1* is actively repressed in the central stele, similar to the expression of *PEARs*. It would thus be interesting to investigate whether the HD-ZIP IIIs also repress *DOF2.1* expression. Although both the *PEARs* and *DOF2.1* are activated by cytokinin, *DOF2.1* expression is excluded from the phloem, suggesting that these genes have different upstream regulators in the vasculature. Furthermore, the *DOF2.1* and *LOG4* expression patterns are remarkably

similar, but they are not identical to the *TMO5/LHW* expression domain, suggesting *DOF2.1* and *LOG4* could have another upstream regulator in common.

The analysis of PEAR targets revealed that the PEARs play a significant role in promoting procambial cell identity in addition to controlling cell division activity. Promoter reporter analysis demonstrated that the targets we identified varied in their expression pattern within the procambium/phloem domain, which is in line with the idea that the procambium is a diverse population of cells. Furthermore, the procambium genes seem to be actively suppressed in the central domain, similar to the *PEARs* and *DOF2.1*, which cannot be attributed to the absence of PEAR expression since some of these genes are active even in the absence of the PEARs. This raises the possibility that HD-ZIP IIIs could have a major role in repressing not only the PEARs but also a large number of procambial cell-identity genes in the central domain. A cluster of PPP-expressed genes was also identified downstream of the PEARs, which suggests that the movement of PEARs to this domain has developmental significance and further emphasizes the non-cell autonomous action of the PEARs in conferring identity to the surrounding cells. Characterizing these PPP genes could potentially reveal regulators important to the recently discovered unloading function of PPP cells. Furthermore, as a result of the downstream analysis, we discovered that *SMXL3* is a regulator of periclinal cell division that is likely directly activated by PEAR2. One of the most intriguing questions for the future is the mechanism that determines cell division orientation downstream of the *PEARs*, *DOF2.1* and *SMXL3*.

Our work also identified *SMXL3*, *4* and *5* as completely novel regulators of phloem identity that are essential for all aspects of phloem development. *smxl4 smxl5* double mutants display a general failure of SE formation. This suggests that SMXLs act very early, a conclusion supported by their very early expression, which can already be seen at the initial stage. The finding that only *SMXL3* acts downstream of the PEARs and also plays a role in periclinal divisions indicates that *SMXL3*, *4* and *5* have differences in their function. This is also supported by the broader expression pattern of *SMXL3* and its phylogenetic distance from *SMXL4* and *5*. The convergence of the early phloem identity and periclinal cell division pathways is highly interesting and confirms the interconnectedness of these two processes. A recent study showed that SMXL4 and 5 are negatively regulated on a translational level by the RNA-binding protein JULGI, which thus negatively regulates phloem development (Cho et al. 2018). Whether translation of SMXL3 is also regulated remains to be determined.

All in all, our data revealed a complex network operating downstream of the hormonal and miRNA inputs that pattern procambium. We uncovered a dual regulatory mechanism acting in boundary formation in which HD-ZIP IIIs inhibit both *PEAR* transcription and PEAR movement. This increases our understanding of the nature of the feedback mechanisms that operate during these patterning processes. Moreover, our results show that the root apical meristem contains a strictly controlled radial meristem which is organized by actively dividing PSE cells on either side of the vascular cylinder that direct the neighboring cells touching the pericycle to divide. Thus, the PSE cells prime cambial growth by organizing the production of the procambial cells that will later become the cambium.

It was recently demonstrated that vascular cambium divisions are initiated next to the xylem and that these divisions are organized by the quiescent xylem cells with high auxin and HD-ZIP III levels (Smetana et al. 2019). Therefore, radial growth during primary and secondary development is organized from different positions, but both processes rely on the exchange of positional information. An intriguing question for the future is to understand how the strictly quiescent xylem-associated internal procambial cells begin to divide as the cambium is activated and whether the *PEAR* genes also play a role during secondary development. Together, these findings provide the most comprehensive understanding yet of vascular tissue formation and radial growth. This knowledge can potentially be used to increase the biomass of trees and storage organs, which has significant economic and environmental implications.

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