# Novel Regulators of Vascular Development in *Arabidopsis thaliana*

Robertas Ursache

Faculty of Biological and Environmental Sciences
Department of Biosciences
Division of Plant Biology
and
Institute of Biotechnology
and
Integrative Life Science Doctoral Program
University of Helsinki

ACADEMIC DISSERTATION

To be presented for public examination, with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki, in the auditorium 1041 at Viikki Biocenter (Viikinkaari 5), on December 5th 2014, at 12 o'clock noon.

Helsinki 2014

**Supervised by**

Professor Yrjö Helariutta
Institute of Biotechnology
Department of Biosciences
University of Helsinki

**Members of the follow up group**

Dr. Ari Pekka Mähönen
Institute of Biotechnology
Department of Biosciences
University of Helsinki

Dr. Mikael Brosché
Institute of Biotechnology
Department of Biosciences
University of Helsinki

Dr. Shunsuke Miyashima
Nara Institute of Science and Technology
Japan

**Reviewed by**

Professor Hely Häggman
Department of Biology
University of Oulu

Dr. Kristiina Himanen
Department of Agricultural Sciences
University of Helsinki

**Opponent**

Professor Catherine Bellini
Department of Plant Physiology
Umeå University
Sweden

**Custos**

Professor Yrjö Helariutta
Institute of Biotechnology
Department of Biosciences
University of Helsinki

ISSN 2342-317X (Online)
ISBN 978-951-51-0464-9 (paperback)
ISBN 978-951-51-0465-6 (PDF)
http://ethesis.helsinki.fi
Hansaprint Helsinki 2014

“When you come out of the storm, you won’t be the same person who walked in. That’s what this storm’s all about” – Haruki Murakami

# TABLE OF CONTENTS

# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles. The publications are referred to in the text by Roman numerals. Publications are reprinted with the kind permission of their copyright holders.

I. **Ursache R**, Miyashima S, Chen Q, Vatén A, Nakajima K, Carlsbecker A, Zhao Y, Helariutta Y, Dettmer J. (2014) Tryptophan-dependent auxin biosynthesis is required for HD-ZIP III-mediated xylem patterning. Development 141, 1250–1259.

II. Dettmer J[1], **Ursache R[1]**, Campilho A[1], Miyashima S, Belevich I, O'Regan S, Mullendore DL, Yadav SR, Lanz C, Beverina L, Papagni A, Schneeberger K, Weigel D, Stierhof Y-D, Moritz T, Knoblauch M, Jokitalo E, Helariutta Y. (2014) CHOLINE TRANSPORTER-LIKE1 is required for sieve plate development to mediate long-distance cell-to-cell communication. Nature Communications 5, 4276.

III. **Ursache R**, Dettmer J, Helariutta Y. Local root-based auxin biosynthesis promotes HD-ZIPIII expression and metaxylem specification. (Manuscript).

[1] These authors contributed equally to this work

## AUTHOR'S CONTRIBUTION

I. RU identified the *TSB1* gene through positional cloning, generated the transgenic *pTSB1::TSB1-YFP*, *pTSB1::GFP/GUS*, *pTSB1::iaaH*, *pWOX5::iaaH* and *pCRE1::PHB* lines, histological analyses, genetic crosses, confocal analysis of the molecular markers described in the manuscript. RU wrote the manuscript together with YH, JD and SM.

II. RU identified the *CHER1* gene through the positional cloning, performed the phylogenetic analysis of CHER1, generated most of the genetic crosses, constructed *pCHER1::CHER1-YFP*, *pEPM::CHER1(cDNA)-YFP* transgenic lines, contributed to analyzing the molecular markers with confocal microscope and performed the histological analysis of wild-type, *cher1* and *xipotl* mutant roots. RU wrote the manuscript together with JD and YH.

III. RU will be the first author in this manuscript, he generated the *pTSB2::GFP-GUS* and *pTSB3::GFP-GUS* transgenic lines to show the root-specific expression patterns of *TSB2* and *TSB3* genes. RU analyzed the combinatorial effect of auxin transport and auxin biosynthesis inhibitors on metaxylem and protoxylem formation. RU plays a major role in writing this manuscript.

# ABBREVIATIONS

| | |
|---|---|
| HD-ZIPIII | class III homeodomain-leucine zipper |
| SE | sieve elements |
| CC | companion cells |
| DT | desmotubule |
| EMS | ethylmethane sulfonate |
| ER | endoplasmic reticulum |
| miRNA/miR | microRNA |
| PD | plasmodesmata |
| TGN | trans-Golgi network |
| PX | protoxylem |
| MX | metaxylem |
| PM | plasma membrane |
| QC | quiescent center |
| CFDA | carboxyfluorescein diacetate |
| KYN | L-kynurenine |
| Trp | tryptophan |
| NPA | 1-N-Naphthylphthalamic acid |
| SBEM | serial block-face scanning electron microscopy |

# ABSTRACT

Plant vascular tissues are supporting and conductive tissues composed of two major components, xylem and phloem. These tissues transport water, food, hormones and minerals within the plant. In my thesis work, I used the *Arabidopsis* root as a model system to study vascular tissue formation.

The first part of my thesis work is focused on the formation of xylem, the water transporting tissue. In the *Arabidopsis* root, the xylem is organized as an axis of cell files with two distinct cell fates: the central metaxylem and the peripheral protoxylem. It has been previously reported that high and low expression levels of the class III HD-ZIP transcription factors promote metaxylem and protoxylem identities, respectively. In this work, we provide evidence that auxin biosynthesis promotes HD-ZIP III expression and metaxylem formation. We observed that plants with mutations in auxin biosynthesis genes, such as *trp2-12*, *wei8 tar2*, or the quintuple *yucca* mutant, as well as plants treated with a pharmacological inhibitor of auxin biosynthesis, show reduced expression of the HD-ZIP III genes accompanied by specific defects in metaxylem formation. We were able to induce a partial rescue of the metaxylem defects by introducing an endogenous auxin supply. In addition, some of the patterning defects can be suppressed by synthetically elevating HD-ZIP III expression in the stele of the *Arabidopsis* root.

The second part of my thesis work is focused on phloem tissue formation. Phloem is the tissue responsible for long-distance molecular transport and signaling. The conductive components of the phloem, the sieve elements, rely on specific junctions between the conducting cells in the form of highly perforated sieve areas. We identified mutations in the *CHER1* (*CHOLINE TRANSPORTER LIKE 1*) locus of *Arabidopsis* which result in altered phloem conductivity, reduced sieve pore density, and defects in sieve pore formation. *CHER1* encodes a member of a poorly characterized choline transporter-like protein family in plants and animals. We provide data showing that CHER1 facilitates choline transport, localizes to the trans-Golgi network, and is associated with the late stage of phragmoplast formation during cytokinesis. Interestingly, CHER1 has a sustained, polar localization in forming sieve plates, which is consistent with its function in the elaboration of the sieve areas.

# 1. INTRODUCTION

## 1.1 PLANT VASCULAR TISSUE SPECIFICATION

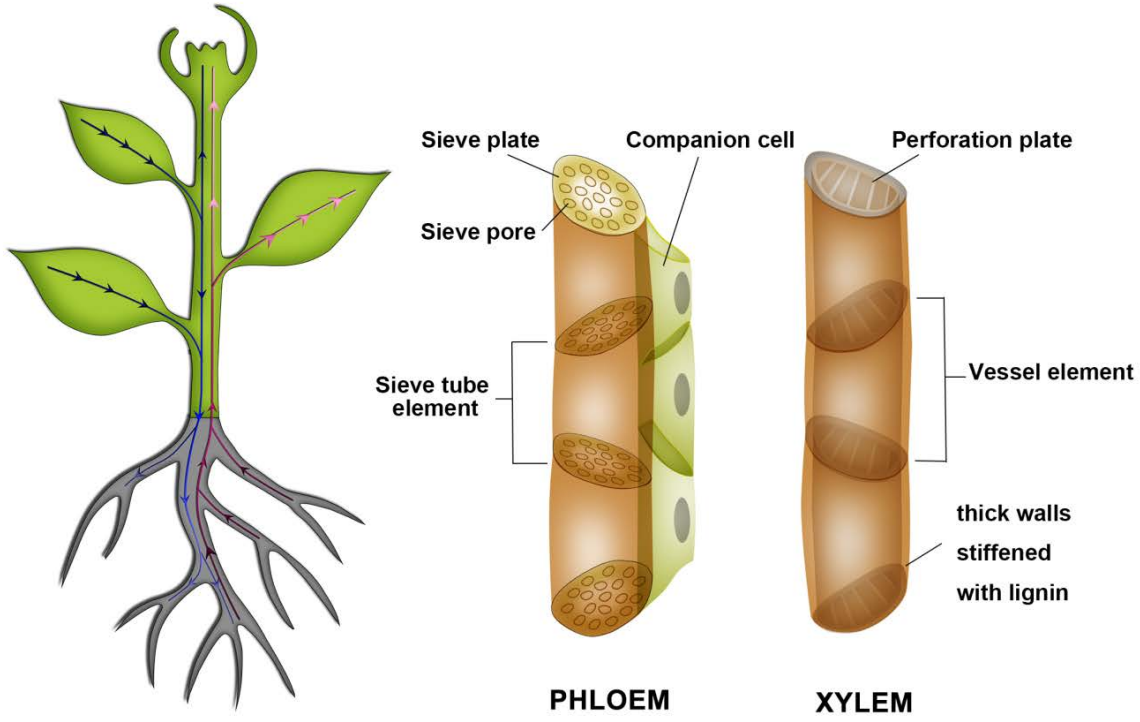


**Figure 1** Schematic representation of the plant vascular system. On the right sight a whole plant picture is presented with blue arrow indicating phloem transport and red arrows – xylem transport. On the right side the transporting elements of phloem and xylem are presented. Modified from Ursache et al, 2014.

As part of their adaptation to various environmental conditions, vascular plants have developed a specialized tissue system, the vascular system, which provides mechanical support and functions as a delivery system for various resources, such as water, mineral nutrients, sugars and amino acids, throughout the plant body. The vascular system connects all of a plant's organs, from the shoot to the root, and is composed of two tissue types, xylem and phloem (**Figure 1**). In addition, the vascular tissues serve as an efficient long-distance communication system, responsible for the

delivery of various signals to coordinate developmental and physiological processes at the whole-plant level (Lucas et al., 2013).

The development of vascular tissues starts during embryogenesis. At the early globular stage in *Arabidopsis* embryos, the cells located inside the protoderm divide and give rise to the ground tissue precursors and the vascular stem cell initials, the procambium. Later, at the late globular stage, four procambial cells undergo periclinal divisions resulting in the formation of the future pericycle and vascular primordium. Subsequently, during the late stages of embryonic development, the procambium continues to divide until it establishes a radial vascular pattern identical to the one observed in the post-embryonic primary root (De Rybel et al., 2014). As individual procambial cells undergo periclinal divisions, they give rise to the procambium tissue, which divides to produce specialized xylem and phloem. At the same time, some of the procambium cells remain undifferentiated. These cells, located between the differentiating xylem and phloem, ensure the continuous formation of vascular tissues in rapidly growing organs such as young roots, stems, and leaves during primary growth.

The organization of the vascular tissues is distinct in each organ of the postembryonic plant body. In the root, these tissues form a vascular cylinder, a structure that is surrounded by the radial tissues – pericycle, endodermis, cortex and epidermis. Xylem develops in the central axis and branches toward the pericycle, whereas the two poles of phloem develop perpendicular to the xylem axis (**Figure 2**).

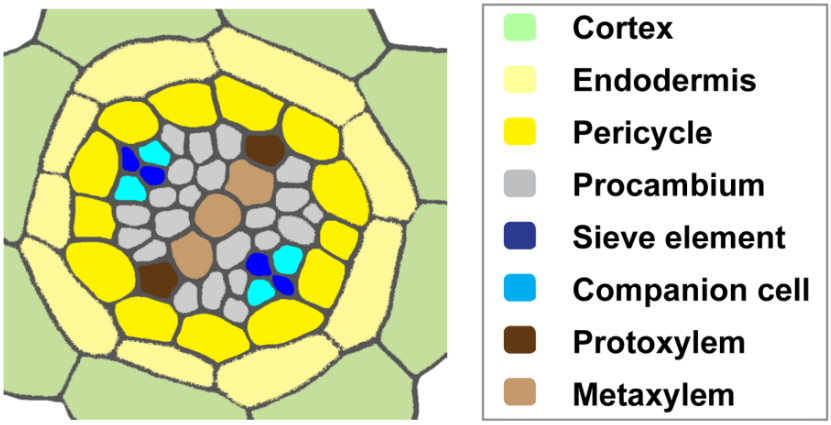


**Figure 2** Schematic representation of the *Arabidopsis thaliana* root vascular cylinder.

In the aboveground parts of a seedling, the vascular initials first form and start to differentiate in the cotyledon mid-veins and cotyledonary nodes; only later does the vasculature progress towards

the hypocotyl and roots to form a continuous network of functional vascular cells (Bauby et al., 2007).

Two distinct phases of growth and development are characteristic for gymnosperms and many dicotyledons. During primary development, primary xylem and phloem differentiate from cells that are derived from the procambium. At this stage, plants increase their biomass mainly in the apical dimension. After the primary vascular tissues are formed, cambium tissue generated from the procambium promotes the formation of secondary xylem and phloem, which undergo secondary growth to increase the biomass of stems and roots in the radial dimension (Ursache et al., 2013).

## 1.2 XYLEM TISSUE SPECIFICATION AND DIFFERENTIATION

Xylem, the water transporting tissue, differentiates from the procambium during primary development, as mentioned above. In the *Arabidopsis* root, 4–5 cell files of xylem vessels are formed in the central axis of the vascular cylinder (**Figure 2**). During differentiation, the xylem vessels develop lignified secondary cell walls and undergo programmed cell death. This leaves behind a sturdy, lignified and hollow cell wall structure suited for water conduction (Fukuda, 1997; Oda and Fukuda, 2012). Two types of xylem vessels form in the root – protoxylem and metaxylem. Protoxylem strands, which develop first, form at the periphery of the xylem axis and have a characteristic spiral pattern of lignin deposition. The metaxylem is specified later in the center of the axis; it has wider vessels and is characterized by a reticulate pattern of lignin deposition. The lignin strengthens the xylem cell walls for water transport and provides support to the stems and roots.

Xylem development and specification in the *Arabidopsis* root is hormonally and transcriptionally regulated (Kondo et al., 2014; Schuetz et al., 2013).

The plant hormone cytokinin has been shown to be involved in the specification of protoxylem vessels in *Arabidopsis* roots (Mahonen et al., 2000). The reduction of cytokinin by expressing a cytokinin degradation enzyme results in the formation of extra protoxylem (Mahonen et al., 2006). This indicates that the number of protoxylem vessels depends on cytokinin levels. Cytokinin signaling pathway is initiated by the receptors ARABIDOPSIS HISTIDINE KINASE 2–4 (AHK2, AHK3, and CRE1/AHK4/WOL) (Kieber and Schaller, 2014). AHK mutants develop extra protoxylem files due to the reduced cytokinin sensitivity (Kondo et al., 2011; Mahonen et al., 2006).

Furthermore, the *wol* mutant exhibits a more severe xylem phenotype, in which phloem cells are completely lost and only protoxylem vessels are formed in the root stele (Mahonen et al., 2006; Scheres et al., 1995). As described previously, the cytokinin signal is transduced via phosphotransfer from AHKs to ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN (AHP). The quintuple AHP mutant *ahp1 ahp2 ahp3 ahp4 ahp5* exhibits the extra protoxylem phenotype similar to that of *ahk*. (Hutchison et al., 2006). All together this indicates that cytokinin negatively regulates protoxylem specification via AHKs and AHPs. On the other hand, high level of another important plant hormone, auxin, directly up-regulates the expression of AHP6, a negative regulator of cytokinin signaling, in the protoxylem vessel position via auxin-responsive elements in its promoter. AHP6 differs from other AHP's, because it lacks the conserved histidine residue which is required for phosphotransfer (Mahonen et al., 2006). The inhibition of polar auxin transport blocks AHP6 expression in the protoxylem vessel position, resulting in the loss of protoxylem vessels (Bishopp et al., 2011a). PIN-mediated polar auxin transport induces AHP6 expression, which in turn attenuates cytokinin signaling at the protoxylem position (Bishopp et al., 2011b).

The regulation of xylem development and specification involves the action of *SHORT ROOT* (*SHR*) and *SCARECROW* (*SCR*), which are members of the GRAS family of transcription factors, and the Class III HD-ZIPs, *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*), *ATHB8* and *CORONA/ATHB15*. Carlsbecker et al (2010) showed that SHR proteins produced in the root vascular cylinder move to the endodermis layer, where they activate the expression of SCR. SHR and SCR then form a complex which activates two microRNAs (miR), miR165 and miR166, which are expressed in the endodermis. Subsequently, miR165/166 move back into the stele to target the mRNAs of stele-specific HD-ZIP III TFs and control the specification of meta- and proto-xylem (Carlsbecker et al., 2010; Miyashima et al., 2011). The lack of miR-mediated mRNA degradation leads to increased levels of HD-ZIP III expression and the formation of metaxylem in the place of protoxylem. By contrast, in multiple HD-ZIP III knockout mutants, the central metaxylem is replaced by protoxylem. Moreover, quintuple HD-ZIP III mutants fail to develop any xylem in the root (Carlsbecker et al., 2010). Together, these data indicate that high and low expression levels of the HD-ZIP III transcription factors determine the metaxylem and protoxylem identities, respectively.

The essential role of the HD-ZIP III genes in xylem formation has also been demonstrated in other organisms. Expression of the HD-ZIP III genes is induced during xylogenesis in *Zinnia* mesophyll cell cultures (Demura et al., 2002; Ohashi-Ito et al., 2002; Ohashi-Ito and Fukuda, 2003). In poplar, the ectopic expression of a microRNA-resistant version of the *Populus REVOLUTA* gene induces xylem formation towards the stem periphery and ectopic cambium formation in cortex layers (Robischon et al., 2011).

Recently it was reported that the boundary between the procambium domain and xylem axis is determined by transcription factors called AT-HOOK MOTIF NUCLEAR LOCALIZED PROTEINs (AHLs). The *ahl3* and *ahl4* single mutants develop ectopic protoxylem vessels and ectopic metaxylem vessels in the place of procambium adjacent to the xylem axis. AHL4 is produced in the procambium and moves into the xylem axis where it is required for correct boundary formation between the procambium and the xylem (Zhou et al., 2013). *ahl* mutants exhibit altered auxin and cytokinin responses, but the relationship between these proteins and hormonal control of xylem formation is unclear.

Two NAC-domain transcription factors, *VASCULAR-RELATED NAC-DOMAIN6* (*VND6*) and *VND7*, have been shown to be the master regulators of xylem cell fate determination. *VND6* has been reported to promote the differentiation of metaxylem and *VND7* to promote protoxylem cell fate. Furthermore, the ectopic expression of *VND6* and *VND7* is able to switch the fate of various cells to metaxylem and protoxylem, respectively (Kubo et al., 2005; Ohashi-Ito et al., 2010; Yamaguchi et al., 2008). VND6 and VND7 directly up-regulate the genes involved in the processes essential for xylem tissue maturation, such as programmed cell death and secondary cell-wall thickening (Ohashi-Ito et al., 2010; Yamaguchi et al., 2011).

## 1.3 PHLOEM DEVELOPMENT

The phloem tissue in plants can be thought of as analogous to the networks of the circulatory and nervous systems found in animals. In plants, phloem is the tissue that carries photosynthetic products, hormones, proteins and miRNAs throughout the body. Primary phloem consists of conductive sieve elements (SE) and their neighboring companion cells (CC). At the interface between individual SE are the sieve plate areas with multiple sieve pores, perforations which facilitate the movement of various macromolecules (**Figure 3**).

Both SE and CC originate from phloem precursor cells in the procambium. However, the SE undergo dramatic changes in their morphology, unlike the CC. During maturation, the SE experience degradation of their nucleus, vacuoles, rough endoplasmic reticulum (ER) and Golgi. These changes all help to establish an efficient route for trafficking through the SE. Unlike xylem, SE cells remain living, as they retain a plasma membrane and some of their organelles, such as the smooth ER, mitochondria and plastids.

The maturation and survival of SE requires a close developmental and functional association with their neighboring CC (Esau, 1950; Sjolund, 1997; Wu and Zheng, 2003).

The CC and SE are connected with each other by numerous branched plasmodesmata (PD) located on the lateral cell walls in specialized areas known as lateral sieve areas. By traversing the wall between SE and CC, PD allow communication between them and supply the mature and enucleate SE with energy, assimilates and macromolecular compounds, such as proteins and RNA (Baker et al., 2013).

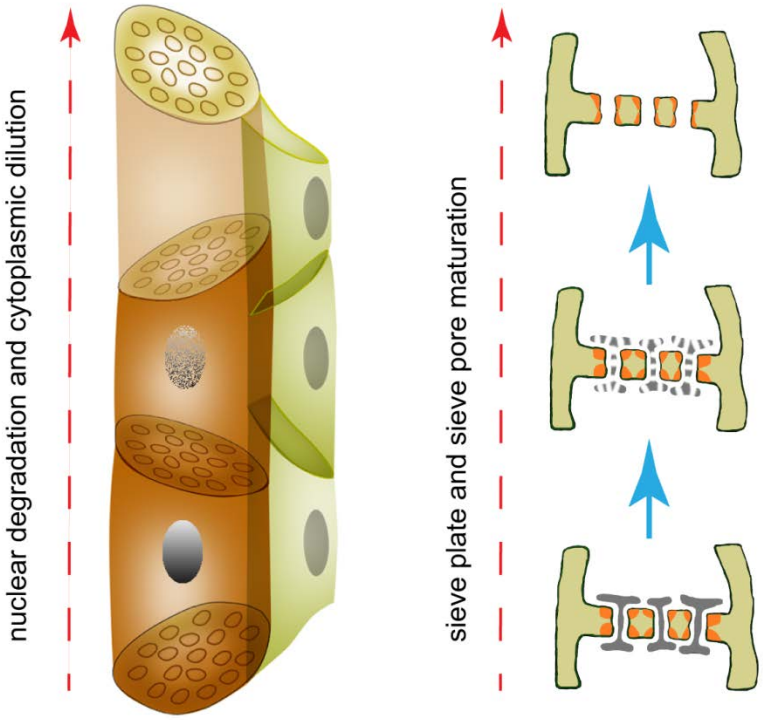


**Figure 3** A graphical image showing the developmental stages of phloem sieve element maturation; modified from Ursache et al (2014).

The PD develop in the newly forming walls of dividing SE and CC, though they can also form across existing walls. Each PD consists of a plasma membrane-lined channel at the center of which is found an appressed form of ER, the desmotubule. The space between the plasma membrane (PM) and the desmotubule (DT) is referred to as the cytoplasmic annulus or symplastic space. This space serves as a channel through which various molecules can diffuse from cell to cell. Proteins located on the ER lumen and plasma membrane divide the symplastic space into smaller microchannels which have the ability to dilate significantly in order to allow the passage of larger molecules (Benitez-Alfonso, 2014; Maule et al., 2011). It has also been proposed that the desmotubule may act as a pathway for the intercellular movement of certain molecules through PD, though it is not the main route for plasmodesmatal transport (Cantrill et al., 1999). Furthermore, certain proteins, such as chaperones, have been suggested to localize at the DT lumen, where they unfold the macromolecules passing through PD and allow them to fit through the narrow passage (Lucas et al., 2001).

PD also form in the sieve plates, where they connect the cytoplast of adjacent SE. During development, the PD in sieve plates undergo significant structural changes. One of the critical factors initiating these changes is callose, a β1,3-glucan which is normally deposited around the PD. During the late stages of SE development, when the nucleus and most of the cell's organelles are degraded, callose is removed from the necks of PD, leading to a significant increase of the PD lumen. Recent studies have demonstrated that two callose synthases, *CALS3* and *CALS7*, are involved in depositing callose at the neck region of the PD, thus regulating the PD size exclusion limit (Vaten et al., 2011; Xie et al., 2011).

Finally, the process of sieve pore formation is complete when the DT disintegrates and the pore becomes completely open. The exact mechanism of sieve pore formation from PD remains elusive and awaits further investigation. Several studies have provided evidence that the sieve pores are essential in determining the transport velocity of phloem contents and have a major impact on the resistance inside the sieve tube. It is predicted that phloem conductivity is highly dependent on the density, length and diameter of the sieve pores (Thompson, 2006; Thompson and Holbrook, 2003).

Phloem SE and CC differentiation is one of the least understood developmental processes in plants. One of the key players in phloem development is *ALTERED PHLOEM DEVELOPMENT* (*APL*), a MYB transcription factor which has been shown to play an essential role in SE and CC differentiation. Loss of *APL* function in the *apl* mutant results in the absence of phloem SE and CC

(Bonke et al., 2003). Additionally, *apl* mutants develop ectopic xylem vessel-like cells, hybrids between xylem and phloem, at the phloem poles (Truernit et al., 2008). Ectopic expression of *APL* in the vascular bundles inhibits the formation of xylem. Together, these findings indicate that *APL* possesses two roles: one is to promote phloem differentiation and the other is to repress xylem differentiation (Bonke et al., 2003). Recently a regulatory mechanism was discovered where APL is transcriptionally targeting another transcription factor, NAC45, which in turn orchestrates several phloem SE-specific processes, such as SE enucleation, perinuclear organelle clustering and cytosol degradation (Furuta et al., 2014).

Another important player involved in phloem differentiation is *OCTOPUS* (*OPS*). *OPS*, a polarly localized membrane-associated protein of unknown function, has been shown to be initially expressed in the provascular cells at the heart stage of embryogenesis. Later, during vascular tissue differentiation, it becomes restricted to the phloem cell lineage (Bauby et al., 2007; Truernit et al., 2012). *OPS* expression is detected earlier than *APL*, in the phloem and procambium initials near the QC. The loss of function of *OPS* results in reduced cotyledon vein complexity and discontinuous protophloem formation, leading to impaired long-distance transport. In *ops*, the file of protophloem cells with thickened cell walls is interrupted by cells that have not undergone the characteristic cell wall thickening. By contrast, overexpression of *OPS* leads to accelerated phloem differentiation and increased cotyledon vein complexity (Truernit et al., 2012). These phenotypes all indicate that *OPS* is involved in the process that leads to the differentiation of a continuous phloem network.

Another transcription factor that affects vascular stem cell activity and phloem formation was recently identified. *HCA2* (*HIGH CAMBIAL ACTIVITY2*) has been shown to be highly expressed in the cambium, phloem and interfascicular parenchyma. Ectopic expression of *HCA2* causes the upregulation of phloem and cambium marker genes. However, *HCA2* seems to act mainly as a transcriptional activator promoting the formation of interfascicular cambium. When *HCA2* was suppressed, the arrest of interfascicular cambium formation was observed with no major phenotype in phloem formation (Guo et al., 2009).

*LATERAL ROOT DEVELOPMENT 3* (*LRD3*) is another important gene which regulates early phloem development and controls phloem transport. It encodes a LIM-domain protein of unknown function. It is expressed specifically in the CC, and loss of *LRD3* function leads to a reduction of callose levels in the root meristem, early SE, and the PD that connect CC to SE. In addition, the

unloading of p*SUC2::GFP* and carboxyfluorescein diacetate (CFDA) dye at the root tip is severely limited in *lrd3* roots, suggesting reduced phloem delivery to the root tip. Interestingly, exogenous auxin treatment is able to rescue the defects in phloem development and unloading in *lrd3* roots, suggesting that auxin plays an important role in early phloem development (Ingram et al., 2011).

## 1.4 AUXIN TRANSPORT AND SIGNALING

Auxin is a small signaling molecule which is required for nearly every aspect of the plant growth and development, including embryonic formation of the apical–basal axis, vascular development, postembryonic organogenesis, tropistic growth, fruit development, stress response, senescence, and apical dominance (Zhao, 2010). Experiments performed by Charles Darwin and his son, Francis, on phototropic bending of canary grass and oat coleoptiles led to the discovery of this plant hormone. The Darwins suggested that a mobile "influence" produced at the tip of the coleoptile moves to the bending or differentially growing region and causes the coleoptile to grow towards light (Darwin, 1880). The compound was later identified and named auxin (from the Greek word "auxein" which means "to grow") (Went 1928).

Indole-3-acetic acid (IAA), the most abundant naturally occurring auxin, regulates various aspects of plant development by modulating the transcription of auxin responsive genes to control the balance between cell division, elongation, and differentiation. An important advance in our understanding of how IAA affects the expression of various genes was the identification of the TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) nuclear receptors, members of the F-box family of proteins. When bound to IAA, TIR1/AFBs form a complex with AUXIN RESISTANT/INDOLE- 3-ACETIC ACID INDUCIBLE (Aux/IAA) repressor proteins. At low auxin levels, the Aux/IAA proteins, together with the transcriptional co-repressor TOPLESS (TPL), bind to and inhibit the transcriptional activity of the auxin response factors (ARFs), a group of transcription factors that regulate auxin responsive genes. High auxin levels promote the formation of the TIR1/AFB-IAA-Aux/IAA complex which, in turn, targets the Aux/IAA proteins for degradation (Hayashi, 2012). This frees the ARFs to bind to genes containing auxin response elements in their promoters, thus activating or repressing their transcription.

An alternative signaling pathway that does not involve transcriptional regulation has also been proposed. This pathway has been suggested to act through *ABP1*, a putative auxin receptor located

at the lumen of the endoplasmic reticulum (ER) and partially secreted to the cell walls. ABP1 was identified as an auxin binding protein (Timpte, 2001) and, as shown in several studies, it plays an essential role in early embryogenesis, root development, leaf expansion, cell morphogenesis, and the subcellular distribution of the PIN auxin efflux carriers. The molecular function of ABP1 and its associated players has been recently demonstrated (Robert et al., 2010; Xu et al., 2014). The actions of the canonical TIR1/AFB pathway and the proposed transcription-independent ABP1 pathway can be coordinated to regulate gene transcription (Tromas et al., 2013).

The regulation of cellular processes by auxin signaling is highly dependent on auxin levels. Both auxin metabolism and cell-to-cell auxin transport contribute to the spatiotemporal distribution of auxin. Auxin movement from cell to cell is carried out by auxin influx and efflux carriers located at the plasma membrane. The most predominant auxin, IAA, is protonated in the apoplast and is able to diffuse into cells. However, the difference in pH between apoplast (pH 5.5) and cytosol (pH 7) causes IAA to become deprotonated in the cytoplasm, after which it can no longer diffuse out of the cell. Efficient cell-to-cell auxin movement therefore requires directed and active efflux out of cells (Benjamins and Scheres, 2008).

Three major families of auxin carriers have been reported so far. The AUXIN-RESISTANT1 (AUX1)/AUX1-LIKE (LAX) proteins are the auxin influx carriers, while the PINFORMEDs (PINs), together with ATP-binding-cassette B (ABCB)/P-glycoproteins (PGP), are responsible for auxin efflux (Grunewald and Friml, 2010). Recently, a study described a new group of proteins, the PIN-LIKES (PILS), as auxin transporters mediating intracellular auxin transport (Barbez et al., 2012). The PIN proteins are particularly critical in defining the directionality of auxin flow and the formation of local auxin gradients. These proteins are asymmetrically localized on the plasma membrane and have the ability to dynamically change their intracellular distribution in response to developmental and environmental signals (Grunewald and Friml, 2010).

## 1.5 AUXIN BIOSYNTHESIS AND ITS ROLE IN PLANT DEVELOPMENT

Auxin homeostasis has a major impact on plant growth and development. However, auxin production and degradation remain poorly understood.

Traditionally, auxin has been thought to be produced primarily in the aerial parts of the plant, from where it is delivered to the rest of the body (Ljung et al., 2001). However, local auxin biosynthesis

has been shown in other tissues, such as the meristematic region of the root tip (Ljung et al., 2005), where it has a major developmental impact. Many genes involved in auxin (IAA) biosynthesis are strongly expressed in the root apical meristem, where the highest auxin concentration has been detected (Ljung et al., 2005). Together with polar auxin transport, local auxin biosynthesis is involved in the formation of an auxin gradient within the root apex (Petersson et al., 2009). In the root, auxin biosynthesis plays an essential role in processes such as cell division, differentiation, and elongation, as well as lateral root initiation and development (Overvoorde et al., 2010).

IAA can be produced via tryptophan (Trp)-independent and Trp-dependent pathways. The Trp-independent auxin biosynthesis pathway is not well characterized, and its biological relevance is unclear. Evidence for its existence consists primarily of feeding studies using labelled IAA precursors and different tryptophan biosynthesis mutants in *Arabidopsis* and maize. Although little is known about the intermediates in the proposed Trp-independent pathway, indole and indole-3-glycerol phosphate (IGP) have been suggested to act as potential precursors for IAA synthesis in the absence of L-Trp (Chandler, 2009; Normanly et al., 1993; Zhao, 2010).

Trp-dependent IAA biosynthesis is better characterized and has been shown to be the developmentally important source of auxin. The Trp-dependent auxin biosynthesis pathways can be divided into the indol-3-yl pyruvic acid (IPA), indole-3-acetamide (IAM), tryptamine (TAM) and indole-3-acetaldoxime (IAOx) pathways (Korasick et al., 2013). Although many components have already been discovered, debates about the auxin production routes in the Trp-dependent pathways still continue.

Four main putative Trp-dependent routes contribute to auxin biosynthesis in plants, and they are defined by the first metabolic intermediate produced from Trp:

1. The indole-3-pyruvic acid (IPyA) pathway.

This two-step pathway seems to be the major source of IAA in plants. Here, the tryptophan aminotransferase TAA1 and its homologues TAR1 and TAR2 convert L-Trp into IPyA. Subsequently, the YUCCA enzymes synthesize IAA from IPyA (Stepanova et al., 2008; Stepanova et al., 2011; Won et al., 2011) (**Figure 4**). In addition, indole-3-acetaldehyde (IAAld) is thought to be an intermediate in this pathway (Mashiguchi et al., 2011; Normanly, 2010).

The *TAA1* gene (also known as *WEI8*) was identified simultaneously in two independent screens, one for shade avoidance (Tao et al., 2008) and the other for root-specific ethylene sensitivity (Stepanova et al., 2008). The level of IAA in *wei8 tar2* double mutants, mutated in both TAA1 and

its close homologue TAR2, is strongly reduced, causing defects in root gravitropism, root meristem maintenance, apical hook formation and gynoecium development (Stepanova et al., 2008). Moreover, treatment with L-kynurenine (L-Kyn), a compound that specifically inhibits TAA1/TAR activity, phenocopies the defects observed in *wei8 tar2* mutants (He et al., 2011).

The *YUCCA* genes have been demonstrated to be important for the development of the *Arabidopsis* embryo, leaf (Cheng et al., 2007; Wang et al., 2011), vasculature, flower (Cheng et al., 2006), and fruit (Eklund et al., 2010), as well as phyllotaxis (Pinon et al., 2013). They also play an important role in normal development and the response to various stress conditions in other plant species, such as rice, maize and potato (Bernardi et al., 2012; Gallavotti et al., 2008; Kim et al., 2013; Ohashi-Ito et al., 2002; Yamamoto et al., 2007).

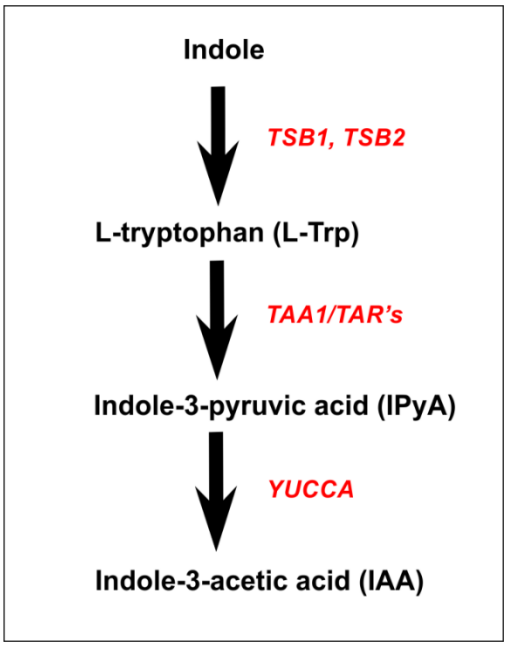


**Figure 4** The main Trp-dependent auxin biosynthesis pathway; see Korasick et al. (2013) for more detailed information on auxin biosynthesis pathways.

Recently, Chen and colleagues (Chen et al., 2014) demonstrated that a group of *YUCCA* genes (*YUC3*, *YUC5*, *YUC7*, *YUC8* and *YUC9*) are essential for *Arabidopsis* root development. These genes are specifically expressed in the root and inactivation of all five genes (*yucQ*) results in reduced auxin levels and agravitropic root growth. The *yucQ* mutant phenotype is rescued on media supplemented with auxin or by overexpressing a YUC gene in *yucQ* shoots. This suggests that local

root-based auxin production is critical to maintain sufficient auxin levels for proper root development.

2. The indole-3-acetamide (IAM) pathway

IAM has been found in many plant species, including *Arabidopsis*, maize, rice and tobacco (Novak et al., 2012; Sugawara et al., 2009). IAM hydrolases, enzymes which catalyze the conversion of IAM into IAA, have been isolated from *Arabidopsis* and tobacco (Nemoto et al., 2009; Pollmann et al., 2006). The *iaaM/iaaH* genes from *Agrobacterium tumefaciens* have been widely used to create endogenous auxin sources in different plant species.

3. The tryptamine (TRA) pathway

TRA is found in low levels in pea and *Arabidopsis*. It has been suggested that tryptophan decarboxylases (TDCs) catalyze the conversion of L-Trp into TRA (Mano and Nemoto, 2012). How exactly TRA is converted into IAA and which enzymes are catalyzing this step, is currently unknown.

4. The indole-3-acetaldoxime (IAOx) pathway

In this pathway, the cytochrome P450 monooxygenases CYP79B2 and CYP79B3 catalyze the conversion of L-Trp to IAOx, the common precursor for camalexin, indole glucosinolates and IAA (Sugawara et al., 2009).

Indole-3-acetonitrile (IAN) is a putative IAA precursor that is synthesized from IAOx (Sugawara et al., 2009) and is found in very high concentrations in *Arabidopsis* tissues (Novak et al., 2012; Sugawara et al., 2009). Nitrilases (NITs) have been suggested to convert IAN into IAA (Normanly et al., 1997; Park et al., 2003), but conclusive evidence for the role of these genes in IAA biosynthesis is still missing.

In addition to above compounds, indole-3-butyric acid (IBA) has been suggested to function as an IAA precursor and to have a significant impact on *Arabidopsis* seedling development. It undergoes peroxisomal β-oxidation to form IAA (Strader and Bartel, 2011).

The levels of IAA are regulated not only by *de novo* synthesis, but also by conjugation and degradation. The function of auxin conjugates and their effect on plant development remains unclear, though they have been found to be stored in seeds. Genes involved in IAA conjugation include members of the auxin-inducible GH3 family of amido-synthases and amido-hydrolases. The major products of IAA degradation are 2-oxoindole-3-acetic acid (oxIAA) and oxIAA-glucose

(oxIAA-Glc) (Korasick et al., 2013). However, the genes involved in IAA catabolism have not been identified so far.

## 1.6 METABOLISM AND TRANSPORT OF CHOLINE

Choline is a water soluble quaternary ammonium compound. It is needed to synthetize the major membrane lipid, phosphatidylcholine, and so is an essential compound in plants and other eukaryotes. Choline is an essential nutrient for humans and is required for the normal function of all of our body's cells, the brain and nervous system, liver function and the transport of nutrients throughout the human body.

Choline is also found in homogenized tissues of a variety of plants. The biosynthesis pathway of choline in plants is different from what has been described in other eukaryotes. The key enzyme in the plant choline synthesis pathway is phosphoethanolamine N-methyltransferase (PEAMT), which catalyzes the three methylation steps required to convert phosphoethanolamine into phosphocholine (Cruz-Ramirez et al., 2004; Datko and Mudd, 1988; Nuccio et al., 2000). Phosphocholine, in turn, can be dephosphorylated to free choline, as observed in some plant species, such as spinach (Summers and Weretilnyk, 1993), or incorporated into phosphatidylcholine and then metabolized to free choline, as observed in tobacco and *Arabidopsis* (Cruz-Ramirez et al., 2004; McNeil et al., 2000). Free choline can also be directly phosphorylated by a choline kinase to produce phosphocholine.

*XIPOTL1 (XPL1),* a gene encoding a PEAMT in *Arabidopsis*, has been previously identified and shown to be important for proper root development. Mutation of *XPL1* causes various defects in root development, such as increased lateral root formation, a reduced number of root hairs, and cell death in the root epidermis (Cruz-Ramirez et al., 2004). This indicates that the PEAMT-dependent phosphocholine biosynthesis pathway plays an important role in root development and that the components of this pathway act as signals for cell integrity.

Another important compound produced from choline is glycine betaine, which plays a significant role in the response to various environmental stress conditions, such as high levels of salts and low temperatures. However, plant species vary in their capacity to synthesize glycine betaine. Some plants, such as spinach and barley, accumulate high levels of glycine betaine, while others, such as *Arabidopsis* and tobacco, do not synthesize this compound (Weretilnyk et al., 1989).

Several studies indicate the involvement of another choline-derived metabolite, acetylcholine, in various developmental processes in plants. In many animals, including humans, acetylcholine acts as a neurotransmitter. The presence of acetylcholine and enzymes involved in acetylcholine synthesis and decomposition has been detected in a variety of plants (Momonoki, 1992; Momonoki et al., 1998; Wessler et al., 2001). For example, acetylcholinesterase is suggested to be involved in a myriad of physiological processes in plants, such as heat tolerance, phytochrome signal transduction (Jaffe, 1970), regulation of the stomatal aperture (Madhavan et al., 1995), gravitropism (Momonoki, 1997), germination (Beri and Gupta, 2007), and root development (Bamel et al., 2007).

It was recently demonstrated that free choline and some choline-derived metabolites are released by germinating seeds and move to the extracellular space to affect the interaction of plants and plant-associated microbes, such as *P. syringae*. The extracellular release of these compounds allows their interception and exploitation by plant-associated microbes, providing a selective advantage for microbes that are adapted to maximally exploit choline (Chen et al., 2013).

In animals, a number of studies have demonstrated that inter- and intracellular choline transport is performed by different transporter systems which can be divided in three major groups: polyspecific organic cation transporters (OCTs) with a low affinity for choline, high affinity choline transporters (CHTs) and the intermediate-affinity choline transporter like proteins (CTLs) (Traiffort et al., 2005). Our knowledge concerning choline transport in plants is very limited. Early studies using isotope-labeled choline indicated that the compound can be transported into plant cells by carriers. Furthermore, phosphocholine, a choline derivative, is found in xylem exudate (Gout et al., 1990). This organic phosphate ester might serve as storage for phosphate and nitrogen, and it is distributed throughout the plant by the vascular system. The idea that choline can be taken up from the media and distributed within the plant is further supported by the observation that externally supplied choline can rescue the developmental defects of the *xipotl* choline biosynthesis mutant (Cruz-Ramirez et al., 2004). In addition, choline can be transported by the proline transporter BvBet/ProT1, which is specifically expressed in phloem and xylem parenchyma of sugar beet (Yamada et al., 2011).

# 2. AIMS OF THE STUDY

Two genetic screens based on *pAtSUC2::GFP* and *pAHP6::GFPer* misexpression were conducted in our laboratory to find novel regulators of vascular development in *Arabidopsis*. The screens resulted in the identification of the *trp2-12*, *trp2-13*, *cher1-1*, *cher1-2* and *cher1-3* mutants with an altered vascular phenotype. The *trp2-12* and *trp2-13* mutants show defects in metaxylem specification, whereas the *cher1-1* and *cher1-2* mutations result in altered phloem transport and abnormal formation of sieve plates and sieve pores.

The specific aims of the study were:

1. To identify the genes behind the *trp2-12, trp2-13, cher1-1* and *cher1-2* phenotypes;
2. To understand how auxin biosynthesis regulates HD-ZIP III expression and metaxylem specification.
3. To understand the role of CHER1 and choline transport in phloem development, specifically in sieve plate and sieve pore formation.

# 3. MATERIALS AND METHODS

The materials and methods used are described in the corresponding publications. The methods used in this study are summarized in Table 1 with references to the publications in which they have been used.

**Table 1** Methods used in this study. Those in brackets were used by co-authors in the indicated publications.

| Method | Publication |
|---|---|
| Ethyl methane sulfonate (EMS) mutagenesis | (I), (II) |
| Mutant screen based on molecular marker misexpression | (I), (II) |
| Gene identification through positional cloning | I, II |
| DNA sequencing | I, II |
| Whole genome Illumina sequencing | (II) |
| Phylogenetic analyses | I, II |
| Genetic crossing of *Arabidopsis* | I, II, III |
| Polymerase chain reaction (PCR) analysis | I, II, III |
| Plasmid construction | I, II, III |
| *Agrobacterium* mediated transformation of *Arabidopsis* | I, II, III |
| Histological sectioning of plastic embedded samples | I, II |
| Fuchsin staining | I, II, III |
| Light microscopy | I, II, III |
| Confocal light microscopy | I, II, III |
| Histological staining of GUS activity | I, II |
| 4',6-diamidino-2-phenylindole (DAPI) staining | II |
| *In situ* RNA hybridization | I |
| Serial block-face scanning electron microscopy (SBEM) | (II) |
| 3D reconstruction of confocal stack images | II |
| *Xenopus* oocyte uptake assay | (II) |
| Structural Electron Microscopy (SEM) | (II) |
| Immunogold labelling | (II) |

# 4. RESULTS AND DISCUSSION

## 4.1 FORWARD GENETICS SCREENS TO IDENTIFY NOVEL REGULATORS OF VASCULAR DEVELOPMENT IN *ARABIDOPSIS THALIANA*

To identify novel regulators of plant vascular tissue specification, we carried out two independent ethyl methanesulfonate (EMS) mutagenesis screens based on misexpression of two vascular-specific molecular markers, *pSUC2::GFP* and *pAHP6::GFPer*. The *Arabidopsis SUC2* gene encodes a sucrose transporter which is expressed in the phloem companion cells and is essential for the loading of sucrose into phloem sieve elements and its long-distance transport. The free and mobile version of GFP expressed under the control of the companion cell-specific *pSUC2* promoter moves into the sieve elements, gets transported rootwards, and is unloaded symplastically in the root tip (Stadler et al., 2005).

*AHP6* encodes a pseudo-phosphotransfer protein which counteracts cytokinin signaling and allows the formation of protoxylem. Endoplasmic reticulum (ER) targeted *pAHP6::GFPer* is expressed in a highly specific pattern associated with protoxylem (Mahonen et al., 2006). Furthermore, the expression of *pAHP6::GFPer* has been shown to be promoted by auxin transported via the phloem (Bishopp et al., 2011b).

Both screens resulted in the isolation of several mutants with altered vascular development (Publications I and II). In my thesis work, I identified and characterized two of the isolated mutants. This allowed me to uncover novel genes and pathways critical for proper vascular tissue differentiation.

## 4.2 FROM AUXIN BIOSYNTHESIS TO DEVELOPMENTAL EVENTS

Several mutant seedlings that failed to unload GFP into the root tip were isolated from the *pSUC2::GFP* screen. Two of the mutants, *trp2-12* and *trp2-13*, exhibited an obvious and severe phenotype which included agravitropic root growth and small leaves with dark venation. A detailed examination of root xylem axis in these mutants revealed defects in xylem patterning. In the vascular cylinder of wild-type *Arabidopsis* roots, the xylem axis consists of two central metaxylem (MX) files flanked by two protoxylem (PX) poles. The identified mutants differed from wild-type,

exhibiting severely impaired MX formation. These mutant roots either lacked MX or formed discontinuous MX files. Consistent with a MX-specific vascular phenotype, expression of the PX-specific marker *pAHP6::GFPer* was unaffected in *trp2-12* roots, whereas expression of the MX/procambium marker *pACAULIS5 (pACL5)::GFPer* (Muniz et al., 2008) was severely reduced or absent. To identify the mutation causing such severe changes in MX specification, we used PCR-based positional cloning to establish a mapping window containing several potential candidate genes. Subsequent sequencing of the candidate genes led to the identification of point mutations for two alleles in the *At5g54810* locus, which encodes *TRYPTOPHAN SYNTHASE BETA-SUBUNIT 1* (*TSB1/TRP2*). As Trp is a precursor for auxin biosynthesis and TSB1 is specifically responsible for the conversion of indole into tryptophan, we next analyzed whether the auxin response might be altered in *trp2-12* seedlings. As we expected, the auxin signaling reporters *pDR5::GFPer* and *pIAA2::GFP/GUS* were detected at reduced levels in the root meristem and xylem axis of *trp2-12* seedlings. Based on these observations, we speculated that Trp biosynthesis or Trp-dependent auxin biosynthesis might play an important role in metaxylem specification (Publication I).

Trp is a precursor not only for auxin, but also for a variety of other compounds essential to plant life. To check if the MX-specific defects were directly due to a reduction in Trp synthesis or resulted from reduced Trp-derived IAA production, we decided to check if other mutants in the Trp-dependent auxin biosynthesis pathway exhibited similar defects in metaxylem specification. We screened the *Arabidopsis* eFP Browser (Winter et al., 2007) to find genes expressed in the root tip and found complex expression patterns of genes involved in Trp-dependent auxin biosynthesis, with several being preferentially expressed in the quiescent center and its surrounding tissues. We decided to investigate whether the *TAA1 (WEI8)*, *TAR2* and *YUCCA* genes might function in vascular tissue development, as *TAA1/TAR* and *YUCCA* act downstream of *TSB1* in the same biosynthetic pathway. We checked the status of xylem formation in the combinatorial mutants of both the *TAA1/TAR* and *YUCCA* genes, the double mutant *wei8 tar2* (mutations in both *TAA1* and *TAR2*) and the multiple combinatorial mutant *yuccaQ* (mutations in *YUCCA3, YUCCA5, YUCCA7, YUCCA8* and *YUCCA9*). Interestingly, while PX formed properly at the peripheral position of the xylem axis in *wei8 tar2* and *yuccaQ*, ectopic PX formation was observed in the central MX position. In addition, we observed a dramatic reduction of *pACL5::GFPer* expression in the *wei8 tar2* mutant. These observations suggest that Trp-dependent auxin biosynthesis via the TAA1/TAR-

YUCCA pathway in the non-vascular tissues is required for MX differentiation in the central domain of the xylem axis in the *Arabidopsis* root (Publication I).

L-Kynurenine (Kyn), a recently discovered auxin biosynthesis inhibitor, acts as an alternate substrate for TAA1/TAR and competitively blocks the activity of these enzymes. To test whether we could pharmacologically block MX differentiation and mimic the *wei8 tar2* phenotype, we treated wild-type seedlings with Kyn. Following Kyn treatment, we observed a reduction of *pACL5::GFPer* expression, reduced auxin perception and elevated levels of the auxin-downregulated reporter DII-VENUS (Brunoud et al., 2012) in the xylem axis, confirming the inhibitory effect of Kyn on auxin biosynthesis. As we expected, Kyn mimics the *wei8 tar2* phenotype and leads to the conversion of MX into PX in the central domain of xylem axis, where MX normally develops. Altogether, these results suggest that the xylem phenotype in *wei8 tar2* and Kyn-treated plants is due to a reduction of auxin levels in the xylem axis.

To further confirm that reduced auxin levels cause the conversion of MX into PX, we generated several transgenic lines expressing the bacterial auxin biosynthesis gene *iaaH*. We cloned *iaaH* under promoters specific for the root meristem (*pTSB1::iaaH*) and QC (quiescent center) (*pWOX5::XVE>>iaaH*). We observed reduced responsiveness to Kyn when seedlings were additionally supplied with the substrate of IAAH, IAM (indoleacetamide). This suggests that iaaH-mediated endogenous auxin production can restore sufficient auxin levels to maintain MX formation under conditions inhibiting the TAA1/TAR auxin biosynthetic pathway.

Interestingly, none of the analyzed genes have metaxylem specific expression. *TSB1* and its homologous genes are expressed in the ground tissue initials cells (Publications I and III), the ground tissue and the columella. *TAA1* is strongly expressed in the QC and the protoxylem. The expression of *TAR2* comes very late in the elongation zone. The analyzed root-specific *YUCCA* genes are mostly expressed in QC and its surrounding region (Publication I). The difference between the expression domain of these genes and their place of action, the metaxylem, suggests the need for an efficient transport system to distribute auxin and its precursors in order to maintain proper metaxylem formation.

## 4.3 TRP-DEPENDENT AUXIN BIOSYNTHESIS VIA THE TAA-YUCCA PATHWAY IS REQUIRED FOR PROPER HD-ZIP III EXPRESSION AND METAXYLEM SPECIFICATION

The HD-ZIP III family of transcription factors plays a critical role in a variety of developmental processes essential to plant life. One such process is xylem differentiation. As explained in the introduction, the dosage of HD-ZIP III determines whether xylem cells will become metaxylem or protoxylem. A high dose of HD-ZIP III specifies metaxylem and low dose protoxylem (Carlsbecker et al., 2010).

Given that our auxin biosynthesis mutants could all form PX but not MX, we wondered whether they had lower HD-ZIP III levels in the xylem. Indeed, we discovered reduced transcript levels of *PHB, AtHB8, AtHB15* and *PHV* in root tips of the *trp2-12* mutant and Kyn-treated plants. These findings led us to propose a model wherein auxin regulation of the activity of the HD-ZIP III genes leads to lower HD-ZIP III levels following inhibition of auxin biosynthesis, and the reduced HD-ZIP III activity results in protoxylem formation. In Kyn-treated plants and the *trp2*-12 mutant, the level of auxin and thus HD-ZIP III is not sufficient to maintain proper MX formation.

Several gain of function mutants, such *phb-7d* and *phb-1d*, have been shown to have increased HD-ZIP III expression levels as a result of mutations in the miRNA165/166 targeting sites. These mutations allow the HD-ZIP III to escape miRNA165/166-mediated degradation, resulting in the upregulation of the HD-ZIP III gene in the stele. As these mutants exhibit high HD-ZIP III levels, they develop ectopic metaxylem (Carlsbecker et al., 2010). We used these mutants as a tool to check our model by testing whether the ectopic MX phenotype of the *phb7-d* mutant could be reverted by inhibiting auxin biosynthesis with Kyn. We observed a partial rescue of PX development in *phb7-d* mutants after Kyn treatment (Publication I). This supports our hypothesis that Trp-dependent auxin biosynthesis controls xylem specification by regulating the activity of the HD-ZIP III genes.

## 4.4 AUXIN BIOSYNTHESIS AND AUXIN TRANSPORT: BOTH ARE ESSENTIAL, BUT THEY HAVE DIFFERENT EFFECTS ON XYLEM SPECIFICATION

As described above, all of our identified auxin biosynthesis mutants exhibit MX-specific defects: *trp2-12* forms protoxylem but fails to form metaxylem, and the *wei8 tar2* and *yuccaQ* mutants form ectopic PX in place of MX (Publication I). By contrast, it has been previously shown that inhibition of polar auxin transport (PAT) by NPA only affects cells at the PX position and does not have an effect on MX. Seedlings treated with NPA often lack PX or develop incomplete PX files (Bishopp et al., 2011a). This is an interesting observation, because it suggests a functional and spatial separation between auxin biosynthesis and PAT in xylem type specification (Publication III). The metaxylem position seems to be more dependent on changes in auxin levels produced via the Trp-dependent pathway, whereas PX requires active auxin transport to maintain its identity (**Figure 5**). Such a separation could be partially due to spatially isolated regulatory pathways for MX and PX. PX formation is dependent on an auxin-cytokinin regulatory loop. In this loop, *AHP6*, a cytokinin signaling inhibitor, is promoted by auxin actively delivered by the PIN auxin transporters. In turn, *AHP6* inhibits cytokinin signaling and allows PX to form. At the same time, *AHP6* acts downstream of the HD-ZIP III genes, since in multiple HD-ZIP III mutants ectopic PX is formed followed by expansion of the AHP6 domain (Bishopp et al., 2011a; Carlsbecker et al., 2010). By contrast, the MX position lacks *AHP6* expression, and the fact that NPA removes only PX suggest that MX is less dependent on active PAT and more dependent on a different transport system. In addition, the formation of PX and MX might be highly dependent on the levels of IAA and HD-ZIP III in the stele. Since PX formation requires only a low dose of HD-ZIP III, the level of auxin in the xylem axis after the inhibition of IAA biosynthesis might still be sufficient to maintain PX formation. MX, on the other hand, requires higher dose of HD-ZIP III and therefore might be very sensitive to even subtle changes in IAA levels.

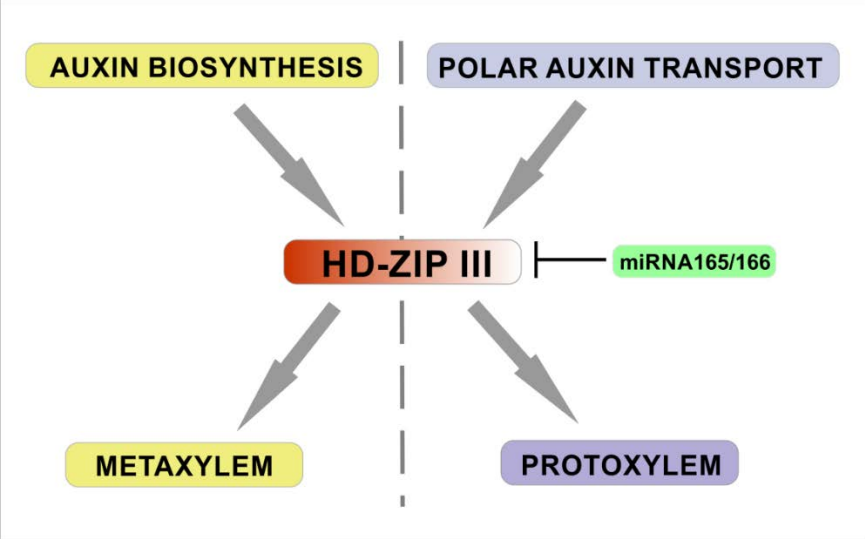


**Figure 5** Proposed functional separation between Trp-dependent auxin biosynthesis and polar auxin transport in xylem specification.

## 4.5 CHER1 (CHOLINE TRANSPORTER RELATED 1) – A NOVEL REGULATOR OF PHLOEM SIEVE PLATE AND SIEVE PORE DEVELOPMENT

In the second project of this thesis, we carried out a misexpression screen of *pAHP6::GFPer*, an ER-targeted GFP fused to the promoter of *AHP6*, a cytokinin signaling inhibitor. *pAHP6::GFPer* is normally expressed in a highly specific pattern associated with protoxylem and the adjacent pericycle cells, and its expression pattern is known to respond to the transport status of the phloem. The screen resulted in the isolation of a mutant with altered, often expanded, *pAHP6::GFPer* expression. We mapped the mutant and used whole genome sequencing to identify the causal mutation in one of the *Arabidopsis* CTL homologues, At3g15380, referred to here as *CHER1*.

In *Arabidopsis*, seven genes share sequence similarity with the *CTL* genes of animals and yeast. In this study, we show that one of the CTL homologues, *CHER1*, mediates choline transport and that mutation of this gene leads to an altered choline metabolite profile and severe defects in phloem sieve plate and sieve pore formation. As a result of these defects, phloem transport is impaired.

In our study, we observed that *CHER1* is expressed in several tissues, where it localizes to the trans-Golgi network (TGN) and transiently to the forming cell plate during cell division. In addition, during phloem development, *CHER1* has a polar localization pattern associated with young and developing phloem sieve plates. The expression pattern suggested to us that *CHER1* may function

in early phloem development. To follow up on this idea, we analyzed vascular conductivity in the *cher1-1* mutant by using *pSUC2-GFP* as a marker. In contrast to wild-type, where *pSUC2::GFP* is translocated from CC to SE and unloaded symplastically into the root meristem, unloading of GFP from the protophloem into the root meristem was blocked in the *cher1-1* mutant. In addition, reduced symplastic transport was observed in *cher1-1* mutants expressing *pSUC2::SpoGFP*, a Sporamin fused to GFP to increase the protein's size (Publication II). In order to understand why phloem transport was so severely affected, we investigated phloem development in the *cher1-1* mutant. We observed severe phloem patterning defects in cross sections of mutant roots, as well as a disrupted pattern of the phloem-specific marker *pAPL::GFPer*. This confirmed our suspicion that the disruptions in phloem transport result from defects in the continuous differentiation of phloem cells.

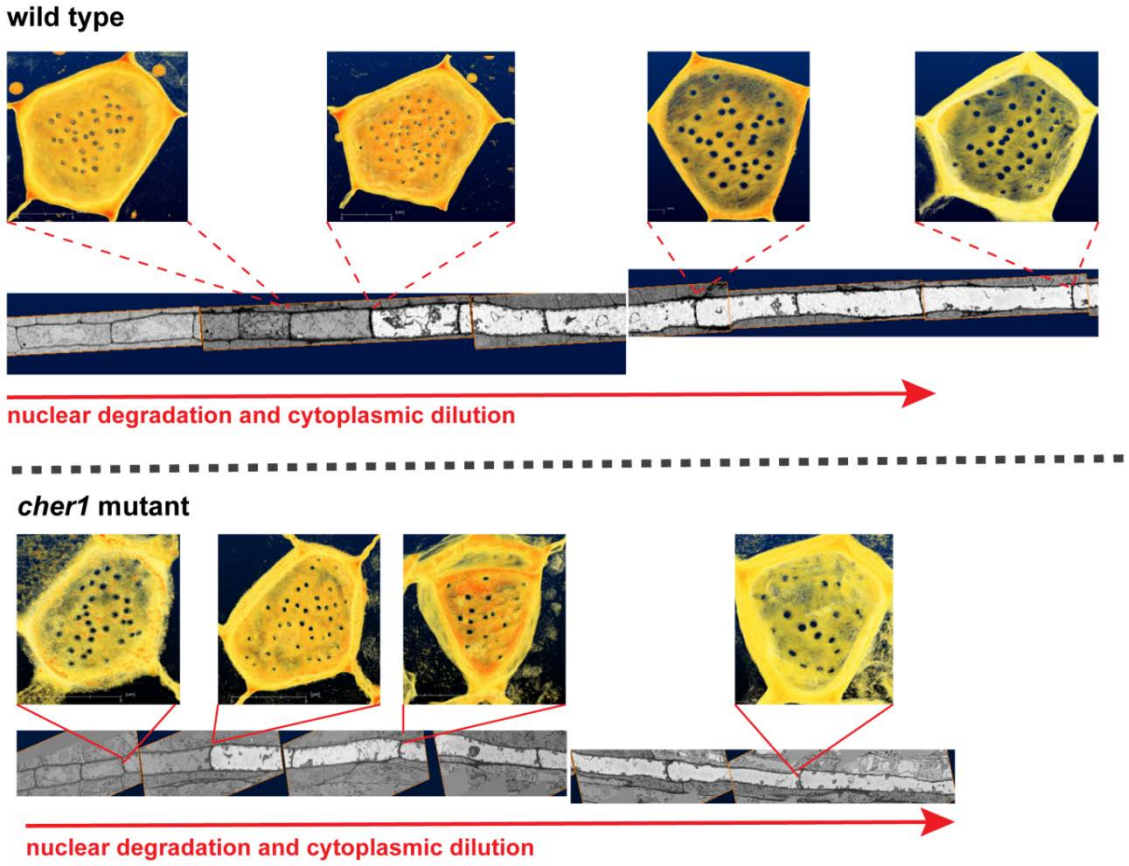


**Figure 6** SBEM images of sieve tubes and reconstructed sieve plate images of wild type and cher1. "White cells" in the longitudinal images represent sieve elements with degraded nuclei and diluted

cytoplasm. Sieve plates were taken from different developmental stages of phloem sieve element development.

The intriguing polar localization of CHER1 in early phloem sieve elements suggested that it may play a role in the elaboration of sieve plates. Using serial block-face scanning electron microscopy (SBEM), we compared serially-sectioned roots of wild-type and *cher1* (**Figure 6**).

We focused specifically on the forming sieve plates, where CHER1 expression was observed. We found that *cher1* plants have a reduced sieve plate area and reduced sieve pore density when compared to wild-type. Sieve pores are essential for the transport of various molecules from one sieve element to the next, thereby enabling long distance transport throughout the plant body. A reduction in sieve pore number in *cher1* is therefore likely to explain the impaired transport of GFP in the phloem observed with *pSUC2::GFP*. Interestingly, we observed that the differentiation of sieve pores is also affected. In wild-type roots, the sieve pores lose the desmotubules located in their symplastic space during maturation. This process is severely affected in the *cher1* mutant, where most of sieve pores still retained their desmotubules even at very late stages of development (**Figure 7**).

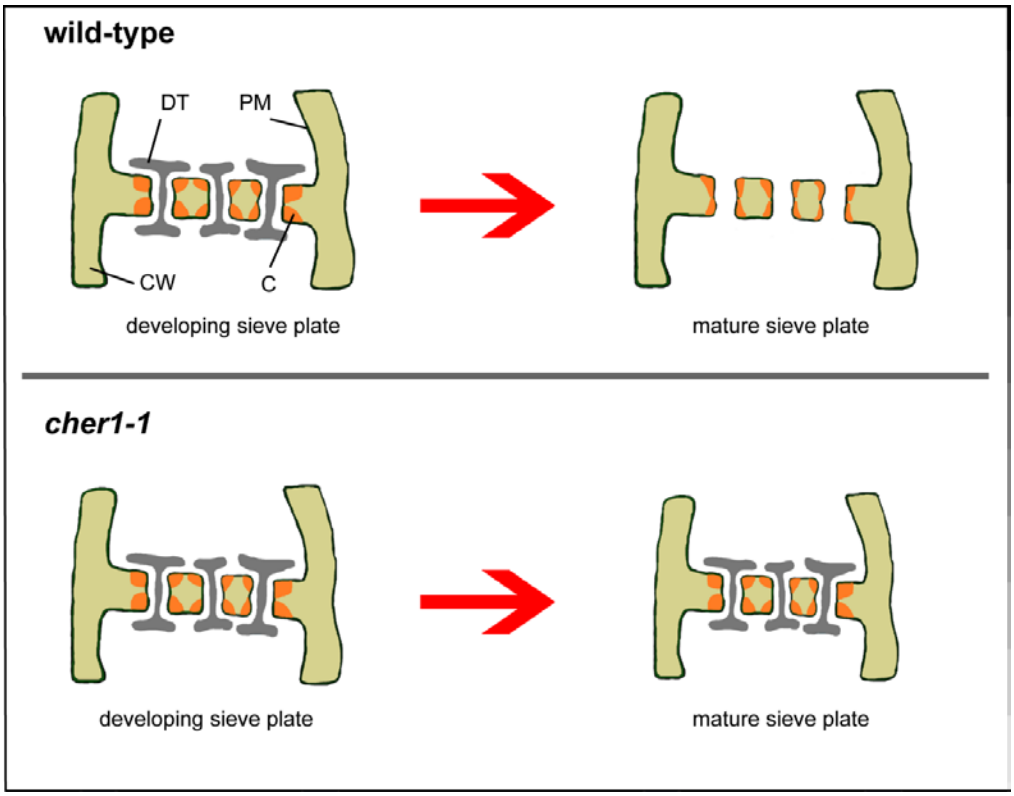


**Figure 7** Sieve pore formation is affected in *cher1* mutant

## 4.6 CHER1 FACILITATES CHOLINE TRANSPORT

The CHER1 protein consists of 700 amino acids, with 10 predicted trans-membrane spanning domains similar to the predictions of most CHOLINE TRANSPORTER LIKE (CTL) proteins from other organisms. In *Arabidopsis*, seven genes have been annotated as encoding CTL proteins based on their DNA sequence. We took a closer look at the CHER1 protein and compared it with other known CTL proteins from various organisms. The *in silico* analysis using multiple alignment with other CTLs revealed that CHER1 is closest in homology to the animal CTL proteins and is distinct from the other six *Arabidopsis* CTL-like proteins. We also tested whether CHER1 is able to transport choline in oocytes, a system which has served as a very efficient test of the transport capability of various proteins form different organisms. We observed that expression of *in vitro* transcribed, tagged *CHER1* RNA was able to increase choline uptake 1.3-fold, which is similar to the uptake rates observed with tagged human CTL1. Moreover, our analysis of the transport kinetics revealed that CHER1 increased the $V_{max}$ of a high affinity component of choline transport in the oocytes. A similar effect on $V_{max}$ has been previously observed with the rat rCTL1a and rCTL1b proteins. We used auxin (IAA) and glucose as controls, and the uptake of both compounds by oocytes was not affected by the expression of *CHER1*.

Mutation of the *CHER1* gene leads to disturbed homeostasis of choline and its derivative, phosphocholine, in the *Arabidopsis* root, as shown by measurement of the total content of these molecules in the roots of wild-type and *cher1* seedlings. Furthermore, we analyzed the expression of *pAPL::GFPer* and the distribution of freely moving GFP driven by the *pSUC2* promoter during phloem development in the biosynthetic *xipotl* mutant, which also shows reduced levels of choline and phosphocholine. Similar to *cher1-1*, we observed reduced movement and unloading defects of *pSUC2::GFP*, in addition to the fragmented expression pattern of *pAPL::GFPer* in the phloem of *xipotl* and an increased number of sieve element-like cells in the root cross-section. Taken together, these data indicate that CHER1 facilitates choline transport and maintains choline homeostasis during root (and phloem) development in *Arabidopsis* (Publication II).

## 4.7 CHER1 EXPRESSION PATTERN – BUILDING SIEVE PLATES AND CELL PLATES

As indicated above, we observed that CHER1 localizes to the TGN in most root tissues. In the phloem, CHER1 is polarly localized at the forming sieve plates. In dividing cells, CHER1 has a cell plate-specific expression pattern associated with the phragmoplast, a specific structure that forms during late cytokinesis. We were curious to see if there was a difference between the CHER1 expression pattern in transverse sections of sieve plates and cell plates. Using confocal z-sectioning to reconstruct a 3D image, we observed that CHER1 forms a sustained pattern in the early sieve plates. By contrast, in the dividing tissues of the outer layers of the root, CHER1 forms a ring-like pattern coinciding with very late stages of phragmoplast formation. The different localizations of CHER1 in the early phloem and dividing cells of other tissues suggest a different mode of action of CHER1 during sieve plate and cell plate formation. In the forming sieve plates, the sustained pattern of CHER1 suggests that a continuous recycling of CHER1 from the TGN to the sites of forming plates is required. This pattern can be easily disturbed by the application of BFA, a drug commonly used to inhibit endocytotic recycling. Moreover, we observed interesting behavior of two dynamin-related proteins, DRP1A and DRP1C, which are involved in clathrin-mediated endocytosis in plants. Both DRP1A and DRP1C localize to the cell plate and the plasma membrane of expanding and fully expanded interphase cells. Interestingly, we found that both proteins exhibit a polarized and sustained pattern similar to that of CHER1 at sieve plates. Taken altogether, our observations suggest that a BFA-sensitive endocytotic recycling mechanism is involved in the maintenance of the polar CHER1 pattern in developing sieve element cells (Publication II). Further investigations will be required to clarify the exact mechanism and function of CHER1 in sieve plate formation.

CHER1 was only detected during the late stages of phragmoplast formation in dividing cells. This suggests that it has a very specific function at this stage of cell division. We hypothesize that CHER1 might be involved in providing building materials for the construction of the cell plate, especially during the late steps of phragmoplast formation. As CHER1 is expressed in both the TGN and the forming phragmoplast, it is likely that CHER1 is delivered via vesicular transport to the site of the forming plate, where it provides material for building the plate (Publication II). Perhaps choline-derived compounds could serve as building material, since CHER1 mediates the

transport of choline. However, the exact mechanism through which CHER1 contributes to cell plate formation is currently unclear and awaits further investigation.

# 5. CONCLUDING REMARKS

In the first part of my thesis work, I have shown that TRP-dependent auxin biosynthesis via the TAA-YUCCA pathway is required for proper HD-ZIP III expression and metaxylem tissue specification. This work, together with several other recent studies, highlights the developmental role of auxin biosynthesis. Polar auxin transport is often not sufficient to establish and maintain certain developmental events; local auxin biosynthesis is additionally required (Publications I and III).

We were also able to reveal the essential role of Trp-dependent auxin biosynthesis in the specification of meta- and protoxylem, although many questions still remain unanswered. One important question is why the inhibition of Trp-dependent auxin biosynthesis affects only the metaxylem, while blocking polar auxin transport leads to very specific protoxylem defects.

It would be also interesting to investigate whether the regulatory pathway described in this thesis is conserved throughout the plant kingdom. Taking into account that the HD-ZIP III transcription factors and auxin biosynthetic enzymes described above are present in the majority of plants, including basal species, it seems likely that the metaxylem specification pathway is a highly conserved process.

In the second part of my thesis work, I identified *CHER1*, a novel regulator of phloem sieve plate and sieve pore formation. The expression pattern of *CHER1* in the early phloem correlates with the sieve plate and sieve pore phenotype we found in the corresponding loss of function *cher1* mutant. Furthermore, our choline uptake experiments in *Xenopus* oocytes indicated that CHER1 facilitates choline transport (Publication II). However, the exact mechanism by which CHER1 functions in phloem sieve plate and sieve pore maturation is still unclear. In the future, it would be interesting to identify and study components which are active upstream and downstream of CHER1 and investigate exactly how choline and its derivatives contribute to sieve plate and sieve maturation. Further studies of the function of CHER1 will also contribute to our currently limited understanding of how phloem sieve plates and sieve pores form.

*CHER1* also has a very interesting expression pattern in the dividing cells of the outer layers. This pattern is associated with the later stages of cell plate formation, when the phragmoplast forms a ring-like structure. Why CHER1 is required specifically at this stage is currently unknown. As CHER1 acts as choline transport facilitator, certain components synthesized from choline might be

specifically required to finalize the cell plate building process. What these components are and how CHER1 participates in the process of cell plate formation remains to be discovered in the future.

# SUMMARY IN FINNISH

Kasvien johtojänteet koostuvat pääosin kahdesta solutyypistä, puu- ja nilasoluista, jotka tukevat kasvia ja ovat erilaistuneet veden, ravintoaineiden, kasvihormonien sekä mineraalien kuljettamiseen kasvin eri osien välillä. Väitöstyössäni tutkin johtosolukon muodostumista käyttäen mallina lituruohon juurta.

Väitöstyöni ensimmäinen osio pureutuu puusolukon eli ksyleemin, vettä kuljettavan putkiston muodostumiseen. Lituruohon pääjuuressa kahdenlaiset puusolut muodostavat keskuslieriöön poikittaisen akselin jonka kummassakin päädyssä on yksi ns. protoksyleemi solu ja niiden välissä metaksyleemi soluja. Aiempien tutkimusten perusteella on tiedetty että HD-ZIP III transkriptiofaktorin korkea ilmenemistaso edistää metaksyleemin erilaistumista kun taas alhainen taso on kytköksissä protoksyleemiin. Väitöstyöni puitteissa selvitimme että kasvihormoni auksiinin tuotanto edistää HD-ZIP III ilmenemistä ja siten myös metaksyleemin muodostumista. Poikkeavan alhainen auksiinin määrä kasveissa joiden auksiinin tuotantoa oli heikennetty joko geneettisin tai kemiallisin menetelmin alensi HD-ZIP III ilmenemistä, mikä puolestaan johti poikkeavuuksiin metaksyleemin erilaistumisessa. Näitä poikkeavuuksia voitiin osittain korjata nostamalla joko auksiinin määrää tai HD-ZIP III ilmenemistasoa juuren keskuslieriössä.

Väitöstyöni toisessa osassa tutkin nilan muodostumista. Nila on erikoistunut useiden molekyylien pitkänmatkan kuljetukseen, mikä perustuu siiviläputkien huokosellisten poikkiseinien eli siivilälevyjen ominaisuuteen yhdistää peräkkäiset siiviläputkisolut toisiinsa pitkäksi solujonoksi. Havaitsimme että lituruoholla siivilälevyjen huokoisuus ja siten siiviläputkien kuljetuskyky poikkeaa normaalista jos CHER1 (CHOLINE TRANSPORTER LIKE 1) geenin toiminta on estynyt. CHER1 proteiini paikallistuu kehittyviin siiviläputkiin polaarisesti ennakoiden siivilälevyn sijaintia, ja on siis siivilälevyjen kehittymisen kannalta tärkeä jo varhaisessa vaiheessa. Lisäksi osoitimme että CHER1 osallistuu koliinin kuljetukseen, paikallistuu solutasolla Golgi verkostoon, ja liittyy tiettyjen rakenteiden muodostumiseen solunjakautumisen yhteydessä.

# ACKNOWLEDGEMENTS

This work was carried out at the Division of Plant Biology, Department of Biosciences and Institute of Biotechnology, University of Helsinki. For financial support I thank The Integrative Life Science Doctoral Program (ILS), University of Helsinki and Academy of Finland.

I would like to express my deepest gratitude to my supervisor, Professor Ykä Helariutta, for giving me the chance to work in his group under his excellent scientific guidance. It has been a pleasure and a great experience to start my career by working with you. Thank you for all your support and encouragement during these years.

I thank all the co-authors of my thesis publications for their contributions.

I would like to thank the members of my follow-up group Dr. Ari Pekka Mähönen and Dr. Mikael Brosche for the fruitful discussions in the meetings and for all their advices. I am grateful to Dr. Kristiina Himanen and Professor Hely Häggman for reviewing my thesis and Sedeer el-Showk for proofreading it. Additionally, I would like to thank the Integrative Life Science Doctoral Program for accepting me as a graduate student, as well as coordinator Erkki Raulo and Anita Tienhaara for all the help during all these years.

My dear friends and colleagues - Jung-ok, Jiajia, Kaori, Hanna, Eva, Raquel, Kamil, Gugan, Juha, Juan, Fulgencio, Pawel, Karolina, Kaisa, Annakaisa, Ondrej, Riccardo, Iris, Jing, Raffael, Sedeer, Tony, Shri Ram, Dawei, Raili, Anne — for sharing the office and the lab, for lovely discussions and for creating a nice working atmosphere. I would like to acknowledge Dr. Jan Dettmer who was my supervisor when I just joined the group, also during the last years of my Master thesis work and during early years of my PhD work. I would like to express my deepest gratitude to Dr. Shunsuke Miyashima who was not only my supervisor and an example of an enthusiastic and creative scientist, but also a very good friend I could always talk to and whom I trusted the most.

I wish to thank Katja Kainulainen and Mikko Herpola for all your help with everything and for your patience.

I would like to thank all the people working in the Plant Biology division in 6th floor of Biocenter 3 for creating a pleasant working environment.

I wish to thank Marjukka, Maritta, and Markku for technical help, and gardener Leena for taking care of plants.

My most heartfelt thanks go to my family and my friends. I thank my mother Janina and my sister Alina for all the support and care during these years, my nephew Damian and my niece Eliza, and all the others – thank you for being there for me during all these years!

Helsinki, December 2014

# REFERENCES


**Baker, R. F., Slewinski, T. L. and Braun, D. M.** (2013). The tie-dyed pathway promotes symplastic trafficking in the phloem. *Plant signaling & behavior* **8**, e24540.

**Bamel, K., Gupta, S. C. and Gupta, R.** (2007). Acetylcholine causes rooting in leaf explants of in vitro raised tomato (Lycopersicon esculentum Miller) seedlings. *Life sciences* **80**, 2393-2396.

**Barbez, E., Kubes, M., Rolcik, J., Beziat, C., Pencik, A., Wang, B., Rosquete, M. R., Zhu, J., Dobrev, P. I., Lee, Y., et al.** (2012). A novel putative auxin carrier family regulates intracellular auxin homeostasis in plants. *Nature* **485**, 119-122.

**Bauby, H., Divol, F., Truernit, E., Grandjean, O. and Palauqui, J. C.** (2007). Protophloem differentiation in early Arabidopsis thaliana development. *Plant & cell physiology* **48**, 97-109.

**Benitez-Alfonso, Y.** (2014). Symplastic intercellular transport from a developmental perspective. *Journal of experimental botany* **65**, 1857-1863.

**Benjamins, R. and Scheres, B.** (2008). Auxin: the looping star in plant development. *Annual review of plant biology* **59**, 443-465.

**Beri, V. and Gupta, R.** (2007). Acetylcholinesterase inhibitors neostigmine and physostigmine inhibit induction of alpha-amylase activity during seed germination in barley, Hordeum vulgare var. Jyoti. *Life sciences* **80**, 2386-2388.

**Bernardi, J., Lanubile, A., Li, Q. B., Kumar, D., Kladnik, A., Cook, S. D., Ross, J. J., Marocco, A. and Chourey, P. S.** (2012). Impaired auxin biosynthesis in the defective endosperm18 mutant is due to mutational loss of expression in the ZmYuc1 gene encoding endosperm-specific YUCCA1 protein in maize. *Plant physiology* **160**, 1318-1328.

**Bishopp, A., Help, H., El-Showk, S., Weijers, D., Scheres, B., Friml, J., Benkova, E., Mahonen, A. P. and Helariutta, Y.** (2011a). A mutually inhibitory interaction between auxin and cytokinin specifies vascular pattern in roots. *Current biology : CB* **21**, 917-926.

**Bishopp, A., Lehesranta, S., Vaten, A., Help, H., El-Showk, S., Scheres, B., Helariutta, K., Mahonen, A. P., Sakakibara, H. and Helariutta, Y.** (2011b). Phloem-transported cytokinin regulates polar auxin transport and maintains vascular pattern in the root meristem. *Current biology : CB* **21**, 927-932.

**Bonke, M., Thitamadee, S., Mahonen, A. P., Hauser, M. T. and Helariutta, Y.** (2003). APL regulates vascular tissue identity in Arabidopsis. *Nature* **426**, 181-186.

**Brunoud, G., Wells, D. M., Oliva, M., Larrieu, A., Mirabet, V., Burrow, A. H., Beeckman, T., Kepinski, S., Traas, J., Bennett, M. J., et al.** (2012). A novel sensor to map auxin response and distribution at high spatio-temporal resolution. *Nature* **482**, 103-106.

**Cantrill, L. C., Overall, R. L. and Goodwin, P. B.** (1999). Cell-to-cell communication via plant endomembranes. *Cell biology international* **23**, 653-661.

**Carlsbecker, A., Lee, J. Y., Roberts, C. J., Dettmer, J., Lehesranta, S., Zhou, J., Lindgren, O., Moreno-Risueno, M. A., Vaten, A., Thitamadee, S., et al.** (2010). Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. *Nature* **465**, 316-321.

**Chandler, J. W.** (2009). Local auxin production: a small contribution to a big field. *BioEssays : news and reviews in molecular, cellular and developmental biology* **31**, 60-70.

**Chen, C., Li, S., McKeever, D. R. and Beattie, G. A.** (2013). The widespread plant-colonizing bacterial species Pseudomonas syringae detects and exploits an extracellular pool of choline in hosts. *The Plant journal : for cell and molecular biology* **75**, 891-902.

**Chen, Q., Dai, X., De-Paoli, H., Cheng, Y., Takebayashi, Y., Kasahara, H., Kamiya, Y. and Zhao, Y.** (2014). Auxin overproduction in shoots cannot rescue auxin deficiencies in Arabidopsis roots. *Plant & cell physiology* **55**, 1072-1079.

**Cheng, Y., Dai, X. and Zhao, Y.** (2006). Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in Arabidopsis. *Genes & development* **20**, 1790-1799.

---- (2007). Auxin synthesized by the YUCCA flavin monooxygenases is essential for embryogenesis and leaf formation in Arabidopsis. *The Plant cell* **19**, 2430-2439.

**Cruz-Ramirez, A., Lopez-Bucio, J., Ramirez-Pimentel, G., Zurita-Silva, A., Sanchez-Calderon, L., Ramirez-Chavez, E., Gonzalez-Ortega, E. and Herrera-Estrella, L.** (2004). The xipotl mutant of Arabidopsis reveals a critical role for phospholipid metabolism in root system development and epidermal cell integrity. *The Plant cell* **16**, 2020-2034.

**Datko, A. H. and Mudd, S. H.** (1988). Phosphatidylcholine synthesis: differing patterns in soybean and carrot. *Plant physiology* **88**, 854-861.

**De Rybel, B., Breda, A. S. and Weijers, D.** (2014). Prenatal plumbing--vascular tissue formation in the plant embryo. *Physiologia plantarum* **151**, 126-133.

**Demura, T., Tashiro, G., Horiguchi, G., Kishimoto, N., Kubo, M., Matsuoka, N., Minami, A., Nagata-Hiwatashi, M., Nakamura, K., Okamura, Y., et al.** (2002). Visualization by comprehensive microarray analysis of gene expression programs during transdifferentiation of mesophyll cells into xylem cells. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 15794-15799.

**Eklund, D. M., Staldal, V., Valsecchi, I., Cierlik, I., Eriksson, C., Hiratsu, K., Ohme-Takagi, M., Sundstrom, J. F., Thelander, M., Ezcurra, I., et al.** (2010). The Arabidopsis thaliana STYLISH1 protein acts as a transcriptional activator regulating auxin biosynthesis. *The Plant cell* **22**, 349-363.

**Esau, K.** (1950). Development and Structure of the Phloem Tissue .2. *Bot Rev* **16**, 67-114.

**Fukuda, H.** (1997). Programmed cell death during vascular system formation. *Cell death and differentiation* **4**, 684-688.

**Furuta, K. M., Yadav, S. R., Lehesranta, S., Belevich, I., Miyashima, S., Heo, J. O., Vaten, A., Lindgren, O., De Rybel, B., Van Isterdael, G., et al.** (2014). Arabidopsis NAC45/86 direct sieve element morphogenesis culminating in enucleation. *Science*.

**Gallavotti, A., Barazesh, S., Malcomber, S., Hall, D., Jackson, D., Schmidt, R. J. and McSteen, P.** (2008). sparse inflorescence1 encodes a monocot-specific YUCCA-like gene required for vegetative and reproductive development in maize. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 15196-15201.

**Gout, E., Bligny, R., Roby, C. and Douce, R.** (1990). Transport of phosphocholine in higher plant cells: 31P nuclear magnetic resonance studies. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 4280-4283.

**Grunewald, W. and Friml, J.** (2010). The march of the PINs: developmental plasticity by dynamic polar targeting in plant cells. *The EMBO journal* **29**, 2700-2714.

**Guo, Y., Qin, G., Gu, H. and Qu, L. J.** (2009). Dof5.6/HCA2, a Dof transcription factor gene, regulates interfascicular cambium formation and vascular tissue development in Arabidopsis. *The Plant cell* **21**, 3518-3534.

**Hayashi, K.** (2012). The interaction and integration of auxin signaling components. *Plant & cell physiology* **53**, 965-975.

**He, W., Brumos, J., Li, H., Ji, Y., Ke, M., Gong, X., Zeng, Q., Li, W., Zhang, X., An, F., et al.** (2011). A small-molecule screen identifies L-kynurenine as a competitive inhibitor of TAA1/TAR activity in ethylene-directed auxin biosynthesis and root growth in Arabidopsis. *The Plant cell* **23**, 3944-3960.

**Hutchison, C. E., Li, J., Argueso, C., Gonzalez, M., Lee, E., Lewis, M. W., Maxwell, B. B., Perdue, T. D., Schaller, G. E., Alonso, J. M., et al.** (2006). The Arabidopsis histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling. *The Plant cell* **18**, 3073-3087.

**Ingram, P., Dettmer, J., Helariutta, Y. and Malamy, J. E.** (2011). Arabidopsis Lateral Root Development 3 is essential for early phloem development and function, and hence for normal root system development. *The Plant journal : for cell and molecular biology* **68**, 455-467.

**Jaffe, M. J.** (1970). Evidence for the regulation of phytochrome-mediated processes in bean roots by the neurohumor, acetylcholine. *Plant physiology* **46**, 768-777.

**Kieber, J. J. and Schaller, G. E.** (2014). Cytokinins. *The Arabidopsis book / American Society of Plant Biologists* **12**, e0168.

**Kim, J. I., Baek, D., Park, H. C., Chun, H. J., Oh, D. H., Lee, M. K., Cha, J. Y., Kim, W. Y., Kim, M. C., Chung, W. S., et al.** (2013). Overexpression of Arabidopsis YUCCA6 in potato results in high-auxin developmental phenotypes and enhanced resistance to water deficit. *Molecular plant* **6**, 337-349.

**Kondo, Y., Hirakawa, Y., Kieber, J. J. and Fukuda, H.** (2011). CLE peptides can negatively regulate protoxylem vessel formation via cytokinin signaling. *Plant & cell physiology* **52**, 37-48.

**Kondo, Y., Tamaki, T. and Fukuda, H.** (2014). Regulation of xylem cell fate. *Frontiers in plant science* **5**, 315.

**Korasick, D. A., Enders, T. A. and Strader, L. C.** (2013). Auxin biosynthesis and storage forms. *Journal of experimental botany* **64**, 2541-2555.

**Kubo, M., Udagawa, M., Nishikubo, N., Horiguchi, G., Yamaguchi, M., Ito, J., Mimura, T., Fukuda, H. and Demura, T.** (2005). Transcription switches for protoxylem and metaxylem vessel formation. *Genes & development* **19**, 1855-1860.

**Ljung, K., Bhalerao, R. P. and Sandberg, G.** (2001). Sites and homeostatic control of auxin biosynthesis in Arabidopsis during vegetative growth. *The Plant journal : for cell and molecular biology* **28**, 465-474.

**Ljung, K., Hull, A. K., Celenza, J., Yamada, M., Estelle, M., Normanly, J. and Sandberg, G.** (2005). Sites and regulation of auxin biosynthesis in Arabidopsis roots. *The Plant cell* **17**, 1090-1104.

**Lucas, W. J., Groover, A., Lichtenberger, R., Furuta, K., Yadav, S. R., Helariutta, Y., He, X. Q., Fukuda, H., Kang, J., Brady, S. M., et al.** (2013). The plant vascular system: evolution, development and functions. *Journal of integrative plant biology* **55**, 294-388.

**Lucas, W. J., Yoo, B. C. and Kragler, F.** (2001). RNA as a long-distance information macromolecule in plants. *Nature reviews. Molecular cell biology* **2**, 849-857.

**Madhavan, S., Sarath, G. and Herman, P. L.** (1995). Acetylcholinesterase activity in plants. *Enzymes of the Cholinesterase Family*, 291-292.

**Mahonen, A. P., Bishopp, A., Higuchi, M., Nieminen, K. M., Kinoshita, K., Tormakangas, K., Ikeda, Y., Oka, A., Kakimoto, T. and Helariutta, Y.** (2006). Cytokinin signaling and its inhibitor AHP6 regulate cell fate during vascular development. *Science* **311**, 94-98.

**Mahonen, A. P., Bonke, M., Kauppinen, L., Riikonen, M., Benfey, P. N. and Helariutta, Y.** (2000). A novel two-component hybrid molecule regulates vascular morphogenesis of the Arabidopsis root. *Genes & development* **14**, 2938-2943.

**Mano, Y. and Nemoto, K.** (2012). The pathway of auxin biosynthesis in plants. *Journal of experimental botany* **63**, 2853-2872.

**Mashiguchi, K., Tanaka, K., Sakai, T., Sugawara, S., Kawaide, H., Natsume, M., Hanada, A., Yaeno, T., Shirasu, K., Yao, H., et al.** (2011). The main auxin biosynthesis pathway in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 18512-18517.

**Maule, A. J., Benitez-Alfonso, Y. and Faulkner, C.** (2011). Plasmodesmata - membrane tunnels with attitude. *Current opinion in plant biology* **14**, 683-690.

**McNeil, S. D., Rhodes, D., Russell, B. L., Nuccio, M. L., Shachar-Hill, Y. and Hanson, A. D.** (2000). Metabolic modeling identifies key constraints on an engineered glycine betaine synthesis pathway in tobacco. *Plant physiology* **124**, 153-162.

**Miyashima, S., Koi, S., Hashimoto, T. and Nakajima, K.** (2011). Non-cell-autonomous microRNA165 acts in a dose-dependent manner to regulate multiple differentiation status in the Arabidopsis root. *Development* **138**, 2303-2313.

**Momonoki, Y. S.** (1992). Occurrence of acetylcholine-hydrolyzing activity at the stele-cortex interface. *Plant physiology* **99**, 130-133.

**----** (1997). Asymmetric distribution of acetylcholinesterase in gravistimulated maize seedlings. *Plant physiology* **114**, 47-53.

**Momonoki, Y. S., Hineno, C. and Noguchi, K.** (1998). Acetylcholine as a signaling system to environmental stimuli in plants. III. Asymmetric solute distribution controlled by ACh in gravistimulated maize seedlings. *Plant production science* **1**, 83-88.

**Muniz, L., Minguet, E. G., Singh, S. K., Pesquet, E., Vera-Sirera, F., Moreau-Courtois, C. L., Carbonell, J., Blazquez, M. A. and Tuominen, H.** (2008). ACAULIS5 controls Arabidopsis xylem specification through the prevention of premature cell death. *Development* **135**, 2573-2582.

**Nemoto, K., Hara, M., Suzuki, M., Seki, H., Muranaka, T. and Mano, Y.** (2009). The NtAMI1 gene functions in cell division of tobacco BY-2 cells in the presence of indole-3-acetamide. *FEBS letters* **583**, 487-492.

**Normanly, J.** (2010). Approaching cellular and molecular resolution of auxin biosynthesis and metabolism. *Cold Spring Harbor perspectives in biology* **2**, a001594.

**Normanly, J., Cohen, J. D. and Fink, G. R.** (1993). Arabidopsis thaliana auxotrophs reveal a tryptophan-independent biosynthetic pathway for indole-3-acetic acid. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 10355-10359.

**Normanly, J., Grisafi, P., Fink, G. R. and Bartel, B.** (1997). Arabidopsis mutants resistant to the auxin effects of indole-3-acetonitrile are defective in the nitrilase encoded by the NIT1 gene. *The Plant cell* **9**, 1781-1790.

**Novak, O., Henykova, E., Sairanen, I., Kowalczyk, M., Pospisil, T. and Ljung, K.** (2012). Tissue-specific profiling of the Arabidopsis thaliana auxin metabolome. *The Plant journal : for cell and molecular biology* **72**, 523-536.

**Nuccio, M. L., Ziemak, M. J., Henry, S. A., Weretilnyk, E. A. and Hanson, A. D.** (2000). cDNA cloning of phosphoethanolamine N-methyltransferase from spinach by complementation in

Schizosaccharomyces pombe and characterization of the recombinant enzyme. *The Journal of biological chemistry* **275**, 14095-14101.

**Oda, Y. and Fukuda, H.** (2012). Secondary cell wall patterning during xylem differentiation. *Current opinion in plant biology* **15**, 38-44.

**Ohashi-Ito, K., Demura, T. and Fukuda, H.** (2002). Promotion of transcript accumulation of novel Zinnia immature xylem-specific HD-Zip III homeobox genes by brassinosteroids. *Plant & cell physiology* **43**, 1146-1153.

**Ohashi-Ito, K. and Fukuda, H.** (2003). HD-zip III homeobox genes that include a novel member, ZeHB-13 (Zinnia)/ATHB-15 (Arabidopsis), are involved in procambium and xylem cell differentiation. *Plant & cell physiology* **44**, 1350-1358.

**Ohashi-Ito, K., Oda, Y. and Fukuda, H.** (2010). Arabidopsis VASCULAR-RELATED NAC-DOMAIN6 directly regulates the genes that govern programmed cell death and secondary wall formation during xylem differentiation. *The Plant cell* **22**, 3461-3473.

**Overvoorde, P., Fukaki, H. and Beeckman, T.** (2010). Auxin control of root development. *Cold Spring Harbor perspectives in biology* **2**, a001537.

**Park, W. J., Kriechbaumer, V., Moller, A., Piotrowski, M., Meeley, R. B., Gierl, A. and Glawischnig, E.** (2003). The Nitrilase ZmNIT2 converts indole-3-acetonitrile to indole-3-acetic acid. *Plant physiology* **133**, 794-802.

**Petersson, S. V., Johansson, A. I., Kowalczyk, M., Makoveychuk, A., Wang, J. Y., Moritz, T., Grebe, M., Benfey, P. N., Sandberg, G. and Ljung, K.** (2009). An auxin gradient and maximum in the Arabidopsis root apex shown by high-resolution cell-specific analysis of IAA distribution and synthesis. *The Plant cell* **21**, 1659-1668.

**Pinon, V., Prasad, K., Grigg, S. P., Sanchez-Perez, G. F. and Scheres, B.** (2013). Local auxin biosynthesis regulation by PLETHORA transcription factors controls phyllotaxis in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 1107-1112.

**Pollmann, S., Neu, D., Lehmann, T., Berkowitz, O., Schafer, T. and Weiler, E. W.** (2006). Subcellular localization and tissue specific expression of amidase 1 from Arabidopsis thaliana. *Planta* **224**, 1241-1253.

**Robert, S., Kleine-Vehn, J., Barbez, E., Sauer, M., Paciorek, T., Baster, P., Vanneste, S., Zhang, J., Simon, S., Covanova, M., et al.** (2010). ABP1 mediates auxin inhibition of clathrin-dependent endocytosis in Arabidopsis. *Cell* **143**, 111-121.

**Robischon, M., Du, J., Miura, E. and Groover, A.** (2011). The Populus class III HD ZIP, popREVOLUTA, influences cambium initiation and patterning of woody stems. *Plant physiology* **155**, 1214-1225.

**Scheres, B., Dilaurenzio, L., Willemsen, V., Hauser, M. T., Janmaat, K., Weisbeek, P. and Benfey, P. N.** (1995). Mutations Affecting the Radial Organization of the Arabidopsis Root Display Specific Defects Throughout the Embryonic Axis. *Development* **121**, 53-62.

**Schuetz, M., Smith, R. and Ellis, B.** (2013). Xylem tissue specification, patterning, and differentiation mechanisms. *Journal of experimental botany* **64**, 11-31.

**Sjolund, R. D.** (1997). The phloem sieve element: A river runs through it. *The Plant cell* **9**, 1137-1146.

**Stadler, R., Wright, K. M., Lauterbach, C., Amon, G., Gahrtz, M., Feuerstein, A., Oparka, K. J. and Sauer, N.** (2005). Expression of GFP-fusions in Arabidopsis companion cells reveals non-specific protein trafficking into sieve elements and identifies a novel post-phloem domain in roots. *The Plant journal : for cell and molecular biology* **41**, 319-331.

**Stepanova, A. N., Robertson-Hoyt, J., Yun, J., Benavente, L. M., Xie, D. Y., Dolezal, K., Schlereth, A., Jurgens, G. and Alonso, J. M.** (2008). TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. *Cell* **133**, 177-191.

**Stepanova, A. N., Yun, J., Robles, L. M., Novak, O., He, W., Guo, H., Ljung, K. and Alonso, J. M.** (2011). The Arabidopsis YUCCA1 flavin monooxygenase functions in the indole-3-pyruvic acid branch of auxin biosynthesis. *The Plant cell* **23**, 3961-3973.

**Strader, L. C. and Bartel, B.** (2011). Transport and metabolism of the endogenous auxin precursor indole-3-butyric acid. *Molecular plant* **4**, 477-486.

**Sugawara, S., Hishiyama, S., Jikumaru, Y., Hanada, A., Nishimura, T., Koshiba, T., Zhao, Y., Kamiya, Y. and Kasahara, H.** (2009). Biochemical analyses of indole-3-acetaldoxime-dependent auxin biosynthesis in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 5430-5435.

**Summers, P. S. and Weretilnyk, E. A.** (1993). Choline Synthesis in Spinach in Relation to Salt Stress. *Plant physiology* **103**, 1269-1276.

**Tao, Y., Ferrer, J. L., Ljung, K., Pojer, F., Hong, F., Long, J. A., Li, L., Moreno, J. E., Bowman, M. E., Ivans, L. J., et al.** (2008). Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. *Cell* **133**, 164-176.

**Thompson, M. V.** (2006). Phloem: the long and the short of it. *Trends in plant science* **11**, 26-32.

**Thompson, M. V. and Holbrook, N. M.** (2003). Application of a single-solute non-steady-state phloem model to the study of long-distance assimilate transport. *Journal of theoretical biology* **220**, 419-455.

**Timpte, C.** (2001). Auxin binding protein: curiouser and curiouser. *Trends in plant science* **6**, 586-590.

**Traiffort, E., Ruat, M., O'Regan, S. and Meunier, F. M.** (2005). Molecular characterization of the family of choline transporter-like proteins and their splice variants. *Journal of neurochemistry* **92**, 1116-1125.

**Tromas, A., Paque, S., Stierle, V., Quettier, A. L., Muller, P., Lechner, E., Genschik, P. and Perrot-Rechenmann, C.** (2013). Auxin-binding protein 1 is a negative regulator of the SCF(TIR1/AFB) pathway. *Nature communications* **4**, 2496.

**Truernit, E., Bauby, H., Belcram, K., Barthelemy, J. and Palauqui, J. C.** (2012). OCTOPUS, a polarly localised membrane-associated protein, regulates phloem differentiation entry in Arabidopsis thaliana. *Development* **139**, 1306-1315.

**Truernit, E., Bauby, H., Dubreucq, B., Grandjean, O., Runions, J., Barthelemy, J. and Palauqui, J. C.** (2008). High-resolution whole-mount imaging of three-dimensional tissue organization and gene expression enables the study of Phloem development and structure in Arabidopsis. *The Plant cell* **20**, 1494-1503.

**Ursache, R., Nieminen, K. and Helariutta, Y.** (2013). Genetic and hormonal regulation of cambial development. *Physiologia plantarum* **147**, 36-45.

**Wang, W., Xu, B., Wang, H., Li, J., Huang, H. and Xu, L.** (2011). YUCCA genes are expressed in response to leaf adaxial-abaxial juxtaposition and are required for leaf margin development. *Plant physiology* **157**, 1805-1819.

**Vaten, A., Dettmer, J., Wu, S., Stierhof, Y. D., Miyashima, S., Yadav, S. R., Roberts, C. J., Campilho, A., Bulone, V., Lichtenberger, R., et al.** (2011). Callose biosynthesis regulates symplastic trafficking during root development. *Developmental cell* **21**, 1144-1155.

**Weretilnyk, E. A., Bednarek, S., McCue, K. F., Rhodes, D. and Hanson, A. D.** (1989). Comparative biochemical and immunological studies of the glycine betaine synthesis pathway in diverse families of dicotyledons. *Planta* **178**, 342-352.

**Wessler, I., Kilbinger, H., Bittinger, F. and Kirkpatrick, C. J.** (2001). The biological role of non-neuronal acetylcholine in plants and humans. *Japanese journal of pharmacology* **85**, 2-10.

**Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G. V. and Provart, N. J.** (2007). An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. *PloS one* **2**, e718.

**Won, C., Shen, X., Mashiguchi, K., Zheng, Z., Dai, X., Cheng, Y., Kasahara, H., Kamiya, Y., Chory, J. and Zhao, Y.** (2011). Conversion of tryptophan to indole-3-acetic acid by TRYPTOPHAN AMINOTRANSFERASES OF ARABIDOPSIS and YUCCAs in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 18518-18523.

**Wu, H. and Zheng, X. F.** (2003). Ultrastructural studies on the sieve elements in root protophleom of Arabidopsis thaliana. *Acta Bot Sin* **45**, 322-330.

**Xie, B., Wang, X., Zhu, M., Zhang, Z. and Hong, Z.** (2011). CalS7 encodes a callose synthase responsible for callose deposition in the phloem. *The Plant journal : for cell and molecular biology* **65**, 1-14.

**Xu, T., Dai, N., Chen, J., Nagawa, S., Cao, M., Li, H., Zhou, Z., Chen, X., De Rycke, R., Rakusova, H., et al.** (2014). Cell surface ABP1-TMK auxin-sensing complex activates ROP GTPase signaling. *Science* **343**, 1025-1028.

**Yamada, N., Sakakibara, S., Tsutsumi, K., Waditee, R., Tanaka, Y. and Takabe, T.** (2011). Expression and substrate specificity of betaine/proline transporters suggest a novel choline transport mechanism in sugar beet. *Journal of plant physiology* **168**, 1609-1616.

**Yamaguchi, M., Kubo, M., Fukuda, H. and Demura, T.** (2008). Vascular-related NAC-DOMAIN7 is involved in the differentiation of all types of xylem vessels in Arabidopsis roots and shoots. *The Plant journal : for cell and molecular biology* **55**, 652-664.

**Yamaguchi, M., Mitsuda, N., Ohtani, M., Ohme-Takagi, M., Kato, K. and Demura, T.** (2011). VASCULAR-RELATED NAC-DOMAIN7 directly regulates the expression of a broad range of genes for xylem vessel formation. *The Plant journal : for cell and molecular biology* **66**, 579-590.

**Yamamoto, Y., Kamiya, N., Morinaka, Y., Matsuoka, M. and Sazuka, T.** (2007). Auxin biosynthesis by the YUCCA genes in rice. *Plant physiology* **143**, 1362-1371.

**Zhao, Y.** (2010). Auxin biosynthesis and its role in plant development. *Annual review of plant biology* **61**, 49-64.

**Zhou, J., Wang, X., Lee, J. Y. and Lee, J. Y.** (2013). Cell-to-cell movement of two interacting AT-hook factors in Arabidopsis root vascular tissue patterning. *The Plant cell* **25**, 187-2