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YEB HANNA HELP-RINTA-RAHKO The Interaction Of Auxin and Cytokinin Signalling Regulates Primary Root Procambial Patterning 1/2016

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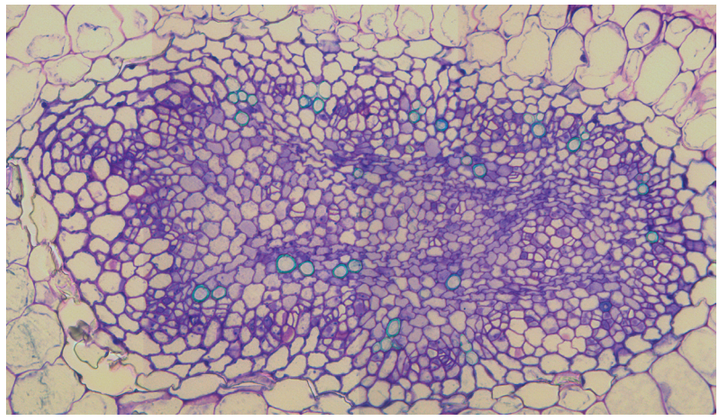
HANNA HELP-RINTA-RAHKO

The Interaction of Auxin and Cytokinin Signalling Regulates Primary Root Procambial Patterning, Xylem Cell Fate and Differentiation in *Arabidopsis thaliana*



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Doctoral Thesis

The interaction of auxin and cytokinin signalling regulates primary root procambial patterning, xylem cell fate and differentiation in *Arabidopsis thaliana*

Hanna Help-Rinta-Rahko

UNIVERSITY OF HELSINKI

ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki in the Auditorium 1041 at Biocenter 2, Viikinkaari 5, Helsinki, Finland on April 22nd 2016 at 12 o'clock noon.

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For my F1 - Hertta.

“Tosiasioiden tunnustaminen on kaiken viisauden alku”

-J.K. Paasikivi

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List of Original publications

I. **Help**, Mähönen, Helariutta and Bishopp, 2011 Bisymmetry in the embryonic root is dependent on cotyledon number. *Plant Signaling & Behavior*, Volume 6, Issue 11, Pages 1837-1840.

II. Bishopp1, **Help**1, El-Showk, Weijers, Scheres, Friml, Benková, Mähönen and Helariutta, 2011. A Mutually Inhibitory Interaction between Auxin and Cytokinin Specifies Vascular Pattern in Roots. *Current Biology*, Volume 21, Issue 11, 7 June 2011, Pages 917–926.

III. Bishopp, Lehesranta2, Vatén2, **Help**, El-Showk, Scheres, Helariutta, Mähönen, Sakakibara and Helariutta, 2011. Phloem-Transported Cytokinin Regulates Polar Auxin Transport and Maintains Vascular Pattern in the Root Meristem. *Current Biology*, Volume 21, Issue 11, 7 June 2011, Pages 927–932.

IV. Wrzaczek1, Vainonen1, Stael2, Tsiatsiani2, **Help-Rinta-Rahko**2, Gauthier, Kaufholdt, Bollhöner, Lamminmäki, Staes, Gevaert, Tuominen, Van Breusegem, Helariutta and Kangasjärvi, 2015. GRIM REAPER peptide binds to receptor kinase PRK5 to trigger cell death in *Arabidopsis*. *The EMBO Journal*, January 2015, Volume 34, Issue 1, Pages 55-66.

1. Authors concuded equally to this work as first authors
2. Authors concuded equally to this work as second authors

Author's contribution

I. HHRR participated in designing the experiments and carried out all experiments presented in the paper. HHRR participated in writing the manuscript with APM and AB.

II. HHRR participated in designing experiments. HHRR did the crosses and selected the material used in analyses (excluding *CRE1::XVE>>axr3-1 AHP6::GFP*, *AHP6mut::GFP*, *CRE1::XVE>>CKI PIN7::PIN7-GFP* and *CRE1::XVE>>PIN7 DR5::GFP* lines). HHRR participated in *agrobacterium* transformations of published lines. HHRR conducted confocal analysis of the published lines excluding the abovementioned lines. HHRR performed all histological analysis including GUS-stainings, fuchsin stainings and plastic cross sections and did all anatomical phenotypings. HHRR wrote the manuscript with AB and YH.

III. HHRR participated in designing the experiments and carried out part of the confocal analysis, fuchsin stainings and anatomical phenotypings. HHRR selected *pAPL::icals3m* lines in the T2 generation and performed the analysis of the marker genes in wt and transgenic *pAPL::icals3m* lines and participated in the analysis of the patterning defects in *apl* and *CKX1:YFP* lines. HHRR performed all histological GUS-stainings and plastic sections. HHRR participated in writing the manuscript.

IV. HHRR designed and carried out all protein subcellular localization assays with *Arabidopsis* protoplasts. HHRR participated in writing the manuscript.

The unpublished data presented in thesis parts I, II and III:

Part I: HHRR produced all the unpublished shown data in Figure 4 (of which panels A, B, C, F, G, K, Q, V and W were published in OP II), Figure 5, Figure 7, Figure 8 and Figure 9.

Part II: AB cloned and transformed the *pCRE1-XVE::CRE1* construct into *wol* mutant background. HHRR selected the primary transformants and performed the consequtive selection of lines for analysis, did the crosses to marker lines (*ARR5::GUS, PIN7::PIN7-GFP*, both published in OPII) and performed all histological, anatomical and marker gene expression imaging analysis (presented in figures 11, 12, 13, 14, 15 and 16).

Part III: MW cloned and produced the *pGRI::GUS* line, cloned and transformed protoplasts with the PRK5-CFP, At3g20190-CFP and At2g07040-CFP constructs (published in OP IV) and did the statistical analysis of the unpublished internalization data presented in figure 22. HHRR did all the histological analysis and GUS- plastic sections of the GRI::GUS and AtMC9::GUS lines, anatomical sections of gri1 mutants, and did the receptor localization and internalization confocal imaging assays on protoplasts expressing FLS2-GFP and PRK5-CFP, At3g20190-CFP and At2g07040-CFP.

Abreviations

BA	benzyl adenine, 6-Benzylaminopurine
CK	cytokinin
cZ	cis-zeatin
DNA	deoxyribonucleic acid
dpg	days post germination
dpi	days post induction
ER	endoplasmatic reticulum
GUS	β-glucuronidase
GFP	green fluorescent protein
H_2O_2	hydrogen peroxide
hpi	hours post induction
iP	isopentenyl-adenine
IAA	indole-3-acetic acid
LR	lateral root
miRNA	micro RNA
NPA	N-1-Naphthylphthalamic Acid
PAT	polar auxin transport
PIN	PIN FORMED protein
PCD	programmed cell death
QC	quiescent center
RAM	root apical meristem
RNA	ribonucleic acid
ROS	reactive oxygen species
SAM	shoot apical meristem
TE	tracheary element
TF	transcription factor
tZ	trans-zeatin

Abstract

The interaction of auxin and cytokinin signalling regulates primary root procambial patterning, xylem cell fate and differentiation in *Arabidopsis thaliana.*

Plants contribute to the Earth's atmosphere by binding carbon dioxide and releasing oxygen. Trees produce biomass, which is a renewable source of energy. The *Arabidopsis* root vasculature is a good model system for studying biomass formation, as it contains the same cell types that are also found in trees: xylem, phloem and intervening pluripotent procambial cells. In *Arabidopsis thaliana* roots, these cells arise from stem cells within the root meristem. The wild type root radial pattern is bisymmetric, and the regulation of xylem formation is controlled by phytohormones, especially auxin and cytokinin.

Our findings show that the vascular pattern is set by a symmetry-breaking event during embryogenesis and is initiated by auxin accumulation and signalling at the cotyledon initials. As the embryo grows, the high auxin signalling promotes the expression of AHP6. Upregulation of AHP6 in specific cells leads to inhibition of cytokinin signalling and might be a key factor in symmetry breakage. Mutants with altered cotyledon numbers or altered cotyledon anatomy fail to establish the bisymmetric pattern and often show altered root symmetry. In growing roots, the bisymmetric pattern is actively reinforced by polar auxin transport and long distance cytokinin transport/translocation from the apical parts of the plant. Cytokinin movement via the phloem and unloading at the root apical meristem promotes cytokinin signalling in the procambial cells in the proximal meristem. Both cytokinin and auxin are required during root procambial patterning, and the interaction of these two phytohormones is mutually inhibitory. According to our model (described in the first part of this thesis), auxin signalling is critical for protoxylem identity formation. In turn, the results from the procambial re-patterning experiments (second part of this thesis) show that cytokinin is the key hormone in promoting cell proliferation in the proximal meristem. Epistasis experiments illustrate that a fine balance between these two hormones affects the fate of all vascular cells.

We are beginning to understand the complexity and interdependencies of signalling pathway interactions during proximal meristem vascular patterning, yet the temporal aspect is still largely unexplored. In the last part of this thesis, I discuss the role ROS signalling might have in stele patterning and temporal regulation of programmed cell death. While our published GRI-MC9-PRK5 module might not be directly linked to primary root proximal meristem procambial patterning, one cannot exclude the possibility that it might be required in the final stages of protoxylem differentiation or that a similar signalling mechanism could regulate initial stele patterning and meristem growth dynamics.

This thesis describes the auxin-cytokinin interaction in vascular initial patterning and the mechanism by which the hormonal signalling domains are maintained in the proximal meristem. The unpublished data demonstrate how procambial cells can be manipulated to generate new tissues by affecting the homeostasis of auxin and cytokinin signalling. The last part of the thesis describes a cell death signalling module and speculates that it (or similar module) might be involved with primary root meristem maturation.

Tiivistelmä

Auksiini-sytokiniini-signaloinnin vuorovaikutus *Arabidopsis thalianan* primääri-juuren johtojänteen solutyyppien identiteetin muodostumisen ja puusolukon erilaistumisen säätelyssä.

Kasvit vaikuttavat maapallon ilmakehän koostumukseen sitomalla itseensä hiilidioksidia sekä vapauttamalla happea. Puut tuottavat biomassaa, joka on uusiutuva energianlähde. Puusolukon muodostumista säätelevät kasvihormonit, erityisesti auksiini ja sytokiniini. *Arabidopsis thalianan* eli lituruohon juuren johtojänne on hyvä malli puunmuodostuksen tutkimiselle, sillä se sisältää samat solutyypit kuin suuremmat puuvartiset kasvit. Nämä keskeiset solutyyppit ovat ksyleemi (puu), nila ja jälsi, joka sijaitsee ksyleemi- ja nilasolujen välissä. Nämä solutyypit kehittyvät lituruohon juurissa kärkikasvupisteiden kantasoluista. Villityypin lituruohon johtojänne on rakenteeltaan bisymmetrinen. Johtojänteen rakenne muodostuu varhain alkiokehityksen aikana, ja juurten symmetria määräytyy verson sirkkalehtien perusteella. Auksiini akkumuloituu sirkkalehtien aiheisiin. Kun alkio kasvaa suuremmaksi, korkea auksiini-pitoisuus edistää AHP6-geenin ekspressiota sirkkalehdissä ja alkion juuren johtojänteessä. Tämä soluspesifinen AHP6 ilmentyminen johtaa sytokiniinisignaloinnin inhibitioon, mikä on kriittistä bisymmetrian muodostumiselle. Mutanteilla joiden sirkkalehtien lukumäärä tai muoto poikkeaa normaalista, on havaittu ongelmia sekä bisymmetrisen rakenteen muodostumisessa alkionkehityksen aikana, että juuren normaalin rakenteen ylläpidossa itämisen jälkeen. Kasvavien juurten rakennetta pidetään aktiivisesti yllä auksiinin ja sytokiniinin kuljetuksella versoista juuriin. Sytokiniinin liikkuminen nilan mahlavirtauksen mukana juurten kärkiin edistää sytokiniinisignalointia kärkikasvupisteen kantasoluissa ja niiden tytärsoluissa. Sekä sytokiniinia että auksiinia tarvitaan johtosolukon erilaistumiseen ja nämä hormonit vaikuttavat toisiinsa inhiboivasti.

Esittämämme mallin mukaan (kuvattu ensimmäisessä osiossa) auksiinisignalointi on kriittistä protoksyleemin identiteetin muodostumiselle. Sytokiniini on puolestaan tärkeää juuren kärkikasvupisteen solujen jakautumisen ja erilaistumattomien jälsisolujen identiteetille, kuten tulokset johtojänteen uudelleen-järjestäytymiskokeista osoittavat (väitöskirjan toisessa osuudessa). Näiden kahden hormonin välinen epistasia säätelee kaikkien johtojänteen solujen kehitystä. Tämän väitöskirjan viimeinen osuus keskittyy happiradikaali-signaloinnin ja kontrolloidun solukuoleman rooliin juuren meristeemin kehityksessä. Vaikka julkaisemamme GRI-MC9-PRK5-moduuli ei vaikuta liittyvän kärkikasvupisteen kantasolujen identiteetin ja johtojänteen rakenteen säätelyyn, on mahdollista että sitä tarvitaan protoksyleemin erilaistumisessa myöhemmissä vaiheissa. Väitöskirjan viimeisessä osuudessa spekuloidaan sillä, mikä rooli solukuolemaan liittyvällä signaloinnilla on juuren kärkikasvupisteen kypsymisen säätelyssä.

Tämä väitöskirjatyö havainnollistaa auksiini-sytokiniini-vuorovaikutuksen roolia johtojänteen kantasolujen identiteetin muodostumisessa ja mekanismin, jolla hormonisignalointidomeenit vuorovaikuttavat toisiinsa. Tulokset osoittavat, että juuren rakennetta voidaan muuttaa keinotekoisesti manipuloimalla auksiini-sytokiniini hormonisignalointia. Ymmärryksemme eri hormonisignalointireittien monimutkaisuudesta ja niiden välisistä vuorovaikutuksista juuren johtojänteen eri solutyyppien identiteettien muodostumisessa on lisääntynyt merkittävästi viime vuosien aikana, mutta juuren kärkikasvupisteen eri solujen kypsymisen ajallinen säätely kaipaa lisää tutkimusta.

1. Introduction

1.1. Arabidopsis thaliana as a model species

Arabidopsis thaliana is a small dicotyledonous annual flowering plant in the *Brassicaceae* family. Many of its cousins are well known for their nutritional value, including species like *Brassica oleraceae* (kale), *Sinapis* spp. (mustard), *Brassica napus* and *Brassica rapa* (rapeseed and turnip, respectively), *Brassica junkea* (mustard green), and *Raphanus sativus* (radish). Some family members, such as *Isatis indigotica, I. tinctoria* and *Camelina sativa*, are also used for traditional medicinal purposes *(*Qin and Xu, 1998*). Arabidopsis thaliana* ecotypes grow all over the northern hemisphere (*The Arabidopsis Information Resource*), ranging from warm and temperate to arctic climates; even Finland has its own wild populations of *Arabidopsis thaliana* in the Southern and South-western areas (Kasviatlas). *Arabidopsis* has a relatively small diploid genome (~125 Mbp) arranged in five chromosome pairs. The genome is compact, as the intragenic regions are small and the amount of repetitive DNA is low compared to several other genomes (C-value Database). *Arabidopsis* can be genetically modified via random mutagenesis (by single nucleotide point mutations caused by EMS or larger T-DNA insertions) and targeted genome editing (CRISPR-Cas9, TALEN) and is effortlessly transformed by floral dipping (for generation of stable genome integrated plant lines) or transfection in cell cultures (for transient expression lines). The generation time of *Arabidopsis* is rather short (about 8-12 weeks), and plants can be grown in greenhouses all year round. Healthy *Arabidopsis* plants make hundreds or even thousands of seeds under good growth conditions, and since the plants are self-pollinating, desired mutants and marker lines can be easily maintained as pure homozygous lines (as long as the mutation is not embryo lethal). Many tools and techniques developed from other systems have been adapted for *Arabidopsis* research, enabling an incredible range of analysis. Open-access and commercial tools for various analyses (many of which are specific to *Arabidopsis*) can be readily found online and a cornucopia of information is in public databases. In short, *Arabidopsis* is a wonderful model species for plant molecular biology used by developmental biologists, plant stress researchers, ecologists, cell biologists, modellers and bioinformaticians. Basic research done on this humble weed is increasingly being extrapolated and applied to plant species of agronomical importance (e.g., rice, maize, pulp trees), and encouraging results have emerged with respect to increasing biofuel and biomass production, improving yield quality and quantity, and helping breed environmentally hardier crops.

Let us start our journey through this thesis at the very beginning: meristems, hormones and primary growth patterning. The literature cited in this thesis is from *Arabidopsis thaliana*, unless stated otherwise.

1.2. Plant stem cells

The fertilized *Arabidopsis* egg cell is totipotent. It has the capacity to become any cell type; its genetic differentiation potential is limitless. As this totipotent stem cell divides, its new daughter cells retain a great deal of differentiation potential and are pluripotent. These daughter cells divide further to give rise for multipotent

cells, which, as the embryo matures, differentiate and sometimes die to make distinct shapes and structures – tissues and organs. In plants, tissues are generated by meristematic stem cells that divide and give rise to new daughter cells. The position of a plant cell is permanently fixed due to the existence of cell walls, so positional cues are critical for cell patterning. These positional signals include cell autonomous and non-cell autonomous (cell-to-cell signalling) mechanisms and are primarily conveyed by mobile signalling molecules, such as peptides, transcription factors and hormones. In animals, hormones are secreted signalling molecules produced by specific cells in specific organs (e.g., testosterone or adrenaline). In plants, hormones are synthetized in various locations; some are produced locally in the meristems – similar to animals – whereas others are produced more widely around the plant body.

1.3. Meristems

The shoot apical meristem (SAM), located at the top of the plant, has a unipolar growth manner and grows upwards (Schweingruber, Bärner and Schulze, 2006). Root apical meristem (RAM) is located at the tip of the primary root (thesis Figure 1). It grows in a bipolar manner, producing primary growth not only towards the root itself but also towards the root cap (Schweingruber, Bärner & Schulze, 2006). The RAM produces all of the different cell types in roots (thesis Figure 2), including vascular tissues (which are discussed further below). Both the RAM and the SAM are primary meristems established during embryogenesis, and mutations that affect the formation or maintenance of these primary meristems can be devastating (Berleth and Jürgens, 1993, Mayer *et al.,* 1993 and 1999, Scheres *et al.,* 1995, Hamann *et al.,* 1999). *Arabidopsis* embryonic development has been characterized in detail (Weigel and Glazebrook, 2002). In wild type plants, embryonic development is very robust, due to signalling networks that control the rate and direction of cell divisions reliably ensuring precise patterning. Minor changes in embryonic cell divisions can affect the morphology and identity of the daughter cells, which in turn can have far reaching effects on the plant's entire architecture.

Apical growth takes place at the centre of the SAM, called the apical dome, and organ formation occurs at the margins of the SAM. The shoot apical and axillary meristems are responsible for both the vegetative and generative growth, as they can switch to a reproductive phase and become inflorescence meristems. In *Arabidopsis,* the vegetative shoot growth phase is easy to distinguish from the reproductive phase. During vegetative growth, the SAM makes rosette leaves which are all stacked on top of each other, and the stem is practically indistinguishable; when the SAM switches to reproductive fate, it makes a long inflorescence stem with several nodes, cauline leaves and multiple branches. The indeterminate inflorescence meristems produce flowers that get pollinated and make seeds (Shannon and Meeks-Wagner, 1991). As an annual plant, flowering is a one way street for *Arabidopsis*; once the plant starts making flowers and siliques it will end its life cycle, senesce and die.

While the SAM and RAM form during embryonic development, secondary meristems form and activate post-embryonically throughout the plant body as it matures. In *Arabidopsis*, secondary meristems include lateral root meristems, adventitious root meristems, axillary branch meristems, cambium and cork cambium. Cambial tissue can be found both in roots and in shoots. The cambial

region is a narrow layer of cells wedged between the xylem and phloem; when these stem cells divide, they give rise to phloem cells and xylem vessel elements (with tracheid cells, fibres and parenchyma cells), producing secondary phloem towards the bark and secondary xylem cells towards the pith (reviewed by Nieminen *et al.,* 2015). The *Arabidopsis* shoot cambium is similar to other annual dicot plant species, consisting initially of primary vascular bundles that eventually fuse together to form a continuous cambial ring. Rather remarkably, the *Arabidopsis* root cambium is structurally and functionally highly similar to cambium found in trees, such as birch and aspen This similarity makes the *Arabidopsis* root an attractive system to study procambial and cambial pattering, as the results can be compared with and extrapolated to much larger species for optimizing biomass production.

Cork cambium, or phellogen, is a thin layer of meristematic cells which produces cork (phellem) towards the surface of the shoot, or epidermis, for protection and phelloderm as an inner layer (Schweingruber, Bärner & Schulze, 2006). The phelloderm below the phellogen consists mainly of cortical and living phloem cells and sclereid cells. Data about the phellogen in *Arabidopsis* is quite limited, as it is mainly studied in trees.

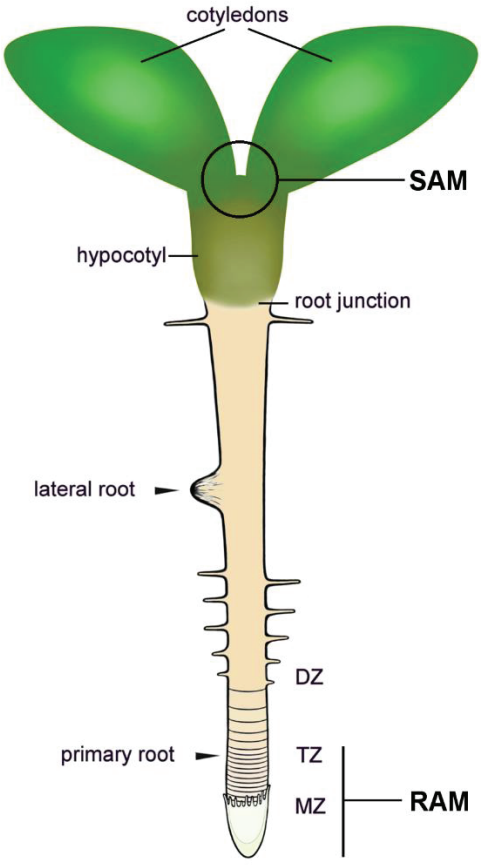

Figure 1: A schematic of a young *Arabidopsis thaliana* seedling. The schematic illustrates the shoot apical meristem (SAM) positioned between the two cotyledons, the primary root apical meristem (RAM), and the lateral root meristem (LR). The hypocotyl and root junction separate the shoot and root. The RAM contains three distinct zones: the meristematic zone (MZ), the transition zone (TZ) and the differentiation zone (DZ). Root hairs form in the differentiation zone.

1.3.1. Primary root proximal meristem

The *Arabidopsis thaliana* root apical meristem is a compact and robustly organized organ (Dolan *et al.,* 1993). It can be divided to three distinct zones: the meristematic zone, closest to the root tip, the transition zone and the differentiation zone. Cells in the meristematic zone are actively dividing, maintaining root growth. In the transition zone, meristematic cells switch to elongation growth before maturing and differentiating in the differentiation zone. The start of the differentiation zone can be distinguished by root hair formation (thesis Figure 1). The *Arabidopsis* proximal meristem is positioned just above the quiescent centre (QC) and contains a few dozen cells (with a total length of roughly 100μm) from the first meristematic initial cells to the transition zone. The proximal meristem is the region where pluripotent meristematic initials divide, producing daughter cells shootward. Growth regulators, such as phytohormones, nutrients, micro-RNAs, mobile transcription factors, peptides and other signalling molecules, converge in the proximal meristem and pattern the different cell types in the tissue (thesis Figure 2). While the *Arabidopsis* root proximal meristem is fairly simple and elegant in structure, it contains all the cell types (thesis Figure 2) that are present in larger and architecturally more complex plants. Due to its small size, the *Arabidopsis* primary root is a convenient model system for studying root cell type specification and differentiation.

At the very heart of the proximal meristem rests the quiescent centre (QC), which acts as an organizing centre for the different cell layers in the meristem. The QC is a cluster of four cells which act as stem cells (Dolan et al., 1993). In contrast to the actively dividing meristem initials, the QC cells are mitotically inactive and divide very seldom (thus retaining their totipotency). The link between division and genetic potential was reinforced by a recent study in which the length of telomeres was analysed in the different cell lineages of the *Arabidopsis* root. The results showed that the meristematic stem cells in the proximal meristem had the longest telomeres (González-García et al., 2015); since telomeres can be regarded as biomarkers for genetic longevity and potency, this indicates that the regeneration potential of plant stem cells is maintained in the meristems. Research has shown that cell divisions and differentiation rates are differentially regulated at the distal vs. proximal parts of the RAM, and that QC fate is actively promoted by mitotic suppression (Vanstraelen et al., 2009).

While the QC acts as a static organizer, continuous growth of the proximal meristem is fuelled by cell divisions in meristematic founder cells, or meristem initials. As these pluripotent initial cells divide, they produce daughter cells above them which in turn divide and differentiate into various cell types. This differentiation occurs a few cell layers away from the QC, maintaining separation of the stem cells. If an injury (such as an insect bite) damages the meristem initials, the QC cells can divide and replace those cells. It is noteworthy that not only the QC cells but the entire root meristem can regenerate under certain conditions, if necessary. Studies have shown that if the QC is destroyed by laser ablation, the proximal meristem initials around the damaged area can re-organize themselves and form a new, functioning QC (Sabatini *et al.,* 1999). Even if the entire root tip, including the QC, is chopped off, plants can overcome the damage and re-grow a RAM, including meristem initials, QC and columella cells. The meristem regeneration after cell ablation or cleavage is possible provided that the damage occurs within the meristematic zone, where the cells are still actively

proliferating and retain their tissue origin memory (Sena and Birnbaum, 2010). During this regeneration process, the root vasculature appears to function as an organizing centre.

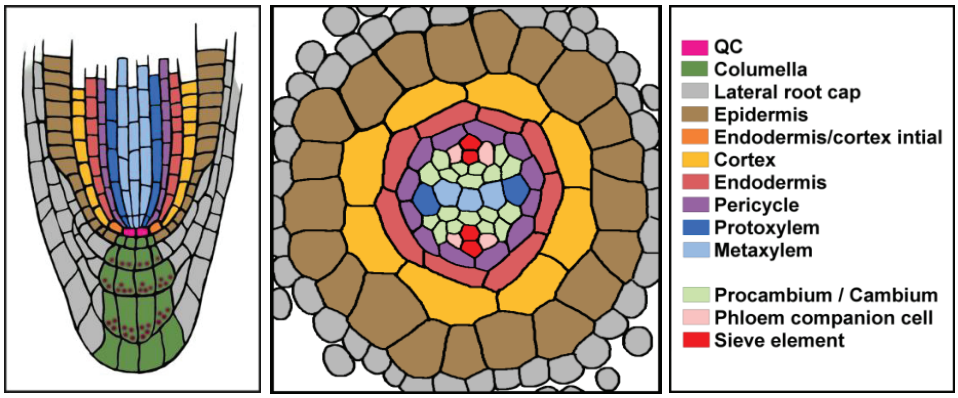

Figure 2: **Graphical illustration of the *Arabidopsis thaliana* primary root proximal meristem.** The longitudinal section on the left passes through the xylem axis, showing metaxylem cells (light blue) and protoxylem cells (dark blue) and pericycle (purple), endodermis (brick red), cortex (yellow), endodermis cortex initials (orange), QC (fuchsia), columella cells (green w/o purple statoliths) and lateral root cap cells (light grey). The transverse section of the proximal meristem in the middle illustrates all the cell types of the root, and also shows procambium (light green), phloem sieve element cells (bright red) and phloem companion cells (light pink). Modified from publication by Bishopp *et al.,* 2009.

The cells below the QC are the columella initials, which provide new cells for the root cap, the columella (thesis Figure 2). Columella cells contain amyloplasts (Kiss *et al.,* 1989), which are modified starch granules. These granules, or statoliths, are gravity anchors that move in the cells in response to gravity, and their movement triggers gravistimulus sensing (Kiss *et al.,* 1989; Takahashi *et al.,* 2003; Herranz *et al.,* 2014), possibly by transforming their kinetic energy to membrane deformations at the ER, triggering mechanosensing (Leitz *et al.,* 2009). This, in turn, leads to altered auxin signalling, resulting in a change of root growth dynamics (reviewed by Sato *et al.,* 2015). Adjacent to the QC are the epidermal and lateral root cap (LRC) initials, both of which provide lateral protection for the fragile, undifferentiated, thin-walled meristematic cells as the root grows in soil. Once mature, the LRC layers die and peel off at the transition zone of the meristem (thesis Figure 1) (Fendrych *et al.,* 2014) only to be replaced by new cells at the base of the meristem. The epidermal cells located just inwards of the LRC cells form the outermost cell layer in mature roots. These cells may take one of two identities, differentiating into hair cells (trichoblasts) or non-hair cells (atrichoblasts). The formation of root hairs from trichoblasts marks the beginning of the differentiation zone. Root hairs are thought to participate actively in water and nutrient harvesting from the soil, as well as acting as adhesive and rhizosphere sensing structures (Gilroy and Jones, 2000, Walker *et al.,* 2003). Each hair cell is in contact with two underlying cortex cells, whereas non-hair cells usually are in contact with only one cortical cell. The reason for this is a bit of a mystery. Perhaps the trichoblasts require some input from two adjacent cortex cells via cell-to-cell

communication for proper differentiation – in addition to the transcriptional feedback between them and the neighbouring atrichoblasts (Wada *et al,*. 2002).

Cortex cells lie beneath the epidermal cells (Dolan *et al.,* 2003). They are large cells that have a role in water and nutrient uptake from the soil (Javot and Maurel, 2002). The underlying endodermal cells are smaller in size and have a specific cell wall structure called the Casparian strip. The strip is made of lignin and is positioned at the centre of the longitudinal cell wall between two neighbouring endodermal cells (Naseer *et al.,* 2012). The Casparian strip is critical to nutrient and water uptake, since it forces these components and other molecules located in the apoplastic space (outside the cell plasma membrane) into the cells for symplastic transport. Older endodermal cells also form suberin lamellae, which give them extra insulation (reviewed by Geldner, 2013). Suberization occurs only further from the root tip, allowing younger roots to harvest water and nutrients before turning into tougher insulated structures. When fully suberized, the endodermal cells form a protective cylinder around the stele. However, this cylinder is not fully sealed, as there are sites, called passage cells, where the suberin is missing (Peterson and Enstone, 1996). It has been suggested that these passage cells might be required for transport of calcium and magnesium into the stele (Peterson and Enstone, 1996). Endodermal cells also play a role in stele patterning via miRNA-mediated signalling (Carlsbecker *et al.,* 2010, Vatén *et al.,* 2011), acting as a source for specific miRNA species that move via the plasmodesmata across the pericycle into the stele, where they control the fate of xylem and phloem cells. Thus, the endodermis acts as an insulating layer where components from the apoplastic space are channelled into the symplastic space where they can move between cells via: 1) active transport through highly selective and/or general transporters; or 2) diffusion or selective transport through the plasmodesmata, connective structures between different cells. The aperture of plasmodesmata can be modified to exclude molecules above a size threshold (Kim and Zambryski, 2005), and their number and aperture varies in different cell types, providing plants an effective but selective route for both long distance (from shoots to roots via the phloem) and short distance transport (e.g., between different meristematic cells).

The pericycle is located underneath the endodermis. Pericycle cells seem to have different gene expression profiles and might even have different identities based on their location within the stele (for example, phloem-pole pericycle and xylem-pole pericycle cells show different gene expression patterns). Although not much is known about the regulation of pericyclic identities, this cell layer is under intense investigation, especially in the context of lateral root development, since the protoxylem-associated pericycle cells can regain meristematic behaviour and give birth to lateral root primordia (Dubrovsky *et al.,* 2000).

The stele, or vascular bundle, is located at the centre of the root. The *Arabidopsis* stele is bisymmetric: one plane of symmetry is aligned along the xylem axis (thesis Figure 2) – the other plane along the phloem poles, which are located at a ~90 degree angle to the axis. The xylem axis is surrounded on both sides by undifferentiated procambial cells, which separate the xylem cells from the phloem poles. Maintenance of this bisymmetry and proper positioning of the tissues is critical for proper meristem growth, and mutants with disorganized proximal

meristem tissue often exhibit growth defects (Mähönen *et al.,* 2000; Bonke *et al.,* 2003).

The procambial cells function as a physical barrier of apparently non-differentiated cells between xylem and phloem, which have very different developmental programming and cell fates, and help maintain the properties of these tissues through spatial isolation. The status of auxin and cytokinin signalling and transport, cell-to-cell signalling and the recruitment of new cells to the xylem and phloem via cell division (as in the case of phloem companion cells) are controlled in the procambial cells. Procambium plays an essential role in regulating of the overall size and position of auxin and cytokinin hormone domains and cell identities in the proximal meristem stele. As the root matures, the procambial cells start proliferating through periclinal cell divisions, and the secondary growth phase is activated, turning procambium cells into cambium (Matsumoto-Kitano *et al.,* 2008; Zhang *et al.,* 2011).

In wild type *Arabidopsis* proximal meristems, the xylem axis is one cell layer wide and contains both protoxylem and metaxylem cells. The protoxylem cells are located at the ends of the axis touching pericycle cells (normally each protoxylem cell is connected to two pericyclic cells). Protoxylem cells are the first xylem cells to differentiate, coinciding with the emergence and elongation of root hairs. Protoxylem identity is already established in the first initial cells above the QC, as illustrated by the AHP6 marker (AFig. 1 in appendix). This early protoxylem identity gene expression sets in motion a genetic cascade that proceeds from: 1) establishment of the meristem initial's identity via repression of an inhibitory hormone (cytokinin) to 2) activation of differentiation promoting transcription factors (Kubo *et al.,* 2005; Yamaguchi *et al.,* 2010a and 2011), all the way to 3) promotion of cell death (Bollhöner *et al.,* 2013) and clearing of the protoxylem cell into a conductive empty vessel. When mature and differentiated, the protoxylem cell embodies a unique secondary cell wall structure, a lignified spiral inside the vessel (Mähönen *et al.,* 2000). The metaxylem cells are located between the protoxylem cells. Metaxylem cells differentiate considerably later than protoxylem cells, and their secondary cell walls have a very different lignification pattern, resembling pitted tubes. These proto- and metaxylem cell wall patterns are robust in wild type *Arabidopsis*. However, in some mutants with altered marker gene expression in the proximal meristem (such as expansion of AHP6 into metaxylem cells), protoxylem-like lignification patterns can also be detected higher up in the metaxylem position (Help-Rinta-Rahko, unpublished data). This suggests that xylem cell fates are not fixed at the meristem initials, and that maintenance of cell fate is an ongoing, multi-layered patterning process required until differentiation has taken place and the cells have died. Interestingly, the genetic cascades that define meta- and protoxylem cell fates are regulated by homologous genes (*VASCULAR-RELATED NAC-DOMAIN* genes) (Kubo *et al.,* 2005; Yamaguchi *et al.,* 2010a and 2011) that share regulatory components (such as *VNI2*, Yamaguchi *et al.,* 2010b).

The phloem poles consist of sieve elements and companion cells, all of which originate from the phloem initial cells in the proximal meristem. Like the protoxylem initials, the phloem pole initials touch the QC, indicating that their identity is established very early on. It remains to be seen whether the phloem initials are established already during embryogenesis, similar to the xylem. The

phloem initials give rise to all phloem cell types found in roots; protophloem, metaphloem and companion cells, through a tightly regulated cell division and patterning process which depends on various cell-to-cell signalling components (Miyashima & Sevilem unpublished data). As the phloem sieve elements differentiate, they lose their nucleus and other cellular organelles (Miyashima Furuta *et al.,* 2014) in an autolytic morphogenetic process called enucleation; unlike xylem, phloem sieve elements are not dead, and they depend on the companion cells for sustenance. Differentiated phloem strands are the major top-down transporting tissues in roots, and individual phloem sieve element cells are connected to each other via sieve tube plates, which contain both larger sieve pores and smaller plasmodesmata. The sieve element cells in wild type *Col-0* plants begin to enucleate ~200-300 μm above the QC (Miyashima Furuta *et al.,* 2014). The area around this region is called the phloem unloading zone. Here, molecules and compounds that are transported downwards from the shoot via phloem sap flow are unloaded and continue to move downwards to the root tip and laterally from one tissue to another via diffusion and active transport. If the cell-to-cell connections are blocked in phloem sieve elements or in the undifferentiated meristematic region, the vascular pattern is disturbed (Vatén *et al.,* 2011).

1.3.2. Main roles of the stele

1) Transport: The stele functions as the motorway for macro- and micro molecular transport and can be considered the "veins and arteries" of the plant. Individual xylem vessels are connected to one another via perforation plates, forming long, hollow tubes which can be tens of meters long in adult trees. Xylem cells conduct water from the roots up to the shoots via capillary action. The capillary action is based on negative water potential (Ψ), which is maintained by the continuous evaporation of water through leaf stomata. The negative water potential literally sucks the water from the root system to the over ground parts of the plants. Roots take up macro- and micro-nutrients from the soil, and these assimilated minerals and ions are also distributed around the plant through the xylem. The phloem is responsible for majority of the top-down transport from leaves to roots. Phloem sap is rich with sugars, minerals, amino acids, RNAs, phytohormones, small proteins and other putative signalling molecules (such as peptides). Phloem transport is thought to occur via different solute-specific mechanisms, which include passive gradient dependent diffusion, active transport via membrane transporter proteins and cytoplasmic cell-to-cell connections via plasmodesmata.

2) Support: Xylem cells (also termed tracheary elements (TE) in tracheophytes have thick secondary cell walls which contain cellulose, lignin, hemicellulose, xyloglucan, pectin and cell-wall-associated proteins, and other molecules (Carpita and Gibeaut, 1993). Accumulation and arrangement of these compounds in xylem vessels gives plants mechanical support and enables upright growth. These macromolecules are essential for the bioenergy and wood industries.

3) Generation of new tissues: Specific cell types within the stele can also act as sources for new meristems outside the meristematic zone of the primary root. These cell types are procambium and pericycle. As was briefly mentioned, activation of secondary growth occurs in procambial cells (reviewed by Zhang *et al.,* 2011; Miyashima *et al.,* 2013; Nieminen *et al.,* 2015), leading to the formation of secondary xylem and phloem. The pericycle cells touching the xylem axis (two cells on each side of the stele) can divide anticlinally and make a lateral root

primordium. This process is very robust and well characterized and under tight control through auxin and cytokinin signalling (Himanen *et al.,* 2004, reviewed by Aloni *et al.,* 2006, Laplaze *et al.,* 2007, Chang *et al.,* 2013; Marhavý *et al.,* 2014). After initiation, lateral root primordia emerge though the root endodermis, cortex and epidermis in a process called lateral root emergence (LRE). During LRE, a lateral root meristem gains its independence, and the lateral root starts to grow autonomously from the primary root.

1.4. Plant hormones

Phytohormones are the plant equivalent of animal hormones. Phytohormones are produced in small quantities, yet they are capable of regulating critical developmental programs and responses, such as the transition to flowering (sexual reproduction), seed germination ("birth"), and senescence ("death"). Like animal hormones, phytohormones are produced in different parts of the body, from which they are transported to target tissues by various mechanisms. The following compounds have been classified as plant hormones: auxins, cytokinins, gibberellins, brassinosteroids, strigolactones, karrikins, abscisic acid, salicylic acid, jasmonic acid (jasmonates) and ethylene. Certain plant peptides (*CLE, CLEL*) are developmentally critical and regulate cell-to-cell signalling within meristems (Strabala *et al.,* 2006; Kondo *et al.,* 2011; Meng *et al.,* 2013), and their signalling pathways can interact with other hormonal pathways (Kondo *et al.,* 2011). Peptides can thus be regarded as a novel class of plant hormones, along with nitric oxide species (NOS), reactive oxygen species (ROS) and polyamines, which also affect various developmental processes in plants and can be regarded as growth regulators, if not actual plant hormones themselves (Urano *et al.,* 2003; Wendehenne *et al.,* 2004; *Kwak et al.,* 2006; Matsubayashi and Sakagami, 2006).

Some hormones, such as auxin and cytokinins, can be considered "broad spectrum hormones" which affect an array of processes, whereas others are highly specific (e.g., karrikins (Nelson *et al.,* 2009a and 2009b; Chiwocha *et al.,* 2010)). While certain hormones are actively transported from cell to cell with specific transporters (e.g., auxins), others appear to move mainly through diffusion via cell-to-cell connections through plasmodesmata. Some hormones are small, volatile, gaseous molecules (e.g., ethylene), while others are larger, more complex compounds (auxins, cytokinins, gibberellins and brassinosteroids). Hormonal signalling pathways are highly linked to one another and have counteracting effects on development and stress responses (which will be discussed in more detail in chapters 1.7 and 1.8). The focus of this thesis is on the role of two of the most pleiotropic, developmentally important and actively studied plant hormones, auxins and cytokinins.

1.5. Auxins

The phytohormone auxin regulates plant growth and development through the establishment of local auxin signalling maxima. The primary targets of auxin signalling control the expression of downstream components which vary depending on the tissue, cell type and developmental process in question. In shoots, auxin promotes apical dominance and shade avoidance (Müller and Leyser, 2011). In leaves, it promotes vascular tissue formation and differentiation via canalization (Mattsson *et al.,* 2003). In roots, auxin transport and signalling is required for meristem growth (Blilou *et al.,* 2005) and gravistimulus response

(Ottenschläger *et al.,* 2003). In callus cultures, auxin promotes tissue regeneration (Ckurshumova and Berleth, 2015).

The genes that regulate auxin metabolism and action *in planta* make up large gene families with overlapping and distinct expression patterns. Auxin response elements (AuxRE) in the genome are recognized by various AUXIN RESPONSE FACTORS (ARFs) which promote or inhibit the expression of their targets genes (Guilfoyle and Hagen, 2007). The auxin response regulators and their downstream targets can also interact with different signalling pathways. In turn, components of other signalling pathways can regulate the biosynthesis, transport, signalling and degradation of auxins.

Different types of auxins have been extracted from plants, including indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA), 2-phenylacetic acid (PAA), and indole-3-butyric acid (IBA), which are all naturally occurring auxins. In addition, several synthetic auxins have been discovered. Some of them are potent chemicals used in agriculture as growth inhibitors, like 2,4-dichlorophenoxyacetic acid (2,4-D). Since high auxin concentrations can inhibit plant growth, endogenous levels are tightly controlled to maintain an optimum. Various mechanisms regulate auxin homeostasis, including biosynthesis, transport, degradation and conjugate formation (Ludwig-Müller 2010).

1.5.1. Auxin biosynthesis

IAA is the most abundant naturally occurring active auxin. The majority of IAA is synthetized via two main pathways: tryptophan-dependent and tryptophan-independent (Woodward and Bartel, 2005). Altogether, four separate auxin biosynthesis pathways have been proposed in plants, though the activity, components and regulation of all four are not yet fully characterized (Mashiguchi et al, 2011). IAA can also be released from conjugates by hydrolysis (reviewed by Bartel 1997; Ludwig-Müller 2010).

The tryptophan-dependent pathway is well characterized (and described in a review by Mano and Nemoto, 2012). Genetic studies have demonstrated that the two key enzyme families for IAA biosynthesis, TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) genes and YUCCA (YUC) flavin monooxygenase-like genes, act on the same pathway. These gene families show synergistic interactions (Mashiguchi *et al.,* 2011). While the TAAs convert tryptophan to IPA (indole-3-pyruvate), YUCCAs are required for converting IPA to IAA, in collaboration with other IAA-pathway enzymes, including the CYP79Bs, iaaM, iaaH and nitrilases (Won *et al.,* 2011). Members of the CYP79B gene family have only been found in Brassicaceae species, indicating that the IAOX (which is a metabolic auxin intermediate) -dependent IAA biosynthesis pathway is not conserved in plants (Mano and Nemoto, 2012).

The *TAA1*, *TAR1* and *TAR2* genes belong to a small gene family in plants (Mano and Nemoto, 2012). TAA1 is required for a rapid increase in auxin levels through *de novo* IAA biosynthesis (Mano and Nemoto, 2012). The expression of genes in the TAA-family is regulated temporally and spatially, yet they appear functionally redundant (Stepanova *et al.,* 2008; Tao *et al.,* 2008). The TAA1 protein localizes to the vasculature and QC in the proximal meristem (Stepanova *et al.,* 2008). Loss of TAA1 in *wei8-1* mutants leads to altered ethylene sensitivity and reduced root

gravitropic response (Stepanova *et al.,* 2008). Very little information is available regarding the localization of *TAR1*, but it is known to be required for IAA biosynthesis and plant development, acting redundantly with *TAA1* and *TAR2* (Stepanova *et al.,* 2008; Mashiguchi *et al.,* 2011; Brumos *et al.,* 2014). *TAR2* is expressed in the root vasculature (Ursache *et al.,* 2014). Its expression could be stimulated by ethylene treatments, demonstrating interaction between the auxin biosynthesis and ethylene signalling pathways (Stepanova *et al.,* 2008). Double *wei8-1 tar2-1* and *wei8-1 tar2-2* mutants show reduced apical dominance and defective flower development, and growth of their primary root meristem arrests post-embryonically (Stepanova *et al.,* 2008; Mashiguchi *et al.,* 2011). Triple *wei8-1 tar1-1 tar2-1* mutants have severely defective embryonic development and cannot not form primary roots altogether (Stepanova *et al.,* 2008). Overexpression of TAA1 alone does not lead to an auxin overproduction phenotype, indicating that TAA1 is not a rate limiting enzyme in IAA biosynthesis. Consistent with this, root development is not dramatically altered in *TAA1ox* plants. However, when the inducible *TAA1ox* line was combined with a dominant YUCCA mutant (*yuc1D*), the roots were severely affected; overexpression of these key IAA biosynthesis genes leads to an auxin-related phenotype, with a decrease in primary root length and an increase in the number of lateral roots (Mashiguchi *et al.,* 2011). *TRANSPORT INHIBITOR RESPONSE2 (TIR2)* is identical to *TAA1* (Yamada *et al.,* 2009). *TIR2::GUS* is expressed in roots, with the strongest signal in the stele. The protein localization is slightly different, as the signal was also seen in lateral root cap cells and epidermis cells at the transition zone. Loss of function *tir2* mutants have weaker auxin maxima in the root tips.

In *Arabidopsis*, the YUCCA gene family has 11 members (Mano and Nemoto, 2012). Different YUC genes are required for auxin biosynthesis in shoots and roots. YUC1, 2, 4, 6, 10 and 11 control embryo patterning and vascular development in flowers and leaves (Cheng *et al.,* 2006, Robert *et al.,* 2015). Accordingly, triple *yuc1 yuc2 yuc4* and *yuc1 yuc4 yuc6* mutants show leaf venation phenotypes (Cheng *et al.,* 2006). Plants overexpressing *YUCCA6* and dominant *yucca6-1D* mutants have elevated auxin levels, increased expression of auxin regulated genes and severe shoot phenotypes, but their root development is unaltered (Kim *et al.,* 2007). The *YUCCAs* required for root auxin biosynthesis are *YUC3, 5, 7, 8 and 9* (Won *et al.,* 2011). When *yuc3 yuc5 yuc7 yuc8 yuc9* quintuple mutants were treated with the auxin export inhibior naphthylphthalamic acid (NPA), the roots failed to show root tip swelling. The loss of NPA response was similar to *wei8-1 tar2-1* biosynthesis mutants or *tir1* auxin signalling mutants (Won *et al.,* 2011 and Ruegger *et al.,* 1998, respectively). Higher order *yuc* mutants also show severe auxin phenotypes, with reduced rosette size and a loss of apical dominance (Mashiguchi et al, 2011). The most extreme auxin biosynthesis mutants, such as the *yuc1 yuc4 yuc10 yuc11* and *yuc1 yuc4 wei8-1 tar2-1* quadruple mutants, fail to form functional root meristems during embryogenesis (Cheng *et al.,* 2007, Won *et al.,* 2011). Their phenotype is similar to that of *wei8-1 tar1-1 tar2-1* triple mutants and mutants of *AUXIN RESPONSE FACTOR 5/MONOPTEROUS (MP)*, which also fail to establish roots (Stepanova *et al.,* 2008, Berleth and Jürgens 1993, respectively). These phenotypes show that embryonic root development and post-embryonic root growth is highly dependent on IAA biosynthesis and auxin signalling.

TRYPTOPHAN SYNTHASE B1 (TSB1) is an enzyme that catalyses the conversion of indole-3-glycerol phosphate to tryptophan. *TSB1,* together with its downstream components, *TAR1, TAR2* and the root *YUCCAs* (*YUC3, 5, 7, 8 and 9)*, was shown to regulate local auxin biosynthesis in primary root tips (Ursache *et al.,* 2014). This local biosynthesis is required for the correct expression of the HD-ZIP class III transcription factors, which regulate root growth and xylem axis patterning (Ursache *et al.,* 2014). Loss of function *trp2-12* mutants grow very poorly on growth media and show variable xylem patterning defects. The most striking and consistent xylem phenotype was the loss of metaxylem cell identity. However, protoxylem differentiation is not inhibited, indicating that local auxin biosynthesis is required mainly for metaxylem cell fate establishment. The *trp2-12* phenotype was rescued both by exogenous application of TRP and by *pTSB1::iaaH*-driven local auxin biosynthesis in the proximal meristem.

1.5.2. Auxin transporters and their role in primary root development

In *Arabidopsis* roots, auxin gradients and local domains of high auxin signalling that control developmental processes are established by the translocation and transport of auxin. While non-polar long distance translocation of auxin can occur via symplastic diffusion in the phloem, the majority of auxin is transported actively against a gradient by plasma membrane localized transporters (H^+-symporters). *Arabidopsis* auxin transporters can be classified into different categories based on their mode of action. *AUXIN RESISTANT 1 (AUX1), LIKE AUX1 (LAX1)* , *LIKE AUX2 (LAX2)* and *LIKE AUX3 (LAX3)* are auxin importers. The ARABIDOPSIS THALIANA PIN-FORMED (PIN) genes, PIN-LIKE (PILS) genes and ATP-BINDING CASSETTE B (ABCB) genes encode auxin exporters. Some genes in the ABCB family can function as facultative exporters *(ABCB4 and 21)*. While most of the characterized key transporters are localized to the plasma membrane and contribute to polar auxin transport (PAT), others are localized to endomembranes, such as the endoplasmic reticulum (ER) and vacuole tonoplasts.

Auxin importers

The *AUX1* and *LAX1, LAX2* and *LAX3* genes belong to the same gene family. They are proton-gradient driven secondary importers that pump auxin into the cells against a gradient by co-transport with protons (Blakeslee *et al.,* 2005). While *AUX1, LAX1* and *LAX3* were shown to localize to the plasma membrane, the targeting and localization of *LAX2* to the plasma membrane remains to be verified (Carrier *et al.,* 2008, Péret *et al.,* 2012). AUX1, LAX1 and LAX3 localize at the plasma membrane (Péret *et al.,* 2012). These importers are required in several different developmental contexts, such as embryo sac development (Aneesh *et al.,* 2015), embryogenesis (Robert *et al.,* 2015), apical hook formation (Vandenbussche *et al.,* 2010), SAM phyllotaxis maintenance (Bainbridge *et al.,* 2008) and in primary and lateral root growth (Bennett *et al.,* 1996; Swarup *et al.,* 2001; Marchant *et al.,* 2002; Overvoorde *et al.,* 2010 and Péret *et al.,* 2012). In roots, *AUX1* is expressed in columella cells, in the lateral root cap and in the stele (El-Showk *et al.,* 2015). Protein immunolocalization data show that the AUX1 protein is localized in two specific cell files. Radial cross sections of GUS stained *AUX1::uidA* plants showed that these cells were protophloem (Swarup *et al.,* 2001). In a more recent paper (El-Showk *et al.,* 2015) the expression of *AUX1* was demontrated both in protoploem and protoxylem positions. Loss-of-function *aux1* mutants grow agravitropically, indicating that *AUX1* is required for the gravitropic

response (Bennett *et al.,* 1996). Like *AUX1*, *LAX1, LAX2* and *LAX3* are also expressed in the primary roots; *LAX1* is expressed in protoxylem initiatals, whereas *LAX3* is expressed higher up in mature stele cells. *LAX2* is expressed in all vascular initials, whereas *LAX2* is only expressed in the stele of proximal meristems and lateral root initials (Péret *et al.,* 2012). Higher order loss-of-function importer mutants show abnormal embryonic development (Robert *et al.,* 2015), indicating that they are redundantly required for embryogenesis.

Auxin exporters

The *Arabidopsis* ABCB transporters belong to a large gene family of ATP-binding cassette (ABC) transporters (Kang *et al.,* 2011). They contain an ATP-binding domain required for pumping protons against a trans-membrane gradient. Some ABCB genes encode proteins that transport auxin and contribute to auxin homeostasis. Among these genes are *ABCB1, ABCB4, ABCB19, ABCB14, ABCB15* and *ABCB21* (Kang *et al.,* 2011; Kamimoto *et al.,* 2012; Cho and Cho, 2013). These auxin-transporting ABCBs are all localized to the plasma membrane and are NPA sensitive (Kim *et al.,* 2010; Kang *et al.,* 2011; Henrichs *et al.,* 2012; Kamimoto *et al.,* 2012; Cho and Cho 2013). The ABCB proteins associated with root development are *ABCB1, ABCB4, ABCB19* and *ABCB21*. They show cell-type specific expression patterns (Kamimoto *et al.,* 2012; Cho and Cho, 2013). *ABCB1* is expressed in the RAM, showing strongest expression in the ground tissues (Henrichs *et al.,* 2012). Its closest homolog is *ABCB19* (Kang *et al.,* 2011). Loss-of-function *abcb1* and *abcb19* mutants show decreased apical dominance and impaired PAT (Kang *et al.,* 2011). Compared to the single mutants' phenotypes, *abcb1 abcb19* double mutants have a more extreme phenotype, with reduced rootward auxin transport and dwarfed shoots (Cho and Cho, 2013). ABCB19 was shown to stabilize PIN1 in membrane micro-domains (Titapiwatanakun *et al.,* 2009), illustrating the co-regulation of different types of auxin exporters. The *abcb4* loss-of-function mutant is defective in root hair formation and *abcb14* mutants show minor alterations in shoot vascular development (Cho and Cho, 2013). *ABCB21* is expressed in leaves, abscission zones and flowers. In roots, its expression was detected in both mature vasculature and the root tip, with the highest expression in the pericycle (Kamimoto *et al.,* 2012). Unlike the other family members, ABCB4 and ABCB21 are facultative transporters which can act as an exporters of IAA at high auxin concentrations and as importers when the cytoplasmic IAA concentration is low (Yand and Murphy, 2009; Kang *et al.,* 2011; Kamimoto *et al.,* 2012; Kubes *et al.,* 2012). The ABCB proteins interact with the PIN proteins to regulate auxin homeostasis and PAT. This is supported by their overlapping expression domains, membrane co-localisation and protein-protein interactions (Vieten *et al.,* 2007; Titapiwatanakuni 2009; Yand and Murphy, 2009; Kang *et al.,* 2011).

The *ARABIDOPSIS THALIANA PIN-FORMED (PIN)* genes encode plasma membrane localized transporters required for cellular auxin efflux (Petrásek *et al.,* 2006). Unlike *Arabidopsis* ABCB proteins, the PINs do not contain an ATP binding domain and are thus regarded as secondary transporters (Blakeslee *et al.,* 2005). *PIN1, PIN2, PIN3, PIN4* and *PIN7* are expressed in the primary root, and the proteins localize to the plasma membranes (Blilou *et al.,* 2005). Each of these PINs has a specific expression pattern (some of which overlap), and their subcellular localization varies depending on the cell type (showing variable patterns of basal, apical and lateral PM localization). Analysis of single and

combinatorial higher-order knock-out mutants has revealed a high rate of genetic redundancy among the PINs (Blilou *et al.,* 2005). PIN1 is expressed in the stele of the primary root proximal meristem (Peer *et al.,* 2004). Accordingly, this is where the highest level of PIN1 was detected by protein immunostaining (Blilou *et al.,* 2005). The subcellular localization of PIN1 in the stele is mostly basal and directed towards the centre of the stele (Peer *et al.,* 2004; Blilou *et al.,* 2005; Zhang *et al.,* 2014). PIN2 is expressed in the cortex and epidermis of the meristematic zone. The subcellular localization of PIN2 depends on the tissue. In the epidermis, PIN2 localization is apical, directing auxin flow towards the shoot at the outer cell layer; in the cortex, PIN2 localizes to the basal membrane, directing auxin back towards the root tip (Blilou *et al.,* 2005). If the polar localization of PIN2 is switched, the direction of auxin flow changes, which results in an altered gravitropic response (Wisniewska *et al.,* 2006). Loss-of-function *pin1 pin2* double mutants show a severe reduction in root elongation (Vieten *et al.,* 2005). The expression of PIN3 is strongest in the root tip, and the protein localizes to the QC and meristem initials. Some protein localization can be seen higher up in the vasculature. According to the model by Blilou *et al.* (2005), PIN3 is thought to pump auxin away from the QC, thus contributing to cycling auxins shootwards through the epidermis. PIN4 is most strongly expressed and localized around the QC and in the proximal meristem initials. PIN7 is expressed in the stele and columella (Vieten *et al.,* 2005). Based on mutant phenotypes and redundant PIN protein localization patterns in various *pin* mutants, it can be concluded that all plasma membrane localized PINs contribute to PAT in primary roots and can complement one another (Aida *et al.,* 2002, Benková *et al.,* 2003, Furutani *et al.,* 2004, Blilou *et al.,* 2005, Vieten *et al.,* 2005).

Intracellular auxin transporters

To date, three *Arabidopsis* genes have been identified that encode intracellular PIN transporters: *PIN5, PIN6* and *PIN8*. They all localize to the ER (Mravec *et al.,* 2009; Dal Bosco *et al.,* 2012) and are thought to regulate subcellular auxin homeostasis. Loss-of-function *pin5* mutants show dramatically reduced numbers of lateral roots and decreased sensitivity to exogenous auxin in root elongation assays. In turn, lines overexpressing *PIN5* show reduced primary root growth, misspecification of columella cells, abnormal rosette leaf phenotypes and stunted shoot growth overall (Mravec *et al.,* 2009). *PIN6* is expressed in all of the vascular tissues of embryos, seedlings and mature plants, where expression can be seen in the rosette leaf veins and the inflorescence stem's vascular bundles. *PIN6* is also expressed in floral organs and silique abscission zones. Loss-of-function *pin6* mutants show abnormal floral phenotypes and delayed emergence of the primary root compared to wild type plants. Overexpression of *PIN6* results in reduced primary root elongation and a lack of root hairs, formation of fewer lateral roots, smaller rosettes and stunted inflorescence stem growth (Cazzonelli *et al.,* 2013, Nisar *et al.,* 2014). *PIN6* overexpression lines also show a bizarre root waving phenotype (Cazzonelli *et al.,* 2013). *PIN8* is expressed in male gametophytes during pollen maturation (Dal Bosco *et al.,* 2012; Ding *et al.,* 2012). While endogenous *PIN8* is not expressed in seedlings, leaves or roots, overexpression of PIN8 leads to stunted shoot growth, altered cotyledon shape, and enhanced primary root growth. The overexpression phenotypes illustrate the functional similarity between the intracellular PINs, regardless of their endogenous expression patterns (Dal Bosco *et al.,* 2012; Ding *et al.,* 2012).

Arabidopsis also has another class of intracellular exporters, the PIN-LIKES (PILS) genes, which regulate intracellular auxin accumulation and endogenous IAA levels. The PILS gene family has seven members. They have trans-membrane domain structures similar to those of PIN5 (Feraru *et al.,* 2012). The PILS localize to the ER and are suggested to regulate auxin compartmentalization from the cytoplasm to the ER lumen, which might be functionally important for various developmental processes (Barbez *et al.,* 2012; Feraru *et al.,* 2012). The phenotypes of PILS loss-of-function mutants include enhanced growth of the hypocotyl and primary root and increased lateral root density. In turn, overexpression of the PILs causes severe growth reduction in shoots, abnormal flower development, decreased hypocotyl length, and defects in root hair elongation and lateral root density. Overexpression of the PILs also decreases primary root length and enhances tolerance to exogenous auxin treatments (Barbez *et al.,* 2012). *WALLS ARE THIN1 (WAT1)* is a vacuolar auxin transporter localized to the tonoplast membrane (Ranocha *et al.,* 2013). *WAT1* is expressed in the vascular tissues of shoots and roots. Loss-of-function *wat1* mutants show secondary cell wall formation defects which can be rescued with exogenous auxin application. WAT1 is predicted to be structurally similar to PILS2, PILS5, and PIN5 (Ranocha *et al.,* 2013). The existence of several types of intracellular auxin transporters that localize to *different* endomembranes demonstrates that intracellular auxin homeostasis maintenance is important for plant development.

Regulation of PINs

NPA was first shown to bind to plasma membrane localized transporters in zucchini (Bernasconi *et al.,* 1996). In *Arabidopsis,* NPA inhibits auxin transporter via competitive binding to the ABCB and PIN family auxin efflux carriers (Thomson and Leopold, 1974; Kim *et al.,* 2010; Kang *et al.,* 2011; Henrichs *et al.,* 2012; Kamimoto *et al.,* 2012; Cho and Cho, 2013). NPA treatment stimulates PIN expression (Vieten *et al.,* 2005), possibly to compensate for reduced PAT. Long term NPA treatments cause the loss of cell polarity and can lead to the proliferation of QC-like cells at the flanks of the meristem, leading to RAM re-organization and ectopic cell proliferation (Sabatini *et al.,* 1999 and Himanen *et al.,* 2002). The fact that higher order auxin biosynthesis, transport or signalling mutants do not respond to the auxin transport inhibitor NPA in the same manner as wild type plants indicates that auxin biosynthesis, PAT and signalling at the RAM are all required for QC identity maintenance. NPA also blocks the auxin ABCB transporter (Kim *et al.,* 2010), but not AUX1 importer (Yang *et al.,* 2006), so inhibition of auxin transport by NPA appears to be a shared feature of the auxin exporters.

The level of all of the PIN proteins is increased by mild concentrations of auxins and inhibited by high doses of exogenously applied auxins (Vieten *et al.,* 2005). Cytokinins have also been shown to modulate auxin transport by regulating gene expression (Ruzicka *et al., 2009;* Della Rovere *et al.,* 2013) and subcellular localization and endocytosis of the PIN proteins (Marhavý *et al.,* 2011 and 2014; Stepanova and Alonso, 2011). Several studies have addressed the effect of exogenous cytokinin treatments on the expression of different *PINs* (Dello Ioio *et al.,* 2008; Pernisová *et al.,* 2009; Ruzicka *et al.,* 2009; Zhang *et al.,* 2011 and Burgess 2012). According to Dello Ioio *et al.* (2008), levels of PIN1, PIN3 and PIN7 transcripts and proteins decrease in the stele upon exogenous trans-zeatin (tZ) treatment. Zhang *et al.* (2011) reported similar changes in expression patterns for

the PIN-GFP markers, although their time scale was completely different. The *PIN1* data in both publications is consistent with Ruzicka *et al.* (2009) and Della Rovere *et al.* (2013), who showed that *PIN1* transcription decreases after cytokinin treatment. However, the reported decrease in *PIN7* expression and protein localization reported by Dello Ioio *et al.* (2008) and Zhang *et al.* (2011) is not consistent with the material published by Ruzicka *et al.* (2009) and Della Rovere *et al.* (2013), leaving room for speculation. In 2012, Burgess addressed this matter by combining the published material and the various experimental set-ups. According to the compiled data, expression of *PIN1* can be either up- or downregulated by CKs, leaving its status unresolved. In the compared assays, *PIN2* and *PIN3* expression levels were either slightly reduced unaltered by CK. This is supported by Ruzicka *et al.* (2009) and Zhang *et al.* (2011), which show only a minor reduction in longitudinal PIN2-GFP and PIN3-GFP signals even in very harsh cytokinin treatments. By contrast, *PIN4* and *PIN7* appear to be promoted by cytokinins, according to most published datasets. This is in agreement with Persinová *et al.* (2009), who showed that *PIN4* expression levels are reduced in transgenic plants with depleted endogenous cytokinin, providing further evidence for CK promoting *PIN4* expression *in planta*. Reduction of PIN7-GFP in some assays (Zhang *et al.,* 2011) could possibly be attributed to very high cytokinin doses and long exposure times, as earlier time points showed an increase in protein level instead. Perhaps the described discrepancies in these published PIN expression and protein localization responses can be partially explained by cytokinin signalling promoting post-translational regulation and endocytosis of PINs (Marhavý *et al.,* 2011 and 2014; Stepanova and Alonso, 2011), which might in turn feed back to gene expression levels. It is noteworthy that different types of cytokinins were used in the PIN expression assays, as well as variable concentrations and different exposure times, which may partially explain the conflicting results. However, it is quite certain that the PIN genes are not all regulated in the same way by cytokinins. The reason for this remains to be discovered. In addition to the PM-localized PINs, intracellular PINs also respond to cytokinin. *PIN6* expression was promoted by kinetin, and it seemed to be more tolerant to transcriptional inhibition caused by high cytokinin than the plasma membrane localized PINs (Pernisová *et al.,* 2009).

PIN protein polarity, stability and turnover are regulated by several genes, such as *PINOID (PID)* and *GNOM*. PINOID is a serine/threonine kinase (Christensen *et al.,* 2000) that phosphorylates plasma membrane localized PINs. PID co-localizes to the plasma membrane with the PIN proteins (Kleine-Vehn *et al.,* 2009) and is required for polar auxin transport via the PINs. *PID* is expressed in the cotyledon initials in embryos (Christensen *et al.,* 2000) and in young vascular tissues in both roots and shoots (Benjamins *et al.,* 2001). It is also expressed in floral organs, where it regulates organ development. Loss-of-function *pid* mutants are phenotypically similar to *pin1* mutants, with severe morphogenetic defects, including abnormal cotyledon development, reduced numbers of lateral organs and defective formation of leaf vasculature (Bennett *et al.,* 1995). *PID* overexpression lines show reduced primary root elongation, delayed lateral root formation, a reduction in root hair length and collapse of the primary root meristem, which indicates that *PID* can also negatively regulate auxin signalling (Christensen *et al.,* 2000; Lee and Cho, 2006; Kleine-Vehn *et al.,* 2009). GNOM is a vesicle transport regulator that localizes to endosomes and regulates their structure and function. The GNOM-dependent ARF-GEF pathway controls the

recycling rates of PINs from apical to basal membranes via protein transcytosis, thus affecting cell polarity (Geldner *et al.,* 2003). Loss-of-function *gnom* mutants have reduced numbers of lateral roots (Kleine-Vehn *et al.,* 2009), which is phenotypically similar to PID overexpression lines. Based on genetic evidence, PID and GNOM are thought to affect auxin accumulation and the formation of vascular tissues in an opposing manner (Kleine-Vehn *et al.,* 2009). PID regulates the subcellular trafficking of PINs by modulating their phosphorylation status; when phosphorylated, the PINs escape the GNOM-dependent endosomal trafficking and become polarized to apical membranes. PID phosphorylation can be counteracted by PROTEIN PHOSPHATASE 2A (PP2A) proteins that de-phosphorylate PINs, removing them from the apical membranes and subjecting them to recycling by the endosomal GNOM dependent ARF-GEF pathway (Kleine-Vehn *et al.,* 2009).

1.5.3. Auxin signalling maximum in the primary root

The auxin importers and exporters establish and maintain polar auxin transport (PAT) from the shoot to the root meristem (Blilou *et al.,* 2005, Kang *et al.,* 2011). This transport leads to the formation of local auxin signalling maximum in the proximal meristem and QC. The auxin signalling maximum in the protoxylem position was first reported and illustrated by Swarup *et al.* in 2001. Several computational models on PAT have been created that recapitulate the observed auxin signalling pattern based on the reported expression patterns of the PINs (Grieneisen *et al.,* 2009; Mironova *et al,* 2012; Clark *et al.,* 2014). The published IAA2::GUS expression pattern and the PAT models are further supported by the map of auxin distribution in different root tissues generated by Petersson *et al.* in 2009. Perturbations of the PAT models have verified published experimental mutant data, demonstrating that the auxin signalling maxima in the root tip are dependent on PAT and are required for proper regulation of root patterning and growth. Apart from IAA2::GUS cross section data showing high auxin signalling in the protoxylem position (Swarup *et al.,* 2001), no lateral expression data had been published for auxin transporters when we started our work. These results are presented in this thesis. Our published work and other publications have since led to the creation of new computational models that recapitulate lateral patterning mechanisms (Muraro *et al.,* 2013 and El-Showk *et al.,* 2015).

1.5.4. Auxin signalling pathway

Auxin binds to auxin receptors, *TRANSPORT INHIBITOR RESPONSE 1 (TIR1)* and AUXIN F-BOX PROTEINs (AFBs) (Teale, Paponov and Palme, 2006; Mockaitis and Estelle, 2008 and Overvoorde *et al.,* 2010). TIR1 and its close homologs the AFBs are F-box proteins that bind to CULLIN1 and to the ARABIDOPSIS SERINE/THREONINE KINASE 1 & 2 proteins (ASK1 and ASK2) to form an SCF ubiquitin protein ligase (E3) complex (Gray *et al.,* 2001). The highly complex auxin responses of plants are mediated by two large gene families, the Aux/IAAs and the ARFs. The *Arabidopsis thaliana* genome contains 29 Aux/IAA genes that inhibit the expression of auxin responsive genes. *Arabidopsis* also has 23 ARF genes that encode positive regulators of transcription. Auxin is required for the SCF^{TIR1} complex to bind to AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) proteins, leading to their rapid ubiquitin-dependent degradation. In the absence of auxin, the Aux/IAAs are free to bind to AUXIN RESPONSE FACTORS (ARFs), inhibiting their function. The ARFs are DNA binding

transcription factors that bind to auxin-responsive elements (AuxREs) in the promoter sequences of auxin-regulated genes (*Grey et al., 2001*; Woordward and Bartel, 2005; Teale, Paponov and Palme, 2006; Mockaitis and Estelle, 2008 and Overvoorde *et al.,* 2010). Once the Aux/IAA proteins are degraded, the ARFs are released from their suppression and can bind to AuxREs and promote gene expression (reviewed and illustrated by Teale, Paponov and Palme, 2006). In 2013, Yu *et al.,* reported mutations in the TIR1 receptor which enhanced the its affinity for Aux/IAAs, leading to enhanced degradation and increased auxin sensitivity. These mutants exhibited auxin hypersensitive phenotypes, including elevated expression of auxin target genes, enhanced suppression of root elongation, increased formation of lateral roots, a reduction in shoot branching (indicating increased apical dominance) and smaller rosettes.

AUXIN BINDING PROTEIN 1 (*ABP1*) can also bind auxin, yet its role in auxin signalling has been debated over the yearsAccording to the literature, ABP1 is mainly localized to the ER, although low levels of the protein appear to be secreted to the apoplast, where they remain associated with the plasma membrane, able to mediate auxin responses. While ABP1 might not relay the majority of auxin signalling, it may be involved in auxin-mediated activation of Rho of Plant (ROP) GTPases (Vanneste and Frilm 2013), which control polar growth of the root epidermis and root hair cells (Molendijk *et al.,* 2001). ABP1 thus might play a role in auxin-regulated calcium dependent mechanical signalling and be required for mechanical perception and gravitropic responses (Vanneste and Frilm 2013). Recent publication by Gao *et al.,* (2015) show data from loss-of-function *abp1* alleles generated with the CRISPR genome editing technology. As these new null alleles fail to show phenotypes characterized in previous publications, this strongly supports a thorough re-examination of the role of ABP1 in developmental context.

Several Aux/IAAs that regulate root development have been identified, among which are *IAA2/ AUXIN RESISTANT 2 (AXR2), IAA3/ SHORT HYPOCOTYL 2 (SHY2), IAA12/ BODENLOS (BDL)* and its sister *IAA13, IAA14/SOLITARY ROOT (SLR) and IAA17/ AUXIN RESISTANT 3 (AXR3)* (Rouse *et al.,* 1998; Tian and Reed, 1999; Nagpal *et al.,* 2000; Weijers *et al.,* 2005; Muto *et al.,* 2007). Semi-dominant gain-of-function alleles of Aux/IAA genes also have been identified. These alleles contain mutations in the conserved domain required for protein degradation by the SCF^{TIR1} complex. Such mutations affect the stability of the repressors, rendering the plant insensitive to auxins and causing severe auxin-related phenotypes (Rouse *et al.,* 1998; Nagpal *et al.,* 2000). Mutants like *arx1, arx2* and *arx3* can be used as effective genetic tools for supressing auxin signalling. Depending on their expression pattern, Aux/IAA semi-dominant mutations can have adverse effects on plant growth (Grey *et al.,* 2001). The gain-of-function *axr1-12* mutant shows defects in cotyledon vasculature formation, with shoots that are highly branched (loss of apical dominance) and siliques that do not elongate properly. The roots of *axr1-12* mutants show enhanced elongation and increased tolerance to hormones compared with wild type plants (Nakasone *et al.,* 2012). Gain-of-function *arx2-1* roots are extremely agravitropic and develop more lateral roots than wild type plants; however their root hair formation is supressed (Nagpal *et al.,* 2000; Muto *et al.,* 2007). The *arx2-1* mutant also occasionally shows cotyledon defects (Muto *et al.,* 2007). The gain-of-function *arx3-1* mutant has an extreme agravitropic phenotype and severely reduced

primary root growth (Nagpal *et al.,* 2000). The *axr6* mutation was shown to cause severe growth defects early in development; the mutants cannot form primary roots and have sporadic vasculature in their cotyledons. Their shoot phenotype is also striking; the rosette leaves are twisted and fail to elongate properly, the inflorescence stem architecture is altered (upright siliques, shorter internodes), and the plants show a clear loss of apical dominance. In addition, lateral root numbers are reduced and primary root elongation shows resistance to exogenous 2,4-D treatments (Hobbie *et al.,* 2000). Overexpression of *IAAs* under the cauliflower mosaic virus *35S* promoter results in similar phenotypes as the gain-of-function mutations (Li *et al.,* 2009).

SHY2 was shown to control primary root meristem size via direct interaction with *ARR1*, a cytokinin signalling response gene (Dello Ioio *et al.,* 2008). Loss-of-function alleles of *IAA3/SHY2* (such as *shy2-24*) cause increased auxin transport to the roots, resulting in enhanced formation of lateral roots (Tian and Reed, 1999), which is possibly linked to their increased root curvature (wavy phenotype). The *shy2-31* loss-of-function mutant has larger primary root meristems than wild type plants and stronger expression of *PIN1*, *PIN3* and *PIN7*. By contrast, gain-of-function *shy2-2* mutants have shorter root meristems and roots than wild type plants and exhibit a constitutive auxin response phenotype (Tian and Reed 1999; Weijers *et al.,* 2005; Dello Ioio *et al.,* 2008). *IAA12/BODENLOS (BDL)* and *IAA13* are functional paralogs. *BDL and IAA13* are both expressed in embryos, where they localize to the pre-vascular tissues. Both *bdl* and stabilized *iaa13* mutants fail to develop roots during embryogenesis. *IAA14/SOLITARY ROOT (SLR)* gain-of-function mutants do not form lateral roots *(De Smet et al., 2007).*

Auxin signalling appears to be converted to tissue- and cell-type-specific responses by the pairing of *Aux/IAA* and *ARF* genes (Weijers *et al.,* 2005, Overvoorde *et al.,* 2010). For example, *IAA12/BDL* and *IAA13* regulate root meristem establishment during embryogenesis and control meristem maintenance after germination, together with *IAA3/SHY2* and *ARF5/MP,* (Weijers *et al.,* 2005; Dello Ioio *et al., 2008;* Schlereth *et al.,* 2010). Like *IAA12/BDL e*mbryos, *ARF5/MP* embryos do not form root apical meristems and the seedlings of *mp* do not have a primary root (Schlereth *et al.,* 2010; De Rybel *et al.,* 2013). *MP* regulates the expression of TARGET OF MONOPTEROS genes, including *TMO5,* which was shown to be required for xylem axis patterning during embryogenesis (De Rybel *et al.,* 2013). ARF7 and ARF19 were shown to interact with several Aux/IAAs, including IAA2/SHY2, IAA12/BDL, IIA14/SLR, IAA17/EXR3 (reviewed by Overvoorder *et al.,* 2010).

Even though most ARFs are thought to promote downstream targets, some have been suggested to inhibit transcription, at least in transient protoplast assays (Ulmasov *et al.,* 1999). In addition, some ARFs, such as ARF8, were shown to regulate auxin degradation by promoting the expression of IAA-conjugating enzymes, such as the GH3 genes (Woordward and Bartel, 2005). Loss-of-function *gh3-9* mutants have shorter roots and increased sensitivity to auxin-regulated root growth compared with wild type plants (Khan and Stone, 2007). By contrast, GH3-5 overexpressing lines (gh3.5-1D) show an altered root phenotype, with reduced primary root length and fewer lateral roots (Zhang *et al.,* 2007), as has

been seen in various auxin-related mutants (Zhang *et al.,* 2007), suggesting that *GH3* genes may have a role in root development.

1.6. Cytokinins

Cytokinins promote cell division in the shoot but inhibit cell cycle progression in the root. Like many other plant hormones, endogenous cytokinins are generally thought to be synthetized in meristematic tissues. Naturally occurring cytokinins are N^6-subtstituted compounds that are synthesized in plants from adenine. Cytokinins can be classified as 1) isoprenoid derived iso-pentenyladenines (*iP-group),* trans-zeatins *(tZ-group),* cis-zeatins *(cZ-group)* and dihydrozeatins (*DZ-*group); and 2) aromatic cytokinins that contain a benzyl group at the N^6 position, including benzyl adenine (BA) and its metabolites (Sakakibara, 2006). Based on the chemical structure of the side chains, it has been suggested that the biosynthesis of aromatic cytokinins occurs likely from a different precursor than isoprene, possibly from phenolics (Strnad, 1997). The free base cytokinin species, such as *iP* and *tZ*, are generally considered the most abundant and biologically active cytokinin forms in the plant kingdom, and they have a high binding affinity to cytokinin receptors (reviewed by Kieber and Schaller, 2014; *Sakakibara, 2006*). However, some evidence has emerged indicating that cis-zeatin (cZ) may be the predominant active cytokinin in some species (Frébort *et al.*, 2011). Accordingly, a recent high-resolution study of the distribution of cytokinin species in the root apical meristem of *Arabidopsis* revealed that the most predominant cytokinins in the root meristem are *cZ-types,* followed by *iP*-types (Antoniadi *et al.,* 2015). While the precursors, active and conjugated forms of *cZ*-type cytokinins were most abundant in the meristem initial cells, QC, columella and lateral root cap cells (matching the published pattern on the synthetic cytokinin reporter *TCS,* Zürcher *et al.,* 2013), the levels of active *tZ*-types were considerably lower and appeared uniform in all analysed root apical tissues and the active *iP*-type cytokinins showed accumulation in the stele.

Unlike *iP, tZ* or *cZ* cytokinins, dihydrozeatins have been reported to be predominantly present in storage organs (Frébort *et al.,* 2011), indicating that they might function as a degradation resistant reservoir of cytokinins for young germinating plants. Ribosylated-, acylated- or glycosylated conjugates of *iP*, *tZ* and *cZ*-type cytokinins are considered less active or inactive storage species (Sakakibara 2006; Lomin *et al.,* 2012; Kieber and Schaller 2014), making them better suited for long-distance transport (Lomin et al., 2015) and activation at the target site by tissue/cell-type specific biosynthesis enzymes (such as the IPTs and LOGs). However, some conjugated ribosides have been reported to display affinity to cytokinin receptors, and – equally puzzling – some compounds lacking receptor binding affinity (such as CK-O-glucosides) have shown activity in bioassays (Antoniadi *et al.,* 2015). Thus, there seems to be a discrepancy between the molecular structure and the expected activity of some cytokinins, an issue that requires further work to be fully addressed. In addition to the endogenous, plant-derived cytokinins, synthetic cytokinins exist, and many naturally existing compounds can be synthetized biochemically. The synthetic compounds mimic the structure of endogenous cytokinins; they have a high affinity to CK receptors and can activate cytokinin signalling (Lomin *et al.,* 2012).

1.6.1. Components of cytokinin biosynthesis and activation in *Arabidopsis*

Biosynthesis of the isoprenoid family cytokinins can begins with one of two pathways, the methylerythritol phosphate (MEP) pathway for the *iP-, tZ-* and *DZ-*types and the mevalonate (MVA) pathway for *cZ*-type cytokinins. Despite starting with different substrates, the pathways contain similar biosynthesis steps, including dephosphorylations, phosphorylations, conjugations, conversions and interconversions (as reviewed and illustrated by Sakakibara 2006), leading to the formation of free, active cytokinins and their inactive storage forms. The enzymes catalysing the first steps of *iP* (and *tZ*) biosynthesis from their precursor molecules are adenosine phosphate-isopentenyltransferases (IPTs). The *Arabidopsis* IPT gene family contains 9 members, which display distinct expression patterns (Takei *et al.*, 2001; Miyawaki *et al.,* 2006). While *IPT1, IPT3, IPT4, IPT5, IPT6, IPT7* and *IPT8* are required for *iP*- and *tZ*-type biosynthesis, *IPT2* and *IPT9* are required for synthetizing *cZ*. No root phenotypes have been reported for the single *ipt* mutants, but the root growth of *ipt3 ipt5 ipt7* triple mutants is slightly enhanced (Miyawaki *et al.,* 2006; Matsumoto-Kitano *et al.,* 2008). The higher order *ipt1 ipt3 ipt4 ipt7* quadruple mutants have considerably thinner roots with reduced cambial activity (Matsumoto-Kitano *et al.,* 2008). When IPT levels were globally elevated in tobacco plants, this led to pleiotropic growth reduction and changes in branching, called the "cytokinin syndrome" (Hewelt *et al.,* 1994). Interestingly, in contrast to the negative effects of global IPT overexpression, tissue specific overexpression of IPTs can result in the enhancement of agronomically useful traits in several plant species, such as larger organs and seeds, increased silique number, or a delay in senescence (reviewed by Nick and Opatrny, 2014).

The *CYP735A1* and *CYP735A2* genes encode enzymes that convert *iP*-type cytokinins into trans-zeatins (Takei *et al.,* 2004). These CYP genes might also play a role in the synthesis of aromatic cytokinins (Sakakibara 2006). The LONELY GUY *(*LOG*)* genes are critical for cytokinin biosynthesis in *Arabidopsis*, catalysing the formation of free, active cytokinin from inactive ribosides (Kuroha *et al.,* 2009). The LOG family contains 9 genes (Kurakawa *et al.,* 2007). Higher order *log* mutants show reduced root growth rates, and the heptuple *log1 log2 log3 log4 log5 log7 log8* mutant is dwarfed, failing to show any signs of cytokinin activity in its vascular meristem (Tokunaga *et al.,* 2012; De Rybel *et al.,* 2014). The vasculature of the heptuple mutant consists entirely of protoxylem, resembling mutants with severely impaired cytokinin signalling, such as *WOODEN LEG* mutants (*wol)* or the triple *ARABIDOPSIS RESPONSE REGULATOR* mutants *arr1-3 arr10-5 arr12-1* (Mähonen *et al.,* 2006, Argyros *et al.,* 2008, respectively). While *LOG7* appears to be a key contributor to cytokinin biosynthesis on the whole-plant scale, only *LOG3* and *LOG4* have been specifically linked to root proximal meristem development, with effects on both root length and lateral root number (Tokunaga *et al.,* 2012). Indeed, the regulation of periclinal cell divisions in the proximal meristem appears to be controlled by local LOG-driven cytokinin biosynthesis at the xylem position, the protoxylem-associated pericycle cells and the overlaying endodermis (De Rybel *et al.,* 2014).

1.6.2. Cytokinin transport

The plant hormone research community has long debated the existence of cytokinin transporters. Until recently, it seemed that no such proteins existed and that cytokinin moved via passive diffusion only. However, *ATP-binding cassette (ABC) transporter G14* (*ABCG14*) was shown to be required for the transport of cytokinins from the roots to the shoots (Ko *et al.,* 2014). *ABCG14* encodes a plasma membrane-localized, dimerizing protein expressed in the procambial cells of the root stele. The *abcg14* mutant is small and dwarfed, with reduced tZ levels in the shoot; the shoot phenotype could be rescued by exogenous tZ treatment. The *abcg14* mutant root, on the other hand, accumulates tZ; however, when the xylem sap was analysed, it was had no tZ, DZ or cZ cytokinins, indicating that shootwards flow and the loading of cytokinins into TEs is *ABCG14* dependent.

To date, only limited data exists on active cytokinin transport into phloem cells. Members of the PURINE PERMEASE *(*PUP*)* family have been shown to take up cytokinins in cell cultures (Bürkle *et al.,* 2003; Cedzich *et al.,* 2008), and at least one family member, *PUP2,* is expressed in phloem cells of the *Arabidopsis* leaf (Bürkle *et al.,* 2003). The *Arabidopsis* genome contains 15 PUP genes (Gillissen *et al.,* 2000), many of which show a high affinity to purines, the starting material for cytokinin biosynthesis. The evolutionary conservation of the PUPs (Jelesko*,* 2012) and their role in other plant species suggests that they may act as cytokinin transporters. In addition to the PUPs, *EQUILIBRATIVE NUCLEOSIDE TRANSPORTER (ENT)* gene family members *ENT3* and *ENT8* have been proposed to transport nucleoside-type cytokinins in *Arabidopsis* (Sun *et al.,* 2005). While the intense research done in this area seems quite promising, no major breakthrough on cytokinin transport has been published yet.

While it is generally thought that the bulk of CK biosynthesis occurs primarily in the roots and that the dispersal of cytokinins into the aerial organs is due to transpiration from the xylem (as was shown by Aloni *et al.,* 2005), analyses of several different plant species have shown that xylem and phloem have very distinctive cytokinin profiles (Lejeune *et al.,* 1994; Mok and Mok, 1994). This has led to speculation that the rootward flow in the phloem and shootward flow in the xylem might distribute different cytokinin species from their biosynthesis sites to source tissues (reviewed by Hwang *et al.,* 2012)*,* indicating that a finely tuned hormonal signalling mechanism might be balancing the pace of root and shoot growth (as was also suggested by Hitoshi Sabakibara, 2006 and Bishopp *et al.,* 2013). Recent evidence supports this speculation. Shoot-derived cytokinins were shown to promote root nodule development in *Lotus japonicus* (Sasaki *et al.,* 2014), and mutants of the root-specific *ABCG14* transporter gene failed to provide *Arabidopsis* shoots with *tZ*, *DZ* and *cZ* cytokinins. When combined with the reported expression pattern of *PUP1* in leaf hydathodes (Bürkle *et al.,* 2003) [where it has been postulated to prevent loss of cytokinin during guttation (Sakakibara 2006)] and *PUP2* in leaf vasculature (Bürkle *et al.,* 2003), these data suggest that the shoots can 1) synthetize cytokinins, 2) convert cytokinins (from *tZ* to *iP*, etc.), and 3) contribute to the translocation of cytokinins between xylem and phloem (via diffusion or transport), and thus 4) provide the roots with different types of cytokinins. Whether shoot-derived phloem-sap cytokinins play a critical role in root development is addressed by research presented in this thesis.

On the tissue level, the lack of cytokinin transporters might not hamper cytokinin accumulation in roots, partially because of high local biosynthesis in these tissues, but also because diffusion via plasmodesmata allows effective shuffling of various small compounds within the meristem (Vatén *et al.,* 2011). Recent modelling work (El-Showk *et al.,* 2015) on root vascular pattering demonstrated that rapid passive diffusion establishes uniform levels of cytokinin in the root tip regardless of local biosynthesis or the source of transport/translocation.

What about cytokinin transport on subcellular level? Research in maize has shown that the cytokinin profiles of subcellular compartments are quite different (reviewed by Sakakibara in 2006). In *Arabidopsis,* the MVA pathway (for *cZ*-type biosynthesis) operates in the cytoplasm and the MEP pathway (for *iP*-, *tZ*- and *DZ*-type cytokinins) functions in plastids (Frébort *et al.,* 2011). Interestingly, the CK receptors were shown to localize to the ER membrane, with their sensor modules facing ER lumen (Wulfetange *et al.,* 2011; Lomin *et al.,* 2015), indicating that cytokinin binding occurs in the ER lumen. The discrepancy between biosynthesis, receptor localization and cytokinin binding locations strongly suggests that intracellular transport of cytokinin from the plasma membrane, cytoplasm, or plastids to the ER is critical for cytokinin signalling *in planta*. However, no CK specific intracellular transporters have been identified to date.

1.6.3. De-activation and degradation of cytokinins

Cytokinins can be deactivated either reversibly or irreversibly. Phosphorylation of the free cytokinin nucleobases to ribosides reduces their biological activity (Sakakibara 2006, Frébort *et al.,* 2011; Kieber and Schaller, 2014) and is reversible. Deactivation can occur either via the CK oxidase/dehydrogenase (CKX) or glycosyltransferase (UGT) enzymes. CK oxidation is an irreversible reaction in which the N6 side chain is cleaved off (Zazímalová *et al.,* 1999), transforming active or riboside cytokinins into inactive metabolites. The CKX gene family contains seven members (Schmülling *et al.,* 2003), which are expressed in different tissues. Where *CKX2* and *CKX3* are expressed primarily in shoots, *CKX4*, *CKX5* and *CKX6* are expressed in young primary and lateral root meristems, and *CKX1* can be seen in older roots (Werner *et al.,* 2003). *CKX7* is expressed in the vascular tissues throughout young plants (Köllmer *et al.,* 2014). In addition to differences in their expression patterns, the CKX enzymes also have different subcellular localizations and substrate preferences (Werner and Schmülling 2009; Kowalska *et al.,* 2010; Frébort *et al.,* 2011). While single *ckx* mutants appear phenotypically normal in terms of root and shoot development, some higher order mutants show an enlarged SAM, thicker inflorescence stems, bigger floral organs and siliques, an increased number of ovules, and greater seed yield (Bartrina *et al.,* 2011). Root phenotypes for the high order combinatorial knock outs have not been reported in literature, since the majority of CKX functions have been identified using overexpression lines. For example, inducible *CRE1-XVE::CKX1* lines formed ectopic protoxylem strands in the vasculature (Mähönen *et al.,* 2006). Similarly, *35S::CKX7* roots showed a severe reduction in meristem size accompanied by the formation of ectopic protoxylem, similar to *wol* mutants (Höllmer *et al, 2014*). In general, *iP*- and *tZ*-type cytokinins are more amenable to degradation by CKX than the *cZ*- and *DZ*-types, due to their chemical structure

(Sabakibara 2006). Interestingly, DZ and the aromatic cytokinin, BA, are resistant to CKX degradation.

The enzymes encoding glycosyltransferases belong to the UGT gene family; to date, 5 members have been characterized in *Arabidopsis* (Kieber and Schaller, 2014). Glycosyltransferases can reversibly inactivate cytokinins by conjugations at the N7 or N9 position. This type of glycosylation leaves the cytokinins "vulnerable" to degradation by CKX. By contrast, inactivation via O-glucosylation transforms active cytokinins into storage forms which cannot be degraded by the CKX enzymes (Frébort *et al.,* 2011; Kieber and Schaller, 2014). UGT loss-of-function mutant phenotypes are highly variable; some grow rather normally, resembling wild type plants, while others are severely dwarfed (Grubb *et al.,* 2014).

The other plant hormones can affect cytokinin biosynthesis and degradation. For example, auxin has been shown to promote cytokinin degradation via the CKXs (Palni *et al.,* 1988). The crosstalk between different hormonal pathways has proven to be quite complicated, with the expression levels of *Arabidopsis* cytokinin biosynthesis and homeostasis genes regulated by at least cytokinin, auxin, and abscisic acid – which often antagonize each other (Sakakibara 2006; Kieber and Schaller 2014). In addition, members of the same gene family (such as the IPTs) may respond differently to a certain hormone, indicating that the regulation of *iP* and *tZ* biosynthesis, conversion and degradation might play a fundamental role in morphogenetic responses (Sakakibara, 2006).

1.6.4. Cytokinin signalling receptors

The known *Arabidopsis* cytokinin signalling pathways are two-component phosphorelay systems. The two pathways are 1) a multi-step AHK-AHP-ARR-pathway, which consists of histidine sensor kinase-receptors (AHKs), histidine kinase proteins (AHPs) and response regulators (ARRs); and 2) a *CKI1*-ARR pathway, in which the *CYTOKININ-INDEPENDENT 1* (*CKI1*) receptor directly activates the downstream response regulators via AHPs.

Arabidopsis has three histidine kinase cytokinin receptors that have been shown to bind cytokinins: *ARABIDOPSIS HISTIDINE KINASE 2 (AHK2), AHK3* and *AHK4/CYTOKININ RESPONSE 1 (CRE1)/WOODEN LEG (WOL)* (Mähönen *et al.,* 2000; Inoue *et al.,* 2001; Suzuki *et al.,* 2001; Yamada et al, 2001; Higuchi *et al.,* 2004). *AHK1* also encodes a histidine kinase protein, but it has been shown to be involved with ABA and osmotic stress sensing, rather than cytokinin signalling, in both *Arabidopsis* and rice (Tran *et al.,* 2007; Kumar *et al.,* 2013; Kushwaha *et al.,* 2014). The AHK2, AHK3 and CRE1 receptors show variable affinity to different cytokinin species, with the highest affinity to free nucleobases (Sakakibara 2006). The AHK3 and CRE1 receptors both have high affinity to tZ, although AHK3 is more sensitive to cZ and DZ; furthermore, AHK3 could also be activated by riboside cytokinins but showed much lower affinity to iP than CRE1 (Romanov *et al.,* 2006; reviewed by Sakakibara 2006 and Bishopp *et al.,* 2009). These data suggest that the *iP*-type cytokinins are the primary activators of CRE1, whereas AHK3 favours *tZ*-type cytokinins (Spíchal *et al.,* 2004; Lomin *et al.,* 2015). The expression patterns of the cytokinin receptors are overlapping yet distinct. While *AHK2* is expressed equally in shoots and roots, *AHK3* expression is highest in the shoot, and *CRE1* is most abundant in the roots (Higuchi *et al.,*

2004; reviewed in Bishopp *et al.,* 2009). Overall, the staining patterns of *AHK2::GUS*, *AHK3::GUS* and *CRE1::GUS* in 5 day old seedlings look very similar, showing expression in the vasculature and SAM, but the patterns are different in the root proximal meristem region (Nishimura *et al.,* 2004); the expression of *AHK2::GUS* and *CRE1::GUS* is clearly visible in the vascular initials above the QC, whereas *AHK3::GUS* expression peaks at the differentiation zone above the proximal meristem. Unlike AHK2 and AHK3, which can only relay the phosphoryl to downstream components (such as AHP2), CRE1 is a bi-directional receptor. When cytokinin is bound to CRE1, the kinase activity promotes signalling; when cytokinin is absent, the phosphatase activity can inactivate the receptor and neatly shut down signalling (Werner and Schmülling, 2009).

Cytokinin receptor mutants show reduced sensitivity to cytokinin in various assays and have ectopic protoxylem. The phenotypes are milder in the single mutant than in the double and triple mutants; the *cre1-12* mutant forms ectopic protoxylem files and has slightly larger meristems than wild type, while the *cre1-12 ahk2-2* and *cre1-12 ahk3-3* double mutants show increased insensitivity towards cytokinins in root elongation assays and have more severe protoxylem phenotypes. Triple *cre1 ahk2 ahk3* mutants show extreme growth reduction, with a stele composed entirely of xylem and the arrest of primary root meristem growth a few days after germination (Inoue *et al.,* 2001; Higuchi *et al.,* 2004; Nishimura *et al.,* 2004; Riefler *et al.,* 2006). *WOODEN LEG* mutants *(wol)* are recessive dominant-negative cytokinin signalling mutants at the CRE1 locus (Mähönen *et al.,* 2000). In *wol*, the ligand binding site in the CRE1 receptor domain contains a single amino acid mutation, inhibiting cytokinins from binding to the receptor. In *wol* mutants, CRE1 is therefore unable to transfer the phosphoryl group to the downstream AHPs, but its phosphatase activity is unimpaired, allowing it to actively suppress cytokinin signalling. As a result, the *wol* mutant is phenotypically similar to the triple receptor *cre1 ahk2 ahk3* mutant.

Over the years, there has been some debate over the localization of the cytokinin receptors, as they were initially assumed to localize to the plasma membrane, similarly to many other plant receptors. Finally, in 2011, two papers were published (Wulfetange *et al.* & Caesar *et al.*) showing that the majority of the AHK receptors localize primarily to the ER. Strangely, the sensor modules – the CHASE domains – of the receptors were predicted to face the ER lumen (Lomin *et al.,* 2015), indicating that cytokinin binding happens within the organelle. When cytokinin binds to a receptor's CHASE-domain, a histidine residue in the protein kinase domain becomes autophosphorylated. Next, presumably via a conformational change of the receptor domain (Kieber and Schaller, 2014), the phosphoryl group is transferred to an aspartate residue in the receptor's receiver domain, across the ER membrane in the cytoplasm; from there, it is transferred to a histidine residue on an AHP protein.

CYTOKININ-INDEPENDENT 1 (CKI1) is a histidine kinase receptor that lacks a cytokinin-binding CHASE domain. Initially, the CKI1 protein was thought to function as a cytokinin receptor (Estelle, 1998 and Kakimoto, 1998); however, no evidence has been presented demonstrating that CKI1 actually binds cytokinins (Pekárová *et al.,* 2011). The protein has distinct sensor-receiver and response regulator domains and is localized to the plasma membrane (Pekárová *et al.,* 2011). CKI1 functions on a parallel pathway independent from the cytokinin

receptors. It is capable of translocating a phosphoryl group from a conserved histidine residue in its transmitter domain to a conserved aspartate residue in the receiver domain of the response regulators, thus activating the transcription of downstream response genes independently from the activity of the CRE1/AHK2/AHK3 cytokinin receptors (reviewed by Chang and Stewart, 1998; West and Stock, 2001 and Hwang *et al.,* 2002). Accordingly, overexpression of *CKI1* can rescue the root phenotype of *wol* mutants (Deng *et al.,* 2010); further evidence that *CKI1* interacts with the cytokinin signalling pathway downstream of the receptors is provided by co-incubation protoplast assays with CKI1 and downstream response regulators (Hwang & Sheen, 2001) and over-expression studies that mimic exogenous cytokinin application (Kakimoto 1996). Interestingly, the *CKI1*-induced phenotype is dependent on the AHPs, as the quintuple *ahp* mutant cannot be rescued by *CKI1* induction (Deng *et al.,* 2010). Accordingly, CKI1 was shown to recognize and bind to several AHP proteins (including AHP2, AHP3 and AHP5) via its receiver domain, independent from plasma membrane localization (Pekárová *et al.,* 2011). Thus, cytoplasmic AHPs can receive and mediate signals from both cytokinin-dependent and independent pathways. Perhaps the AHPs are critical for distinguishing signals from different sources and mediating them to shared downstream response regulators. In this scheme, the AHPs and ARRs could be phosphorylated and activated by, for example, cytokinin, ABA, or ethylene, to generate a co-ordinated response.

It is noteworthy that *CKI1* is a close relative of the ethylene receptor *ETR1* (Chang *et al.,* 1993) and that *CKI1* has a role independent of cytokinin signalling in vascular bundle development in shoots (Hejátko *et al.,* 2009), flowers (Borkovcová *et al.,* 2014) and female gametophytes (Hejátko *et al.,* 2003, Deng *et al.,* 2010). Consistent with this, *CKI1* expression has been detected in the vascular bundle sheath cells of *Arabidopsis* shoots and in the vascular bundles of floral organs (Hejátko *et al.,* 2009, Borkovcová *et al.,* 2014) by transcriptional GUS-reporters, *in-situ* RNA-localization and antibody-immunolocalizations. In roots, *CKI1* is expressed in the vascular tissues and in the root tip (Hejátko *et al.,* 2005), which is in line with the original mutant lines by Kakimoto (*cki1-1, -2, -3,* and *-4*), which were unable to produce roots and also showed abnormal floral development, as well as sterility. The root phenotype of *CKI1* RNAi roots (analysed by Hejátko *et al.,* 2009) resembles wild type roots, leaving the developmental role of *CKI1* a mystery.

*CKI2 (*also known as *AHK5)* has been shown to modulate ROS production and pathogen tolerance under biotic and abiotic stress, including necrotic fungi and soil salinity (Pham and Desikan, 2012a; Pham *et al.,* 2012b). *CKI2* has also been suggested to function as a negative regulator of the ethylene signalling pathway, inhibiting root elongation in collaboration with ABA (Iwama *et al.,* 2007). Based on the protein structures and downstream interactions of the AHKs, CKIs and ETR receptors (Stock *et al.,* 2000; Hwang *et al.,* 2002; Grefen and Harter in 2004), it seems that two-component signalling pathways are a general and ancient signalling mechanism in higher plants; indeed, TCS-signalling pathways are found in fungi and bacteria as well as plants. It is therefore not surprising that ethylene and cytokinin hormone pathways have common targets in the ARR genes (Shi *et al.,* 2012).

The *G-PROTEIN-COUPLED RECEPTOR 1 (GCR1)* was initially named "G-protein coupled putative cytokinin receptor" (Kakimoto, 1998), but in 2001 the link to CK signalling was disproven (Humphrey and Botella 2001, Kanyuka *et al.,* 2001). Data published since have demonstrated that *GCR1* is actually an ABA receptor (Liu et al, 2007). In plants, it is involved in the detection of quorum-sensing signals secreted by bacteria, which affect primary root elongation and cause architectural changes with heterotrimeric G-proteins (Liu *et al.,* 2012). Transcript profiling of *gcr1* mutants has verified the link to ABA signalling, but also revealed other hormone biosynthesis genes, including cytokinin oxidase genes, SA and ethylene response genes, to be under *GCR1* regulation (Chakraborty *et al.,* 2015). These findings conclude that GCR1 might be a G-protein coupled receptor, and that G-protein pathways can affect hormonal signalling networks and root meristem size by integrating biotic and abiotic stress signalling pathways and through secondary metabolite biosynthesis.

1.6.5. Arabidopsis Histidine Phosphotransfer proteins

Arabidopsis has six ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN (AHP) genes. *AHP1, AHP2, AHP3, AHP4* and *AHP5* are positive regulators of cytokinin signalling; cytokinin promotes their phosphorylation and translocation from the cytoplasm to the nucleus (Hwang and Sheen 2001), where the phosphoryl group from their His residue is relayed to downstream response regulators (ARABIDOPSIS RESPONSE REGULATORS, ARRs). The *AHP1-5* genes are redundant, and while single *ahp* single mutants do not show any phenotypes, several higher order mutant combinations show reduced cytokinin responses. The quintuple *ahp1 ahp2 ahp3 ahp4 ahp5* mutant develops ectopic protoxylem cell files, similar to cytokinin receptor mutants (Hutchison *et al.,* 2006). Overexpression of AHPs, such as *AHP2*, has been shown to cause hypersensitivity to cytokinins (Suzuki *et al.,* 2002). Unlike the other *AHPs*, *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6 (AHP6)* is a pseudo-phosphotransferase that lacks the conserved His residue (Mähönen *et al.,* 2006). AHP6 interacts with the phosphorelay machinery and competes with other AHPs. As a result, AHP6 functions as an inhibitor of cytokinin signalling (Mähönen *et al.,* 2006). Loss of function *ahp6* mutants are not only more sensitive to cytokinin than wild type plants in root growth assays but they also exhibit increased cytokinin signalling at the protoxylem position and are defective in protoxylem differentiation (Mähönen *et al.*, 2006). Overexpression of *AHP6* leads to ectopic protoxylem formation in roots (Ren *et al.,* 2013). *AHP6* has homologs in other plant species, indicating that inhibition of cytokinin signalling via a pseudo-AHP protein is a conserved feature in the plant kingdom (Mähönen *et al.,* 2006).

The AHP6 protein can move from cell-to-cell and is an important factor in creating signalling gradients in shoot apical meristem (Besnard *et al.,* 2014a); however, the role of AHP6 movement in root patterning is not clear. While data published by De Rybel *et al.,* (2014) suggests that cytokinin biosynthesis within the xylem axis is necessary for promoting procambial cell divisions (De Rybel *et al.,* 2014), Muraro *et al.,* (2014) proposed that cytokinin signalling must be inhibited at the central xylem position of the stele for proper vascular patterning. According to their model, either a cytokinin degrading/inactivating enzyme, such as a CKX, or a cytokinin signalling repressor is needed for local degradation of cytokinins in the metaxylem. Movement of AHP6 from the protoxylem position inwards to the

metaxylem might serve to repress cytokinin signalling there. Indeed, AHP6 was shown to move from the protoxylem position into the neighbouring cells in young *Arabidopsis* roots (Mähönen *et al.,* 2006). Perhaps AHP6 movement forms a gradient of cytokinin signalling suppression along the whole xylem axis; this movement, together with a CKX in the metaxylem position (promoted by local auxin biosynthesis and an auxin-inducible metaxylem gene, perhaps one of the HD-ZIP class III genes) might be enough to supress cytokinin signalling within the biosynthetically-active xylem cells. As the *aph6* mutant has normal metaxylem development, *AHP6* alone is clearly not enough to block cytokinin signalling in metaxylem position, suggesting the need for a redundant partner. The fact that the *ahp6* mutant also frequently has stretches of protoxylem further reinforces the idea that a redundant partner supports *AHP6* activity.

1.6.6. Arabidopsis Response Regulators

Upon translocation to the nucleus, the AHP proteins phosphorylate response regulators (RR) at a conserved aspartate in their receiver domain (D'Agostino and Kieber, 1999). *Arabidopsis* has different types of response regulators: type-A, type-B and type-C ARABIDOPSIS RESPONSE REGULATORS (ARRs) and CYTOKININ RESPONSE FACTORs (CRFs).

The type-B ARRs *(ARR1, 2, 10, 11, 12, 13, 14, 18, 19, 20 and 21)* are positive regulators of cytokinin signalling that are located in the nucleus (reviewed by Kieber and Schaller, 2014). AHPs translocated to the nucleus phosphorylate the type-B ARRs. This activates them and allows the C-terminal DNA binding domain of the type-B ARRs to bind to DNA and promote the expression of target genes (Sakai *et al.,* 2000), leading to cytokinin signalling output (as illustrated by Werner and Schmülling 2009). Type-B ARR expression does not respond rapidly cytokinin signalling. The type-B ARRs are repressed by the type-A ARRs, creating a feedback loop in the signalling pathway.

The type-B ARRs belong to a large gene family whose members show high redundancy with overlapping yet distinctive expression patterns (Mason *et al.,* 2004; Takajima et al, 2004; Argyros *et al.,* 2008; Hill *et al.,* 2013). The type-B ARRs can be classified to different subfamilies (Mason *et al.,* 2004). Most mutants of subfamily 1 type-B ARRs show cytokinin insensitive phenotypes similar to the heptuple *log* cytokinin biosynthesis mutant, triple cytokinin receptor mutants and higher order *ahp* mutants, all of which form ectopic protoxylem files within the stele (Hill *et al.,* 2013). By contrast, mutants of subfamily 2 and 3 members do not show any pronounced effects on cytokinin sensitivity in any of the analysed tissues (Hill *et al.,* 2013). *ARR1, ARR10, ARR12* belong to subfamily 1, and the single and multiple mutants exhibit clear cytokinin phenotypes. While the *arr1* and *arr1 arr12* mutants show enhanced primary root meristem size (Moubayidin *et al.,* 2010), the triple *arr1 arr10 arr12* mutant is severely dwarfed and fails to show any response to exogenously applied cytokinins. Inducible overexpression via a *35S::ARR1ΔDDK::GR* construct (in which the receiver domain at the N-terminus is mutated) leads to an increase in endogenous cytokinins levels and, interestingly, co-activation of several type-A *ARRs* (Sakai *et al.,* 2001), highlighting that the type-A and type-B families are jointly regulated and that the type-As are required to supress cytokinin signalling.

The type-A response regulators (*ARR3, 4, 5, 6, 7, 8, 9, 15, 16* and *17*) are cytokinin-inducible negative regulators of cytokinin signalling (Imamura *et al,* 1999; D'Agostino *et al.,* 2000; Kiba *et al.,* 2003; Kieber and Schaller, 2014). They are activated by the AHPs in the nucleus, and the proteins become more stable against degradation when phosphorylated (To & Kieber, 2007). Expression of the type-A ARRs is promoted by the type-B response regulators (Werner and Schmülling, 2009). The type-A ARRs lack a DNA binding domain and cannot bind to DNA to modulate transcription of target genes; they are thought to interact with and repress the type-B ARRs to suppress cytokinin signalling. This repression is generally thought to occur via competition over the phosphoryl groups from the AHPs (To *et al.,* 2004). The type-As have also been proposed to repress the type-Bs by binding to the phosphorylated receiver domains and forming inactive protein heterodimers (Kim, 2008). Seedlings of type-A *arr* mutants are more sensitive to repression of root elongation by CK, as well as to inhibition of lateral root formation (To *et al.,* 2004). Loss-of-function analyses show that most of the type-A ARRs are highly redundant (To and Kieber, 2007), although some of their functions are antagonistic (To *et al.,* 2004). In general, higher order mutants of these cytokinin signalling repressors show cytokinin hypersensitive phenotypes (To *et al.,* 2004). While single type-A *arr5* or *arr6* mutants do not show have any xylem phenotype in the primary root, the double *arr5 arr6* mutant has an occasional loss of protoxylem in the lateral roots (Kondo *et al.,* 2011). Due to their rapid cytokinin response and expression in the primary meristem procambium, *ARR5* and *ARR15* have been used as markers for cytokinin signalling in *Arabidopsis* roots (Mähönen *et al.,* 2006) in a similar manner to the synthetic TCS-markers (Müller and Sheen, 2008; Zürcher *et al.,* 2013). Interestingly, some of the type-A ARRs are regulated not only by cytokinin but also auxin signalling (Müller and Sheen, 2008); type-A ARRs, in turn, regulate the abundance of the PIN auxin efflux carrier proteins (Zhang *et al.,* 2011). Cold stress (abiotic stress) and ethylene also crosstalk with cytokinin signalling via the type-A ARRs (Jeon *et al,.* 2010 and Shi *et al.,* 2012), indicating that this large family of negative cytokinin response regulators acts as a hub for the integration hormonal signals (reviewed by O´Brien and Benkova in 2013).

In addition to the type-A and B response regulators, *Arabidopsis* has two type-C response regulators, *ARR22* and *ARR24*. They are structurally similar to the type-A ARRs but are not upregulated upon cytokinin treatment (Kiba *et al.,* 2004; Gattolin *et al.,* 2006; Horák *et al.,* 2008). *ARR22* expression is restricted to the chalaza of developing seeds (Gattolin *et al.,* 2006; Horák *et al.,* 2008). In tobacco leaf cells, ARR22 is localized in the cytoplasm, where it interacts with AHP2, AHP3 and AHP5 (Horák *et al.,* 2008). In onion epidermal cells, transient expression of GFP-ARR22 showed protein localization in both the cytoplasm and the nucleus (Kiba *et al.,* 2004). This pattern is in line with the localization of the AHPs. *ARR24* is expressed in reproductive organs (Gattolin *et al.,* 2006). ARR22 and ARR24 are 66% similar on the amino acid level. It was postulated that they are functionally diverged from the other ARRs and are required for mediating hormonal signals other than cytokinin (Gattolin *et al.,* 2006). Accordingly, *ARR22* expression was induced by wounding and by auxin, ethylene and ABA treatments (Kiba *et al.,* 2004, Gattolin *et al.,* 2006). Overexpression of *ARR22* causes severe dwarfism (Gattolin *et al.,* 2006) in both shoot and roots and have a primary root vascular phenotype identical to *wol* (Kiba *et al.,* 2004). Loss-of-function a*rr22* mutants do not exhibit seed or embryo phenotypes, but genomic

complementation and introduction of an additional gene copy causes a phenotype similar to plants with a cytokinin deficiency syndrome (Horák *et al.,* 2008), indicating that maintaining the proper expression level of *ARR22* is critical during embryonic development.

Arabidopsis thaliana also has several CYTOKININ RESPONSE FACTOR (CRF) genes. To date, 12 CRF genes have been identified in Arabidopsis (Rashotte and Goertzen, 2010). These genes all belong to the AP2/ERF transcription factor gene family. Similarly to the type-A ARRs, the transcription of CRFs is upregulated by cytokinins. Protein localization assays in protoplasts indicate that these cytoplasmic proteins are translocated to the nucleus after cytokinin application (Rashotte *et al.,* 2006*).* However, data from *in planta* analyses show that CRF proteins are primarily localized in the nucleus, and thus cytokinins do not enhance their nuclear localization (Raines, 2013). Nonetheless, CRFs participate in protein-protein interactions with other components of the multi-step cytokinin signalling pathway (AHP1-5) through their CRF domain (Cutcliffe *et al.,* 2011) and are thought to convey cytokinin signalling parallel to the type-B ARRs. The fact that the CRFs might relay messages from other signalling pathways (such as abiotic stresses) explains why the type-B ARRs and CRFs have both shared and unique target genes. This is in line with observations that cytokinin receptors are highly similar to ethylene and osmotic stress receptors. Perhaps some of the CFRs mediate messages from these pathways, and thus their genetic outputs are only partially redundant with type-Bs.

The role of CRF genes have been studied in senescence (Zwack *et al.,* 2013). *CRF6* appears to be the key gene repressing premature senescence and has been suggested to play a role in other stress responses downstream of cytokinin signalling (Zwack *et al.,* 2013). *CRF6* is expressed in leaf veins and in roots, and its expression is promoted by exogenously applied cytokinins (Raines, 2013). Loss of CRF function does not reduce the plant's response to cytokinin in most assays (Rashotte *et al.,* 2006), further indicating that the CRF pathway is functionally parallel to the other cytokinin signalling pathways described earlier.

In 2013, Ren *et al.* demonstrated that the *Arabidopsis* eukaryotic translation initiation factor *eIF5A-2* modulates cytokinin signalling in root protoxylem development. The T-DNA insertion mutant for *eIF5A-2, fbr12*, showed a severe reduction in root elongation growth, reduced cell numbers within the stele and ectopic protoxylem differentiation. Consistent with this*, eIF5A-2* is highly expressed in the RAM vasculature in the elongation zone. The loss-of-function *eIF5A-2* mutants were insensitive to exogenously applied cytokinin, indicating that *eIF5A-2* is required for sensing cytokinin. Indeed, the root elongation rates, callus culture assays and expression of type-A ARR genes verified that *fbr2* mutants were suffering from cytokinin deficiency. The combination of *fbr2* with *cre1* and *wol* in higher order mutants led to enhanced phenotypes with very severe anatomical abnormalities. The *fbr2* mutation was suppressed by *ahp6*, indicating that *eIF5A-2* affects the cytokinin signalling pathway directly. Protein pull-down assays showed that FBR12, CRE1 and AHPs form cytokinin-regulated protein complexes. This data positions *eIF5A-2* upstream of *AHP6*, which also showed increased expression in the *fbr2* mutants. The evolutionary conserved *eIF5A-2* is likely involved in regulating protein stability in the *Arabidopsis* cytokinin signalling pathway.

1.6.7. Cytokinin biosynthesis, translocation and signalling in the proximal meristem

As mentioned earlier, the highest iP concentrations are found in phloem sap, which flows rootwards, suggesting that iP is the most relevant cytokinin species required for stele development and patterning in the root. Accordingly, *CRE1*, which has the highest binding affinity to iP cytokinins, is most abundant in root tips. Conversely, tZ concentrations are highest in the xylem sap, which flows shootwards, and *AHK3* is expressed most abundantly in shoots and has a high affinity to tZ type cytokinins. The expression patterns of these cytokinin receptors and the translocation of activating compounds via phloem and xylem sap on-site appear to coincide, strongly suggesting that the aerial and belowground tissues regulate each other's development.

The different cytokinin profile of xylem and phloem sap and the transport of iP-type cytokinins into the proximal meristem stele, together with procambial *IPT* expression and xylem-localized *LOG3* and *LOG4* expression indicate that xylem-localized LOGs might use *iP*-type cytokinins as a precursor for xylem-specific tZ biosynthesis via iP-to-tZ conversion. This tZ pool is then presumably translocated to the shoot via the xylem. Interestingly, Antoniadi *et al.,* (2015) showed that the most abundant cytokinins in the meristems are the cZ cytokinins, which are likely synthesized at the meristem and required locally for the maintenance of cell divisions. Together, these data suggest a mechanism in which each cytokinin type plays a different role in tissue patterning. An *iP*-type pool is required for stele development and might function as a source for conversion, while *tZ*-types is required to maintain the xylem-procambium and for root-to-shoot signalling, and *cZ*-type cytokinins synthesized all over the meristem maintain cell divisions and RAM growth. The different cytokinin profiles within the proximal meristem likely activate receptors and their downstream response genes (AHPs, ARRs and CRFs) in the stele in a tissue-specific manner.

In addition to radial patterning, cytokinin signalling is also critical for controlling the timing of the maturation of cells in the root meristem. In the meristematic tissue, cytokinins promote mitotic cell divisions, whereas in the transition zone, cytokinins repress divisions and promote cell elongation (Moubayidin *et al.,* 2010) through a transition to endoduplication (Takahashi and Umeda, 2014). These functionally different roles are highly dependent on the cell-specific expression patterns of the various response regulators, but also rely heavily on hormone-signalling interactions in which auxin often acts antagonistically to cytokinin.

1.7. Interaction of auxin and cytokinin in different developmental processes

The auxin and cytokinin signalling pathways interact with each other in developmental processes. In most cases, their interactions are mutually inhibitory and the signalling pathways antagonize one another. In SAM phyllotaxis patterning, auxin signalling maxima position new primordia. These signalling maxima also promote the expression of *MONOPTEROS*, which upregulates *AHP6* expression one plastochron after the auxin signalling maximum is established. Non-cell autonomous movement of the AHP6 protein within the meristem in turn represses cytokinin signalling where the protein is located, imposing a temporal and spatial sequence on organ initiation (Vernoux *et al.,* 2010; Besnard *et al.,*

2014a and 2014b). In shoots, auxin promotes apical dominance by inhibiting cytokinin signalling in dormant axillary buds; the inhibition occurs by the repression of biosynthesis through the IPTs and the promotion of cytokinin degradation/oxidation via CKX. At the same time, auxin signalling from the apical meristem decreases PIN levels in the bud, reducing auxin flow from the bud (Bennett *et al.,* 2006). If cytokinin levels in the bud increase via enhanced xylem sap flow or local biosynthesis, or if exogenous cytokinin is applied directly onto an axillary bud, the repression of apical dominance can be supressed and the axillary bud can grow out (Dun *et al.,* 2006, Ferguson *et al.,* 2009, Shimizu-Sato *et al.,* 2009, Müller and Leyser, 2011). As discussed earlier in chapter 1.5.2.4, cytokinins regulate the expression and localization of the PIN proteins, so axillary bud dormancy breakage can be explained by cytokinin enabling the new bud's vasculature to canalize to the main stem, thus establishing the vascular connection required for bud outgrowth (Balla *et al.,* 2011).

Auxin and cytokinin signalling domains correlate inversely during embryogenesis; in globular embryos, high auxin signalling is localized in the basal cell and high cytokinin signalling in the lens-shaped cell above it, which will later form the QC. A sharp boundary between these hormonal domains forms in the embryonic root meristem, allowing proper formation of the root stem cell niche, which regulates vasculature formation. Interestingly, auxin antagonizes cytokinin output in the basal cell-lineage by direct transcriptional activation of *ARR7* and *ARR15*, which are feedback repressors of cytokinin signalling (Müller and Sheen 2008). This illustrates a convergence of hormonal signalling at the level of response regulators. The establishment of hormonal signalling domains occurs very early in development; the symmetry breakage required for the formation of high auxin and cytokinin signalling domains occurs in embryos between the globular and heart-shaped stages. This patterning process is thought to rely on cellular connectivity via a small "cell wall bridge" between the vascular xylem initials, which are connected in turn to the cotyledons above (De Rybel *et al.,* 2014). The continuous growth of the developing root is maintained by a mechanism in which auxin promotes the expression of members of the TARGET OF MONOPTEROS (TMO) and LONESOME HIGHWAY (LHW) families; these then promote the expression of LOG genes and of *AHP6*, leading to both local cytokinin biosynthesis, which causes cell divisions in the procambial domain, and repression of cytokinin signalling at the xylem position, respectively (De Rybel *et al.,* 2013; Ohashi-Ito *et al.,* 2014).

Auxin signalling positions, primes and initiates lateral root (LR) formation, whereas cytokinin has been shown to inhibit these processes (Himanen *et al.,* 2004, reviewed by Aloni *et al.,* 2006, Laplaze *et al.,* 2007, Chang *et al.,* 2013, Marhavý *et al.,* 2014). While mutants with increased auxin signalling generate more lateral roots than wild type plants, reduced auxin signalling mutants have less lateral roots, a phenotype which can be mimicked by NPA treatments (Ullah *et al.,* 2003) or exogenous cytokinin application (Chang *et al.,* 2013). The pericycle cells which are to become LR founder cells are primed in the meristem by auxin (De Smet *et al.,* 2007); this is proposed to occur by an oscillating pattern of gene expression along the primary root meristem (Moreno-Risueno *et al.,* 2010). The oscillation of gene expression is thought to be critical for specifying the prebranch sites, and thus the spacing, of LRs along the root. As the primary root matures, the LR founder cells initiate transcriptional events which lead to specific periclinal cell

divisions in the pericycle (Himanen *et al,* 2004). Certain environmental stimuli, such as bending, can activate the asymmetric cell divisions in the pericycle and initiate primordia formation (Richter *et al.,* 2009). Cytokinins repress the expression of PINs and modulate their degradation, affecting auxin flow within the meristem. This can cause defects in several stages of LR development, from initiation to emergence through the overlaying endodermal, cortical and epidermal layers (illustrated beautifully by Vilches-Barro and Maizel, 2014).

The interaction of auxin and cytokinin signalling is a general theme in various developmental context and in different plant species. In *Arabidopsis* primary roots, the size of the meristem is regulated by a balance between cytokinin signalling and the expression of auxin response genes and PIN localization (Dello Ioio *et al.,* 2007, Moubayidin *et al.,* 2010). During secondary growth activation in *Arabidopsis*, it seems that the interaction between auxin and cytokinin signalling is required for periclinal divisions to occur properly in the procambium (Siligato *et al.* data not published). In trees such as *Populus*, auxin and cytokinin localize to separate domains in the stem, with a maximum of auxin signalling at the central zone of meristematic procambial cells and cytokinins more towards the phloem cells (Nieminen 2009, Immanen *et al.,* unpublished).

The hormonal signalling domains seen in the primary root and forming in lateral roots can be traced all the way back to the embryo. Some genes (such as *AHP6*) are expressed and regulated similarly in different developmental contexts, demonstrating that certain key genes and their targets are repeatedly harnessed for meristem formation. This is fascinating, because it means that hormonal signalling patterns are not only maintained but also re-iterated in spatially and temporally distant sites throughout the whole life span of the plant. Why reinvent the wheel when you can iterate, with minor modifications, a whole cassette of genes to make an organ upon request? But what then are the minor modifications that explain the range of morphogenesis driven by these key hormones in different tissues? To fully grasp the mechanisms of auxin and cytokinin interaction in vascular patterning, one has to understand that they interact on every imaginable level: biosynthesis, transport, perception and signal transduction, promotion or repression of target genes, translation, post-translational regulation (e.g., phosphorylation) and degradation. The cell-type specific gene expression patterns of their pathway components, downstream target genes and the genetic redundancy of these large gene families complicates matters further. Put simply, the complexity of hormonal interactions and signalling is mindboggling. To complicate matters further, in addition to affecting each other directly, auxin and cytokinin also regulate other signalling pathways which may or may not interact with one another in the proximal meristem.

1.8. Proximal meristem maintenance and tissue patterning mechanisms

Auxin and cytokinin antagonize each other in various developmental contexts and contribute pleiotropically to plant growth. These master regulators have tissue- and cell-specific targets that dictate the genetic outputs of each stimulus. At each meristematic cell division, the identities of the different cell types are re-established. This requires the integration of various overlapping lateral signalling mechanisms, many of which are, not surprisingly, regulated by auxin, cytokinin,

or both hormones. These signalling mechanisms coordinate meristem growth in order to 1) maintain a passive, anchor-like organizing centre around which the stem cell niche and tissue identities are formed; 2) sustain and regulate cell proliferation rates of the stem cell initials; 3) promote daughter cell identities and differentiation rates; and 4) regulate meristem maturation. To highlight the complexity and beauty of root apical meristem maintenance, some of the key processes in RAM function and patterning are presented in the following paragraphs.

The quiescent centre (QC) is the sessile, organizing stem-cell niche of the RAM. One of the key genes in QC identity maintenance is the transcription factor *WUSCHEL-RELATED HOMEOBOX 5 (WOX5)* (Sarkar *et al.,* 2007, reviewed and illustrated by Miyashima *et al.,* 2012). Root meristems of *wox5* loss-of-function mutants differentiate terminally (Sarkar *et al.,* 2007) as the QC identity is lost. Accordingly, *WOX5* suppresses *CYCLIN D*-promoted cell divisions in the QC, and through this repression maintains the root stem cell niche (Forzani *et al.,* 2014). As the *CYCLIN D* genes are primary targets of cytokinin signalling, it is fair to assume that *WOX5* also suppresses cytokinin signalling at the QC position; this is supported by the fact that *TCSn::GFP* is not expressed in these cells (see Appendix). Unlike the QC expressed *WOX5*, *WOX4* regulates the procambial meristem function and mediates signals from CLE-peptides and auxin, ethylene and brassinosteroid signalling (reviewed by Jouannet *et al.,* 2015).

If the mitotic activity of the QC cells is promoted, as occurs in the *BRASSINAZOLE RESISTANT 1 (BZR1)* and *BRI1 EMS SUPPRESSOR 1 (BES1)* mutants, the stem cells can divide, causing severe developmental defects (Lee *et al.,* 2015). *REPRESSOR OF WUSCHEL1 (ROW1)* (Zhang *et al.,* 2015) and *PLEIOTROPIC REGULATORY LOCUS1 (PRL1)* (Ji *et al.,* 2015) also affect *WOX5* expression, QC and root size; the absence of their suppression in *row1* or *prl1* mutants results in the spread of QC markers and the meristem becomes determinate prematurely. The *prl1* mutation was shown reduce auxin transport and signalling, which contributes to the reduction in meristem size and progressive cell death in the proximal meristem. These phenotypes are due to a reduction in the auxin-dependent expression of the *PLETHORA* genes (Ji *et al.,* 2015). The stem niche is also regulated by the *POLTERGEIST (POL)* and *POLTERGEIST LIKE 1 (PLL1)* genes. *POL* and *PPL1* are required for embryonic asymmetric divisions regulated via PIN1 accumulation in embryonic roots. Following germination, the loss-of-function *pol ppl1* double mutant exhibits arrested root meristems resulting in TE differentiation close to the root tip, similar to the *wol* mutant (Song *et al.,* 2008).

The *Arabidopsis* genome contains several loci that encode *CLAVATA3/EMBRYO SURROUNDING REGION-related (CLE)* peptides. Phenotypic characterization of lines overexpressing different CLE peptides is presented in Strabala *et al.,* 2006. According to the study, the CLE peptides have diverse yet redundant functions. Overexpression of the peptides can lead to developmental timing delays, dwarfed shoots, and longer or shorter primary roots, depending on which peptide is overexpressed. Several of the peptides inhibit primary root meristem growth and affect protoxylem formation (as was listed by Kondo *et al.,* in 2011). *CLE10* is especially interesting, as it is expressed in the vasculature and was shown to repress the expression of two type-A response regulators, *ARR5* and *ARR6*

(Kondo *et al.,* 2011). Double *arr5 arr6* mutants are defective in protoxylem formation, mimicking the effect of exogenously applied CLE10 peptide.

CLE40 inhibits cell differentiation in the RAM by promoting ABA signalling and suppressing cytokinin signalling (Pallakies and Simon, 2014). In addition, CLE40 regulates the expression of several auxin-signalling related genes; loss-of-function *cle40* mutants show reduced expression of genes related to the promotion of auxin transport and auxin signalling and increased expression of several auxin response factors and response regulators. Several genes that are associated with CK biosynthesis, signal perception, and response *(LOG1, AHK3, CFR6, ARR1 and ARR15*) were also upregulated in *cle40* mutants (Pallakies and Simon, 2014). Application of CLE40 reduced root length in wild type plants, whereas *CLE40*-receptor mutants showed insensitivity to the peptide. When short, 12 - 13 aa peptides, termed CLE-like (CLEL), peptides were overexpressed in roots, this resulted in an increase in root meristem size and length together with disturbed LR initiation (Meng *et al.,* 2013).

CLE41 binds to the receptor *PHLOEM INTERCALATED WITH XYLEM (PXY)* and their interaction controls the rate and orientation of vascular cell divisions. Overexpression of CLE41 leads to intercalation of xylem and phloem tissues in the shoot (Etchells and Turner, 2010). In the root vascular cambium, CLE41 and CLE44 are both implicated in regulating procambial divisions around the phloem poles through interaction with *PXY* (Miyashima *et al.,* 2012). The *PXY/CLE41* pathway interacts with and functions in parallel to the ethylene signalling pathway (Etchells *et al.,* 2012); while ethylene promotes radial growth and cell division, *PXY* is needed for the co-ordination of cell division. The PXY/CLE41 signalling pathway is also linked with brassinosteroid signalling during xylem differentiation in the proximal meristem. The PXY/CLE41 signalling module is proposed to inhibit xylem differentiation by activating *BRASSINOSTEROID-INSENSITIVE 2*, which leads to the phosphorylation of the transcription factors BRASSINAZOLE-RESISTANT 1 and BRASSINAZOLE-RESISTANT 2. These genes promote brassinosteroid signalling and xylem differentiation, and their phosphorylation inhibits signalling in early xylem cells, delaying their differentiation (Jouannet *et al.,* 2015). As these examples illustrate, the CLE and CLEL peptide families are critical for proximal meristem function, and they co-ordinate cross talk between the auxin, cytokinin, ethylene and brassinosteroid signalling pathways.

Recently, the RING/U-box family member *PLANT U-BOX 4 (PUB4)* was shown to regulate cell proliferation in the primary root downstream of *CLAVATA3/CLE19* (Kinoshita *et al.,* 2015). *PUB4* is expressed in the meristem. It regulates the first periclinal cell division in the cortex/endodermis initials, but is also required for controlling cell proliferation more broadly, as the root meristem of the *pub4-1* mutant is considerably longer and wider than in wild type. *PUB4* regulates the timing of asymmetric cell division, possibly by promoting *CYCD6;1*, yet it does not seem to affect cytokinin signalling, nor does it interact with the known ground-tissue identity genes *SCARECROW* and *SHORTROOT*. The *pub4-1* mutant has increased endogenous auxin levels, though it remains unclear if this is a cause or effect of the increased meristem size.

SCARECROW (SCR) is a transcription factor required for positioning the stem cell niche and regulating radial patterning of the primary root (Scheres *et al.,* 1995; Di

Laurenzio *et al.,* 1996; Sabatini *et al.,* 2003) together with another transcription factor, *SHORTROOT (SHR)*. *SCR* is expressed in the QC, the endodermis/cortex initials and the endodermis. The *scr* mutant shows a loss of QC cell identity and distorted columella stem cells (Sabatini *et al.,* 2003), and the ground tissue identities are perturbed (Di Laurenzio *et al.,* 1996). Loss of *SHR* leads to a stunted phenotype and severely reduced primary root growth. Similar to *scr* mutants, *shr* mutants fail to develop normal ground tissue; one cell layer is lost and the endodermis and cortex identities are mixed together. *SHR* is expressed in the stele (Helariutta *et al.,* 2000), and the protein moves via the plasmodesmata (Vatén *et al.,* 2011) to the ground tissue, where it localizes to the nucleus and interacts with SCR (Cui *et al.,* 2007). In the ground tissue, SHR also promotes the expression of SCR (Helariutta *et al.,* 2000), and SCR, in turn, restricts SHR to the nucleus in endodermal cells (Cui *et al.,* 2007), inhibiting its movement to the outer cell layers. Together, these two transcription factors promote the asymmetric cell divisions which are required for both endodermis and cortex cell determination (Scheres *et al.,* 1995; Di Laurenzio *et al.,* 1996, Cui *et al.,* 2007). Both *shr* and *scr* loss of function mutants show a loss of protoxylem identity and form metaxylem-like TEs at the protoxylem position (Carlsbecker *et al.,* 2010).

SHR was shown to regulate xylem patterning by promoting the expression of microRNA165a/166b in the ground tissue (Carlsbecker *et al.,* 2010). These small RNAs move inwards to the stele via plasmodesmatal connections (Vatén *et al.,* 2011) and restrict the expression of the HD-ZIP class III transcription factors in the target tissues (reviewed by Miyashima *et al.,* 2012). The HD-ZIP class III genes *PHABULOSA (PHB), CORONA (CNA), REVOLUTA (REV), PHAVOLUTA (PHV)* and *HOMEOBOX GENE 8 (ATHB8)* are expressed in the stele (Carlsbecker *et al.,* 2010). Their expression levels regulate xylem cell fate in a dose-dependent manner; if all five genes are knocked out (*athb8-11 cna-2 phb-13 phv-11 rev-6*), the xylem cells fail to differentiate altogether. However, when just four of them are knocked out (*cna-2 phb-13 phv-11 rev-6*), the whole xylem axis differentiates into protoxylem. The key gene for protoxylem fate is *ATHB8*, which is expressed in the whole xylem axis in wild type plants (OP II Fig 2 I). *PHB* is required for metaxylem fate, and its exclusion from the protoxylem, pericycle and ground tissue due to miRNA silencing allows these tissues to develop normally (Carlsbecker *et al.,* 2010, Miyashima *et al.,* 2011). Gain-of-function *phb-7d* mutants that escape the miRNA regulation show a loss of protoxylem, with all xylem cells differentiating into metaxylem (Carlsbecker *et al.,* 2010). *PHB* therefore promotes metaxylem identity and *ATHB8* protoxylem identity, and the local suppression of *PHB* at the protoxylem position allows protoxylem formation through activation of auxin-promoted *AHP6* and *VASCULAR-RELATED NAC-DOMAIN7 (VND7)* expression.

Genes of the *PLETHORA* transcription factor family are expressed in the root proximal meristem, where they mediate patterning and promote cell divisions within the root stem cell niche (Aida *et al.,* 2004; Galinha *et al.,* 2007). The *PLTs* are dose-dependent master regulators of root identity and control root regeneration (Galinha *et al.,* 2007). The PLETHORA gene expression pattenrs and protein stability form a protein gradient that controls the maturation of the meristem (Mähönen *et al.,* 2014). This gradient is not a simple readout of the auxin gradient in the root tip, but rather a result of protein stability, cell-to-cell movement and mitotic segregation originating in the stem cell niche (Mähönen *et*

al., 2014), meaning that the gradient is established by the protein levels decreasing with each cell division. The loss-of-function mutants exhibit aberrant root phenotypes and arrested growth (Aida *et al.,* 2004; Galinha *et al.,* 2007; Kareem *et al.,* 2015) accompanied by a loss of stem cell maintenance, as demonstrated by the abnormal expression pattern of various stem cell markers, including *SCARECROW* and *SHORTROOT* (Aida *et al.,* 2004). Auxin signalling is impaired in *plethora* mutant root apical meristems (Aida *et al.,* 2004). It has been suggested that the meristem defects in *plt* mutants result not only from improper stem cell maintenance but also from poor establishment of meristem boundaries (Galinha *et al.,* 2007). In keeping with this, the PLT protein gradient has been proposed to define zonation boundaries by orchestrating different outputs in different regions of the root (Mähönen *et al.,* 2014). PLT overexpression leads to an enlarged root meristem, underlining the importance of a reduction in protein levels for meristem maturation (Galinha *et al.,* 2007). The enlarged meristem size in these overexpression lines was not due to an increase in PIN levels, but rather due to increased mitotic activity. The *PLETHORAs* also regulate shoot phyllotaxis by affecting the transcription rates of the auxin transport machinery, the PINs (Prasad *et al.,* 2011).

TIME FOR COFFEE (TIC) was suggested to tap into the PLT-auxin loop and affect meristem size by regulating auxin accumulation (Hong *et al.,* 2014). *TIC* is expressed in the RAM and mature parts of the root in *Arabidopsis* seedlings. Loss-of-function *tic-2* mutant plants have weaker auxin maxima, reduced PIN and PLT expression, reduced cell division rates and smaller meristems than wild type plants. Similarly, *ROOT MERISTEM GROWTH FACTOR 1* (*RGF1*) was shown to be critical for the post-translational regulation of PLT patterning in primary roots (Matsuzaki *et al.,* 2010). Triple loss-of-function *rgf1 rgf2 rgf3* mutants have reduced PLT levels and smaller meristem sizes, but PLT expression is rescued and the mutant phenotype complemented by exogenous application of RGF1. The post-transcriptional regulation is thought to occur via protein stabilization via RGF1 signalling (Matsuzaki *et al.,* 2010).

In case of an injury to the RAM, QC cells can divide and replenish the columella and vascular initial cells, a process that is tightly controlled by *RETINOBLASOMA-RELATED (RBR)* and *SCARECROW (SCR)* (Cruz-Ramirez *et al.,* 2013). *RBR* regulates differentiation (Perilli *et al.,* 2013), and under normal growth conditions, it suppresses asymmetric cell division in the QC, maintaining its quiescence. However, stressful conditions such as changes in the cell cycle or genotoxic DNA damage (which can lead to cell death) lead to the suppression of *RBR*, allowing QC cell division to compensate for the damaged cells. Misregulation of the *SCR-RBR* interaction leads to asymmetric cell division of the QC (Cruz-Ramirez *et al.,* 2013). In the root transition zone, *RBR* acts with a cytokinin response regulator, *ARR12*, to activate the auxin signalling required for cell differentiation (and elongation) (Perilli *et al.,* 2013).

VND7 and its homolog, *VND6*, are NAC transcription factors and master regulators of the later stages of xylem differentiation (Kubo *et al.,* 2005; Yamaguchi *et al.,* 2010a and 2011). *VND7* promotes protoxylem cell fate, whereas *VND6* is required for metaxylem fate. Both genes are involved in promoting the formation of secondary cell wall structures and priming the xylem cells for autolytic cell death (Ohashi-Ito *et al.,* 2013). While *AHP6* is regarded as a primary

protoxylem identity gene due to its early and consistent expression, *VND7* expression in the protoxylem position occurs only later, around the transition zone.

VND-INTERACTING2 (VNI2) is a transcriptional repressor that is capable of suppressing the expression of several VND targets, and VNI2 was shown to interact with VND7 (Yamaguchi *et al.,* 2010b). *VNI2* is expressed earlier than *VND7*in both the xylem and the phloem of the proximal meristem. Loss-of-function *vni2* mutants do not exhibit dramatic protoxylem phenotypes, despite upregulation of several of its target genes involved with vessel formation. However, when *VNI2* was expressed under the *VND7* promoter, xylem development was distorted, leading to the loss of vessel elements. The expression pattern of *VNI2* and its repressive function suggest that its role is to prevent premature xylem differentiation (Yamaguchi *et al.,* 2010b). The role of *VNI2* in phloem development remains unclear, yet its capacity to suppress several NAC TFs opens up a possibility that it could also be interacting with and suppressing *ALTERED PHLOEM DEVELOPMENT (APL)* in the proximal meristem.

APL is a TF required for phloem development (Bonke *et al.,* 2003). Its ectopic expression leads to the loss of xylem, whereas loss-of-function *apl* mutants frequently have protoxylem-like cells in the phloem position. Like *VND7, APL* is also expressed outside of the proximal meristem, and therefore probably affectsg cell-type differentiation rather than stem cell identity formation. APL promotes the expression of two NAC-type transcription factors: *NAC045 (NAC45)* and *NAC086 (NAC86)* (Miyashima Furuta *et al.,* 2014). Double *nac45 nac86* mutants showed a phenotype similar to *apl* mutants, with delayed growth, seedling lethality and the loss of sieve elements. Ectopic expression of *NAC45* caused companion cells to form sieve-element-like structures in differentiated cell, culminating in cytosolic degradation and enucleation. The downstream targets and effectors of *NAC45* and *NAC86*, termed *NAC45/86-DEPENDENT EXONUCLEASE-DOMAIN PROTEIN* (*NEN*), are required for completion of the enucleation process. These results show that autolytic cell death in the sieve element position, which is required for the formation of conductive phloem, is regulated by downstream targets of *APL,* the *NAC45/NAC86* and *NEN*-proteins. Early phloem identity genes that precede *APL* expression have also been identified in our laboratory. These genes appear to regulate the early cell divisions of the phloem initials, sieve elements and surrounding procambial cells (Miyashima and Sevilem, unpublished).

AT-HOOK MOTIF NUCLEAR LOCALIZED PROTEIN 3 (AHL3) and *AHL4* are transcription factors, expressed in the procambium (Zhou *et al.,* 2013). Loss-of-function *ahl4* single mutants and *ahl3 ahl4* double mutants show ectopic proto- and metaxylem formation in the procambial domain. AHL3 and AHL4 interact *in vivo* and form a hetero-protein complex. The cell-to-cell movement of these proteins from the procambium to the xylem axis is critical for their function, as tagged protein fusions too large to move were unable to complement the mutant phenotypes. *AHL3* and *AHL4* are required to maintain tissue boundaries between the vascular cells in the *Arabidopsi*s RAM, and accordingly, *ahl*-mutants show misspecification of cell fates in the proximal meristem.

As these few examples show, cell cycle regulation, patterning and tissue boundary maintenance are critical for proximal meristem development. The hormonal signalling networks, TFs and other cell-to-cell signalling mechanisms regulating root apical meristem growth and patterning are complex. Several review articles have addressed these interactions, including excellent recent examples by Jung and McCouch 2013, Kondo *et al.,* 2014 and Drisch and Stahl, 2015.

1.9. Temporal pattern maintenance

There are several requirements for a functional root meristem. First, the root needs to establish (more or less) all the cell identities through an initial patterning process. However, these radial patterning mechanisms, described in the previous pages, are not the whole story, as the primary cell identity patterns set in the proximal meristem initial cells have to be maintained and re-established as the meristem matures. Thus, temporal regulation of meristem maturation is important for root development. This requires tightly-controlled cascades of activation and repression of various cell identity genes and their downstream targets. This brings us to the second requirement: the growth dynamics of the meristem should be in balance, meaning that rates cell division and differentiation/death must be synchronized. This is where reactive oxygen species (ROS) signalling and programmed cell death (PCD) come into the picture as the final mechanisms determing meristem size.

Few papers have reported the effects of delayed differentiation and cell death on meristem dynamics (Bollhöner *et al.*, 2013). Interestingly, none of the genes studied were so-called early identity genes, but rather later developmental genes, such as *METACASPASE 9 (AtMC9)*. If such late identity genes are not properly regulated, this can prolong or advance the differentiation process of a certain cell type. In the case of conductive vessel elements or phloem cells, the last phase is controlled cell death. In the RAM, cell death occurs first in the phloem sieve elements; protoxylem lignification and autolysis happen later. The TFs orchestrating these events appear to be direct downstream targets of the primary patterning mechanisms. Genetic cascades from initial cell identity establishment to controlled autolysis have been identified in both phloem and xylem tissues (Kubo *et al.,* 2005; Bollhöner *et al.,* 2013; Miyashima Furuta *et al.,* 2014). In the case of xylem patterning and differentiation, auxin promotes the expression of all known key players, *ATHB8, AHP6, VND6* and *VND7*. Interestingly, in both the xylem and the phloem, genes regulating the first stages of death belong to the NAC family of transcription factors and are expressed at the transition zone and shootwards (Ohashi-Ito *et al.,* 2013, Miyashima Furuta *et al.,* 2014). Perhaps the changing homeostasis of auxin and cytokinin signalling in maturing meristems promotes the expression of elements usually linked to stress signalling to create controlled “exit strategies” for cells through PCD.

1.9.1. Auxin, cytokinin and ROS regulate meristem maturation

The balance between auxin and cytokinin signalling is critical to maintain the size of the RAM. The expression of gibberellin, cytokinin and auxin signalling components (such as *RGA*, *ARR1* and *SHY2*) regulates the transition from the meristematic stage to elongation and differentiation (Dello Ioio *et al.,* 2007; Moubayidin *et al.,* 2010 and 2013). Auxin promotes cell divisions and inhibits cell elongation in the proximal meristem. In turn, cytokinin is the dominant player in

the transition zone, where it promotes cell elongation and differentiation (Blilou *et al.,* 2005; Dello Ioio *et al.,* 2007; Overvoorde *et al.,* 2010; Perrot-Rechenmann, 2010). *AUXIN UP-REGULATED F-BOX PROTEIN1* (*AUF1*) might be required for fine tuning this process (Zheng *et al.,* 2015). Auxin upregulates *AUF1* expression in the cortical and epidermal cells in the transition zone and shootwards. This promotes *PIN* expression and auxin transport, enhancing RAM maintenance by delaying elongation and differentiation. The loss-of-function *auf1-2* mutant has defective auxin transport and reduced expression of the *PIN*s (apart from *PIN7*) and is hypersensitive to NPA. The *auf1-2* mutant also shows increased cytokinin responses and exhibits reduced primary root elongation upon exogenous cytokinin application, indicating that the it is hypersensitive to cytokinins. Zheng *et al.* (2015) proposed that $SCF^{AUF1/2}$ participates in the crosstalk between cytokinin signalling and auxin transport by targeting a positive cytokinin regulator (such as *ARR1*) for ubiquitylation, thus repressing cytokinin activity in the transition zone. The accumulation of this regulator in *auf1-2* is suggested to lead to enhanced cytokinin responses, upregulation of *SHY2*, a reduction in auxin transport and an increase in meristem size.

Even though several downstream targets of auxin and cytokinin that regulate transition zone position and meristem size have been characterized, the current model of RAM maturation is still far from finished. Data suggest that auxin and cytokinin signalling components can interact with factors that are generally regarded as stress signalling factors, such as nitric oxygen species (NOS) and ROS (Lee *et al.,* 2012). While ROS and NOS signalling molecules are known to trigger the production of stress-related plant hormones (JA, SA, ethylene, ABA), new roles for them are becoming apparent. ROS have been shown to affect auxin biosynthesis, transport, metabolism and signal transduction (reviewed and illustrated by Tognetti *et al.,* 2012), whereas NOS can affect nutrient uptake and translocation and cytokinin biosynthesis (Liu *et al.,* 2012), as well as regulating the activity of cytokinins in cell proliferation and meristem maintenance (Shen *et al.,* 2013).

ROS-induced signalling has been shown to control primary root development by regulating the switch from proliferation to differentiation in the root meristem (Dunand *et al., 2007,* Tsukagoshi *et al.,* 2010; Roberts, 2012). The *UPBEAT1 (UBP1)* transcription factor negatively regulates root meristem size via modulation of peroxidase activity and ROS homeostasis in the meristem. *upb1* mutants with reduced hydrogen peroxide (H_2O_2) increased superoxide ($O_2^{\bullet -}$) levels have larger meristems and show an increased root growth rate compared with wild type roots. In contrast, *UBP1* overexpressing plants have smaller meristems and reduced root elongation rates. Interestingly, while *UBP1* appears to only be expressed in the outer cell layers of the meristematic zone, it can affect the growth of the entire meristem. Several other ROS-hypersensitive mutants have smaller meristems, as do plants suffering from stressful conditions, such as genotoxic double strand DNA breaks (DSB). DSB induce cell death in the proximal meristem (Ciccia and Elledge, 2010) and lead to H_2O_2 accumulation in the elongation and differentiation zones via *FLAVIN-DEPENDENT MONOOXYGENASE 1* (*FMO1*) activity, mediated by the *ATM-SOG1* pathway (Chen and Umeda, 2015). This leads to a reduction in meristem size similar to that observed in *UBP1* overexpressing plants. Whether the accumulation of H_2O_2 and *UBP1* expression are somehow related to cytokinin-promoted chromatin

endoduplication, cell elongation, and the initiation of cell differentiation remains to be clarified (Takahashi & Umeda, 2014, Chen & Umeda, 2015) .

In 2006, Passardi *et al.* presented two cell-wall-associated peroxidases that affect root elongation, *ATPRX33* and *ATPRX34*. Loss-of-function *atprx33* and *atprx34* mutant roots are slightly shorter than wild type, indicating that these peroxidases may be required for proper cell elongation by loosening the cell wall. In addition to regulating cell wall dynamics, peroxidases can also affect auxin signalling status via oxidation of IAA (Savitsky *et al.,* 1999). While auxin represses cell elongation and differentiation, peroxidases can counteract this by suppressing auxin signalling, thus affecting meristem size. Whether the decreased auxin levels at the transition zone are the result of a combination of enzymatic inactivation and decreased auxin transport remains to be seen. Auxin can counteract H_2O_2-dependent cell death, implying that auxin also has a role in stress tolerance (Kerchev *et al.,* 2015). Interestingly, the promoter regions *of ATPRX33* and *ATPRX34* contain binding motifs for auxin, ABA, SA and gibberellin, suggesting that these peroxidases may be regulated by an array of development- and stress-related hormones (Passardi *et al.,* 2006). The suggested link between active hormone levels and the regulation of cell-wall-modifying enzymes controlling meristem size is intellectually appealing and is supported by the notion that various hormonal interactions can occur via enzymatic inactivation regulated by ROS.

It has been suggested that the HD-ZIP III genes can modulate the levels of superoxide and hydrogen peroxide to determine the differentiation status of root meristem cells during primary development (Roberts, 2012). This regulation might function through the transcriptional repression of a number of NADPH oxidase and peroxidase encoding genes. The link between the HD-ZIP III genes and ROS has also been verified in leaf assays, where the redox-sensitive *REVOLUTA* was shown to promote age-induced senescence via the *WRKY53* transcription factor (Xie *et al.,* 2014). *WRKY53* is upregulated by H_2O_2, and regulates pathogen resistance and senescence (Miao *et al.,* 2004; Hu *et al.,* 2012). Since senescence can be regarded as programmed cell death (but in a larger context, as was discussed by van Doorn and Woltering, 2004), the emerging link between early patterning genes and programmed cell death is exciting. If the HD-ZIP III genes can promote ROS production to regulate RAM maturation, it would show that genes which are required for xylem identity during initial meristem patterning can also affect the fate of these cells much later by promoting their differentiation and death.

1.9.2. ROS in xylem formation

While ROS regulate meristem growth dynamics (namely, elongation and differentiation rates), there is also evidence for the involvement of ROS signalling in the expression of specific genes that regulate xylem identity and differentiation. ROS (e.g., H_2O_2) are used by peroxidases for substrate oxidation and lignification (reviewed and illustrated by Marjamaa *et al.,* 2009). H_2O_2 was shown to be produced in bundle sheath cells around the vasculature of *Arabidopsis* leaves (Fryer *et al.,* 2003), possibly fuelling peroxidase-regulated xylem development. This finding is supported by a study in *Zinnia elegans,* where H_2O_2 localization in the vascular bundles was shown to correlate with peroxidase activity and lignin production in xylem vessel elements (Ros-Barceló *et al.,* 2002).

The auxin upregulated TFs *VND6* and *VND7* control cell death in xylem by activating secondary cell wall biosynthesis genes, including metacaspases (*AtMC9,* Tsiatsiani *et al.,* 2011; Bollhöner *et al.,* 2013), cellulose synthases (*IRX* genes, Turner and Somerville, 1997), laccases (*LAC4, LAC11* and *LAC17*, Zhao *et al.,* 2013 and Schuetz *et al.,* 2014) and peroxidases. This makes sense, as the secondary cell walls of xylem cells must be fortified prior to death, since the thickening is critical for the support of the conductive vessels. Protoxylem cells are especially vulnerable to collapse because their lignified spiral structure is not sufficient to resist negative water pressure (Turner and Somerville, 1997). In *irx* mutants, the failure to establish normal cellulose depositions in the secondary cell walls of xylem leads to collapse of TEs (Turner and Somerville, 1997). Similar data were obtained from *VND7*-promoted laccase mutants, such as the *lac4 lac17* double mutants, which are deficient in laccase-dependent polymerization in the protoxylem cells, which therefore cannot synthetize proper secondary cell walls (Schuetz *et al.,* 2014). Triple *lac4 la11 lac17* mutants show even more pleiotropic phenotypes; their rosette size, inflorescence stem length and thickness, and primary root diameter are dramatically reduced and the primary root growth pattern is altered. The *lac4 la11 lac17* mutant also has clear reproductive organ abnormalities, as the vascular tissues in the replum fail to lignify properly and pollen release is inhibited (Zhao *et al.,* 2013).

To summarize, ROS interact with the "classical" plant hormones and can affect their metabolism. ROS interact with primary and secondary cell wall biosynthesis enzymes and affect cell elongation and differentiation rates. ROS are crucial for xylem lignification and autolysis and critical for PCD upon stress (which is essential for pathogen defence), underlining a shared regulatory pathway between defence and developmental processes.

2. Aims of the study

My primary interest has always been to understand the hormonal interactions controlling vascular patterning, with an emphasis on the role of auxin and cytokinin in protoxylem specification and differentiation. Positioned at the centre of the root, protoxylem cells are closely connected with other vascular cells. Even though my initial focus was to understand the hormonal regulation of protoxylem cell fate, during my studies it became apparent that the dynamics of the entire meristem had to be studied in order to fully understand xylem formation. Thus, the focus of my work shifted to understanding the hormonal interactions required to establish and maintain the pattern of the root vasculature through meristem growth and maturation. This thesis is divided to three parts which are linked together experimentally and conceptually. In the first part, I summarize the data we published in our original publications (OP) I, II and III. I focus on describing how auxin promotes protoxylem fate in the proximal meristem and how it interacts with cytokinin, a known repressor of protoxylem formation. *My main research questions are*: 1) When is the auxin-cytokinin signalling interaction critical for procambial patterning and protoxylem identity established? 2) How do these two hormonal signalling domains interact on a cellular level? 3) How are the auxin and cytokinin signalling domains maintained during meristem growth?

All vascular cells derive from the undifferentiated procambial cells in the proximal meristem. Therefore, understanding the role of signalling interactions in procambial cell maintenance is the key to understanding patterning. During my years in the lab, I have contributed to two independent modelling projects focused on the hormonal interactions in the procambial meristem; however, I have not included these articles in this thesis. Rather, inspired by the original research done with hormone treatments and hormone signalling manipulations, I have focused on showing how, given the right hormonal cue, vascular cell identities at the proximal meristem can be manipulated to change into a different cell type, a process we termed “procambial re-patterning”. I aimed to clarify whether the homeostasis of auxin and cytokinin – not their absolute levels – is the determinant factor for xylem fate. Thus, part two of the thesis is focused on answering one main question: Can stele patterning be affected by modifying procambial cell signalling status? The data presented in the second part of this thesis is unpublished and supports the published work of OP II and OP III.

While the first two parts of this thesis are focused on “early” patterning mechanisms that establish different cell identities, the final part of this thesis is devoted to the final stages of development: cell differentiation and death. ROS regulate secondary cell wall biosynthesis, cell elongation, differentiation and programmed cell death, which are crucial for root meristem maturation and are key steps in both xylem and phloem differentiation. In the last part of this thesis, I present a novel peptide-ligand-receptor signalling pathway involved in cell death regulation upon oxidative stress (OP IV). My focus was to understand whether GRIM REAPER has a role in root meristem development and tissue patterning. Unpublished data from the individual components of this module are presented, and the putative role of this signalling pathway in context of the meristem growth is discussed.

3. Materials and methods

The materials and methods are described in detail in publications I, II, III and IV. The publications in which they appear are indicated in Table1.

Table: Methods used in this study. Those in parentheses were performed by co-authors in the respective publications.

Method	**Publication**
Agrobacterium mediated transformation of *Arabidopsis*	II, III, (IV)
Anatomical analyses	I, II, III, IV*
Aniline blue staining	(III)
Confocal light microscopy	II, III, IV*
Fuchsin staining	II, III, IV*
Genetic crossing of *Arabidopsis*	II, III, (IV)
Histological sectioning of plastic embedded samples	I, II, III, IV*
Histological staining for GUS activity	II, III, IV*
Hormone / induction/ peptide assays	II, III, IV
Immunolocalization	(II)
In vitro interaction analysis and protein kinase assays	(IV)
In vitro peptide cleavage and separation with RP-HPLC	(IV)
In-situ RNA hybridization and histological cross sections	II, III
Light microscopy	I, II, III, IV
Mass spectrometric analysis	(III), (IV)
Peptide radioiodine-labelling	(IV)
Plasmid construction	(II), (III), (IV)
Polymerase chain reaction (PCR) analysis	(I) II, III, (IV)
Promoter deletion assays	(II)
Protein structure predictions	(IV)
Quantitative real-time PCR analysis	II, III, (IV)
Radiolabelled hormone transport assays	(III)
Radiolabelled peptide assays	(IV)
Radio-ligand binding assays	(IV)
RNA extraction	II, III, (IV)
Scintillation assays	(III)
Sequence analysis	II, (III), (IV)
Statistical analysis	II, (III), (IV)*
Trypan blue staining	(IV)
Western blot analysis	(IV)

*data shown in thesis results section

Materials and Methods for the unpublished data presented in thesis Introduction in Parts II and III:

Part II: Genetic crosses, confocal microscopy analyses, anatomical and histological analysis of roots were all done by HHRR, as described in Original Publications I, II and III.

Part III: Anatomical and histological assays and PRK5 internalization in protoplast confocal imaging were all done by HHRR, as described in OPI, II, III and IV, respectively.

4. Results and discussion

4.1. Part I: Auxin – cytokinin interaction regulates vascular patterning

4.1.1. Cotyledons position the xylem poles in the embryonic root

To address the question of when the hormonal interaction critical for procambial patterning and protoxylem identity is established, we examined auxin response markers in young embryos and compared the cotyledon numbers and xylem poles of various mutants. We found that the bilateral symmetry of the embryonic vasculature is established an oriented by auxin maxima in the cotyledons and initiated by the downwards progression of *AHP6* expression (OP II).

During embryonic development, auxin maxima are seen at the tips of the forming cotyledon primordia (OPII, Fig4C and SFig4). As an auxin inducible gene (OPII), *AHP6* is expressed at the auxin maxima in the globular embryo. *IAA2,* an auxin response regulator and another primary auxin response gene, has a slightly different expression pattern. It is expressed in a radially symmetric pattern in the root pre-vascular cells and hypophysis at the globular and early heart stages. As the embryo matures and enters the late heart stage, *AHP6* expression increases and expands towards the embryonic root. This can be seen as a read-out of canalization, during which auxin flow and transport enhance vascular identity formation. As *AHP6* expression reaches downwards from the cotyledons, this, in turn, promotes a local increase in auxin response, and the expression pattern of *IAA2* changes from radial to bilateral symmetry, leading to xylem axis establishment. In short, the cotyledons, with high auxin signalling domains, function as a positional cue for xylem formation in the embryonic root tissue below.

In wild type plants, the bilaterally symmetric embryonic roots have two protoxylem poles, matching the number of cotyledons. Hypocotyls are established during embryogenesis; following germination, sections of hypocotyls can provide a glimpse of the early patterning events and defects in some mutants. In *Arabidopsis* mutants with an altered number of cotyledons (*pin1,* quadruple *HD-ZIPIII athb8, corona, phabulosa, phavoluta* or the dominant *phb-d7)* or altered cotyledon anatomy (*cuc2 cuc3*), the number and/or arrangement of the xylem poles in the hypocotyl changes accordingly, and the symmetry of primary root can differ from the bilateral symmetry of wild type plants. In many cases, plants with additional cotyledons (i.e., three or four) showed tri- or tetra-arch symmetry in the hypocotyl, whereas plants with fused cotyledons often failed to form a bilateral pattern (*pin1*, data now shown). Notably, the number of protoxylem poles in sectioned mutant hypocotyls was frequently lower than the actual number of cotyledons, while none of the analysed mutants had more xylem poles than the cotyledons. These observations indicate that the vascular strands can fuse below cotyledons, and by doing so, regain normal bilateral root symmetry.

It seems that auxin signalling is critical for initializing developmental patterns and cytokinin signalling is needed for regulating the patterns. While the positional auxin cue from the cotyledons appears to be normal in the most severe cytokinin signalling mutants (*wol, cre1 ahk2 ahk3* triple mutants*, arr1 arr10 arr12* triple

mutants), symmetry breakage does not occur and the vascular cells all differentiate into xylem. In turn, plants with very limited or no auxin signalling (such as the dominant-negative *axr3-1* mutant) have the opposite phenotype, with protoxylem failing to differentiate properly in the primary root. Thus, altering auxin-cytokinin signalling disturbs the hormonal interaction required for patterning the embryonic root/proximal meristem. In the most severe cases, mutants which are unable to generate a normal, bilaterally symmetric vascular pattern during embryogenesis cannot correct the pattern in the primary root post-embryonically, which can lead to an arrest in growth and the death of the primary root meristem (e.g., *wol*). However, if the mutations that affect auxin or cytokinin homeostasis during embryonic patterning are not too severe, the plants can re-establish the wild type pattern post-embryonically. We believe this mechanism is self-organizing and mainly dependent on the auxin – cytokinin interaction.

Indeed, even though the initial vascular patterning occurs during embryogenesis, the pattern must be maintained. As seeds germinate and the primary root spurts into growth, the initials in the proximal meristem begin to proliferate extensively, producing new vascular cells shootward. At each division, the identity of each initial cell lineage must be passed on to the newly formed daughter cells. Gaining or maintaining an identity can be viewed as a combination of protein imprints (which the daughter cells might inherit from their mother cells) and the accumulation of external stimuli, leading to the activation of cell-type-specific signalling cascades and transcriptional changes. As a result, cells gain their identity, mature and differentiate along their developmental program.

Auxin and cytokinin and their interaction with other hormonal pathways regulate various events in meristematic cells, depending on the signalling status and activity of various response regulators and their targets genes in a given cell. Our work focused on understanding where these two hormonal signalling domains are located, how they are maintained and interact on a cellular level, and how changes in the hormonal signalling domains affect vascular patterning. The results are shown in detail in the original publications, OPII and OPIII. The key findings and their effect on protoxylem differentiation are presented in the following chapters.

4.1.2. Auxin & cytokinin signalling domains are mutually exclusive

We showed that the auxin and cytokinin signalling domains are positioned in a complementary manner in the proximal meristem, as illustrated in Figure 3. In line with previous reports, the strongest cytokinin signalling was seen in the procambial cells flanking the xylem axis. The cytokinin domain is a consensus of several markers: *TCS::GFP* version I, *ARR5* in-situ, *ARR5::GUS* (OPII, Sfig 1A, B, C), *PIN7::PIN7-GFP* (OPII SFig 3 L and M) and *TCS::GFP* version II (in Appendix A-Fig.1), which all showed the same localization, with minor variations. The auxin signalling maximum, which was visualized with *DR5::GUS* (not shown), *DR5::GFP*, *IAA2::GUS* and *IAA2::GFP* (OP II Sfig1 panels D1, D2, E), spans the xylem axis in the proximal meristem, exactly matching the expression pattern of a known xylem identity gene, *ATHB8* (OP II Fig2I) 20µm position above the QC. It is noteworthy that just a few cells higher, the auxin maximum consistently shifts towards the protoxylem positions, disappearing from the metaxylem position at the centre of the xylem axis (Figure 3, OP II Sfig 1 D and E). Higher up the IAA2 expression shifts into metaxylem position (data not shown).

We believe these positional shifts plays a role in development; first auxin signalling maximum promotes protoxylem differentiation, and as the cell files are established, auxin maximum is repositioned to promote metaxylem differentiation.

It is somewhat puzzling that the *IAA2::GUS* and *ARR5::GUS* markers overlap at the meristem initials (Fig. 3), whereas the *TCS::GFP*, *DR5::GFP* and *IAA2::GFP* expression patterns are more confined and do not overlap (Appendix, AFig.1). The slightly differing patterns should be interpreted and compared keeping in mind that the *IAA2::GUS* and *ARR5::GUS* markers illustrate the patterns of primary response regulators, whereas *DR5::GFP* and *TCS::GFP* are synthetic reporter lines and may not necessarily depict the actual hormone signalling domains, but rather artificial promoter activity. However, what is clear and repeatable in all imaged GUS and GFP markers is that auxin and cytokinin signalling are restricted to two complementary domains in the proximal meristem initials.

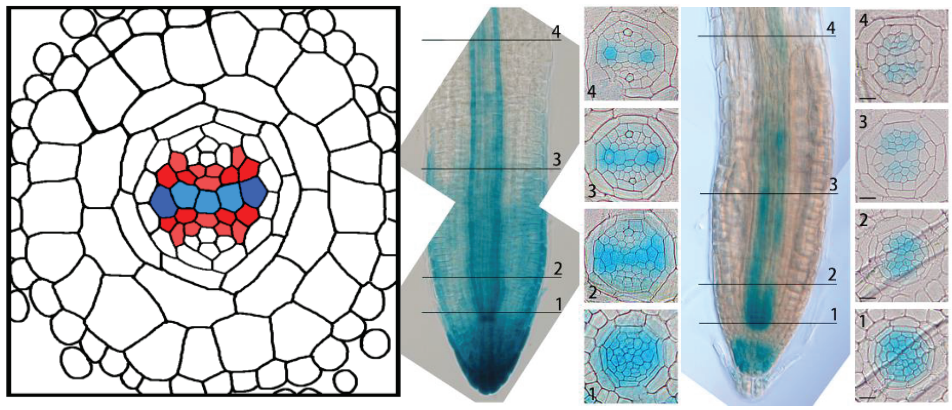

Figure 3: Auxin and cytokinin signalling in the proximal meristem. A graphical illustration of the auxin (blue) and cytokinin (red) signalling domains in the *Arabidopsis* proximal meristem stele (left), drawn according to the observed sections showing the *IAA2::GUS* (middle) and *ARR5::GUS* expression (right) domains in 5 d old primary root tips. The cross sections were made just above the QC and at heights illustrated by black horizontal lines in the longitudinal sections.

4.1.3. A high auxin signalling domain positions protoxylem and promotes differentiation

The expression of *DR5::GFP* (OP II Fig1E) and *IAA2::GUS* (Fig. 4) in the xylem axis overlaps with *AHP6::GUS* (Fig. 4C, OP II Fig. 2A) and *VND7::GUS* (Fig. 4D) expression at the protoxylem position. The proper expression of all of these genes promotes normal differentiation of protoxylem (Fig. 4E, OP II Fig. 2F). As Mähönen et al showed in 2006, cytokinin treatment represses *AHP6* expression and protoxylem formation, as we confirmed in our analysis (Fig 4J; OP II Sfig. 2F). Our data show that cytokinin treatments cause *ARR5* expression to expand to include the protoxylem position (Fig. 4F, OP II Fig. 1I). BA treatments and endogenously induced CKI expression both promote *PIN7* expression at the protoxylem position (OP II Sfig. 3B and Fig. 3P), showing that *PIN7* is induced by cytokinin. When *PIN7* expression is induced throughout the stele at abnormally

high levels under the 17-beta estradiol inducible *CRE1-XVE* promoter, mimicking cytokinin treatments, the auxin maximum can be pushed away from protoxylem position (OP II Fig. 3S), showing that *PIN7* acts as a key player in close proximity to the protoxylem.

It is interesting that the auxin maximum at the metaxylem persists despite cytokinin treatment or stele-specific induction of *CKI* or *PIN7* expression (OP II Fig. 1J, Fig. 3S). Thus, protoxylem cell fate is especially vulnerable to cytokinin-regulated changes in auxin signalling levels, which result in abnormal differentiation, while the auxin maximum at the metaxylem position is resistant to such perturbations; as a result, cytokinin treatments do not affect metaxylem differentiation. Data from cytokinin-hypersensitive *ahp6* mutants support this notion, as *ahp6* mutants frequently lack the auxin signalling maxima at the protoxylem, but never at the metaxylem position, and do not have any obvious metaxylem defects. Our data also show that while auxin promotes *AHP6* expression, treating roots with auxin is not sufficient to express *AHP6* outside its domain (not shown). However, treating roots with NPA, an auxin export inhibitor, leads to the loss of the auxin signalling maxima, and consequently *AHP6* expression, at the protoxylem position, resulting in the loss of protoxylem. Following NPA treatment, expression of both *ARR5* and *ARR15* expands to the protoxylem position in wild type plants (Fig. 4N and M, respectively), indicating that increased CK signalling at that position and causing the loss of protoxylem. The loss of protoxylem was NPA dose-dependent. The protoxylem phenotype of roots treated with 5µM NPA resembled that of treatment with 100nM BA (Fig. 4J). A less severe treatment with 2µM NPA (not shown) caused a protoxylem phenotype similar to *ahp6-1* mutants (Fig. 4O), which often show only a partial loss of protoxylem. The loss of *AHP6* expression and protoxylem could be counteracted to a certain degree by simultaneous NAA treatment or by local induction of auxin biosynthesis under the *AHP6* promoter (OP II Sfig. 2D)

We therefore concluded that both BA and NPA cause a loss of protoxylem. Upon further inspection, we discovered, unsurprisingly, that both treatments alter *IAA2* and *AHP6* expression, mostly affecting the protoxylem position, where the signal is frequently lost. The cytokinin hypersensitive *ahp6* mutant shows a loss of *IAA2* expression at the protoxylem position (Fig. 4L), and is phenotypically similar to NPA treated roots (Fig. 4J and O). We analysed mutants defective in protoxylem formation (i.e., *ahp6*, *pin* mutants) and observed that marker genes for auxin (Fig. 4L) and cytokinin (Fig. 4K) are abnormally expressed in these backgrounds, verifying the link between low auxin and high cytokinin signalling in the protoxylem position and the loss of protoxylem. Further evidence for the auxin-cytokinin interaction came from mutants with low cytokinin signalling and ectopic protoxylem phenotypes, such as *cre1-12 ahk3-3* and *wol*, in which *ARR5* expression was undetectable in the stele (Fig. 4P, U), although expression remained in the columella cells (OPII Sfig. 1). In turn, the *IAA2* expression domain expanded laterally in these cytokinin signalling mutants (Fig. 4Q), and *AHP6::GFP* expression also expanded, consistent with previous reports from cytokinin signalling mutants (Mähönen *et al.,* 2006). As a result of these changes in hormonal signalling, these mutants have ectopic protoxylem strands (Fig. 4R, S and T). The auxin maximum of *wol* roots was only slightly affected by NPA treatments, with *IAA2::GUS* showing little or no change in the stele but frequently expanding to include the endodermis (Fig. 4V, W).

We showed that reduced cytokinin signalling leads to either a reduction or loss of PIN expression in the stele (OPII Fig. 3Q, N and O), but also that the subcellular localization of some PINs was altered in cytokinin signalling deficient mutants (OPII Fig. 3F). We concluded that this change in PIN localization leads to reduced auxin export towards the xylem axis, resulting in a wider auxin maximum (OPII Fig. 1H and K). Inspired by the reduced PIN7 domain seen in the cytokinin signalling mutants, we next investigated whether and how each of the PINs responds to CK treatments and if these patterns could be linked to the hormonal signalling domains and the auxin- and cytokinin-responsive cell type markers we identified.

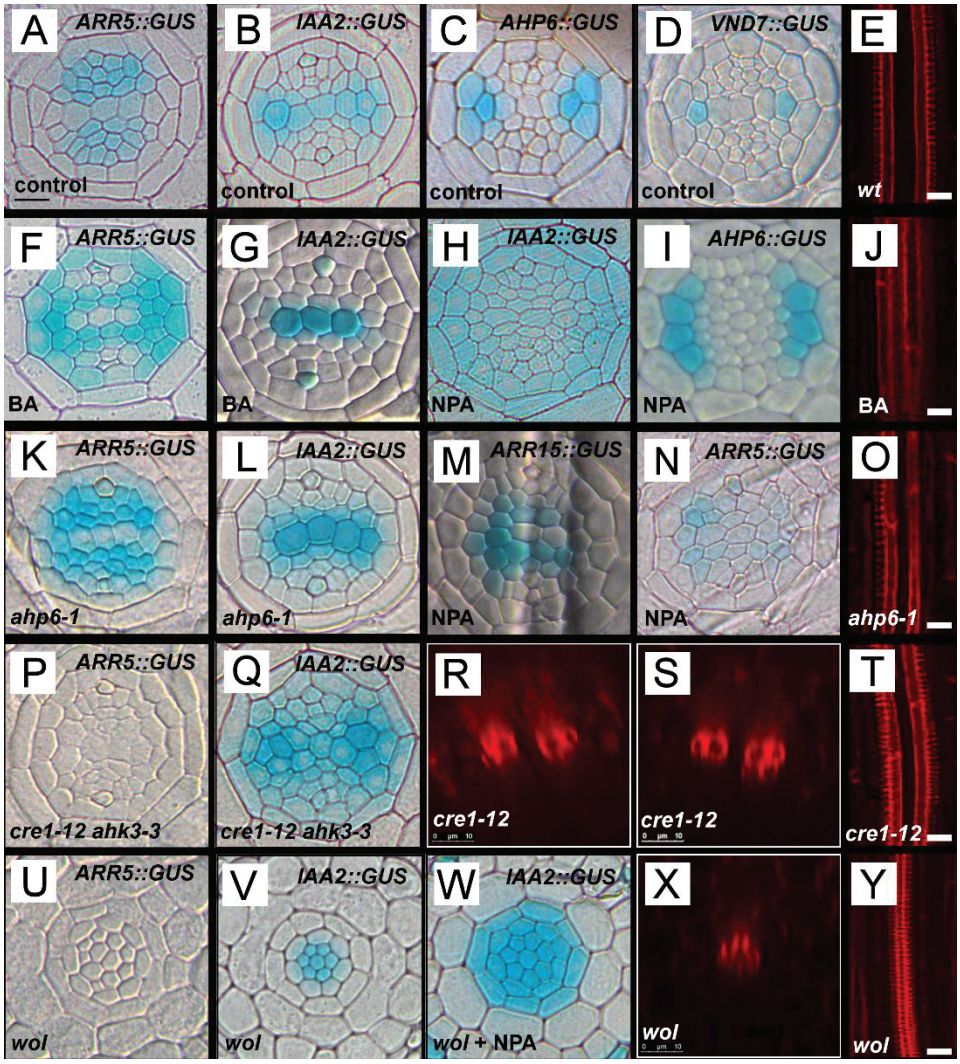

Figure 4: **Complementary signalling domains of cytokinin (illustrated by ARR5::GUS) and auxin (IAA2::GUS).** (A) ARR5::GUS in wild type (wt) Col-0 roots. (B) The auxin signalling maximum in the xylem axis. (C) *AHP6::GUS* expression (D) *VND7*::GUS expression. (E) A fuchsin-stained confocal image showing two protoxylem

strands separated by metaxylem and an undifferentiated cell. (F) *ARR5::GUS* and (G) *IAA2::GUS* expression in roots treated with 100nM BA. (H) *IAA2::GUS* and (I) *AHP6::GFP* expression in roots treated with 5μM NPA. (J) Fuchsin-staining showing a lack of protoxylem following treatment with 100nM BA. (K) *ARR5::GUS* and (L) *IAA2::GUS* expression in the *ahp6-1 mutant*. (M) *IAA2::GUS* and (N) *AHP6::GUS* expression following 5μM NPA treatment. (O) Fuchsin-staining showing only one protoxylem strand in an *ahp6-1* mutant. (P) *ARR5::GUS* and (Q) *IAA2::GUS* patterns in the cytokinin signalling deficient *cre1-12 ahk3-3* mutant. (R, S, T) Fuchsin-stained (R, S) cross- and (T) longitudinal-sections showing variation in extra protoxylem in *cre1-12*, which range from normal protoxylem to (R) 2 strands of protoxylem at each pole or (S, T) two strands at one pole and one at the other. (U) *ARR5::GUS* and (V, W) *IAA2::GUS* expression in *wo*l (V) without and (W) with 5μM NPA treatment. (X, Y) Fuchsin-stained (X) cross- and (Y) longitudinal-sections of *wol* mutants, showing an all-protoxylem stele. Scale bars 10μm.

4.1.4. The PIN transporters are regulated by cytokinin

In order to understand which PINs are critical for the formation of the xylem axis auxin maximum and to clarify the role of cytokinin signalling in their regulation, we observed the expression of the PIN1, 2, 3, 4 and 7 protein-GFP fusions. All of the proteins were detected in the primary root proximal meristem (OP II Sfig. 3A) and showed a tissue-specific pattern. We also examined the subcellular localization of PIN1 and PIN7. We found that PIN1 is purely basally localized in xylem cells, where cytokinin signalling is low (OP II Fig. 3D). In the pericycle cells adjacent to the protoxylem and in the intervening procambial cells, PIN1 is also localized to lateral cell walls (OP II Fig. 3D and E), presumably enhancing auxin transport towards the centre of the stele. *PIN7* is not transcribed in the xylem axis (OP II Sfig. 3D), nor was the protein localized in these cells, which have high auxin signalling (OP II Sfig. 3A). By contrast, PIN7 showed highest level of expression and localization to lateral cell walls in the procambial cells (OP II Fig. 3K), which have high cytokinin signalling.

We next tested the effect of cytokinin treatment on PIN expression and localization. We noticed that the expression domains of *PIN3*, *PIN4* and *PIN7* were most affected, changing from bilateral to radially symmetric patterns (OPII Sfig. 3A and B). The clearest changes were seen at the xylem axis. *PIN3* expression disappeared from the protoxylem-associated pericycle cells and appeared in the central metaxylem position, while expression of both *PIN4* and *PIN7* increased noticeably and spread to include the protoxylem position. These changes increase the auxin flux towards the metaxylem, leading to a loss of auxin signalling at the protoxylem position, as we verified by *IAA2::GUS* sections (OP II Fig. 1J). While cytokinin treatment promotes the expression of *PIN4* and *PIN7*, *wol* mutants, with severely reduced cytokinin signalling, do not show any *PIN4* (data now shown) or *PIN7* expression in the stele (OP II Fig. 3O), and have PIN1 basally localized in all the vascular cells (OP II Fig. 3F), following the uniform auxin maximum throughout the stele (Fig. 4V, OP II Fig. 1K). Thus, high auxin promotes basal PIN localization, and high cytokinin promotes the expression (in the case of PIN4 and PIN7) lateral localization of the stele specific PINs (Figure 5).

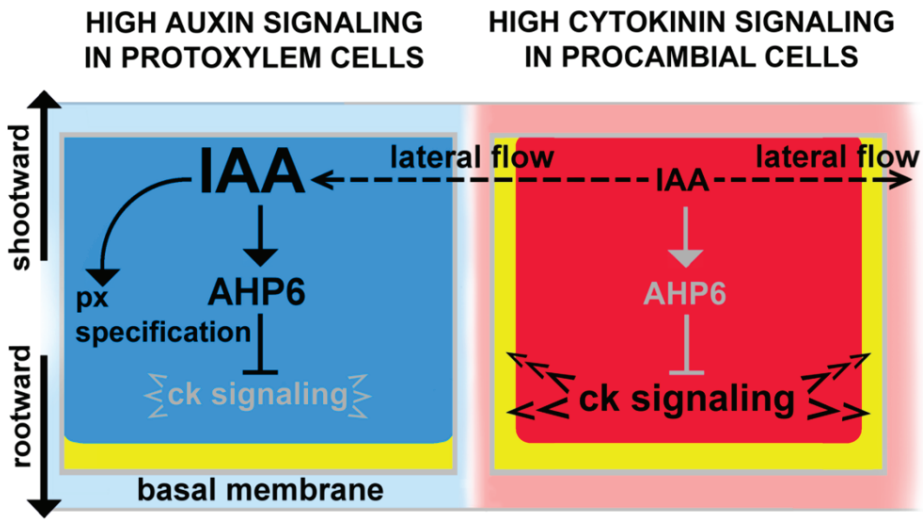

Figure 5: A simplified graphical model of the mutually exclusive interaction between auxin and cytokinin in xylem and procambium cells. Cytokinin signalling in the procambial cells (red) promotes PIN expression and localization (yellow line) to lateral cell walls. This aids auxin flow towards the xylem axis (blue) form a local auxin maximum. In the protoxylem cells, auxin promotes *AHP6* expression and inhibits cytokinin signalling. As a result, PIN localization is predominantly basal, aiding polar auxin transport towards the root tip.

Interestingly, the change we observed in *IAA2* and *DR5* expression overlaps precisely with the appearance of *PIN3* expression in the central xylem position, as can be seen in Figure 6 (and in OP II Sfig. 3C). PIN3 protein localization changes as the distance from the QC increases, appearing in the metaxylem position and expanding to all pericyclic cells. The other stele-specific PIN transporter, *PIN7*, is depicted in the same illustration (for confocal images, see OP II Sfig. 3A). PIN7 levels increase gradually in all procambial cells, but primarily at the positions next to protoxylem cells, closely resembling the reported cytokinin responsive *ARR5* and *TCS::GFP* expression patterns. When the expression patterns of these PINs are overlaid, they mirror the auxin signalling maximum (Figure 6) and overlap with the cytokinin signalling domain observed in confocal and histological marker analysis. Our data show that PIN3 and PIN7 are expressed in a bisymmetric pattern at the proximal meristem; PIN7 flanks the axis and PIN3 is expressed at the xylem pole associated pericyclic cells. In addition, cytokinin signalling in procambium regulates the subcellular lateralization of PIN1 and PIN7 to the lateral cell walls facing towards xylem axis. We believe that these tissue-specific subcellular localization patterns are largely controlled by cytokinin signalling and are the main driver for lateral auxin flux towards the xylem axis. Once in the xylem axis, auxin is funnelled down towards the QC by the strictly basally localized PINs (as was presented by Grieneisen *et al.,* 2007). Confocal analysis of the PIN localization patterns in the proximal meristem suggested that *PIN3*, *PIN4* and *PIN7* might be required promote and maintain xylem identity by preventing auxin from "leaking out" of the xylem. Recently published data showing cytokinin biosynthesis in the xylem axis (De Rybel *et al.,* 2014) are consistent with our hypothesis. If the xylem acts as a source for cytokinin biosynthesis, high auxin

levels would be necessary in order to compensate by preventing the locally synthetized CK from repressing protoxylem identity.

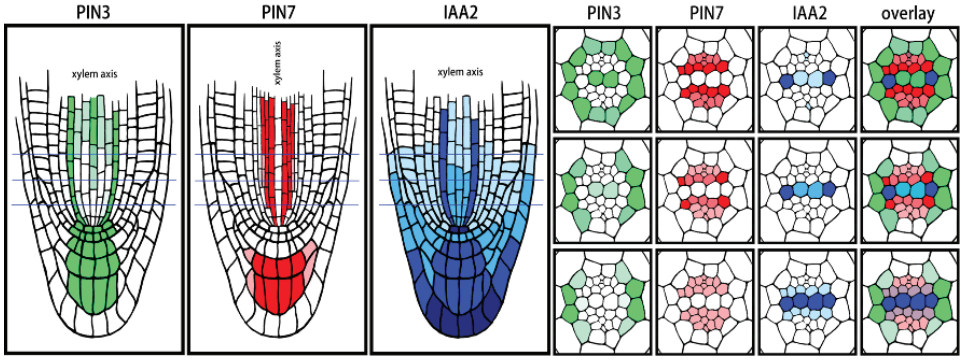

Figure 6: Graphical illustration of PIN3 (green), PIN7 (red) and IAA2 (dark blue) expression and localization patterns in the proximal meristem based on confocal and histological analysis. The light blue lines in the longitudinal images indicate the positions of the cross sections illustrations, which are 20, 40, and 60µm above the QC, from bottom to top. The rightmost panels are an overlay of all three expression patterns, showing how the increased PIN3 levels in the metaxylem position coincide with reduction of IAA2 in the metaxylem, and that the increase in PIN7 levels occurs at the same time as the highly auxin maximum is confined to the protoxylem position.

To illustrate the role of the PINs in protoxylem development, we analysed the protoxylem patterns of various single and multiple *pin*-mutants. As we expected, when the stele-specific *PIN3*, *PIN4* and *PIN7* proteins are not functional, maintenance of the auxin maximum is "unstable", resulting in altered *AHP6::GFP* expression. This is especially pronounced in *pin3pin7* (*DR5* and *AHP6::GFP* data shown in OP II Fig. 2N, O and P). The unstable auxin signalling and *AHP6* domains in *pin3pin7* correlated with variable but pronounced xylem phenotypes (protoxylem scoring in table OP II Sfig. 2). This finding is not surprising, since *PIN3* and *PIN7* appear to be the most influential lateral auxin transporters, with very specific expression patterns in the proximal meristem (Fig. 6).

4.1.5. Cytokinin transport to the proximal meristem and promotion of PAT is critical for vascular pattern maintenance

So, how does cytokinin end up in the root apical meristem in the first place? Recently, genes coding for cytokinin biosynthesis were shown to be expressed at the xylem axis (De Rybel *et al.,* 2014) indicating a role in vascular patterning. However the levels of different types of cytokinin species detected at the root meristem (Antoniadi *et al.,* 2015) are unlikely to result from CK biosynthesis alone. A more likely mechanism for accumulating high amounts of cytokinin would be long-distance transport from the source tissues, the shoot. While auxin moves through the stele from shoots to roots via PAT by the PINs, similar transporters for cytokinin have not been published. In OPIII, we used radiolabelled hormones to demonstrate that cytokinin rapidly moves downwards through phloem and gets unloaded close to the root tip, from where it rapidly disperses throughout the tissues, presumably via plasmodesmatal connections. Auxin is also transported downwards from the shoot (OP III Fig 2B), but radiolabelled hormone transport ceased dramatically when we applied NPA to the

roots, showing that auxin transport occurs almost entirely through the transporters. NPA did not affect cytokinin transport (OP III Fig 2E), as one could expect if the hormone moves mainly via passive diffusion or bulk flow. Conversely, the amount of radiolabelled cytokinin in the meristem decreased significantly when sap flow in the mature phloem was disrupted by inducing callose accumulation in phloem cells to block cell-to-cell movement (OP III Fig 2C). To show that the cytokinins we applied to seedlings were biologically active, we analysed the expression intensity and pattern of the *ARR5* marker in the proximal meristem. We saw a steady increase in the expression of ARR5 after application of BA media onto the cotyledons (OP III, Fig1, Sfig A and B), demonstrating that the cytokinin we applied at the shoot was able to activate signalling in the proximal meristem. The *ARR5* domain within the stele did not expand outside its normal pattern (OP III Sifg1 A), although some *ARR5* expression was visible in the epidermis cell layer after a 24h induction (OP III Fig. 1B). The robustness of the cytokinin signalling pattern in the stele indicates that application of cytokinin through the normal transport channels does not affect vascular patterning if the other components are unaltered. This data is in line with experiments showing that application of auxin does not expand the AHP6 expression pattern, presumably because the PINs rapidly channel auxin to the correct domain.

When inducing callose accumulation to block cytokinin movement from the shoot, we observed reduced expression of the cytokinin response factor *ARR5* in the proximal meristem (OP III Fig. 3P), indicating that a stable source of cytokinin is required for maintenance of the cytokinin signalling domain in the proximal meristem. Since auxin and cytokinin signalling are both required for procambial patterning, we investigated the status of *PIN7::PIN7-GFP, DR5::GFP* and *AHP6::GFP* in various mutant and transgenic lines. These experiments showed a clear correlation between status cytokinin transport and proximal meristem patterning. In *apl* mutants (which cannot form functional phloem), *APL::XVE>>CKX1* lines (which expressed a cytokinin-inactivating enzyme in the phloem position) and *APL::XVE>> cals3m* lines (which accumulate callose in the phloem position), we observed problems with the maintenance of the PIN7 domain, the auxin signalling maxima (*DR5::GFP*) and *AHP6::GFP* expression at the protoxylem position. As a result, the roots showed defective protoxylem differentiation, similar to *pin3pin7* double mutants (OP III Sfig. 3D), demonstrating that continuous cytokinin movement and unloading from the phloem to the meristem is critical for the maintenance of the vascular pattern.

While our published papers only focused on the lateral patterning mechanisms of *PIN1*, *PIN3*, *PIN4* and *PIN7* at the height of meristem initial cells, it is also clear that the longitudinal changes in auxin transporter patterns and the shift of the auxin maximum into the protoxylem positions are linked to the maintenance of xylem identity throughout the meristem, as *AHP6* and *VND7,* early and late protoxylem identity genes, respectively, are both upregulated by *IAA* in the protoxylem position.

4.1.6. Epistasis between cytokinin and auxin signalling in protoxylem formation

Our data demonstrate that a decrease in cytokinin signalling correlates with an increase in protoxylem differentiation. This link is especially clear when comparing wild type roots with single, double and triple mutant combinations containing the *cre1-12* null mutation *(cre1-12*; *cre1-12 ahk2-2 and cre1-12 ahk3-3*; *cre1-1 2ahk2-2 ahk3-3)* and the dominant negative *wol* mutant (Figure 7 and OP II Fig. 2K). By contrast, a decrease in auxin signalling in the dominant negative *axr3-1* mutants (Fig. 7) leads to the loss of protoxylem differentiation, with all cells in the proximal meristem appearing undifferentiated (OP II Fig 2J). Both *axr3-1* and the severe cytokinin signalling mutants (*wol* and *cre1ahk2ahk3* triples) do not form a normal bilateral pattern, indicating that a fine balance between the two hormones is required for normal vascular patterning.

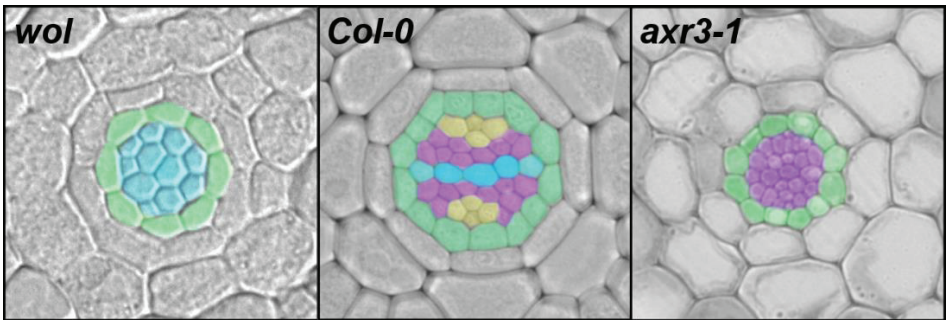

Figure 7: False-coloured cross-sections of cytokinin signalling deficient *wol*, wild type *Col-0* and auxin signalling deficient *axr3-1* roots 5 days post germination. Sections were made at the meristematic zone. Colours are used to mark the different cell types: xylem (light blue), procambium /undifferentiated cells (purple), phloem (yellow), and pericycle (green). The ground tissues and epidermis are grey.

When both cytokinin and auxin signalling are repressed (*wol axr3-1,* Fig. 11 C), the all-xylem phenotype of the *wol* mutant is partially supressed. This suggests that the *ratio* of hormone signalling, rather than the absolute levels, is the determining factor for protoxylem status and the formation of bilateral vascular symmetry. The idea of hormonal homeostasis raises the question of epistasis between these two hormones: which has the final word in xylem fate determination? In order to understand the epistasis between auxin and cytokinin signalling we conducted a series of experiments:

1) We tried to erase the auxin maximum in cytokinin signalling deficient mutants with a dramatic phenotype (*wol* and *cre1 ahk2 ahk3* triple mutants) by long term inductions with NPA, expecting to see a loss of protoxylem. Surprisingly, this did not happen in any of the roots. We next analysed the *IAA2::GUS* marker in *wol* to visualize the auxin response domain. Interestingly, treating *wol* roots with NPA did not reduce the *IAA2::GUS* expression (OP II Sfig. 1G); on the contrary, the *IAA2::GUS* signal increased and spread to the endodermal cells (Fig. 4W). Since these strong cytokinin signalling mutants seemed unresponsive to chemical treatments, we turned to NPA treatments of the phenotypically milder cytokinin double receptor mutants in order to understand the fine dynamics of the hormone balance. We found that protoxylem in the *cre1-12 ahk2-2* and *cre1-12 ahk3-3*

double mutants was extremely resistant to NPA treatments. Whereas wild type *Col-0* seedlings show severe loss of protoxylem when treated with 5µM NPA (see table OP II Sfig. 2L), the double mutants actually made more protoxylem (Fig. 8). The *cre1ahk2* and *cre1ahk3* double mutants were then treated with increasing concentrations of NPA (from 5µM up to 1mM NPA), but still failed to show any loss of protoxylem. Interestingly the *ahk2-2 ahk3-3* double mutant phenocopied wild type *Col-0* plants treated with NPA, indicating that the *cre1-12* mutation might cause apparent resistance to NPA by dampening the CK signal to allow even the small amount of auxin that accumulates in NPA treatments to promote protoxylem differentiation in the stele.

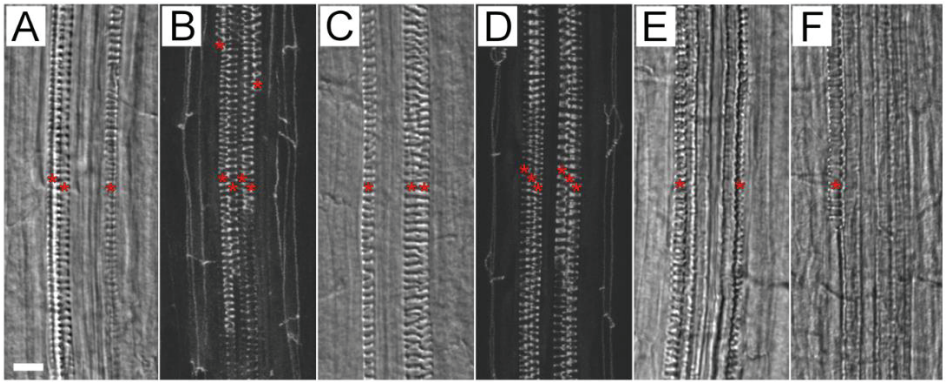

Figure 8: Protoxylem phenotypes of the cytokinin receptor double mutants 5pg without and with 5µM NPA. (A,B) *cre1-12 ahk2-2* (A) without and (B) with NPA. (C,D) *cre1-12 ahk3-3* (C) without and (D) with NPA. (E,F) The *ahk2-2 ahk3-3* double mutant (E) without NPA and (F) with NPA. Only *ahk2-2 ahk3-3* mutants show sensitivity to NPA (loss of protoxylem). The red asterisks indicate protoxylem cell files. Scale bar 10µm.

2) We tested the effect of locally reducing cytokinin levels in the root vasculature by inducing the expression of *CKX1* under the *CRE1* promoter (Appendix, A-Figure 1). Following induction, the roots formed ectopic protoxylem cell files (Fig. 9K). We analysed the *IAA2::GUS* and *AHP6::GUS* expression patterns with histological sections and showed that they had expanded accordingly (Fig. 9C, G); this preceded the lignification of the vessels, which was visible by DIC-microscopy after 12h of induction (OPII Sfig2 H). Induction of the *CKX1* construct in roots pre-treated with 5µM NPA led to significant ectopic protoxylem formation (Fig. 9L, P), similar to the ectopic protoxylem observed in *cre1-12 ahk2-2* and *cre1-12 ahk3-3* double mutants treated with NPA. NPA treatment also led to reduced *IAA2::GUS* (Fig. 9B) and *AHP6::GUS* (Fig. 9F) levels at the protoxylem position, which correlated with loss of protoxylem (Fig. 9J, N). In roots grown on normal medium for 72h and then induced with 20µM 17B EST for 48 hours, the *IAA2::GUS* and *AHP6::GUS* patterns spread laterally (Fig. 9C and G, respectively), leading to the formation of ectopic protoxylem (Fig. 9K, O). When the roots were grown on 5µM NPA for 5d followed by growth on NPA and 20µM EST for 48h, *IAA2::GUS* and *AHP6::GUS* expression got stronger and the expression pattern spread greatly, with *IAA2::GUS* accumulating in almost all stele cells, as well as the endodermis and cortex (Fig. 9D), and the *AHP6::GUS* domain spreading to all procambial cells touching the pericycle, reaching phloem

companion cells (Fig. 9H). This dramatic spread of marker genes was followed by formation of supernumerary ectopic protoxylem cells (Fig. 9L, P). This experiment demonstrates that procambial cells can be triggered to proliferate, by NPA, and if CK signalling is simultaneously reduced, the new procambial cells adjacent to the pericycle can differentiate into protoxylem.

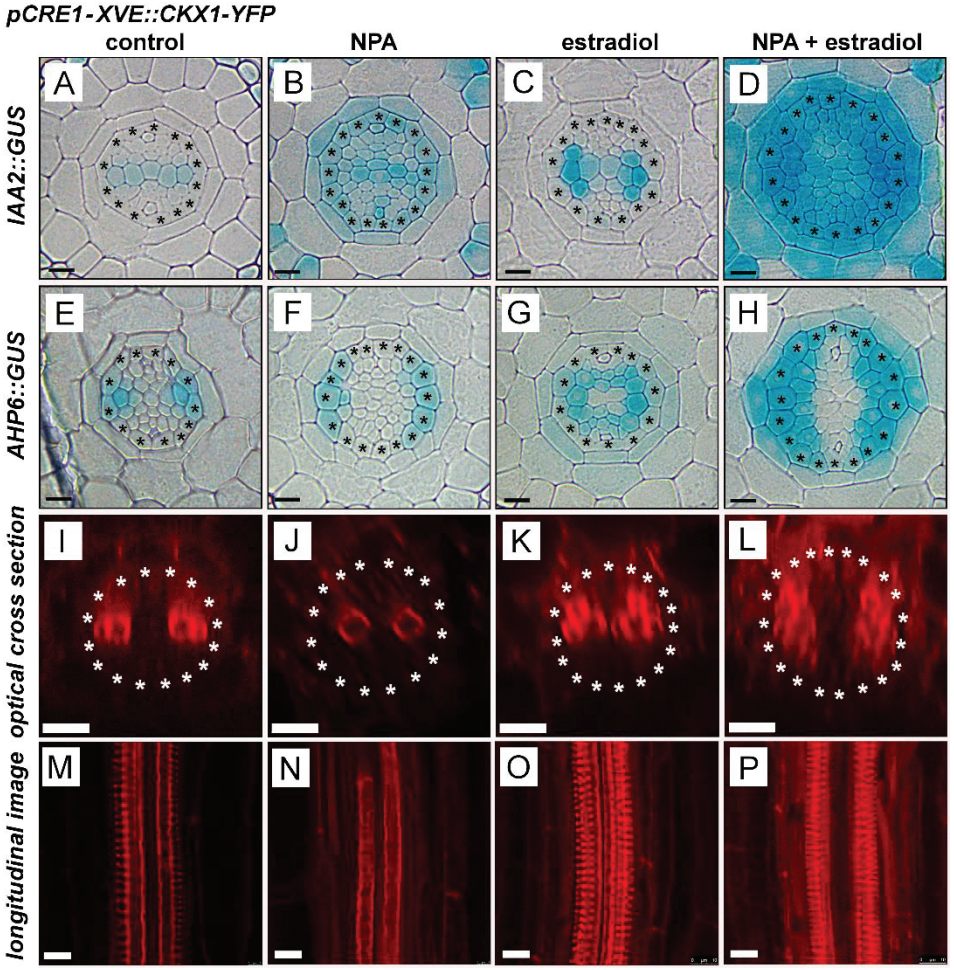

Figure 9: The combinatory effect of NPA and CKX1 induction leads to ectopic protoxylem formation. Panels A, E, I and M present untreated in *pCRE1-XVE::CKX1-YFP* control roots with (A-D) *IAA2::GUS* and (E-H) *AHP6::GUS* expression in (A,E) untreated roots and roots grown on (B, F) 5μM NPA for 5 days, (C, G) normal media for 72h followed by 20μM 17B EST for 48 hours, and (D,H) 5μM NPA for 5d and co-induced with NPA and 20μM EST for 48h. (I-P) Confocal images of fuchsin stained roots; optical (I-L) cross sections and (M-P) longitudinal images of (I, M) untreated control roots and roots grown with (J, N) 5μM NPA for 5 days, (K, O) normal media for 72h followed by 20μM 17B EST for 48 hours, and (L,P) 5μM NPA for 5d and co-induced with NPA and 20μM EST for 48h. Black and white asterisks illustrate pericyclic cells. Scale bars in all panels are 10μm.

3) We also treated roots with NPA and cytokinin simultaneously. Treatment with NPA led to the loss of *AHP6* at the protoxylem position and a lateral spread of the signal in the pericycle (Fig. 9F, OP II Sfig. 2C). In roots treated with BA, the *AHP6* signal was lost entirely (OP II Sfig. 2F). In order to see how *AHP6::GFP* responds to NPA and BA together, we treated roots with the two simultaneously. Rather surprisingly, *AHP6::GFP* expression was not lost at the pericycle. To understand the effect of this treatment on auxin signalling status, we examined *IAA2::GUS* expression in cross sections. The *IAA2::GUS* pattern on BA+NPA was similar that on NPA treatment alone (data not shown). Thus, while cytokinin promotes *PIN7* expression throughout the stele (except the metaxylem) and thus forces the auxin maximum to a central position in the metaxylem, the inhibitory effect of NPA on the PIN transporters appears to be sufficiently strong to overcome auxin transport stimulation by exogenously applied BA.

Why do the pericycle cells express *AHP6* in the proximal meristem, and more intriguingly, why do they maintain auxin signalling on NPA treatment? Could this have some biological role outside the stele? Perhaps the pericyclic cells function as positional cues for the xylem axis and protoxylem identity. Our data show that *AHP6::GFP* expression is dependent on auxin signalling (OP II Fig. 2C, D, H, Sfig. 2E), and consequently, *AHP6::GFP* is expressed in the pericycle cells as long as they have auxin. One possibility is that even though NPA blocks auxin exporters, the importers in the stele (Péret *et al.,* 2012; El-Showk *et al.*, 2105) remain functional and are capable of transporting auxin into the pericycle from the ground tissue. While the biological role of AHP6 expression in the pericycle remains unclear, current knowledge of the priming of lateral root initials (Xuan *et al.,* 2015), the role of *AHP6* in early periclinal cell divisions in lateral root development (Moreira *et al.,* 2013) and auxin maximum modelling data (El-Showk *et al.,* 2015) suggest a link between the auxin maximum, *AHP6* expression and lateral root initiation independent of protoxylem identity formation, possibly in lateral root initial priming. Further studies would be required to unravel the role of pericyclic *AHP6* expression in NPA (and CK) treated roots, as lateral root initiation and lateral root emergence was not analysed in our experiments.

The data presented in OP II and OP III, on previous pages of this thesis and in publications from other research groups can be summarized in a few key points:

1) In wild type plants, NPA treatment blocks auxin transport efficiently, and as a result, the xylem axis maximum cannot be maintained. However, auxin signalling and biosynthesis are not affected, leading to the dispersed auxin signalling patterns seen in the stele of NPA-treated wild type plants. Because cytokinin mobility is not affected by NPA, as demonstrated by our radiolabelled cytokinin assay, cytokinin movement and signalling are still able to repress *AHP6* at the protoxylem position, resulting in a loss of protoxylem.

2) When cytokinin signalling levels are very low, as they are in *cre1ahk3* and CKX1-induced roots, the *PIN7* expression domain shrinks. As a result, the auxin maximum becomes wider than in wild type roots. This expansion of the auxin signalling domain is accompanied by an expanded, laterally spread *AHP6* expression domain. According to our findings, *AHP6* expression persists in the pericycle following NPA treatments. While the *AHP6* protein has been shown to move non-cell autonomously (Mähönen *et al,*. 2006, Besnard *et al.,* 2014), which

may affect the hormonal signalling domains and vascular patterning, the amplitude and role of *AHP6* movement in NPA-treated plants with low ck signalling remains unclear. Without NPA, the PIN proteins in the stele remain active, despite low cytokinin signalling, and while they fail to restrict *IAA2* to a one-cell wide axis (due to their reduced expression level), they can still create a domain of auxin accumulation which becomes specified as xylem .

3) It is logical to assume that NPA-treated *cre1ahk2* and *cre1ahk3* or CKX1 induced mutants have practically no lateral PIN-mediated auxin transport towards the xylem axis. Together with the almost total lack of cytokinin repression on auxin signalling and intact auxin import into the protoxylem position (as suggested by El-Showk *et al.,* 2015), this results in an enhancement and expansion of the auxin maximum compared with control plants. Notably, with cytokinin signalling so low, even low levels of auxin are sufficient to reach the threshold required to promote *AHP6* expression and ectopic xylem formation. While the contribution of the laterally expanded, NPA-insensitive pericyclic expression of *AHP6* and *AHP6* mobility remain unclear in this scheme, I would like to propose that it can, in part, promote supernumerary protoxylem formation. Evidence suggests that in the context of proximal meristem vascular patterning, the hormone signalling homeostasis between auxin and cytokinin is more relevant than the actual hormone levels, and that auxin is the rate-limiting factor for protoxylem development.

4.1.7. Conclusions of Part I

Our data show that protoxylem identity and differentiation is tightly and directly linked to the high auxin maximum in the xylem axis; however, epistasis experiments show that a fine balance between auxin and cytokinin is also required. Based on our published findings and additional data discussed in this thesis, I propose the following model (Figure 10): The breakage of radially symmetry in the embryo establishes the vascular pattern, which in turn is actively reinforced by polar auxin transport and long distance cytokinin transport and unloading in the meristem. Cytokinin transport/translocation from the apical parts of the plant via phloem transpiration promotes cytokinin signalling in the procambial cells, where cytokinin promotes the expression of *PIN7* and enhances its localization to the lateral membranes. Lateral PIN localization results in auxin efflux towards the xylem axis, leading to the formation of an auxin signalling maximum in the axis. This auxin signalling maximum promotes the expression of *AHP6* in the protoxylem cells, which in turn represses CK signalling on site. According to our data, the PINs expressed in the xylem axis are basally localized, reinforcing PAT towards the QC and presumably maintaining the high auxin signalling maximum in the proximal meristem stem cell initials, which promotes *AHP6* expression. The mutually repressive auxin and cytokinin interaction maintains the spatial pattern of their signalling domains in the proximal meristem (and upwards), which is required for the specification and maintenance of various vascular cell types.

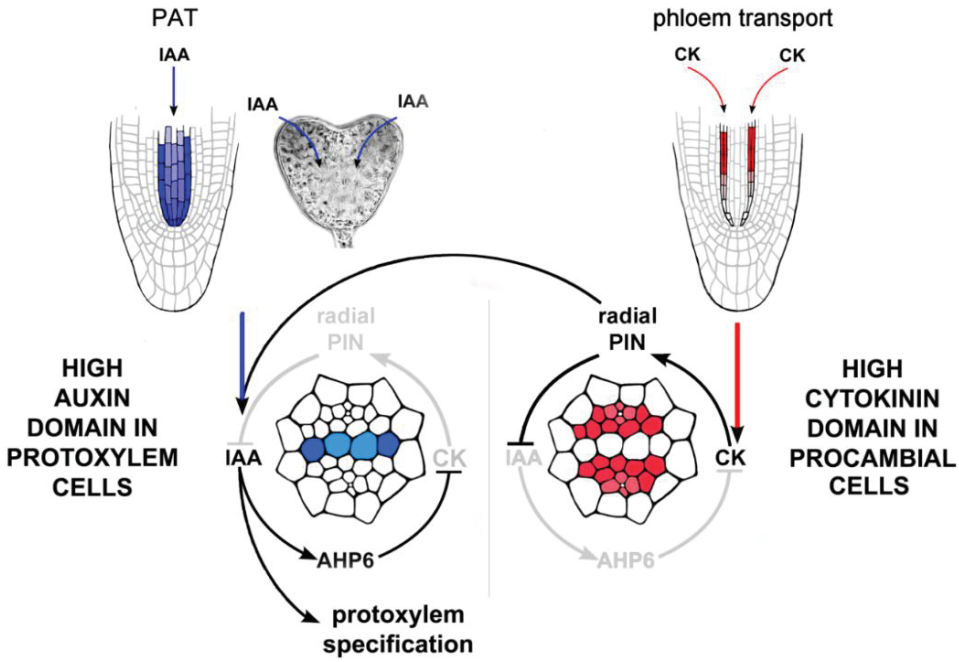

Figure 10: The model. Auxin input from the cotyledons during embryogenesis patterns the embryonic root axis and vasculature. As the plant germinates and root growth continues – guided by this initial pattern – auxin transport is maintained with PAT. Cytokinin flows through the phloem from the shoot to the root. In the proximal meristem, auxin and cytokinin signalling domains are in continuous interaction, inhibiting one another through the expression of downstream components. Cytokinin in the procambium promotes lateral localization of the PINs, which push auxin out of the procambium towards the xylem axis, where an auxin signalling maximum is maintained. The accumulation of auxin promotes the expression of *AHP6* at the protoxylem position , inhibiting cytokinin signalling on site, and promotes protoxylem formation via *ATHB8*. Low cytokinin signalling in the protoxylem position does not promote lateral localization of PIN transporters. The limited lateral auxin transport at the xylem axis and strong basal PAT directs auxin towards the root tip and xylem initials, further aiding in xylem pattern generation and cell identity establishment.

4.2. Part II: Hormonal regulation of procambial re-patterning

4.2.1. Procambial regeneration and re-patterning

Root regeneration assays have shown that damaged root tips can re-generate themselves, including the QC, RAM organizing centre and columella, as long as the damage occurs in the meristematic region (van den Berg *et al.,* 1997; Sena and Birnbaum, 2010), implying that the stele cells have enormous regeneration potential. With our auxin-cytokinin loop in mind (figure 10), we wondered whether we could somehow alter the seemingly pre-set fate of proximal meristem cells in certain mutants with proper stimulus *in planta*. Specifically, we investigated whether the all-xylem pattern of *wol* could be reverted to generate undifferentiated procambial cells by modifying hormonal signalling in the meristem initials.

The gain-of-function auxin response regulator *axr3-1* mutant (Fig. 11A) fails to make xylem, as they lack the big, light blue xylem cells visible in toluidine blue stained plastic sections of *wol* roots (Fig. 11B). The *axr3-1* mutants do seem to form phloem or phloem-like cells, indicating that lack of auxin signalling only represses xylem development. The *axr3-1* mutants can thus be regarded as the phenotypic opposite of the *wol* mutant (Fig. 11B) as all cells in the wol mutant primary root stele are xylem and cell proliferation is clearly reduced compared with the axr3-1 mutant. We therefore crossed these two mutants to generate a double mutant, in which we observed undifferentiated cells which stain dark purple with toluidine blue (Fig. 11C), indicating that the all-protoxylem *wol* mutant phenotype can be alleviated by reduced auxin signalling. However, the *wol axr3* mutant plants are extremely dwarfed, and the primary root arrests after a few days. As one might expect, their shoot is also extremely small and their inflorescences are dwarfed, producing only a few siliques and practically no seeds, highlighting the devastating effects of suppressing two major hormone signalling pathways. Even though this approach showed us that procambial cell fate can be affected by altering the hormone signalling level in roots, these double mutants were too dramatically affected in other ways for further work. Thus, we took an alternate approach and reduced the auxin signalling levels in the primary root stele by inducing the gain of function alleles of *axr2-1* and *axr3-1* under the *CRE1-XVE* promoter in the *wol* background (Fig. 11D, E). Interestingly, all the lines we analysed gave us similar results; *wol* roots in which *axr2-1* or *axr3-1* expression was induced showed phenotypes similar to the axr3-1 wol double mutant as the all-protoxylem phenotype of *wol* was alleviated and the roots were able to re-generate undifferentiated cells. Regardless of whether auxin signalling was inhibited constitutively (*wol axr3*) or inducibly (*wol CRE1-XVE::axr2-1* and *wol CRE1-XVE::axr3-1*), none of mutant lines showed procambial cell proliferation. This indicates that only the fate of existing cell files could be modified by altering the hormonal signalling homeostasis and that the restoration of cell proliferation would require something more.

To restore cell proliferation in the combinatorial hormone signalling mutants, we took a different approach and introduced cytokinin signalling back into *wol* with an estrogen-inducible stele-specific *CRE1-XVE::CRE1* construct. This *CRE1-XVE::CRE1 wol* line, or "*wol* complementation line", is not defective in auxin signalling. By inducing a functioning, wild type cytokinin receptor in the *wol*

background, we were able to reverse xylem specification and re-generate undifferentiated procambial cells and also succeeded in enhancing cell proliferation.

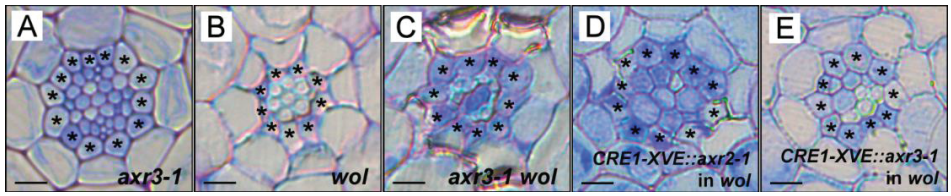

Figure 11: Altering auxin and cytokinin signalling allows fate respecification. (A) *axr3-1*, (B) *wol*, (C) *axr3-1 wol* double mutants, (D) *CRE1-XVE::axr2-1* and (E) *CRE1-XVE::axr3-1* in the *wol* background. Roots were sectioned 5 dpg at the differentiation zone and stained with toluidine blue. Scale bars are 10μm.

4.2.2. Anatomical analysis of procambial re-patterning

To better understand these changes, we analysed the appearance or disappearance of cell identities by observing anatomical changes in fuchsin-stained (Fig. 12) and plastic cross-sections (Fig. 13). Upon the restoration of cytokinin signalling, the *wol* complementation roots first showed a disappearance of protoxylem (and metaxylem) cells, seen in longitudinal confocal images of stele (Fig. 12) and the appearance of undifferentiated cells around 1 dpi (day post induction) in plastic sections (Fig. 13B). The loss of xylem cell fate was followed by increased cell proliferation around 2 dpi (Fig. 13C-G). Some roots also developed cells that anatomically resembled phloem sieve elements and were similarly located (Fig. 13E-G). Between 2 and 3 dpi, the proliferation of undifferentiated cells increased further, resulting in the loss of essentially all xylem cells (Fig. 13F, G). Around 3dpi, the *wol* complementation roots also showed a range of vascular symmetries that are never seen in radially symmetrical *wol*-roots: bilateral symmetry, bisymmetry and tri-symmetry (Fig. 13E, F and G, respectively). The appearance of phloem-like cells (yellow arrows in Fig. 13) along with procambial cell proliferation indicated that the stele-specific re-introduction of cytokinin signalling in the *wol*-mutant was able start a self-organizing process which culminated in the formation of all vascular cell types, without the need for prior positioning from the shoot.

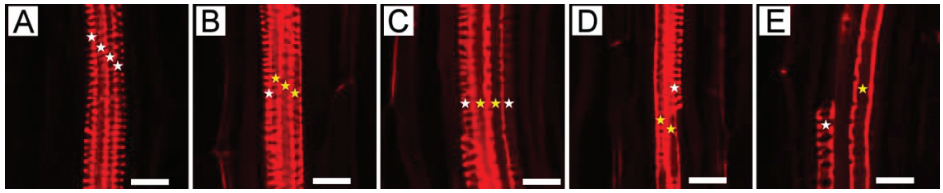

Figure 12: Fuchsin stainings of the *wol* complementation line 5 dpg. (A) Non-induced *CRE1-XVE::CRE1 wol* roots show an all xylem phenotype, similar to *wol* mutants, with four protoxylem cell files aligned on the same focus plane. (B) After 24hours of induction, the protoxylem lignification pattern starts to resemble the characteristic metaxylem pattern, continuing in (C), (D) and (E). After 48h induction, the *wol* complementation line roots showed variable phenotypes ranging from (C) a rather normal bilateral pattern with two protoxylem and two metaxylem cells to (D, E) a more severe loss

of protoxylem. White asterisks indicate protoxylem and yellow indicate metaxylem cells. Scale bars are 10μm.

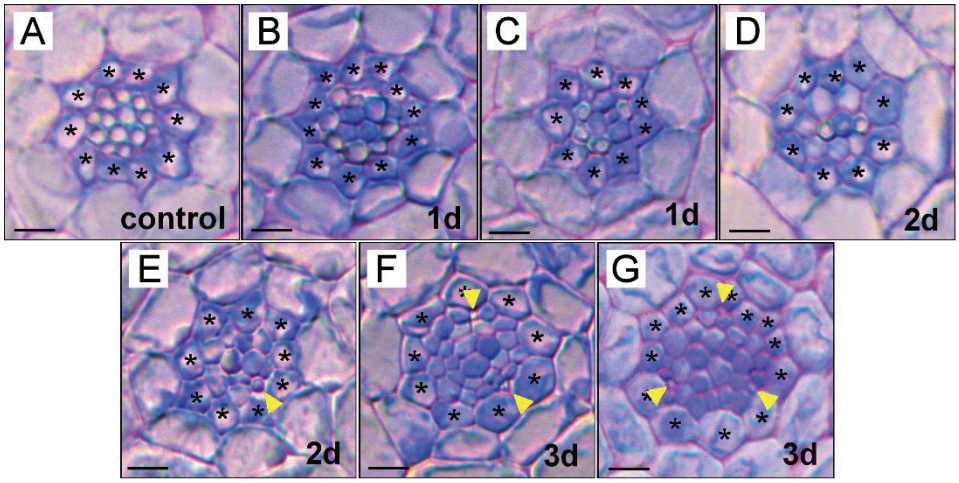

Figure 13: Cross sections of the *wol* complementation line 5 dpg. (A) Control root without induction. (B, C) Roots induced for 1 d begin to show a reduction in the number of xylem cells and an increase of undifferentiated cells. (D, E) Roots induced for 2 d show a progressive loss of xylem. (F, G) Roots induced for 3 d begin to show altered symmetries, with (F) two phloem-like cells (bisymmetry) or (G) three phloem-like cells (trisymmetry). Sections were made at the differentiation zone and show secondary cell wall lignification in xylem cells (light blue). Black asterisk mark pericycle cells and yellow arrowheads emerging phloem-like cells. Scale bars are 10μm.

4.2.3. Cytokinin signalling and auxin transport during procambial re-patterning

Re-introducing a functional cytokinin signalling receptor (*CRE1*) into the *wol* mutant under an inducible promoter gave us an opportunity to observe not only progressive anatomical changes but also early patterning events, such as changes in the hormonal domains and cell-type markers. To validate the identity of the "re-generated" cell files, we introduced the cytokinin signalling marker *ARR5::GUS* and the procambium marker *PIN7::PIN7-GFP* into the *wol*-complementation line. We analysed the appearance or disappearance of these markers at various time points before the observed anatomical changes. The earliest time points at which we observed a robust change in *ARR5::GUS* was at 6 hpi (hours post induction) (Fig. 14C, D). Analysis of *PIN7::PIN7-GFP* yielded similar results, though some plants showed appearance of the GFP signal in the stele as early as 3 hpi (Fig. 15B, G). In accordance with the *ARR5::GUS* data, *PIN7* was clearly upregulated in stele cells 6 hpi (Fig. 15C, H). Cell proliferation and the formation of a distinct PIN7 expression domain within the proximal meristem were visible at 12 hpi in this line (thesis Figure 15D, E and I, J).

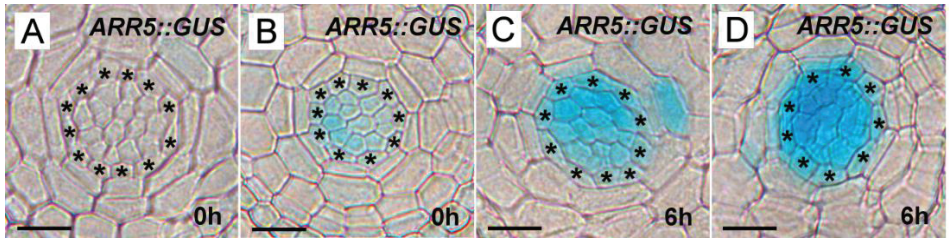

Figure 14: Cytokinin signalling response is recovered in the inducible *CRE1-XVE::CRE1 wol*. Cross sections of *ARR5::GUS* from the meristematic zone of 5 dpg plants (A, B) without induction show (A) no or (B) very little *ARR5::GUS* expression. (C, D) After 6h induction with 17β-estradiol, *ARR5::GUS* expression increases noticeably in the stele of the sectioned roots. Black asterisk mark the pericycle cells. Scale bars are 10μm.

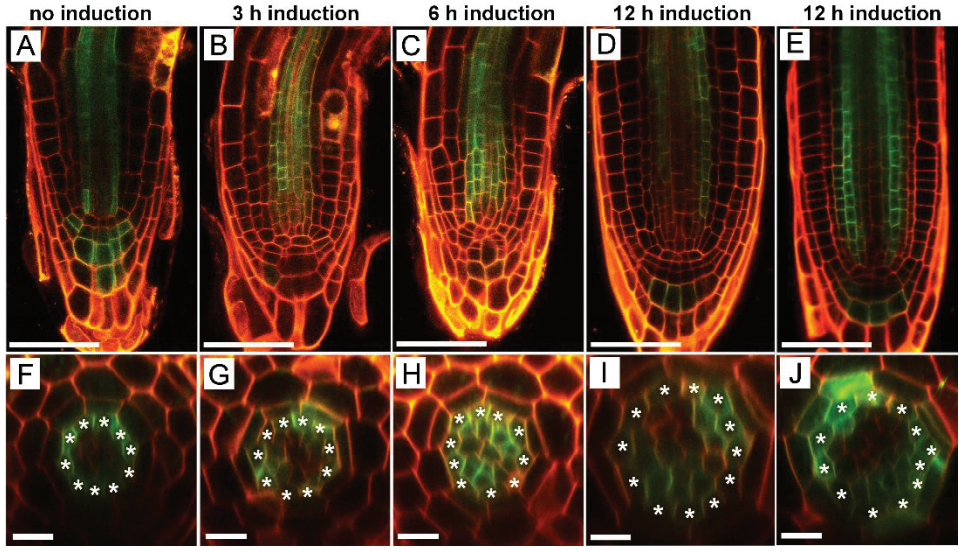

Figure 15: Expression of PIN7::PIN7-GFP in the inducible *CRE1-XVE::CRE1 wol* line. Confocal images of primary root tips of 5dpg plants (A, F) without induction show PIN7::PIN7-GFP expression only in the pericycle, forming a ring like pattern visible in the optical cross section. (B, G) A faint PIN7::PIN7-GFP signal could be seen in the stele 3 hours post induction; however, (C, H) after 6 hours the signal was more visible in most stele cells, excluding the central position. (D, E, I, J) After a 12 hour induction, the majority of plants show cell proliferation in the proximal meristem and distinct PIN7 domains. White asterisks mark the pericycle cells. Longitudinal images were oriented along the centre of the QC in the plane of the stele. The optical cross sections were taken ~50μm above the QC. Scale bars are 50μm in the longitudinal images and 10 μm in the cross sections.

Our published data show that the symmetry breakage in the embryonic root, which is critical for normal root patterning, is dependent on cytokinin signalling. Our data also show that cytokinin transport to the root tip is essential for normal post-embryonic pattern maintenance. With the *wol* complementation line, we were able to show that symmetry breakage can also occur post-embryonically in the primary root, and that local activation of cytokinin signalling can determine the process regardless of the status of cytokinin transport through the phloem (as

wol roots do not have phloem). This re-patterning is rapid, taking place in only a few hours. Re-pattering of *ARR5* expression and an increase and repositioning of *PIN7* precede the anatomical changes by several hours. When *CRE1-XVE::CRE1* induction is maintained for several days, the re-established cytokinin signalling promotes not only procambial cell fate but also cell proliferation, presumably leading to successive symmetry breakage events (from the initial radially symmetric root to bisymmetry and tri-symmetry).

4.2.4. Cell proliferation, cell number and stele patterning are regulated by the auxin-cytokinin loop

Interestingly, when wild type Col-0 roots were treated with NPA for long periods, abnormal stele symmetries similar to the *wol* complementation line were observed. The emergence of extra *AHP6* and *APL* poles was seen in plastic sections prior to and alongside excess cell proliferation, indicating that the presence of xylem and phloem, even though the cells otherwise appeared undifferentiated (OP II Sfig 4). When plants were kept on NPA for 21 days, the root tips became extremely swollen and formed of supernumerary protoxylem and phloem poles (Figure 16). Even though these roots appear chaotic at first glance due to meristem reorganization and elevated cell division rates (as was originally reported by Sabatini *et al.,* 1999), one can see a clear pattern upon careful inspection. The xylem poles are positioned at the periphery of the vascular cylinder and are separated from one another by procambium, and the phloem bundles develop between the xylem poles. Thus, the alternating xylem/phloem pattern that exists in untreated wild type Col-0 roots is still present following long-term NPA treatments, but the poles are more numerous. Published marker data (Sabatini *et al.,* 1999) and our anatomical cross sections demonstrate that NPA treatment results in a massive increase in cell proliferation. As NPA is thought to affect only the auxin exporters and no other components in the auxin-cytokinin signalling loop described above, I propose that long-term NPA treatment acts as a trigger for repatterning. Long-term NPA treatment creates a mass of proliferating undifferentiated cells in which the components of the loop interact with each other in a self-organizing manner, leading to the observed pattern of alternating xylem and phloem poles.

Only a few *Arabidopsis* show reduced differentiation of vascular strands in primary roots, including the phenotypically variable *pin1* and *lhw* (Ohashi-Ito and Bergmann, 2007). The stele of *lhw* roots is thinner, and there are fewer cells than in wild type roots (Ohashi-Ito and Bergmann, 2007). *LHW* is thought to be critical for procambial cell proliferation by affecting CK biosynthesis in the xylem axis (De Rybel *et al.*, 2013 and 2014). The failure to restrict cell divisions [in NPA treated roots, pRPS5A-TMO5/pRPS5A-LHW overexpression lines (De Rybel et al., 2013 and 2014) or the *wol*-complementation line] leads to ectopic cell proliferation and supernumerary poles of xylem and phloem forming in the stele. These examples highlight the importance of establishing a sufficient number of cells for correct bisymmetric vascular patterning. Similar data have been presented in the shoot, where meristem size (which corresponds to cell number) correlates positively with organ permutations and phyllotaxis (Landrein *et al.,* 2015). This suggests that in both roots and shoots an increase in meristem cell numbers can lead to morphological aberrations if the key genetic components regulating meristem

patterning are present (namely, auxin and cytokinin signalling and their mutually inhibitory interaction).

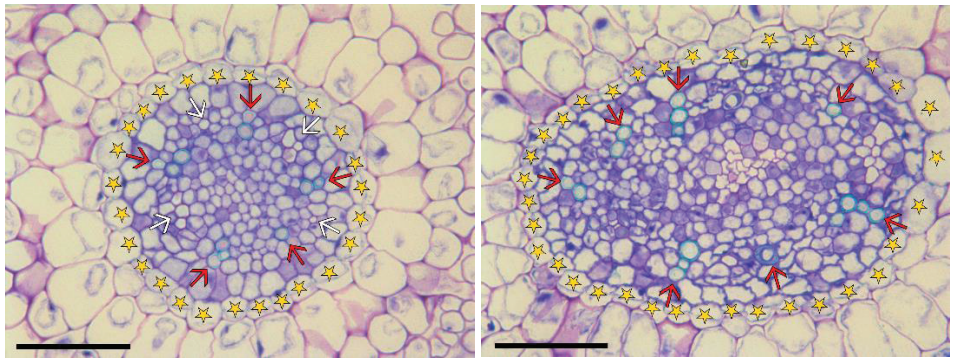

Figure 16: Plastic sections of the primary root meristems of 21 dpg plants grown on 20µM NPA. In these plants, the root meristems swells due to massive cell proliferation, leading to the formation of supernumerary protoxylem (red arrows) and phloem (white arrows) poles. The phenotypes ranged from moderate (left) to extreme (right). Yellow stars mark the innermost endodermal cells. As can be seen in these sections, the endodermal, cortical and epidermal cell layers have proliferated less and still form clear concentric layers. Scale bars in both images are 50µm.

4.2.5. Conclusions of part II

According to the model described in the first part of this thesis, auxin signalling is critical for protoxylem identity formation. The results from the procambial re-patterning experiments show that cytokinin is the key hormone in promoting cell proliferation. My data indicate that the procambium is an extremely flexible, genetically pluripotent tissue; its central role as the boundary, buffer, source and sink for various signalling molecules controlling primary root vascular patterning has been somewhat overlooked. In order to understand how cytokinin induces the de-differentiation and proliferation of procambial cells capable of re-generating all the vascular cell types in the *pCRE1-XVE::CRE1 wol* complementation roots, an in-depth, temporally detailed marker analysis would need to be performed. This would help determine precisely where and when the reintroduction of cytokinin signalling activates the transcription of cytokinin response regulators (*ARR5*), promotes *PIN7* expression and localization in the lateral cell membrane (along with PIN1), eventually leading to the redirection of the auxin maximum in the stele and repression *AHP6* in protoxylem position. The appearance of early and late phloem markers (data not shown) accompanied by cell proliferation would also verify the identity of the phloem-like cells visible in the plastic sections. Even though the all-xylem "plug" below the hypocotyl of *wol* roots probably inhibits the formation of functioning, conductive phloem, the *wol*-complementation line is a useful tool for understanding early signalling events in identity specification and hormonal homeostasis in identity maintenance. Once the relevant time points have been determined, a transcriptome analysis could be done at various stages of induction. This would help identify early-response genes and map the interacting genetic networks and pathways leading to changes in symmetry and the initiation of phloem.

To date, two independent modelling efforts related to OP II and OP III have been made (Muraro *et al.*, 2014 and El-Showk *et al.*, 2015), both of which confirm that the components described in OP II and OP III are sufficient to set up the wild type bilateral vascular pattern. Several open questions remain regarding the relationship between physical space and cell patterning. Does the self-organizing auxin-cytokinin interaction translate physical space and cell number within the stele into a suitable number of xylem and phloem poles and thus effectively control vascular symmetry? Do vascular symmetries become more complex as the stele grow larger, as seen in NPA treated and *wol*-complementation line roots? Answering the questions would require further experiments and is currently beyond the scope of my research. However, it would be interesting to know whether the self-organizing re-patterning mechanism and abnormal symmetries could be generated *in-silico* by modifying the number of cells and/or the physical space occupied by the stele. Future research could also address whether the components in our model have functional orthologs in other plant species and, if so, how they behave and interact in species with larger steles and more complicated vascular patterns.

4.3. Part III: Role of ROS signalling in meristem patterning and protoxylem maturation

4.3.1. *GRIM REAPER* localization and function

GRIM REAPER (GRI) is homologous to the tomato (*Solanum lycopersicum*), petunia (*Petunia x hybrida*) and tobacco (*Nicotiana tabacum*) *STIGMA-SPECIFIC PROTEIN1 (STIG1)* genes. *STIG1* is expressed in pistils and secreted to intercellular spaces (Verhoeven *et al.,* 2005). Loss of *STIG1* does not lead to altered pollen germination or pollen tube outgrowth in either petunia or tobacco; however, both species showed increased amounts of exudates on stigma surfaces, caused by enhanced formation of lipid droplets. Since these exudates are required for pollen viability and germination on the pistil, mutations affecting them can affect reproductive success by reducing female fertility in *Solanaceae* (Verhoeven *et al.,* 2005). In *Arabidopsis thaliana*, *GRI* was shown to be involved in the regulation of ROS-induced cell death (Wrzaczek *et al.,* 2009a and 2009b). The *gri* gain-of-function mutant accumulates a truncated GRI peptide, rendering them hypersensitive to ROS and cell death, thus making the plants more resistant to pathogens. My aim was to understand the developmental impact of *GRI*-regulated ROS signalling, its mode of action, and downstream signalling components.

GRI::GUS is expressed in the lateral root cap and underlying epidermal cells of the meristematic region, and its expression increases considerably in the transition zone (Figure 17), where the epidermal cells undergo elongation and start to differentiate. The expression appears to be initially restricted to non-hair cells (possibly promoting their differentiation prior to root hair cells), and later expand to most epidermal cells. This expression pattern indicates that GRI-mediated ROS signalling might play a role in the death of lateral root cap cells and in the cell wall modifications required for elongation and differentiation of the epidermal cells.

ROS signalling is critical for root hair formation and elongation (Foreman *et al.,* 2003). The lack of *GRI::GUS* expression in hair cells suggests a signalling mechanism similar to the *CAPRICE/GLABRA/WEREWOLF* interaction (Wada *et al.,* 2002); if the expression of *GRI* in non-hair cells and movement of the protein to neighbouring cells acts as a non-cell autonomous signal, *GRI* might promote cell wall modifications in hair cells, that undergo many morphological changes before becoming fully differentiated. Preliminary anatomical data from *Col-0* and *gri* seedlings is quite promising; several sectioned *gri* mutants show an abnormal arrangement of hair and non-hair cells (Figure 18) and, in addition, *gri* mutants have hair cell lineages in which all hairs are morphologically abnormal (data not shown). If GRI also functions in a cell autonomous manner, which cannot be excluded, it could be promoting cell wall modifications in non-hair cells earlier than in hair cells; this would result in differential aging of cells in the epidermis, which could, in turn, act as a morphogen for the hair cells. The mechanism and role of *GRI* in root epidermal cell differentiation should be clarified further and are currently under investigation.

GRI::GUS is also expressed in the shoot, in rosette leaves, inflorescences and epidermal cells neighbouring the trichomes, called the basal cells (Fig. 19A, D-J). The signal can be seen in the midveins and smaller veins in vascular bundles (Fig.

19B, C). Expression in trichome basal cells and non-hair cells in the roots suggests that ROS signalling is relevant to the regulation of epidermal cell wall structures and that the mobile GRI peptide likely moves to the neighbouring cells, which are about to acquire an unusual shape via drastic cell wall modifications. As might be expected based on the expression of the petunia and tobacco homologs in pistils (Verhoeven *et al.,* 2005), *GRI::GUS* is also expressed in the stigma and vascular cells in the silique in *Arabidopsis*, as well as in developing siliques at the base of seed suspensors at the internal septum, indicating a role in reproduction similar to the tomato and tobacco homologs. The expression of *GRI::GUS* in the vasculature of rosette leaves is most relevant to the first two parts of this thesis, as the strongest expression in leaf cross sections is in developing xylem cells that are experiencing secondary cell wall formation and massive synthesis of cell wall components.

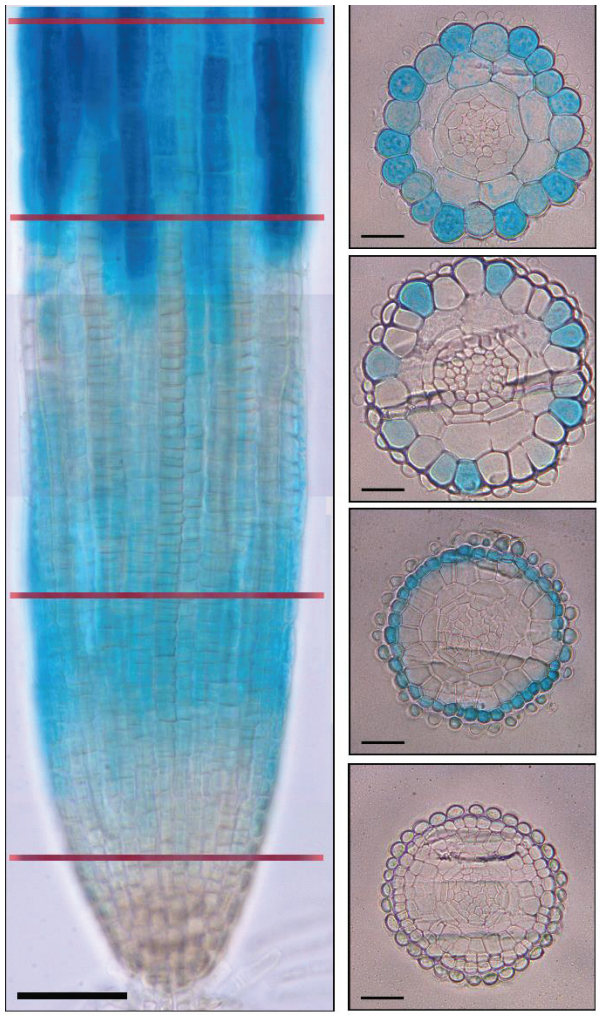

Figure 17: *GRI::GUS* expression in the primary root tip at 5 dpg. *GRI* expression is barely visible in the proximal meristem (lowest cross-section). Expression is first detected in the lateral root cap cells of the meristematic zone (second cross-section). In the

elongation zone, *GRI* is visible in non-hair cells, expanding laterally as the meristem matures (third and fourth cross-sections, respectively). Red lines illustrate the height of the corresponding cross section. Scale bars are 50µm in the longitudinal and 20µm in the cross sections.

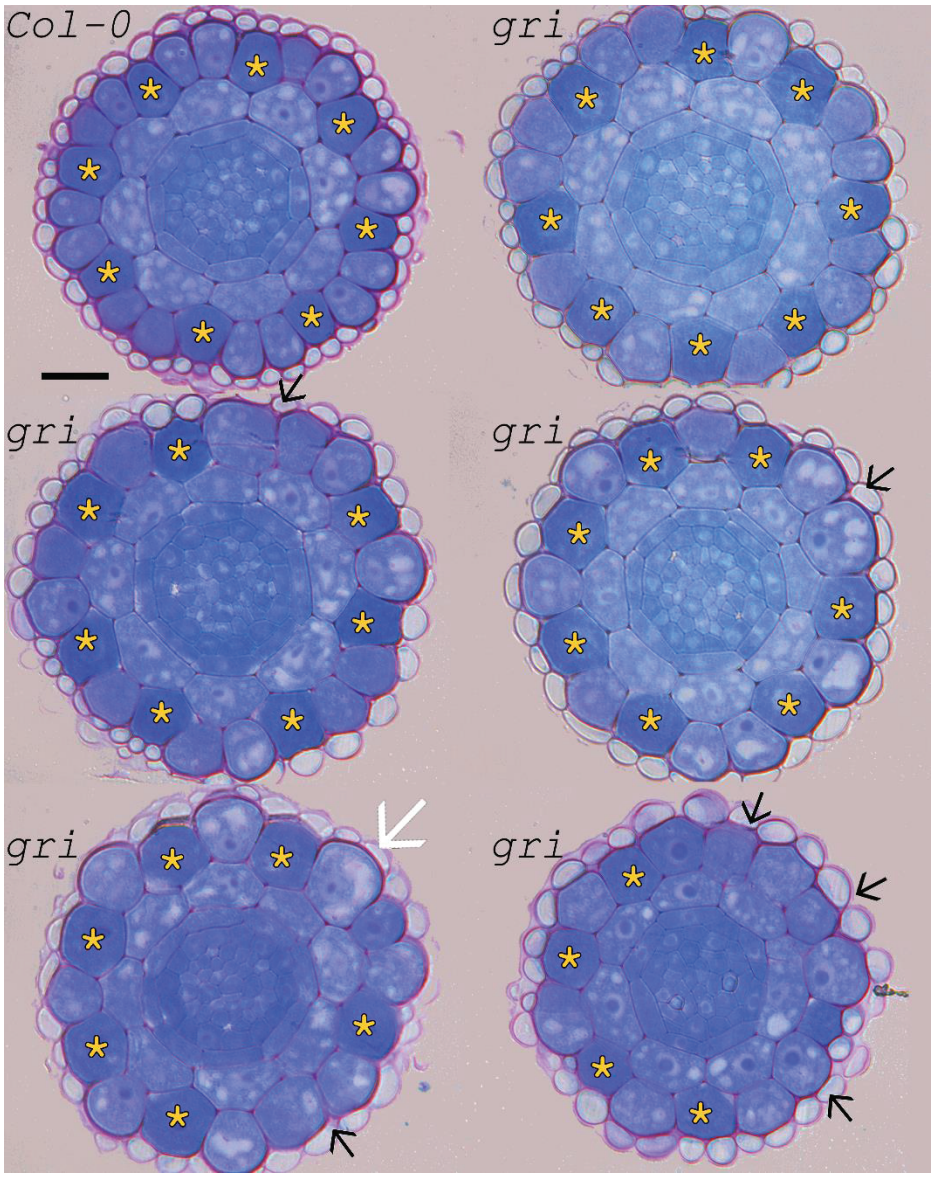

Figure 18: Anatomical sections of primary roots of wild type *Col-0* and *gri* mutants stained with toluidine blue at 5dpg. The root hairs in *Col-0* are regular, alternating with two non-hair cells. This pattern is disturbed in several of the sectioned *gri* roots, which show a lack of roots hairs at the expected position (indicated by black arrows). In some roots, two neighbouring epidermal cells touched two underlying cortical cells (white arrow). Yellow asterisks mark epidermal hair cells, determined based on their position (touching two underlying cortical cells) and darker staining. Scale bar 20µm.

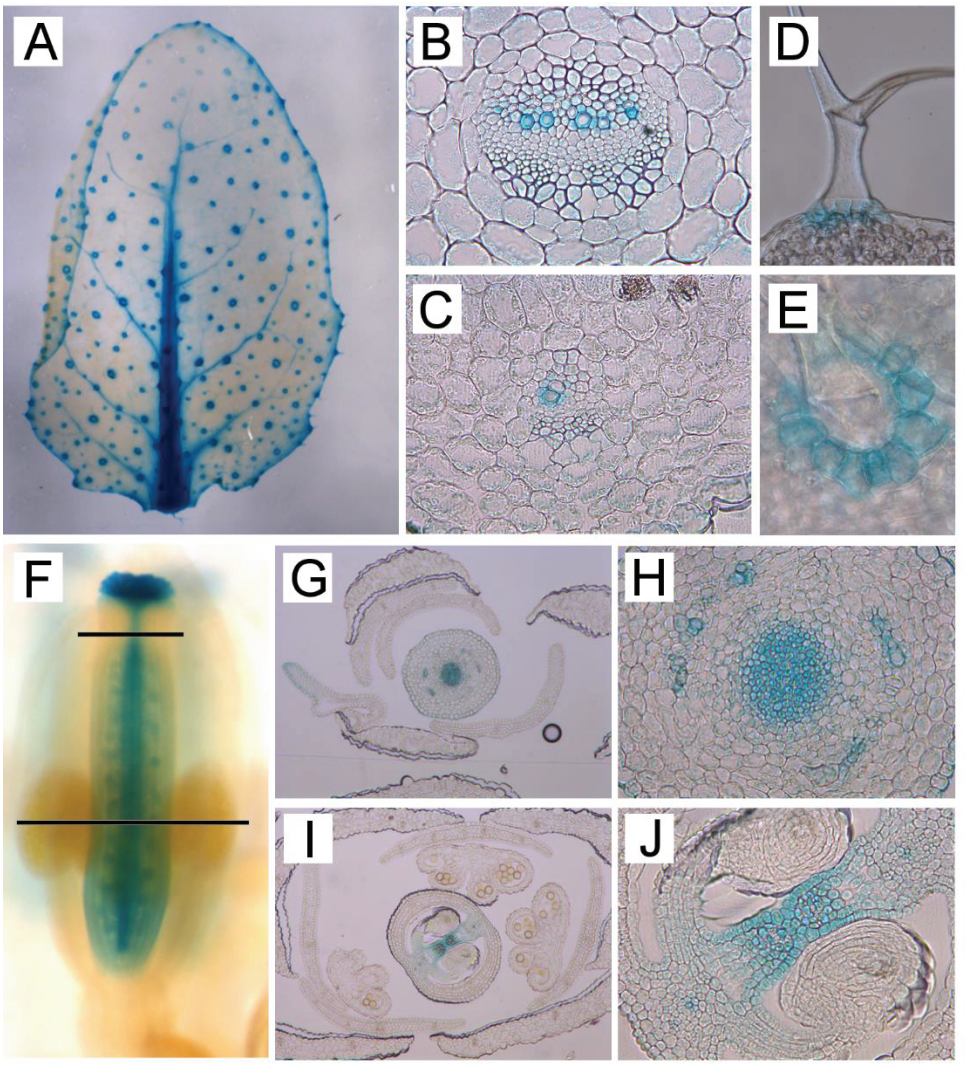

Figure 19: Expression of *GRI::GUS* in the shoot. Expression was detected in several organs, including (A) young rosette leaves, where (B) the expression was strongest in the most recently differentiated xylem cells of the midvein, with (C) similar localization in the side veins. In addition to the vasculature, *GRI::GUS* is also expressed in (D, E) trichome basal cells and (F) flowers, where expression is strongest in the stigma. *GRI::GUS* is also expressed (G, H) in vascular bundles below the stigma and (I, J) in the replum (I and J).

4.3.2. *Arabidopsis* meta-caspase 9 localization and function

The *Arabidopsis meta-caspase 9* has been shown to regulate xylem maturation by promoting PCD (Bollhöner *et al.,* 2013). We analysed the expression pattern of *AtMC9::GUS* and saw signal in the lateral root cap and epidermis in the RAM of young 5-7 d old seedlings (Figure 20) which is similar to that of *GRI::GUS* (Figure 17), and consistent with published expression patterns (Bollhöner et al, 2013 and Tsiatsiani *et al.,* 2013). However, unlike *GRI*, *AtMC9* is also expressed in

developing xylem cells, as was previously reported by Bollhöner *et al.* (2013). While earlier publications reporting the *AtMC9::GUS* pattern do not show strong staining in the stele of the transition and differentiation zone, the signal is clearly visible in Figure 20. However, the published transcriptional fluorescent marker pattern (Bollhöner *et al.,* 2013) matches the signal seen here, with expression in protoxylem cells undergoing differentiation and secondary cell wall formation (spiral lignification), indicating that the difference in the GUS patterns might be due to staining techniques. *AtMC9* has been shown to regulate vacuolated programmed cell death (PCD) of TEs in xylem vessels (Bollhöner *et al.,* 2013). Vacuolated cell death is a slow and controlled process critical for the formation of clear conductive cells, and differs from rapid, necrotic cell death (Minina *et al.,* 2013).

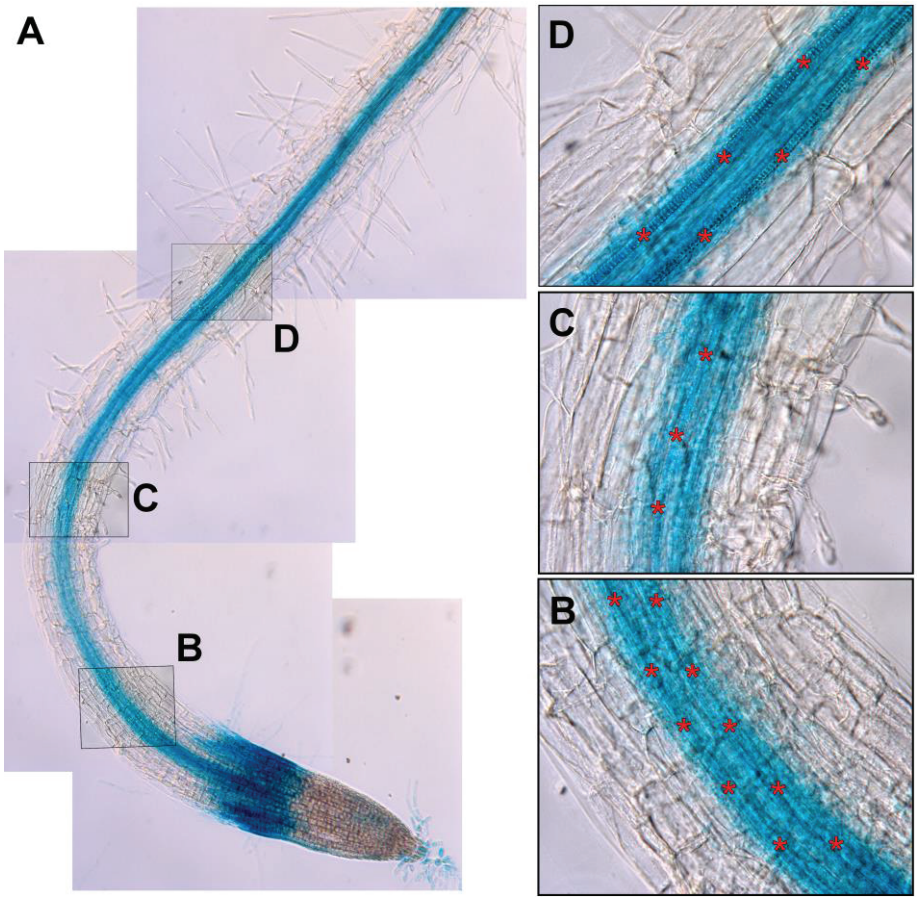

Figure 20. The expression pattern of *AtMC9::GUS* in 7 d old *Arabidopsis* seedlings. (A) A whole mount showing a strong GUS signal in the epidermis of the meristem transition zone and stele-specific expression higher up in the mature root. (B-D) Close ups from the marked positions in the whole mount, showing the stele signal (B) in the elongation zone, (C) at the beginning of the differentiation zone, which is characterized by root hair formation and the first signs of protoxylem lignification, and (D) in the mature root, where protoxylem lignification is complete but metaxylem cells have not yet formed their distinctive secondary cell wall pattern. Red stars mark protoxylem strands.

In roots, *AtMC9* may be required for both clearing and autolysis of protoxylem and metaxylem, as its expression precedes lignification and disappears from both protoxylem and metaxylem positions post-mortem (GUS staining data not shown), as was reported previously (Bollhöner *et al.,* 2013). This changing expression pattern is consistent with reports that *AtMC9* is a downstream target of xylem identity TFs in *Arabidopsis* (Bollhöner *et al.,* 2013). While *AtMC9::GFP* expression is highly specific to xylem cells which are about to die, it appears that *AtMC9* is not required for the initiation of cell death; knock-out lines undergo PCD and TE element differentiation similarly to wild type plants. However, *AtCM9* is required for post-mortem autolysis of TEs and is believed to regulate the function of several proteases required for the final clearing of these cells.

4.3.3. PRK5 localization and function

In tomato, the *GRI* homolog *STIG1* binds to the receptor kinases *LePRK1* and *LePRK2*, promoting *in vivo* pollen tube growth (Tang *et al.,* 2004, Löcke *et al.,* 2010, Huang *et al.,* 2014). The *Arabidopsis* gene *POLLEN RECEPTOR-LIKE KINASE 5* (*PRK5*) was first characterized in 2005 by Wang *et al.* in a paper that focused on its structural resemblance to plant defence proteins. Its expression levels were measured in rosettes, siliques and inflorescence stems. While rosettes and siliques showed low expression levels, the inflorescences had significantly greater *PRK5* expression. Unfortunately, only *PRK5* levels were reported; its localization pattern in tissues was not presented, nor have any publications since 2005 reported its expression pattern, leaving the developmental role of the receptor open to speculation. However, some information about the localization and putative function of *PRK5* can be deduced from its orthologs in other species. Transient overexpression of *LePRK1* leads to altered pollen tube growth (Gui *et al.,* 2014) as the pollen tube tips switch from tubular growth to blebbing growth. This is caused by altered actin cytoskeleton organization. Thus, in tomato, *LePRK1* is involved in pollen tube outgrowth. This in an interesting datum, as pollen tube growth in *Arabidopsis* is also regulated by ROS signalling (Potocký *et al.,* 2007, Duan *et al.,* 2014,). We analysed the subcellular localization of PRK5 in *Arabidopsis* leaf protoplast assays (OP IV Fig. 2B, E and D) and in tobacco epidermal cells. As expected of a receptor that binds apoplastic peptides, PRK5 was expressed at the plasma membrane (OP IV Sfig. 10).

4.3.4. GRI-AtMC9-PRK5-signalling module

The induction of *GRI* by extracellular ROS and its effect on plant stress tolerance has been previously described (Wrzaczek *et al.,* 2009a and 2009b); however, these publications focused only on the truncated, 66-amino acid GRIp$^{31\text{-}96}$ first isolated from ozone-sensitive *gri* mutants. In OP IV, we show that a smaller, synthetically produced 20-amino-acid fragment of the GRI protein (GRIp$^{65\text{-}85}$ peptide) is sufficient to cause elevated ion leakage (cell death) and that the *AtMC9* metacaspase is responsible for cleaving the GRI protein *in vivo* (OP IV Fig. 1A, B). The experimentally confirmed AtMC9 cleavage sites in GRI are shown in OP IV, Fig. 4C. This processing of the GRI protein is dependent on AtMC9 activity, as treatment of *atmc9* mutants with GRIp$^{31\text{-}96}$ did not induce ion leakage. However, infiltration experiments showed that two shorter peptides (20-aa GRIp$^{65\text{-}84}$ and 11-aa GRIp$^{68\text{-}78}$) were sufficient to cause elevated ion leakage in *atmc9* mutants. The GRIp$^{65\text{-}84}$ and GRIp$^{68\text{-}78}$ peptides are biologically active *in vivo*, binding to the receptor *PRK5*, which is a leucine-rich repeat, plasma membrane-localized

protein (OP IV Fig. 2B), though it is an atypical, enzymatically inactive RLK (OP IV Fig. 2G). The receptor-ligand binding triggers cell death. *PRK5* is essential for *GRI* signalling, as the *prk5-1* and *prk5-2* loss-of-function mutants do not undergo cell death upon peptide application.

4.3.5. PRK5 receptor internalization

We also tested whether different GRI peptides can affect the subcellular localization of the *PRK5* receptor. We treated protoplasts with $GRIp^{31-96}$, $GRIp^{31-51}$, $GRIp^{47-68}$, $GRIp^{65-84}$ and $GRIp^{80-96}$. Only the long $GRIp^{31-96}$ and the short $GRIp^{65-85}$ peptides – both of which also cause increased ion leakage – led to internalization of the receptor from the plasma membrane, visible in Figure 21 (middle row) as blue dots inside the protoplasts and, in some cells, leading to regions of the plasma membrane without PRK5-CFP (dark lesions in Figure 21, middle row). The observed internalization was nearly identical to the internalization of FLS2-GFP upon flagellin (flg) treatment (Figure 21, top row), indicating that the receptor gets internalized upon ligand binding.

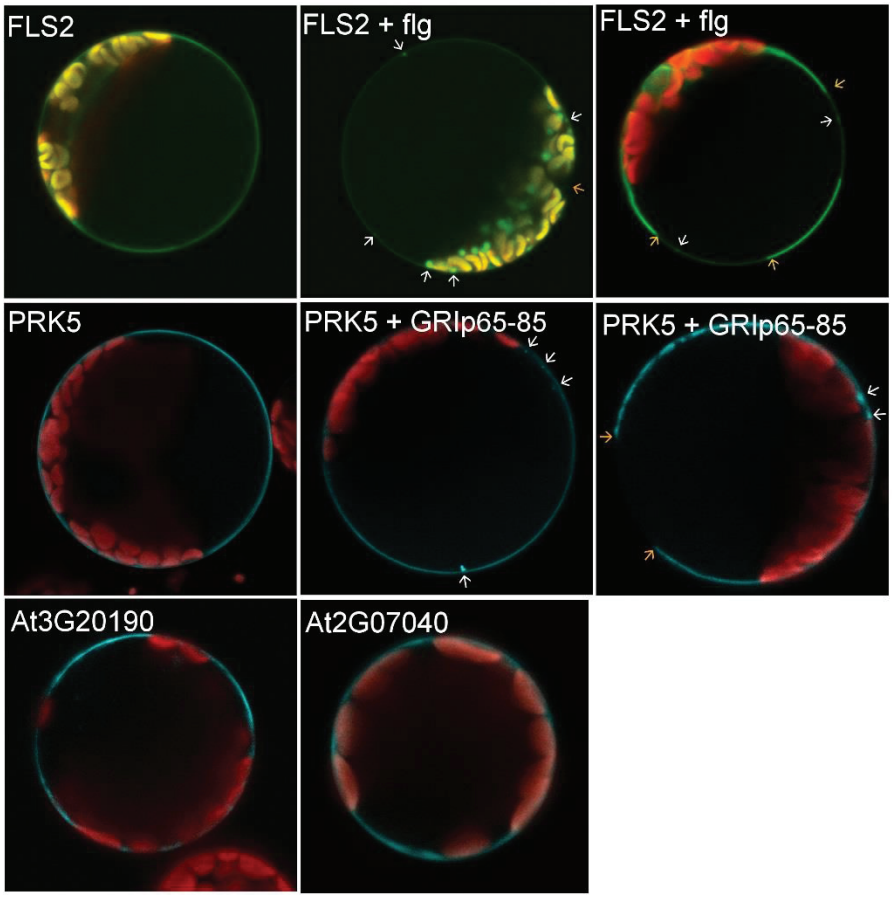

Figure 21. Plasmambrane localization of FLS2, PRK5 and At3G20190 and At2G07040 – PRK5 is internalized opon upon peptide treatments: ***Top row:*** left panel: FLS2-GFP expression at the plasma membrane of an *Arabidopsis* protoplast. Middle and right panels: FLS2::GFP protoplasts incubated with flagellin (flg). FLS2-GFP

disappeared from the plasma membrane (yellow arrows) and was internalized, forming cytoplasmic aggregates (white arrows). ***Middle row:*** left panel: PRK5 localization at the plasma membrane of an *Arabidopsis* protoplast. Middle panel and right panels: PRK5-CFP incubated with GRip$^{65-85}$, showing internalization (white arrows) and the loss of plasma membrane localized signal (yellow arrows) similar to FLS2+flg protoplasts. ***Bottom row:*** Expression of the two *PRK5* homologs, At3g20190-CFP and At2g07040-CFP, which both localize to the plasma membrane.

We measured the percentage of protoplast cells showing internalization (scored by formation PRK5::CFP aggregates) upon incubations with different GRI-peptides. GRip$^{65-85}$ is the only peptide that showed statistically increased internalization (Figure 22). Coupled with the ion leakage data, this indicates that the internalization of PRK5 might be biologically relevant for GRI-mediated cell death signalling. We also tested the effect of peptide dosage on PRK5-CFP internalization and found that increasing the concentration enhances receptor internalization (Figure 22). Further work is needed in order to understand which signalling components interact with PRK5 and whether the receptor internalization is required for signal transduction to the nucleus.

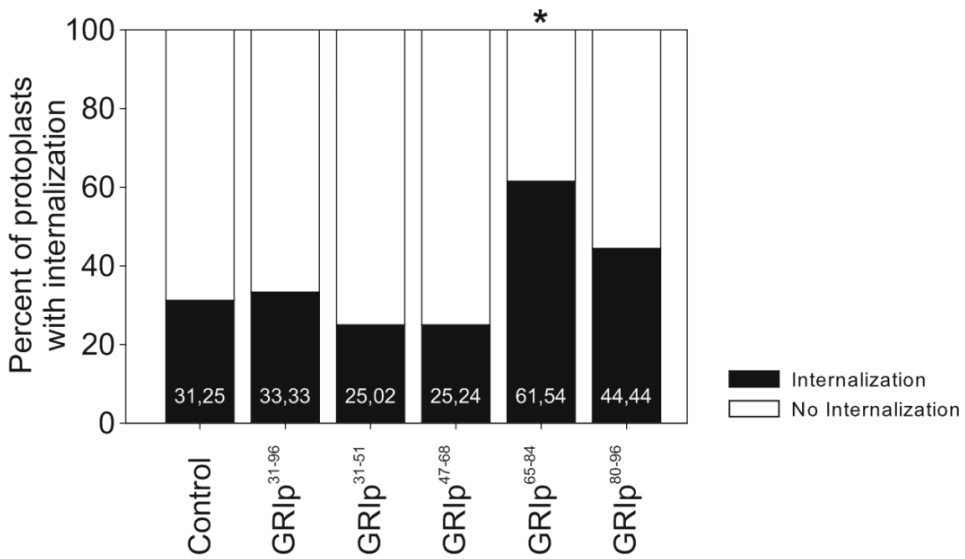

Figure 22. Internalization of PRK5. Quantification of vesicle formation (receptor internalization) of PRK5-CFP in untreated protoplasts and after treatment with GRIp$^{31-96}$, GRIp$^{31-51}$, GRIp$^{47-68}$, GRIp$^{65-84}$ or GRIp$^{80-96}$. Asterisks indicate statistically significant differences from controls according to Tukey's HSD test ($P<0.05$)

4.3.6. Conclusions of part III

Our published research and the unpublished data presented in this thesis illustrate a novel signalling module which consists of three main elements: 1) Upregulation of *GRI* expression upon ROS (which can be mimicked by treating cells with either the truncated 66-amino acid GRIp$^{31-96}$ or the shorter 20- and 11-amino acid GRIp$^{65-85}$ and GRIp$^{68-78}$ peptides). 2) Cleavage of the GRI protein by AtMC9 to form functional signalling peptides. 3) Binding of these peptides to PRK5 at the plasma membrane, conveying an apoplastic signal into the cell,

possibly via receptor internalization, leading cell death (measured by elevated ion leakage levels).

Considering that *GRI* is expressed in the leaf vasculature and cross sections show expression in developing xylem cells, it could very well be that *GRI* (possibly with *AtMC9* and *PRK5*) is involved in vascular development, but only in the later developmental stages and only in shoots. This is supported by the fact that, like *GRI*, *PRK5* is expressed in the leaves (OP IV, SFig. 3, 4, 5, 6, 7, and 8). Even though *GRI* is expressed in the primary root meristem epidermis and might have a role in hair cell formation, the published GRI-MC9-PRK5 module is probably not directly linked to primary root proximal meristem procambial patterning and stele development. That said, we cannot exclude the possibility that a similar signalling mechanism might indeed shape the stele, possibly via ligand-receptor pairing of one of *GRIM REAPER*'s homologs with *PRK5* or its close homologs, At3g20190 and At2g07040 (Figure 21). Further work on the localization, function and ligands on these receptors is needed to verify or disqualify their involvement in proximal meristem vascular patterning.

Our data and published articles show that ROS signalling has a role in RAM development. Following up on this work by focusing on xylem patterning and root growth could give us new insight into root development, adding yet another hormone/signalling mechanism to the already complicated picture of meristem dynamics.

5. Concluding remarks and future perspectives

Our published articles and the additional data presented and discussed in this thesis illustrate the necessity for the maintenance of undifferentiated procambial cells, as their existence is a crucial factor for proximal meristem vascular patterning. Many of the signalling interactions described in this thesis occur in the procambium, and if procambial cell identity is lost (via cell death, loss of cytokinin signalling, etc.), the entire proximal meristem pattern can change via the destabilization and rearrangement of the auxin and cytokinin hormonal signalling domains. Likewise, the re-generation of procambial cell identity through inducible cytokinin perception was shown to repress ectopic xylem formation, enhance PIN-mediated auxin transport and allow de-novo procambial re-patterning, leading to the formation of phloem-like cells in the proximal meristem. Long-term NPA treatments caused hyper-activation of procambial cell proliferation which, in combination with *CKX1* inductions – and through the epistasis of auxin and cytokinin signalling – leads to supernumerary xylem formation. All these data suggest that as long as the plants have the capacity to produce undifferentiated procambial cells and the key components of the auxin-cytokinin loop are functional, vascular patterns can be modified – and even regenerated from seemingly fixed cell fates.

The auxin-cytokinin interaction presented in this thesis work has recently been shown to regulate the vascular cambium activity also in Populus trichocarpa as well (Immanen et al., unpublished). Like As in Arabidopsis, auxin and cytokinin display different distribution profiles across the vascular cambium of the Populus trunk; the auxin maximum is located at in the actively dividing cambial cells, – whereas the cytokinin signalling maximum is located at in the developing phloem (Nieminen, 2009). Modifying these signalling domains – in particular, increasing cytokinin signalling via genetic modifications -– leads to increased cambial activity, which results in increased biomass production (Immanen et al., unpublished). Modifying the hormonal signalling status, and thus the hormonal interactions, in specific cambial zones could potentially be used in as a sophisticated tool in forest tree breeding programs in the future, perhaps by utilizing the CRISPR genome genome-editing technology to avoid generating populations of potentially controversial transgenic forest trees.

While this thesis has mainly focused on describing the radial patterning mechanisms required for proper proximal meristem patterning, temporal control of meristem maturation was also discussed from the point of view of protoxylem differentiation and autolytic PCD. As meristem initials acquire their fate at the proximal meristem, this information has to be maintained up the differentiation zone, which requires a series of parallel genetic cascades in neighbouring tissues. If, for some reason, the genetic pathways that control protoxylem cell identity are not properly maintained, perhaps due to mutations in the components described in our model (auxin signalling, cytokinin signalling, *AHP6,* PINs*)* or downstream (*VND7* or cell wall modifying enzymes), the expected outcome of cell death and continuous vessel formation is not guaranteed, which leads to breaks in the xylem. As antagonizers, auxin and cytokinin are entwined in a mutually repressive interaction loop in which the hormone with the stronger signalling defines cell identity. In the proximal meristem, where most cell identities are established (except the companion cells), auxin has the leading role. At the transition zone,

auxin signalling levels diminish and cytokinin signalling takes centre stage, promoting elongation and differentiation, which leads to sieve element and xylem tracheary element autolysis. This process likely involves the incorporation of ROS signalling, perhaps to decrease local auxin levels via oxidation while simultaneously promoting the switch from replication to endoduplication by boosting cytokinin signalling. It would be thrilling to identify a specific mediator of ROS signalling (such as a GRI-related peptide) as a regulator of xylem identity, possibly working downstream of *AtMC9* or a similar peptide-processing enzyme.

While our understanding of the complexity of the interactions and interdependencies of hormone signalling pathways during proximal meristem vascular patterning has increased considerably in recent decades, the temporal aspect of cell fate maintenance and the interaction between cell-to-cell signalling mechanisms in different meristematic zones during meristem maturation are still mostly unclear. Interestingly, differentiation of protoxylem and phloem sieve elements appear to be similarly regulated (i.e., NAC-family genes are essential for the progression of PCD in both tissues), yet the exact mechanisms regulating primary root maturation on the tissue level are not fully understood. Future research efforts should aim at building a comprehensive picture of the converging radial patterning pathways that create different cell types within the proximal meristem and understanding if the genetic cascades in different tissues that lead to the differentiation of stele cells interact, as well as whether the differentiation rates of various stele cells depend on shared common denominators, such as ROS.

It is important to understand if and how ROS signalling is involved in xylem development and vascular patterning in the proximal meristem of the primary root, and whether ROS is required to synchronize the elongation rates of different cell layers in order to maintain stable growth. During my PhD studies, I have used several parallel approaches to investigate the components required for the initial patterning and the maintenance and temporal regulation of the vascular pattern, some of which are not presented in this thesis. In addition the data presented here, I have performed microarray experiments to identity novel regulators of the auxin-cytokinin interaction affecting xylem fate establishment. The preliminary findings from these experiments support a recent study which identified several genes regulating early xylem patterning (De Rybel *et al.,* 2014). The data also indicate a connection between late xylem-identity transcription factors and several genes involved in cell wall modification and the regulation of oxidative stress prior to xylem autolysis. These data could be used to identify new genes required for auxin-cytokinin interaction and protoxylem identity formation, but also to reveal downstream signalling events and changes in root hormonal homeostasis that control xylem differentiation and PCD. I have also been characterizing mutants from a reverse genetic screen, which promise to shed more light on RAM patterning and maturation and establish a link between the "classical" hormone signalling and stress-related signalling pathways.

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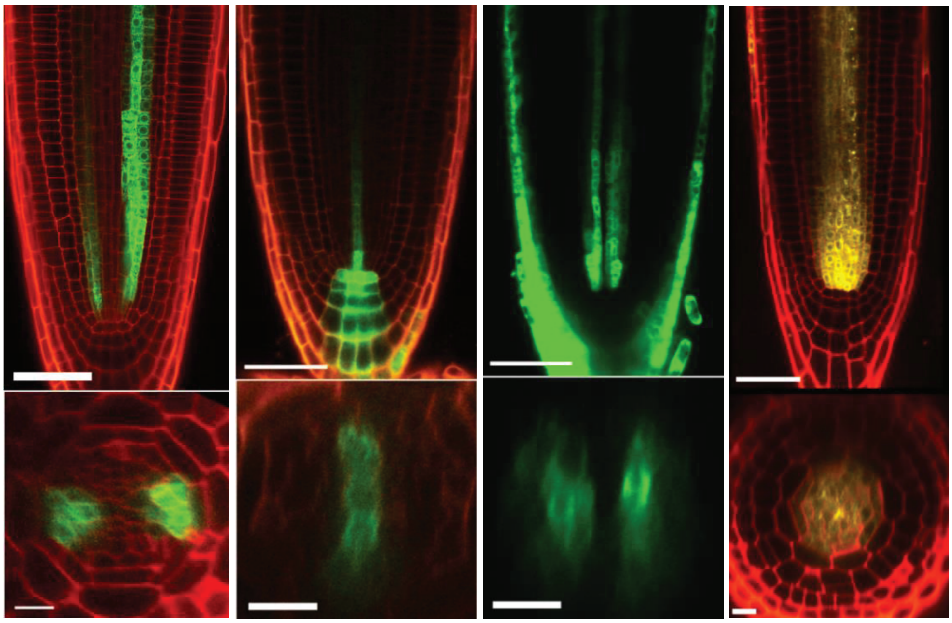
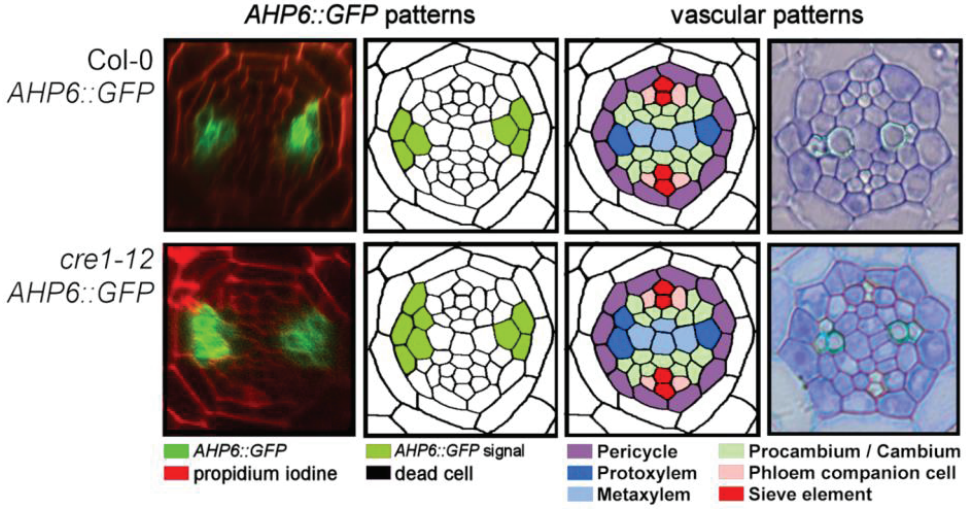
8. Appendix figures



A-Figure 1: Left to right: *AHP6::GFP*, *DR5::GFP*, *TCS::GFP* (version II) and *pCRE1-XVE::CKX1-YFP*. The xylem axis is oriented horizontally in AHP6::GFP cross-section and vertically in the *TCS::GFP* and *DR5::GFP* cross-sections. The rightmost panels show pCRE1-XVE::CKX1-YFP expression in the stele following a 24 h induction with 17-beta-estradiol. Scale bars are 50μm in longitudinal images and 10μm in cross-section.



A-Figure 2: Confocal images and graphical illustrations of *AHP6::GFP* expression in wild type Col-0 and *cre1-12* mutant roots, and graphical illustrations and plastic sections showing cell identities in Col-0 and *cre1-12*.