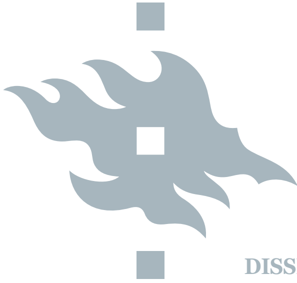

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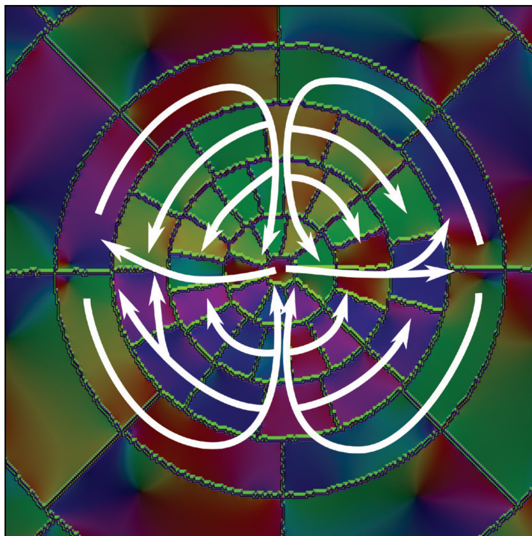
SEDEER EL-SHOWK

Auxin and Cytokinin Interactions Regulate Primary Vascular Patterning During Root Development in *Arabidopsis thaliana*



INSTITUTE OF BIOTECHNOLOGY AND
DEPARTMENT OF BIOSCIENCES
FACULTY OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES
DOCTORAL PROGRAMME IN PLANT SCIENCES
UNIVERSITY OF HELSINKI

Auxin and cytokinin interactions regulate primary vascular patterning during root development in *Arabidopsis thaliana*

SEDEER EL-SHOWK

Faculty of Biological and Environmental Sciences
Department of Biosciences
Institute of Biotechnology
University of Helsinki

ACADEMIC DISSERTATION

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

Thesis advisory committee Dr. Kirk Overmyer
Department of Biosciences
University of Helsinki

Dr. Mika Kotilainen
Department of Biosciences
University of Helsinki

Reviewed by Professor Hely Häggman
Department of Biology
University of Oulu

Dr. Isaac Salazar-Ciudad
Department of Biosciences
University of Helsinki

Opponent Professor Marta Ibañes Miguez
Faculty of Physics
University of Barcelona

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

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To my parents,
who made this possible,
and Hannele,
who saw me through it.

"Love words, agonize over sentences.
And pay attention to the world."
Susan Sontag

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List of original publications

This thesis is based on the following research articles, which are referred to in the text by their Roman numerals:

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IV **el-Showk, S**., Help-Rinta-Rahko, H., Blomster, T., Siligato, R., Marée, A.F.M., Mähönen, A.P. & Grieneisen, V.A. (2015) Parsimonious Model of Vascular Patterning Links Transverse Hormone Fluxes to Lateral Root Initiation: Auxin Leads the Way, while Cytokinin Levels Out. *PLoS Computational Biology* 11(10):e1004450.

Abbreviations

ARF	AUXIN RESPONSE FACTOR
AUX/IAA	AUXIN/INDOLE-3-ACETIC ACID
BA	6-benzylaminopurine
CLE	CLAVATA/EMRYO SURROUNDING REGION-related
CRF	CYTOKININ RESPONSE FACTOR
DNA	deoxyribonucleic acid
ENT	EQUILIBRATIVE NUCLEOSIDE TRANSFERASE
ER	endoplasmic reticulum
GFP	green fluorescent protein
GUS	β-glucoronidase
HD-ZIP	HOMEODOMAIN-LEUCINE ZIPPER
(A)HK	(ARABIDOPSIS) HISTIDINE KINASE
(A)HP	(ARABIDOPSIS) HISTIDINE PHOSPHOTRANSFERASE
IAA	indole-3-acetic acid
IPT	ISOPENTYLTRANSFERASE
LAX	LIKE-AUX1
LOG	LONELY GUY
miRNA	microRNA
NPA	1-naphthylphthalamic acid
NRT	NITRATE TRANSPORTER
PCR	polymerase chain reaction
PGP	phosphoglycoprotein
PIN	PIN-FORMED
PUP	PURINE PERMEASE
QC	quiescent centre
qRT-PCR	quantitative real-time PCR
RNA	ribonucleic acid
(A)RR	(ARABIDOPSIS) RESPONSE REGULATOR
TCS	two-component signalling
TMO	TARGET OF MONOPTEROS
Trp	L-tryptophan
VND	VASCULAR NAC DOMAIN

Abstract

The evolution of vascular tissues was a critical innovation in the colonization of land by plants. We investigated how vascular tissues, in particular xylem, are patterned in the root of the model plant *Arabidopsis*. The vascular tissues of the *Arabidopsis* root tip are consistently patterned as a xylem axis flanked by procambial cells, with phloem poles developing perpendicular to the xylem axis. Cytokinin signalling inhibits the specification of protoxylem; the *AHP6* gene inhibits cytokinin signalling at the protoxylem position during normal vascular development.

We sought to understand the factors regulating *AHP6* expression in the root tip. Cytokinin signalling is known to flank the xylem axis; we discovered a complementary domain of auxin signalling throughout the xylem axis. Based on this, we showed that auxin upregulates *AHP6*, creating a domain of low cytokinin signalling, and also acts to specify protoxylem. We used a combination of mutants and pharmacological treatments to investigate how mutually exclusive auxin and cytokinin signalling domains are maintained in the *Arabidopsis* stele. We discovered a feedback loop between the hormones, in which cytokinin activates auxin exporters, while auxin represses cytokinin signalling. The mutual inhibition between auxin and cytokinin regulates the extent of their domains during vascular patterning.

We turned to computational simulations to investigate the sufficiency, stability, and dynamics of this network. Our simulations confirmed that the network is sufficient to maintain the hormone domains during vascular patterning, but also revealed a role for auxin importers, which we confirmed through experiments. While cytokinin is frequently thought to form gradients guiding developmental processes in the *Arabidopsis* shoot and root, we showed that an informative cytokinin gradient cannot form on the scale of these tissues via diffusion. While auxin is patterned through the activity of polarly localised transporters, there is no evidence for similar transport of cytokinin. Nevertheless, our findings highlight the need for a cytokinin patterning mechanism, such as directed cytokinin transport or patterning of the cytokinin perception machinery, since diffusion cannot form the observed cytokinin patterns.

Finally, we discovered a potential link between the auxin-cytokinin feedback loop in the root tip and the initiation of lateral roots. Since our experimental data are equivocal on whether or not PIN1 is polarly localised in the procambium, we investigated both possibilities in our computational model. We discovered that polar localisation of PIN1 results in a regular flux of auxin towards the centre of the stele and back out via the xylem axis. This circuit privileges pericycle cells flanking the xylem axis to accumulate auxin if they experience a brief activation of an auxin importer; activation of the importer *AUX1* in the xylem-pole pericycle cells is one of the earliest steps in lateral root initiation.

Altogether, my thesis reveals a key role for mutually inhibitory auxin-cytokinin interactions in vascular development and links these findings to other developmental contexts. This work also demonstrates how the combination of experimental & computational approaches enables us to critically evaluate our models and develop more general insights.

Tiivistelmä

Johtosolukon evoluutio oli tärkeä askel kasvien siirtyessä vedestä maalle. Tutkimme, miten juuren johtosolukot muodostuvat mallikasvissa Arabidopsisissa, eli lituruohossa. Johtosolukot lituruohon juuren kärjessä ovat ksyleemi (puu), nila ja esijälsi. Ksyleemisolut sijaitsevat säännönmukaisesti rivissä juuren keskellä ja esijälsi ksyleemi- ja nilasolujen välissä. Sytokiniinisignalointi estää alkuksyleemin määrittelyn. Juuren kehittyessä, *AHP6*-geeni normaalisti ehkäisee sytokiniinisignalointia alkuksyleemissä.

Kysyimme, mitkä tekijät säätelevät *AHP6*:n ilmentymistä juuren kärjessä. Sytokiniinisignaloinnin alue on ksyleemin rivin viereisissä soluissa ja me havaitsimme, että auksiinisignalointi on ksyleemin rivissä. Näin ollen, auksiinisignalointi ja sytokiniinisignalointi ovat toisensa poissulkevia juuren poikkileikkauksessa. Osoitimme, että auksiini edistää *AHP6*:n ilmentymistä ja alkuksyleemin määrittymistä. Sytokiniinisignalointia ei tapahdu *AHP6*:n ilmentymisen alueella. Tutkimme mutanttien ja lääkkeiden avulla miten auksiini- ja sytokiniinisignaloinnin toisensa poissulkevat alueet ylläpidetään litruohon juuren kärjessä. Havaitsimme takaisinkytkennän, joka säätelee kasvihormonien ilmentymisalueita. Sytokiniini lisää auksiinin poistamista soluista, kun taas auksiini ehkäisee sytokiniinisignalointia edistämällä *AHP6*:a. Siten auksiinin ja sytokiniinin molemminpuolinen estäminen säätelee näiden kasvihormonien ilmentymisalueita johtosolukon muodostuessa.

Käytimme tietokonesimulaatioita tutkiaksemme geneettisen verkon vakautta, riittävyyttä ja dynamiikkaa. Simulaatiomme vahvistivat, että verkko on riittävä ylläpitämään kasvihormonien alueita johtosolukon muodostuessa. Simulaatiot osoittivat myös, että auksiinin tuonti soluihin vaikuttaa johtosolukon kehitykseen, ja vahvistimme tuonnin merkityksen kokeilla. Lisäksi, vaikka on ehdotettu että sytokiniinin gradientit ohjaavat kehitystä lituruohon varsissa ja juurissa, osoitimme, että sytokiniinin gradientti ei voi muodostua näiden solukoiden mittakaavassa diffuusiolla. Auksiinin liikkuminen tapahtuu polaarisilla kuljetusjärjestelmillä, mutta sytokiniinin kuljetusjärjestelmiä ei vielä ole selvitetty. Tuloksemme osoittavat, että havaittujen sytokiniinikuvioiden muodostuminen vaativat mekanismin, esimerkiksi suunnattu sytokiniinin kuljetus tai erilainen sytokiniinisignalointi eri soluissa, koska havaitut sytokiniin kuviot eivät voi muodustua ainoastaan diffuusiolla. Havaitsimme myös, että auksiini-sytokiniini takaisinkytkentä juuren kärjessä voisi mahdollisesti vaikuttaa versojuurien kehityksen initiaatioon. Koetuloksemme eivät selvästi osoita, onko PIN1-proteiinin (joka poistaa auksiinin soluista) sijainti esijälsisoluissa polaarinen vai ei. Siksi tutkimme molempia mahdollisuuksia simulaatioilla, ja havaitsimme, että kuljetusjärjestelmän polaarinen sijainti aiheuttaa ensin säännöllistä virtausta juuren keskukseen ja sitten pois ksyleemin kautta. Virtaus suosii auksiinin kertymistä lieriökettosoluihin ksyleemin vieressä, jos auksiinipitoisuus näissä soluissa nousee lyhyesti.

Tässä väitöskirjassa on kerrottu, että auksiinin ja sytokiniinin molemminpuolinen estäminen on tärkeä tekijä johtosolukon muodostumiselle ja että se vaikuttaa myös muihin kehitysprosesseihin. Havainnollistimme myös, että kokeelliset ja laskennalliset menetelmät yhdessä auttavat mallien arvioinnissa ja oivalluksien tekemisessä.

Summary

Introduction

Plant Vascular Development

The problem of development

The fundamental problem of development is how organized multicellular structures are produced from a single founder cell by controlling the processes of replication and growth. Development is a dynamic process involving patterns of genetic and hormonal activity mediated by and acting in cells which grow and divide; the processes that constitute and regulate it must reliably reproduce a structure under variable conditions while retaining the ability to adjust to environmental demands. Multicellularity has evolved independently in several groups of organisms, each of which has found their own solution to the problem of reliably regulating cell division and differentiation to form a body.

The most common recent ancestor of plants and animals was unicellular, so they have evolved very different mechanisms to regulate their development. The life history of plants differs fundamentally from that of animals, a factor which is both a cause and consequence of the profound differences in their development. Plants are sessile, photosynthetic organisms; unlike animals, they cannot respond to environmental changes by moving to a different location. Their development therefore exhibits far greater flexibility in the face of changing conditions. While animal development is restricted to specific life history phases and is buffered against environmental effects, plants develop throughout their life, generating novel organs and structures in a continuous dialogue with their environment. These differing modes of development influence a wide range of life history traits, from tolerance of adverse environments to the ease of hybridization, and thus impact the process of evolution in these two groups of organisms.

Furthermore, unlike animal cells, plant cells are both immobile and totipotent. Cell migration plays a major role in animal development, but rigid cell walls prevent plant cells from moving. Plants grow from regions of actively dividing, undifferentiated cells known as meristems, which may be determinate or indeterminate. Differentiation of plant cells is based on their position within the tissue; environmental, physiological and developmental cues are integrated in the regulation of growth and the initiation of new organs.

Development in plants is controlled by growth regulators or hormones, a group of signalling molecules which regulate cellular processes including growth, division, and

gene expression. Plant hormones are locally produced throughout the organism, and hormone synthesis, metabolism, and transport serve as a computational system for plants, integrating information from a wide range of sources to determine a course of action. Auxin and cytokinins represent two major classes of phytohormones and are involved in regulating nearly every aspect of plant development, often acting antagonistically. In this thesis, I investigate how auxin and cytokinin interact during vascular patterning in the model plant *Arabidopsis thaliana*.

Auxin in plant development

The story of auxin can be traced back over 250 years to experiments investigating the influence exerted by one plant organ on another; early researchers posited special substances which move through plants to control their growth and development, which we now recognize as hormones (Abel and Theologis, 2010). In the late 19th century, Charles Darwin famously discovered that phototropism and gravitropism are mediated by the growing tips of the shoot and root. Darwin demonstrated that covering the tip of a shoot exposed to a directional light source prevented the growing plant from curving towards the light; by contrast, covering the basal portion of the shoot had no effect. He concluded that "some influence is transmitted from the upper to lower part, causing the latter to bend" (Darwin, 1880). Half a century later, Frits Went discovered the substance responsible, which was named auxin from the Greek verb $\alpha\upsilon\xi\varepsilon\iota\nu$ (auxein), meaning "to grow/increase" (Abel and Theologis, 2010). Since its discovery as a regulator of tropisms, auxin has emerged as a central player in nearly every aspect of plant growth and development, from cell division and elongation to senescence, organ formation, and vascular development (Sauer et al., 2013).

Auxin metabolism and perception

Auxin is synthesized from precursors generated via the shikimate pathway, primarily L-tryptophan. Four Trp-dependent biosynthesis pathways involving a wide array of enzymes are known; the names of the pathways (IPyA, IAM, TRA, IAOx) are based on the first metabolite formed after L-Trp. Evidence also exists for a tryptophan-independent auxin biosynthesis pathway branching from the precursor indole-3-glycerol phosphate, although the genes and enzymes involved in this pathway are largely unknown. Auxin is deactivated by being degraded into oxIAA or reversibly inactivated by GH3-mediated conjugation with amides or esters. A thorough review of auxin metabolism has recently been provided by Ljung (2013).

Activation or repression of auxin-responsive genes is mediated by the AUXIN RESPONSE FACTOR (ARF) family of transcription factors. At low auxin concentrations, the ARFs are bound to AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins which inhibit their activity, thereby repressing the auxin response. Intracellular auxin binds to the TIR/AFB family of receptors which are part of the SCF^{TIR1} (SKP1, Cullin, F-box protein TIR1) ubiquitin protein ligase complex; the binding of auxin enables the SCF^{TIR1} complex to ubiquitinate the AUX/IAA proteins, targeting them for 26S-proteasome degradation and thus liberating the ARFs to act on downstream targets. The auxin response pathway therefore represents a "double negative" pathway, in which the presence of auxin removes the AUX/IAA proteins which normally inhibit the ARF transcription factors. The *Arabidopsis* genome

contains 29 AUX/IAA genes and 23 ARFs, leading to high levels of redundancy as well as as creating the potential for extensive combinatorial specificity, enabling auxin to regulate processes differently based on the expression of AUX/IAA and ARF genes (Weijers et al., 2005). The SCF^{TIR1}pathway is more thoroughly reviewed by Abel (2007) and Lau et al. (2008).

An alternative, proteasome-independent pathway was long thought to act via *ABP1* (reviewed by Sauer and Kleine-Vehn (2011); Scherer (2011)), but a recent study found no phenotype in a null *abp1* mutant generated via CRISPR/Cas9 or in a new T-DNA insertion allele of *ABP1* (Gao et al., 2015). The discrepancy between the new study and the *ABP1* data accumulated over previous decades is striking, particularly given that the phenotypes associated with *abp1* in the earlier studies were complemented by wildtype *ABP1* (Liu, 2015). Clearly, the results of the new study will need to be verified and those of the older studies revisited in order to understand the source of this inconsistency.

Auxin movement

Experiments with inhibitors of auxin transport have demonstrated that polar auxin transport provides an important signal during plant development (Liu et al., 1993; Casimiro et al., 2001; Mattsson et al., 1999). Auxin is the only plant hormone known to be transported in a polar manner; an asymmetric distribution of auxin transporters results in directed auxin transport through and within plant tissues. Biochemical and physiological studies in the 1970s led to the proposition of the chemiosmotic model of auxin transport (Rubery and Sheldrake, 1974; Raven, 1975) in which polar auxin transport occurs due to the difference in pH between the inside and outside of plant cells. The apoplast has a pH of 5.5, so auxin indole-3-acetic acid (IAA)—which is a weak acid—is protonated in the extracellular space; the protonated form (IAAH) can enter the cell by diffusing freely across the cell membrane. In the cytoplasm of the cell, which has a higher pH (7), IAA is converted into its charged form (IAA^-). Since the cell membrane is impermeable to IAA^-, it cannot travel out and remains trapped in the cell. Auxin can thus freely enter cells but has to be actively transported out by exporters located in the plasma membrane.

Cande and Ray (1976) abraded the cuticle of maize coleoptiles to expose the apoplast in order to measure the proportion of polar auxin transport occurring in the “free space” (apoplast); based on their measurements, they concluded that the “essentially all” auxin transport occurred via the apoplast and not symplastically via plasmodesmata. In addition, they observed no effect of plasmolysis on polar auxin transport in *Avena* coleoptiles, further arguing against a role for plasmodesmata in polar auxin transport. However, several recent studies have shown auxin-related phenotypes in plants with altered levels of callose, a polymer which controls the permeability of plasmodesmata by occluding the aperture (Vatén et al., 2011; Benitez-Alfonso et al., 2013; Han et al., 2014). Han et al. (2014) identified a feedback circuit between auxin and the callose synthase gene *GSL8*; this interaction could help reinforce the formation of an auxin gradient across a tissue, since increased callose deposition in cells with high auxin levels would reduce symplastic diffusion of auxin out of those cells. Furthermore, the regulation of plasmodesmatal aperture by callose biosynthesis offers an avenue via which auxin might regulate the symplastic movement of other molecules. Auxin has also been shown to regulate callose-

degrading genes during lateral root formation (Benitez-Alfonso et al., 2013). For an overview of the emerging role of plasmodesmata in modulating the distribution of auxin, the reader is referred to a review by Jackson (2015).

Experiments with radioactively labelled auxin derivatives have shown that transport is from the apex of the shoot towards the root (Robert and Friml, 2009). The chemiosmotic theory proposes that polar auxin transport occurs thanks to asymmetric localization of the efflux transporters on the plasma membrane. Over the last few decades, three main classes of proteins mediating auxin transport have been discovered in *Arabidopsis*, although other molecules, such as the NRTs (Guo et al., 2002; Krouk et al., 2010), may modulate auxin movement via interaction with the transporters.

The AUXIN RESISTANT 1/LIKE-AUX1 (AUX1/LAX) proteins serve as influx transporters of auxin. *AUX1* encodes a membrane-localized permease which has been implicated in auxin transport in *Arabidopsis* (Bennett et al., 1996; Marchant et al., 1999; Swarup et al., 2001) and shown to facilitate auxin import when heterologously expressed in *Xenopus laevis* oocytes (Yang et al., 2006). *LAX1*, *LAX2*, and *LAX3* have also been shown to be involved in auxin import (Bainbridge et al., 2008). Acting together with *AUX1*, *LAX3* regulates lateral root initiation and emergence (Swarup et al., 2008; Marchant et al., 2002), while *LAX2* has been implicated in vascular patterning (Péret et al., 2012). Although the chemiosmotic model proposes that auxin influx can occur even in the absence of auxin importers, it has been suggested that influx carriers may be crucial where rapid uptake is required to maintain gradients that would otherwise be lost due to diffusion in the apoplast (Swarup et al., 2005); given the spatial scale of plant meristems, diffusion of auxin through the apoplast would smoothen, or perhaps even eliminate, an auxin gradient unless auxin were somehow sequestered within cells. Computational modelling also indicates that uptake via auxin importers is crucial to maintain a high auxin level in tissues with small cells, such as the vascular tissues (Kramer, 2004).

Transcription of most of the auxin importers has been shown to be induced by auxin. A microarray experiment using *Arabidopsis* root tips reported that *AUX1* is upregulated by auxin (Laskowski et al., 2006); *LAX3* was also shown to be auxin-upregulated by qRT-PCR (Swarup et al., 2008). Observations of promoter-GUS fusions following treatment with the auxin 2,4-D showed an increase in *LAX1* and *LAX3* expression; however, no change was found in the expression of *AUX1* or *LAX2* (Péret et al., 2012).

The phosphoglycoprotein (PGP) subfamily of the ABC transporters were first suggested to have a role in auxin transport based on the observation of specific and tight binding of certain members to the auxin transport inhibitor 1-naphthylphthalamic acid (NPA) and reduced polar auxin transport in mutants (Noh et al., 2001; Murphy et al., 2002). *PGP1* and *PGP19* have been shown to mediate auxin efflux (Geisler et al., 2005; Petrásek et al., 2006); *PGP4* was initially thought to be an influx transporter based on its activity when heterologously expressed in yeast or HeLa cells (Terasaka, 2005), but was later shown to act as an efflux transporter in *Arabidopsis* (Cho et al., 2007).

The PIN-FORMED (PIN) transporters represent a second class of auxin efflux transporters. First identified as candidate auxin efflux transporters at the end of the last century, they exhibit homology to bacterial transporters (Gälweiler et al.,

1998; Luschnig et al., 1998; Müller et al., 1998; Utsuno et al., 1998; Chen et al., 1998), and were later confirmed to be a central component of polar auxin transport (Petrásek et al., 2006; Kramer and Bennett, 2006). The PIN and PGP transporters are known to interact in auxin transport. The expression patterns of *PGP1* and *PGP19* were found to overlap with *PIN1* in the shoot apex and with *PIN1* and *PIN2* the root, and combinatorial *pin* and *pgp* mutants show both additive and synergistic phenotypes (Blakeslee et al., 2007). Furthermore, PGP19 stabilizes the localization of PIN1 on the plasma membrane (Titapiwatanakun et al., 2009). Analysis of PIN and PGP over-expression lines showed that both transporter families are required for patterning during embryogenesis and organogenesis, although they also exhibit complex interactions, acting synergistically and antagonistically in different developmental contexts (Mravec et al., 2008). Despite these interactions, the PINs have been shown to be the rate-limiting efflux transporters in *Arabidopsis* regardless of the activity of the PGPs (Petrásek et al., 2006). The expression pattern of the PIN proteins and their localization on specific regions of the cell membrane is crucial in determining the overall pattern of auxin distribution and flux, which plays an important role in development and patterning processes (Grieneisen et al., 2007; Blilou et al., 2005; Wisniewska et al., 2006).

Physiological experiments in the 1960s suggested that polar auxin transport might be regulated by other phytohormones. Osborne and Mullins (1969) observed a decrease in the polar auxin transport capacity of excised *Phaseolus vulgaris* petiole segments several hours after excision; this was concomitant with a decrease in protein synthesis. Pretreatment with auxin or kinetin (a cytokinin) was found to prevent the decline in auxin transport, while treatment with ethylene enhanced it; furthermore, pretreatment with IAA, but not kinetin, protected the excised segments against the ethylene-induced decline in transport. Since treatment with kinetin increased protein transcription, Osborne and Mullins suggested that cytokinins regulate the synthesis of the (then hypothetical) auxin transporter protein, while ethylene modulates its activity. Recent research has confirmed the role of both ethylene (Růžička et al., 2007) and cytokinins (Dello Ioio et al., 2008; Laplaze et al., 2007; Růžička et al., 2009, Chapter II) in regulating PIN-mediated polar auxin transport.

Cytokinin in plant development

As the 19th century rolled into the 20th, several scientists discovered the existence of substances which could induce cell division when applied to cultured or wounded plant tissue (Wiesner, 1892; Haberlandt, 1913; van Overbeek et al., 1941). Miller et al. (1955) were the first to successfully isolate the active substance (from herring sperm) which was named kinetin; this was quickly followed by the identification of another cytokinin, trans-zeatin (Miller, 1961), and then many more (reviewed in Mok and Mok (2001)). Cytokinins are a class of phytohormones derived from adenine. Naturally occurring cytokinins can be divided into two groups based on their side chain: those with isoprene-derived side chains, which are predominant in plants, and those with aromatic side chains (Sakakibara, 2006). Although cytokinins were named for their ability to promote cytokinesis, they were soon discovered to promote shoot growth, inhibit root growth, stimulate cell division and induce greening in calli (Miller et al., 1956). They have since been found to play a role

in many other developmental processes in plants, including organ formation, leaf senescence, shoot meristem formation and maintenance, apical dominance and seed germination (Mok and Mok, 2001).

Cytokinin metabolism & perception

Research with *Arabidopsis thaliana* has identified a variety of genes involved in cytokinin biosynthesis. The seven ATP/ADP isopentenyltransferase genes (*IPT1*, *IPT3 - IPT8*) encode rate-limiting enzymes that synthesize the cytokinin precursor isopentenyladenine (Kakimoto, 2001; Takei et al., 2001), which is then hydroxylated by the activity of cytochrome P450 monoxygenases *CYP735A1* and *CYP735A2* (Takei et al., 2004b). Finally, enzymes encoded by the LONELY GUY (LOG) gene family convert inactive cytokinins into an active form (Kurakawa et al., 2007). Cytokinin is degraded by the CYTOKININ OXIDASE (CKX) gene family (Pačes et al., 1971; Houba-Hérin et al., 1999; Schmülling et al., 2003). The genes responsible for cytokinin synthesis and degradation are widely expressed throughout the shoots and roots of *Arabidopsis*, although individual genes have been shown to have quite specific expression patterns (Nordström et al., 2004; Miyawaki et al., 2004; Werner et al., 2003). For a comprehensive review of cytokinin synthesis and degradation, the reader is referred to Frébort et al. (2011).

The discovery that over-expression of *CYTOKININ INDEPENDENT* (*CKI*) induced a cytokinin response in plants (Kakimoto, 1996) was the first suggestion that cytokinin signalling is mediated by a two-component signalling (TCS) pathway similar to that found in bacteria, since CKI is homologous to the histidine kinase genes in bacterial TCS. The cytokinin TCS signalling pathway involves several players acting in relay to transfer a signal into the nucleus and activate downstream targets. In brief, cytokinins induce autophosphorylation of a histidine kinase (HK) protein, resulting in the transfer of a phosphoryl group from a phospho-accepting histidine residue in the kinase domain to an aspartate residue. The phosphoryl is then transferred to a conserved histidine on a histidine phosphotransferase (HP) protein. From there, it is finally transferred to an aspartate in the receiver domain of a response regulator (RR) protein; while some of the RRs are transcription factors, others lack the DNA-binding domain and so act as competitive inhibitors of cytokinin response.

The major components of the cytokinin signalling pathway were identified in a series of seminal papers around the turn of the century. The first cytokinin receptor was independently discovered by several research groups. Inoue et al. (2001) identified *CYTOKININ RESPONSE 1* (*CRE1*) as a cytokinin receptor based on the reduced cytokinin response of the loss-of-function mutants, while Mähönen et al. (2000) identified the same gene as a TCS histidine kinase responsible for the *wooden leg* (*wol*) mutation first described by Scheres et al. (1995). Simultaneous research with the same gene under the name *ARABIDOPSIS HISTIDINE KINASE 4* (*AHK4*) demonstrated that it could act as a cytokinin sensor in bacteria and that its histidine kinase activity was cytokinin-dependent (Suzuki et al., 2001; Ueguchi et al., 2001); meanwhile, Yamada et al. (2001) demonstrated the cytokinin-binding activity of *WOL/CRE1 in vitro* and showed that the *wol* mutation abolished this activity. Together with the other cytokinin receptors, *AHK2* and *AHK3* (Hwang and Sheen, 2001), *AHK4* has recently been shown to be localized primarily on the ER membrane, suggesting that this compartment may play an important role in

cytokinin signal transduction (Caesar et al., 2011; Wulfetange et al., 2011).

From the cytokinin receptors, the phosphorelay continues with the transfer of the phosphoryl group to a conserved histidine residue on the HP proteins, which shuffle continuously between the cytosol and the nucleus (Hwang and Sheen, 2001; Punwani et al., 2010). Five HP genes (*AHP1*-*AHP5*) with phosphorelay activity have been identified in the *Arabidopsis* genome (Suzuki et al., 1998; Imamura et al., 2001; Suzuki et al., 2002; Tanaka et al., 2004), along with a sixth pseudo-phosphotransferase (*AHP6*) which lacks the conserved histidine and inhibits cytokinin signalling by competing with the true HPs (Mähönen et al., 2006a).

Within the nucleus the phosphoryl group is transferred to an RR gene. The *Arabidopsis* genome encodes 23 ARABIDOPSIS RESPONSE REGULATOR (ARR) genes divided into three types (A, B, and C), although only the type A and type B ARRs are thought to play a role in cytokinin signalling (Kiba et al., 2004; Schaller et al., 2007). The type A ARRs, which are upregulated by cytokinin, contain a receiver domain fused to a short carboxy-terminal extension (D'Agostino et al., 2000) and are stabilized by phosphorylation (To et al., 2007). They are thought to act as inhibitors of cytokinin signalling, which has been confirmed by genetic analysis in several cases (To et al., 2004), although ARR4 has been shown to also interact positively with phytochrome B (Sweere et al., 2001). Type B ARRs possess a GARP DNA-binding motif in the carboxy-terminal extension in addition to the receiver domain; phosphorylation enables them to bind to DNA and initiate transcription of downstream targets (including the type A ARRs). Unlike the type A ARRs, the type B ARRs are not upregulated by cytokinin (Sakai et al., 1998; Imamura et al., 1999; Sakai et al., 2000, 2001). Studies of over-expression lines (Kiba et al., 2004; Tajima et al., 2004; Ren et al., 2009) and mutants with multiple ARRs knocked out (To et al., 2004; Argyros et al., 2008; Mason et al., 2005) have confirmed this general picture.

The CYTOKININ RESPONSE FACTOR (CRF) family, which consists of six closely related APETALA2 (AP2) transcription factors, was recently identified as a novel class of response regulators (Rashotte et al., 2006). The CRFs were found to migrate to the nucleus in an HK- and HP-dependent manner, although only *CRF2*, *CRF5* and *CRF6* were found to be cytokinin upregulated. Functional type B ARRs were found to be required for this upregulation, and further analysis showed that the CRFs and type B ARRs overlap in activating many cytokinin target genes. More recently, the CRFs have been shown to form both homo- and hetero-dimers with one another (Cutcliffe et al., 2011).

Cytokinin signalling is discussed in more depth in Chapter I and has been the subject of several recent reviews (e.g., Hwang et al., 2012; Heyl et al., 2012; Brenner et al., 2012).

Cytokinin movement

Very little is known about how cytokinin is taken up by cells and transported in plants. While diffusion is likely to be an important factor, evidence from cell cultures indicates that cytokinin can be actively taken up by cells (Fußeder et al., 1989; Cedzich et al., 2008), suggesting that directed cytokinin transport mediated by cytokinin transporters may occur. In addition, grafting experiments in tobacco (Faiss et al., 1997) and between *ipt1;3;5;7* mutants and wild-type *Arabidopsis* plants (Matsumoto-Kitano et al., 2008) have demonstrated the importance of long-distance

cytokinin transport. Furthermore, cytokinins have also been detected in sap and leaf exudates, indicating that they are present in the transport stream (Takei et al., 2002, 2004a).

The PURINE PERMEASE (PUP) gene family encodes broad-affinity transporters that represent the most likely candidates for cytokinin transporters. Direct, active uptake of adenine and various cytokinins by *PUP1* and *PUP2* has been demonstrated; together with their expression pattern, this has led to the suggestion that they may play a role in the loading and unloading of cytokinins for long-distance transport (Gillissen et al., 2000; Bürkle et al., 2003). Another class of potential cytokinin transporters is the EQUILIBRATIVE NUCLEOSIDE TRANSPORT (ENT) gene family, members of which have been implicated in cytokinin uptake in both rice (Hirose et al., 2005, 2008) and *Arabidopsis* (Sun et al., 2005). Although these transporters may play a role in cytokinin loading for long-distance transport, their broad affinity and the lack of strong phenotypes in the mutants suggests that they do not contribute to major processes that regulate plant development.

Auxin and cytokinin crosstalk

Auxin and cytokinin have long been known to play antagonistic roles in many developmental processes (Skoog and Miller, 1957). This antagonism has remained an abiding theme in plant biology (Coenen and Lomax, 1997), although we have only recently begun to unravel its molecular and mechanistic bases. Interactions between auxin and cytokinin play a role in maintaining the meristems of the shoot (Zhao et al., 2010) and root (Dello Ioio et al., 2008), controlling axillary branching (Bainbridge et al., 2005; Chatfield et al., 2000), and initiating lateral roots (Laplaze et al., 2007). Extensive crosstalk between the two occurs at all levels—synthesis (Takei et al., 2004b; Miyawaki et al., 2004; Jones et al., 2010), metabolism (Werner et al., 2006; Jones et al., 2010), and perception (Dello Ioio et al., 2008; Schlereth et al., 2010; Müller and Sheen, 2008; Zhao et al., 2010; Taniguchi et al., 2007)—and we are now beginning to understand how these networks interact to control a wide variety of plant responses (Figure 1). These interactions are reviewed more extensively in Chapter I and their activity in the context of vascular development are the main focus of the research reported in Chapters II-IV.

Experiments by Skoog and Miller over 50 years ago demonstrated that organogenesis in *in vitro* plant tissue cultures could be controlled by varying the relative quantities of auxin and cytokinin in the growth medium (Skoog and Miller, 1957). High auxin:cytokinin ratios induce the formation of roots from callus cultures, while low auxin:cytokinin ratios favour the development of shoots; at intermediate concentrations, the cultures continue to proliferate without differentiating. Experiments by Werner et al. (2003) demonstrated that cytokinin affects the shoot and root meristem in opposite ways. Constitutive over-expression of members of the CKX gene family led to enlargement of the root meristem but a decrease in shoot meristem size; likewise, root length and lateral root number increased while leaf expansion rate and shoot growth decreased. Taken together, these results show that cytokinins act as negative regulators of root growth and lateral root initiation but positively on the shoot meristem.

More recent work has established that the type A ARRs *ARR7* and *ARR15* are a nexus of interaction between auxin and cytokinin in shoot meristem regulation .

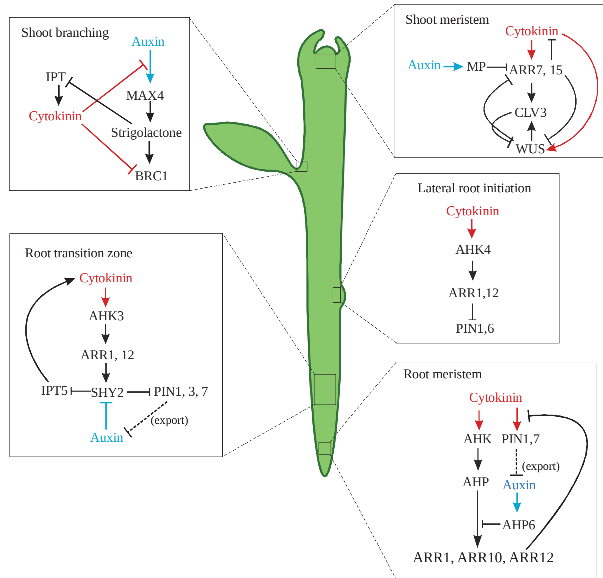

Figure 1: Auxin and cytokinin interact in many aspects of plant development, often through a mutual feedback loop. Auxin and auxin activity are in blue; cytokinin and cytokinin activity are in red. The feedback loop in the "root meristem" box was discovered through research reported in Chapters II-III. AHK: Arabidopsis histidine kinase 4; AHP: Arabidopsis histidine phosphotransferase; ARR: Arabidopsis response regulator ; BRC1: branched 1; CLV3: clavata 3; IPT: isopentenyltransferase; MAX4: more axillary branching 4; MP: monopteros; PIN: pin-formed; SHY2: short hypocotyl 2; WUS: wuschel. Modified from El-Showk et al. (2013).

The size of the shoot meristem is determined by a feedback loop between the genes *WUSCHEL* (*WUS*) and *CLAVATA3* (*CLV3*) (Brand et al., 2000; Schoof et al., 2000). Cytokinin regulates shoot meristem growth by promoting the activity of *WUS* (Gordon et al., 2009); *WUS*, in turn, represses *ARR7* and *ARR15* (Leibfried et al., 2005), which inhibit cytokinin signalling. However, cytokinin also activates *ARR7* and *ARR15* to upregulate *CLV3* (Zhao et al., 2010), a negative regulator of *WUS*. Auxin acts contrary to cytokinin, repressing *ARR7* and *ARR15* in the shoot meristem via the auxin response factor *MONOPTEROS/ARF5*, which was shown to bind to a specific auxin response element in the *ARR15* promoter (Zhao et al., 2010). Together, these interactions form a complex regulatory network around *ARR7* and *ARR15*, with many layers of potential feedback. Furthermore, auxin signalling has been previously shown to activate these RRs during root specification in embryogenesis (Müller and Sheen, 2008), perhaps via interaction of other auxin response elements in the promoter with different ARFs.

Work with auxin transport mutants has shown that auxin plays an instructive role in phyllotaxis (Reinhardt et al., 2003). PIN1-mediated polar transport of auxin leads to accumulation at specific sites and subsequent organ initiation; the auxin-depleted areas around the site serve as zones of inhibition, preventing organ initiation nearby and thus established the phyllotactic pattern.

Recent work has extended this model by showing an interaction between auxin and cytokinin during phyllotaxis. Besnard et al. (2014) observed changes in the arrangement of leaves and flowers in the shoots of *ahp6* mutants and showed that *AHP6* is activated by auxin one plastochron after the primary auxin response genes.

Further examination revealed aberrant ordering of organ initiation in *ahp6*, with organs more frequently initiated out of order or simultaneously. Analysis of an AHP6-GFP protein fusion showed that movement of the AHP6 protein creates gradients centred around primordia in the shoot meristem. Repression of cytokinin signalling by the AHP6 gradients creates differential cytokinin signalling within the meristem, which somehow acts to stabilize the plastochron. No changes were detected in *PIN1* expression or subcellular localization, suggesting that cytokinin's regulation of phyllotaxis is mediated by other genes and may not involve the modulation of auxin transport.

Long before the molecular identity of the polar auxin transporters was known, Osborne and Mullins (1969) observed a positive effect of cytokinin on polar auxin transport and, based on physiological experiments with auxin transport inhibitors, hypothesized that this was due to cytokinin upregulation of hypothetical auxin transport proteins. It is now well-established that cytokinin regulates the polar transport of auxin. In a seminal paper, Dello Ioio et al. (2008) revealed how cytokinin modulation of polar auxin transport regulates the size of the root meristem in *Arabidopsis*. The AUX/IAA gene *IAA3/SHY2* was known to be a target of the type B ARR *ARR1* (Taniguchi et al., 2007), over-expression of which led to a significant reduction in root meristem size. Dello Ioio et al. demonstrated a direct physical interaction between the two proteins and showed that *SHY2* acts downstream of *ARR1* in regulating the size of the root meristem. They then observed that expression of *PIN1*, *PIN3* and *PIN7* in the vascular tissues was downregulated by cytokinin and showed that this was mediated via *AHK3*, *ARR1*, and *SHY2*. Furthermore, *SHY2* was shown to negatively regulate the cytokinin biosynthesis gene *IPT5*. The size of the root meristem is thus determined by an antagonism between auxin and cytokinin mediated by their opposing regulation of *SHY2*, which in turn negatively regulates cytokinin biosynthesis while repressing auxin transport and signalling. This model was later extended by Moubayidin et al. (2010) to include the upregulation of *SHY2* by *ARR12* during meristem growth. Further evidence that cytokinin modulates auxin transport, which is a major theme of this thesis, was independently discovered in lateral roots (Laplaze et al., 2007), tobacco cell cultures (Růžička et al., 2009), and in *Arabidopsis* vascular development (Chapter II).

Root Vasculature

Evolution of roots and vasculature

The successful colonization of land by plants around 500 million years ago required several developmental innovations. Crucial amongst these were roots to anchor the plants and vascular tissue to transport water and nutrients and provide support for an upright body in the competition for light. Support, long-distance transport and anchorage were challenges unfamiliar to aquatic plants, which could float freely and absorbed nutrients throughout their entire surface tissue. Simple vasculature and root-like structures appeared relatively quickly and are amongst the earliest fossils of land plants. Mosses, hornworts, and liverworts lack true roots or vascular tissue, which has constrained their ability to evolve to larger sizes on land.

Roots are polyphyletic in origin, having evolved several times in the history of land plants. Fossil evidence indicates an independent origin of roots in at least two

different plant lineages, first in lycophytes (which dominated the Carboniferous forests that make up today's coal beds) and later in euphyllophytes, a clade which includes spermatophytes (seed plants) and monilophytes (ferns) (Ravens and Edwards, 2011). The evolution of roots and vascular tissues allowed for an elaboration of the plant *bauplan*, enabling the transition from mosses and liverworts to the gigantic trees of the Devonian forests in only 30-40 million years. Although the arborescent lycophytes, ferns, and horsetails of those forests are now extinct, the body plans which emerged are still seen around us today.

Several lines of evidence suggest that roots might be derived from shoots. Like the simple shoots of early vascular plants, roots are protostelic, comprising a central column of primary vascular tissue surrounded by ground tissue (Beck, 2005). Furthermore, analysis of root-like structures found in fossils from the Silurian, shortly after plants moved onto land, indicates that they were, in fact, underground stems which served as roots (Ravens and Edwards, 2011). Scheres and Dolan (1998) identified several developmental parallels supporting the interpretation of roots as modified shoots: (1) Both shoots and roots have a central zone of cells controlling the division and differentiation of neighbouring cells; (2) Shoots and roots use similar genetic modules to regulate epidermal cell fate; and (3) Position is an important determinant of cell fate in both shoots and roots, as shown by laser ablation experiments. Later studies have reinforced the common molecular underpinnings of patterning and proliferation in the shoot and root. *WUSCHEL* (*WUS*) is required for the maintenance of the shoot meristem (Mayer et al., 1998), while *WUSCHEL-related homeobox 5* (*WOX5*), a paralog of *WUS*, was identified as an early marker of the quiescent centre in the root meristem (Haecker, 2004). Furthermore, *SCARECROW* (*SCR*) is required for correct radial patterning in both the shoot (Wysocka-Diller et al., 2000) and the root (Nakajima et al., 2001) and is expressed in both the quiescent centre of the root meristem and the L1 of the shoot apical meristem (Wysocka-Diller et al., 2000). The similarity of the molecular mechanisms underlying development in the shoot and root lends credence to the notion that roots evolved via modification of the shoot developmental programme.

Nevertheless, roots differ from shoots in several important ways. Unlike shoots, roots are positively gravitropic. Their downwards growth into the soil necessitates the presence of an apical root cap, which serves both to protect the root meristem and to lubricate the root's passage through the soil. Furthermore, shoots are segmented by nodes from which exogenous lateral branches often grow; roots, by contrast, are not segmented, and root branches are endogenous in origin (Ravens and Edwards, 2011; Beck, 2005).

Anatomy of the root

Roots, like shoots, grow from an apical meristem; however, unlike the shoot meristem, the root meristem is covered by a root cap which protects and lubricates it. Within the root meristem is a zone of minimal cell division activity called the quiescent centre (QC) (Clowes, 1959), which has been shown to inhibit differentiation of the surrounding cells (van den Berg et al., 1997). These cells are therefore able to divide indefinitely; they give rise to the cell files of the root and so are called initials. Experiments with laser ablation have demonstrated the importance of positional information rather than lineage in determining cell fate in the developing root;

following ablation of initials, adjacent cells will take over their position and begin producing cells of the same type as the ablated initial (van den Berg et al., 1995).

Subsequent divisions push the daughter cells shootwards from the QC and initials (with the exception of the columella cells, which are apically located). The daughter cells undergo several more divisions before they begin to elongate. Although the fate of most cell types is already specified before elongation begins, the cells only complete the differentiation process during or following elongation. The root can therefore be roughly divided into three zones: the meristematic zone; the elongation zone; and the differentiation zone. The presence of root hairs is generally taken to mark the beginning of the differentiation zone, while the boundary between the meristematic zone and the elongation zone is determined by the start of cell elongation.

In *Arabidopsis*, lateral roots are initiated from the pericycle cells adjacent to the xylem (Dolan et al., 1993) and tend to alternate between opposite xylem poles (Dubrovsky et al., 2006) in a manner that is dependent on the activity of the auxin importer *AUX1* (de Smet et al., 2007). Initiation of lateral roots is triggered by auxin accumulation in the xylem-pole pericycle cells (Dubrovsky et al., 2008) and requires repression of cytokinin signalling in these cells (Bielach et al., 2012).

Vascular tissues

The vascular tissues of plants provide support and serve as a long-distance transport network for water, nutrients, and signalling molecules such as hormones. In order to fulfil these functions, the vascular tissues must be appropriately positioned to ensure the continuity of the vascular strands and thus maintain a transport and communication network between distant tissues and organs. Improving our ability to manipulate vascular development will also provide significant practical benefits in areas such as bioenergy and timber production.

The primary vascular bundle in the root is comprised of three cell types (xylem, phloem and procambium) surrounded by an outer layer of pericycle cells; together, these tissues comprise the stele. In the *Arabidopsis* primary root, the vascular tissues are stereotypically organized in a diarch pattern (Dolan et al., 1993), the regularity of which provides an ideal system for the study of vascular patterning processes (Figure 2). Xylem cells are arranged in a single-cell wide axis spanning the vascular bundle; two phloem poles are located at ninety degrees to this axis, separated from the xylem by intervening procambial cells which will later give rise to the vascular cambium (the lateral meristem involved in secondary growth). The *Arabidopsis* stele has two axes of symmetry, one through the xylem axis and another through the phloem poles, and is thus bisymmetric.

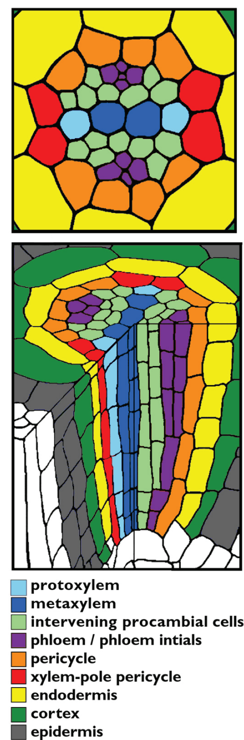

Figure 2: A schematic of the *Arabidopsis* root tip. Modified from el-Showk et al. (2015).

The xylem axis consists of two different types of xylem

cells; the marginal cells (*i.e.*, those adjacent to the pericycle) differentiate into protoxylem, while the central cells differentiate into metaxylem. Protoxylem and metaxylem can be distinguished by their secondary cell wall thickening. Protoxylem cells, which differentiate earlier, have annular thickenings of their secondary cell wall, allowing them to extend during growth; by contrast, metaxylem has a reticulated pattern of secondary cell wall thickening (Esau, 1977). Primary phloem, which consists of conductive sieve elements and the neighbouring companion cells, is likewise divided into protophloem and metaphloem. Protophloem sieve elements initiate at the outer periphery of the vascular cylinder, while metaphloem sieve elements are found adjacent to the protophloem inside the vascular cylinder. Metaphloem elements are initiated later than protophloem and so remain functional, while the protophloem is later obliterated. Differences also exist between pericycle cells; those at either end of the xylem axis have a distinct function, as they are the only cells that are competent to become lateral root primordia.

Embryonic development

The *Arabidopsis* embryo undergoes a well-characterized pattern of cell divisions during its development, allowing for a clear description of the establishment of the vascular initials. The vascular initials are specified early during embryogenesis; all vascular tissues derive from only four initial cells in the globular embryo. These four cells divide periclinally to give rise to two concentric cell layers; the outer cells then divide periclinally again to increase the number of cells in the layer. Later, cells in both the outer and inner layer undergo less regular periclinal divisions to further increase the number of cell files (Scheres et al., 1994). The embryonic vascular cells remain undifferentiated, with properties characteristic of different cell types only appearing post-embryonically (Dolan et al., 1993). β-glucuronidase can be used to create sectors of cells linked by descent; transposon excision in a single cell activates the β-glucuronidase gene, which will remain active in daughter cells and can thus be used to determine the progenitor of the visualized sector. Scheres et al. (1994) used this technique to confirm that the vascular tissues arise from the vascular initials, but also observed the spread of the sectors to multiple cells in the mature root, indicating that later divisions further refine the vascular pattern.

Auxin has been known for some time to play a role in vascular embryogenesis. *PIN1*, *PIN3*, *PIN4*, and *PIN7* are all expressed in specific patterns during embryogenesis, coordinating auxin transport to initiate many developmental processes. For example, when the apical-basal axis is forming, *PIN7* is apically localized in the basal cell, transporting auxin into the apical cell; at the globular stage, it switches to basal localization, transporting auxin into the suspensor cells. The embryos of *pin1 pin3 pin4 pin7* plants are highly abnormal, making it impossible to distinguish the effect of the quadruple mutation on vascular embryogenesis separately from other processes (Friml et al., 2003). The auxin response factor *MONOPTEROS/ARF5* (*MP*) is also known to play a key role in vascular development. Strong *mp* alleles result in failure to form a root at an early stage, while plants with weaker *mp* alleles that do not prevent root development have a reduced stele due to the absence of the stereotypical ordered pattern of cell division in the basal region of the embryo (Berleth and Jürgens, 1993).

The effect of *MP* on vascular development has recently become better understood

and some downstream players have been identified. The *mp* defect could be rescued by driving *TARGET OF MONOPTEROS 5* (*TMO5*) under the *MP* promoter, indicating that TMO5 mediates the effect of MP on division of the vascular initials (Schlereth et al., 2010). *TMO5*, which encodes a bHLH transcription factor, is expressed in all four embryonic vascular initials. The double mutant of *TMO5* and its closest homolog *TMO5-LIKE1*, *tmo5 t5l1*, also has defects in periclinal division of the vascular initials, although the single mutants do not. Furthermore, roots of *tmo5 t5l1* plants show a reduced post-embryonic stele in which the normal bisymmetric pattern is replaced by only one xylem and phloem pole. This phenotype is similar to that of another bHLH transcription factor mutant, *lonesome highway* (*lhw*) (Ohashi-Ito and Bergmann, 2007), though *LHW* is more broadly expressed in embryo and roots than *TMO5* (De Rybel et al., 2013). *TMO5* expression is restricted to xylem precursors at the heart stage, while *LHW* is expressed throughout the root pole and in the cotyledon primordia. Post-embryonically, *LHW* is expressed throughout the root meristem in a decreasing gradient from the QC, while *TMO5* is only expressed in xylem precursors. Immunoprecipitation demonstrated that TMO5 and LHW form a heterodimer where their expression domains overlap; this heterodimer was found to be necessary and sufficient to trigger the periclinal cell divisions which establish the vascular pattern in the early embryo.

Although *TMO5* and *LHW* overlap in the xylem cells, these cells do not undergo periclinal cell divisions; instead, the surrounding procambium and phloem divide. Likewise, ectopic expression of TMO5 in the ground tissue (where LHW is already present) resulted in additional divisions not only in the ground tissue, but also in adjacent pericycle cells. Together, these data suggest that the TMO5/LHW-dependent signal acts in a non-cell autonomous manner to control periclinal divisions in the vascular cells (De Rybel et al., 2013).

Ohashi-Ito et al. (2013) have shown that *LHW* interacts with auxin signalling and transport to regulate vascular development. They observed a more diffuse pattern of *PIN1* expression in *lhw* embryos, as well as altered *TMO5* expression and reduced levels of *MP*. Interestingly, although ectopic expression of *LHW* throughout the root led to increased expression of the synthetic auxin reporter *DR5*, this was not accompanied by changes in the amount of endogenous auxin, suggesting that *LHW* acts to modulate auxin signalling rather than auxin levels, although it remains unclear whether it does so directly or via modulation of cytokinin signalling.

Further work in the *Arabidopsis* embryo revealed the TMO5/LHW heterodimer upregulates the cytokinin biosynthesis gene *LOG4*. Expansion of the expression domain of the two genes leads to excessive periclinal divisions in the developing embryonic vasculature, and this phenotype is partially suppressed in the triple mutant *log3 log4 log7*. Furthermore, treatment of the *lhw* or *t5l1* mutant with cytokinin restored the cell number and diarch vascular pattern (De Rybel et al., 2014).

De Rybel et al. incorporated these findings in a computational model in which auxin acts via TMO5/LHW to promote cytokinin biosynthesis in a growing two-dimensional root cross section. Simulations revealed that the initial cell divisions require a bias from the geometry of the four founder cells in order to consistently form a central xylem axis; subsequent analysis confirmed the bias in growing embryos. Furthermore, they propose that local auxin-driven cytokinin biosynthesis generates a cytokinin gradient centred around the xylem axis and argue that this gradient is

crucial for regulating growth and vascular patterning. In this model, high levels of cytokinin in the xylem axis do not lead to periclinal divisions in those cells because they also have high levels of auxin, which activates *AHP6* to represses cytokinin signalling. The discrepancy between this model and our findings is discussed further in the Results & Discussion, as well as Chapter IV.

Postembryonic development

The last 20 years has seen an explosion in our understanding of postembryonic vascular development. Using *Arabidopsis* as a model system, many key components have been identified, although less is known about the specification of phloem than of xylem and some players likely await discovery. More recently, researchers have begun integrating these components into interacting networks which establish and maintain the vascular pattern.

Phloem specification

The *ALTERED PHLOEM DEVELOPMENT* (*APL*) gene discovered in 2003 encodes a MYB coiled-coil transcription factor which is required for phloem development (Bonke et al., 2003). *APL* is expressed in the cotyledons and hypocotyls of nearly mature *Arabidopsis* embryos. Postembryonically, *APL* is expressed throughout the phloem in the shoots and roots of seedlings. Close to the root meristem, its expression is limited to the protophloem sieve elements; higher up, expression expands into the developing companion cells and metaphloem sieve elements.

Although *APL* is only observed in developing phloem and does not appear to be expressed during the earlier asymmetric divisions, these divisions are delayed in the seedling-lethal *apl* mutant. *apl* plants also fail to form sieve elements and companion cells; instead, ectopic tracheary element-like cells are often found at the phloem position. Ectopic expression of *APL* throughout the vascular cylinder delays metaxylem differentiation and prevents cells at the protoxylem position from differentiating into tracheary elements. The undifferentiated protoxylem cells fail to undergo lignification and autolysis; although these cells undergo a tangential division similar to phloem precursors, they nevertheless retain a nucleus, indicating that APL alone is not sufficient to result in ectopic phloem development (Bonke et al., 2003). The xylem defects seen in these lines indicate that, in addition to specifying phloem, *APL* plays a role in inhibiting xylem differentiation at the phloem poles.

A second gene involved in phloem specification was recently identified. *OCTOPUS* (*OPS*) is a polarly-localized, membrane-associated protein expressed earlier than *APL* in the provascular cells and phloem initials (Truernit et al., 2012) and also in the proto- and meta-phloem (Bauby et al., 2007). Differentiation of the protophloem in the *ops* mutant is discontinuous due to the failure of some protophloem cells to initiate elongation and cell-wall thickening. Over-expression of *OPS* was not sufficient to specify phloem cell identity but led to precocious phloem differentiation within the already-established phloem cell lineage (Truernit et al., 2012). In a later study, an *OPS* over-expression line showed a phenotype similar to constitutive brassinosteroid response. Crosses with the brassinosteroid-insensitive mutants *bri1* and *bin2* and treatment with the brassinosteroid brassinolide or the brassinosteroid inhibitor brassinazole revealed that *OPS* interacts with *BIN2*, sequestering it at the plasma membrane and thus preventing its repression of brassinosteroid signalling.

While treatment of the *ops* mutant with a *BIN2* inhibitor partially rescued the phloem defects, inhibition of brassinosteroid synthesis did not cause phloem defects, nor did brassinosteroid treatment rescue *ops*. These data suggest that brassinosteroid signalling is involved in phloem differentiation, but brassinosteroids themselves are not required for this process (Anne et al., 2015).

Protophloem differentiation is suppressed in wild-type plants treated with the CLE45 peptide, which binds to BARELY ANY MERISTEM 3 (BAM3) (Depuydt et al., 2013). Furthermore, transgenic seedlings with increased *CLE45* activity exhibit a phenotype similar to *ops* mutants, while an increase in *OPS* expression leads to CLE45 resistance (Rodriguez-Villalon et al., 2014). Protophloem specification therefore seems to depend on a balance between pathways mediated by *OPS* and *CLE45*.

Phloem cells mature into sieve elements, elongated cylindrical cells with perforated ends. Sieve element formation consists of enucleation and cellular reorganization. The process has recently been described in detail via scanning electron microscopy of serial 40nm sections, together with microarray experiments identifying the NAC-domain transcription factors *NAC45* and *NAC86* as key regulators of these changes (Furuta et al., 2014). The choline transporter *CHER1* is required for correct formation of the sieve plates; there are sieve pores in the *cher1* mutant and they are structurally altered (Dettmer et al., 2014).

Cytokinin represses protoxylem

The *woodenleg* (*wol*) mutant was identified because of its short, determinate primary root which arrests growth seven days after germination, though the plants recover thanks to the formation of adventitious roots. The vascular bundle of *wol* contains fewer cells than wild-type, all of which differentiate as protoxylem; phloem and procambium are found in the *wol* hypocotyl, which also has more vascular cells (Scheres et al., 1995). *WOL* was found to be allelic with the independently identified loci *CYTOKININ RESPONSE 1* (*CRE1*) and *Arabidopsis HISTIDINE-KINASE 4* (*AHK4*) which encode a cytokinin receptor (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001). *CRE1* acts as a kinase in the presence of cytokinin and a phosphatase in its absence (Mähönen et al., 2006a,b). Since the *wol* mutation is in the cytokinin binding domain, this results in constitutive phosphatase activity in *wol* plants, counteracting the kinase activity of the other cytokinin receptors.

Cytokinin signalling mediated by *CRE1* represses the specification of protoxylem; the stele in *wol* plants, which have severely attenuated cytokinin signalling, is composed entirely of protoxylem cells (Mähönen et al., 2000). Correct specification of protoxylem in wild-type roots requires the suppression of cytokinin signalling at the protoxylem position, which is accomplished by a pseudo-phosphotransferase. In addition to the five functional histidine-phosphotransferase (HP) proteins which relay the phosphoryl group between the HK and RR proteins, *Arabidopsis* has a pseudo-phosphotransferase, *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFERASE 6* (*AHP6*), that lacks the conserved histidine required for phosphotransfer (Mähönen et al., 2006a). *AHP6*, which was discovered in a genetic screen for suppressors of *wol*, is expressed in the protoxylem position and the adjacent pericycle cells, where it inhibits cytokinin signalling by competing with the functional phosphotransferases. Treatment with exogenous cytokinin or a mutation in *AHP6* results in the spread of

cytokinin signalling to the protoxylem position and a concomitant loss of protoxylem (Mähönen et al., 2006a). The mechanism by which cytokinin signalling is excluded from the metaxylem remains unclear, though the weak phenotype of the *ahp6* mutant suggests that it may have an undiscovered redundant partner.

The triple cytokinin receptor mutant *cre1 ahk2 ahk3* shows a more extreme phenotype than *wol* (Mähönen et al., 2006a,b). As in *wol*, the reduction in the number of vascular cell files in these mutants results in the entire stele being specified as protoxylem; in addition, these plants have a determinate root, but unlike in *wol* mutants, this is not rescued by adventitious root formation. Expression of *CYTOKININ OXIDASE* (*CKX*), which degrades free cytokinin, in the vascular cylinder phenocopied *wol*, while treatment with cytokinin led to the loss of protoxylem, further supporting the importance of cytokinin in repressing protoxylem specification (Mähönen et al., 2006a,b). In addition, the *wol* phenotype is also phenocopied by higher order mutants in which several (redundant) members of gene families involved in the downstream relay of cytokinin signalling are knocked out, such as the quintuple mutant *ahp1-5* mutant, which lacks all of the functional histidine phosphotransfer proteins (Hutchison et al., 2006), or the *arr1 arr10 arr12* mutant (Mason et al., 2004), which lacks the three cytokinin-responsive transcription factors which are strongly expressed in the primary root (Argyros et al., 2008; Ishida et al., 2008).

Cytokinin signalling prevents the specification of procambium cells as protoxylem. In normal plants, expression of *AHP6* in the protoxylem position inhibits cytokinin signalling, allowing the correct specification of protoxylem; by contrast, mutants defective in cytokinin perception such as *wol* and *cre1 ahk2 ahk3* show ectopic protoxylem specification (Mähönen et al., 2006a,b). While these experiments show that cytokinin signalling inhibits protoxylem specification, they do not identify the positive factors which specify protoxylem fate, leaving open the question of what factors promote *AHP6* expression and protoxylem specification.

The CLE peptides modulate cytokinin

Tracheary element differentiation factor (TDIF) was identified as a peptide which inhibits tracheary element (xylem) differentiation in *Zinnia* cell cultures (Fukuda and Komamine, 1980); in addition, *Arabidopsis* cultures treated with TDIF show enhanced proliferation of procambial cells (Hirakawa et al., 2008). TDIF is a dodecapeptide whose sequence was found to be similar to the C-terminal amino acids of two *Arabidopsis* genes, *CLE41* and *CLE44*. These genes are members of the CLAVATA/EMBRYO SURROUNDING REGION-related (CLE) family, which is comprised of 32 members in *Arabidopsis*. *CLE41* and *CLE44* are expressed in the phloem and the neighbouring pericycle cells. A screen of candidate *Arabidopsis* T-DNA insertion lines for insensitivity to TDIF led to the identification of the TDIF receptor, *TDR*, which is expressed in the intervening procambial cells. A model has therefore been proposed in which TDIF is produced in the phloem and neighbouring cells but perceived by *TDR* in the procambial cells, where it acts to promote their proliferation and prevent their differentiation into xylem (Hirakawa et al., 2008).

Kondo et al. (2011) treated *Arabidopsis* seedlings with 26 of the CLE peptides and found that 17 of them cause defects in protoxylem and reduced root growth. Since two other CLEs caused a reduction in growth without affecting protoxylem, the protoxylem defects seem to be independent of the effect on root growth. *CLE9* and

CLE10 are expressed in a stele-specific manner and exogenous treatment with either inhibits protoxylem formation. A microarray analysis identified *ARR5*, *ARR6*, *IPT7* and *CKX3* as genes down-regulated by the application of CLE10. While *ARR5* and *ARR6* are type-A ARRs which negatively regulate cytokinin signalling (To et al., 2007), *CKX3* encodes a cytokinin degradation enzyme (Mok and Mok, 2001), and *IPT7* plays a role in cytokinin biosynthesis (Kakimoto, 2001; Takei et al., 2001). Interestingly, although all four are cytokinin-related genes, their contradictory roles suggest that CLE10 may have complex effects on cytokinin status by modulating both signalling and homoeostasis. While the *ahk2 ahk4* mutant, which is defective in two of the three cytokinin receptors, remains responsive to CLE10 treatment, it is less sensitive than wild-type plants (Kondo et al., 2011), suggesting that CLE10 may achieve its effect primarily by affecting cytokinin signalling rather than metabolism, though the full story is likely to be more complex.

Kondo et al. (2011) also showed that treatment of the *arr10 arr12* double mutant with CLE10 did not cause defects in protoxylem. Since type-A ARRs and type-B ARRs are likely to compete for phosphotransfer, it is possible that the down-regulation of *ARR5* and *ARR6* by CLE10 may result in increased activity of the type-B ARRs *ARR10* and *ARR12*, leading to defects in protoxylem development; the insensitivity of the *arr10 arr12* mutant to CLE10 is consistent with this model. Furthermore, they found that application of CLE10 to *ahp6* plants enhances the protoxylem phenotype, suggesting that its mode of action may be independent of *AHP6*.

VNDs and xylem maturation

Using an *in vitro* system to induce the formation of xylem in cell cultures, Kubo et al. (2005) identified several genes involved in xylem morphogenesis. By treating an *Arabidopsis* suspension culture with appropriate hormones, they were able to induce $\sim$ 50% of the cells to differentiate into xylem elements within 7 days; a microarray analysis identified 23 clusters of co-regulated genes. Of these, three clusters (comprising 224 genes) showed upregulation 6 days after induction, coincident with active xylem formation. While these included many genes encoding proteins that would be expected to play a role in developmental events, such as proteins involved in cell wall synthesis and programmed cell death, several putative transcription factors were also identified, including four NAC-domain transcription factors. These four transcription factors showed significant similarity to another NAC-domain transcription factor, *Z567*, previously seen to be upregulated during xylem formation in cell cultures of *Zinnia elegans* (Demura et al., 2002). Based on sequence similarity to *Z567*, three additional NAC-domain transcription factors were identified in the *Arabidopsis* genome; although they had not been recovered in the clusters, these three genes were also found to be upregulated in the microarray data. Collectively, these transcription factors were designated *VASCULAR-RELATED NAC-DOMAIN (VND) 1–7*. All of the VNDs show a vascular-specific expression pattern in the shoot and root, with the exception of *VND1*, *VND4*, and *VND6*, which do not seem to be expressed in the shoot. Within the root vascular tissue, the VND transcription factors show variable expression patterns; *VND1*, *VND2*, and *VND3* are preferentially expressed in the intervening procambial cells adjacent to the root meristem, while the remainder are observed primarily in mature xylem which does not yet show obvious secondary cell wall thickening. *VND6* and *VND7* are nuclear-

localized in the central metaxylem and immature protoxylem, respectively. While over-expression of *VND1–VND5* did not result in any morphological changes, over-expression of *VND6* or *VND7* resulted in transdifferentiation of various nonvascular cells into xylem in both poplar and *Arabidopsis* Kubo et al. (2005). In plants over-expressing *VND6*, the transdifferentiated cells had reticulate, pitted cell walls similar to those found in metaxylem, while those over-expressing *VND7* had annular cell walls with a spiral thickening pattern similar to protoxylem cells. In addition, several xylem-specific genes were found to be ectopically expressed in the *VND7* over-expression plants. Although knock-out lines of *VND6* and *VND7* showed no morphological defects, the dominant repression of either gene (by over-expression of the gene fused to a strong repressor domain) resulted in shorter roots with defects in the formation of metaxylem and protoxylem, respectively. Both *VND6* and *VND7* appear to be positively regulated by auxin and cytokinin.

Yamaguchi et al. (2008) showed that *VND7* can form homo- and hetero-dimers with the other NAC-domain proteins, including the VNDs; in addition, the stability of *VND7* appears to be regulated by proteasome-mediated degradation. Taken together, these data suggest that the transcriptional activity of *VND7* may be regulated by its interactions with other proteins. In later work, Yamaguchi et al. (2010, 2011) began to elucidate these interactions and identify other components regulated by *VND7*. Using a yeast two-hybrid screen, they identified two NAC-domain proteins which interacted with *VND7*, dubbed *VND-INTERACTING* (*VNI*) *1* and *2*. Binding assays demonstrated that *VNI2* interacts with *VND7*, as well as with the other VND proteins with a lower affinity. Furthermore, *VNI2* is expressed in the xylem and phloem precursor cells in the root and shoot; this expression pattern overlaps with that of *VND7*. Contrary to *VND7*, over-expression of *VNI2* results in discontinuities in protoxylem formation. Given the overlapping expression and interaction with *VND7*, it seems likely that *VNI2* affects xylem development by inhibiting the upregulation of *VND7* targets; indeed, a transient Luc reporter assay demonstrated just such repression. Although the *vni2* mutant shows no visible phenotype, the expression level of several genes involved in xylem formation is elevated in the mutant, consistent with *VNI2* acting as a transcriptional repressor during xylem development. Finally, a transcriptome analysis by Yamaguchi et al. (2011) identified 63 putative targets of *VND7*. These targets include a broad range of proteins, such as transcription factors and proteolytic enzymes; this suggests the existence of a transcriptional network downstream of *VN*D7 regulating xylem differentiation.

Protoxylem or metaxylem?

The class III homeodomain leucine zipper genes play a central role in determining the vascular pattern of the mature root. The *Arabidopsis* genome contains five HD-ZIP III genes, *PHABULOSA* (*PHB*), *REVOLUTA* (*REV*), *PHAVOLUTA* (*PHV*), *CORONA* (*CNA*), and *ATHB8*; all five are targets of the microRNAs *miR165* and *miR166* (Emery et al., 2003; Mallory et al., 2004). The miRNAs post-transcriptionally downregulate the HD-ZIP III genes by binding to target sequences in their mRNA. Over-expression of *miR165a* in the stele leads to a reduction in HD-ZIP III expression and thereby the production of ectopic protoxylem; conversely, HD-ZIP III mutants which are resistant to miRNA targeting, such as *phb1-d*, show expansion of the metaxylem into the protoxylem and pericycle positions (Miyashima

et al., 2011). The expression of the *miR165* and *miR166* is regulated by interactions between the transcription factors *SCARECROW* (*SCR*) and *SHORTROOT* (*SHR*). Movement of the SHR protein from the stele, where it is produced, into the adjacent layer leads to the asymmetric division of the cortex/endodermis initial, activates endodermis identity, and promotes the expression of *SCR* (Nakajima et al., 2001). SHR and SCR then form a heterodimer which promotes the expression of *miR165a* and *miR166b*, which re-enter the stele and repress HD-ZIP III activity (Carlsbecker et al., 2010; Miyashima et al., 2011). This mechanism results in high levels of HD-ZIP III proteins in the centre of the stele and lower levels towards the periphery; together with the mutant phenotypes, this suggests that HD-ZIP III dosage regulates xylem identity, with high levels leading to the formation of metaxylem and lower levels resulting in protoxylem.

The HD-ZIP III genes are also known to interact with auxin. *ATHB8* is auxin regulated via the *MP/ARF5* activity (Baima et al., 1995; Donner et al., 2009) and has recently been shown to act as a xylem identity gene (Carlsbecker et al., 2010). Furthermore, the triple HD-ZIP III mutant *phb phv rev* shows an altered pattern of *PIN1* expression in the embryo (Izhaki and Bowman, 2007). However, a reduction in polar auxin transport inhibits the specification of protoxylem but not metaxylem (Chapter III and (Ursache et al., 2014)); conversely, reduced local auxin biosynthesis in the *taa1 tar2* mutant led to the replacement of metaxylem by protoxylem (Ursache et al., 2014). Xylem development in the *Arabidopsis* root therefore seems to depend on a coordination and balance between auxin transport and synthesis.

Regulation of vascular cell number

Post-embryonic divisions in the procambium and phloem normally increase the number of vascular cell files (Mähönen et al., 2000). Several genes have been identified which contribute directly or indirectly to this process. The reduced stele of the *wol* mutant (Scheres et al., 1995) is due to the absence of these divisions (Mähönen et al., 2000). The HD-ZIP III, SHR/SCR, *miRNA* circuit described above also seems to regulate the number of vascular cell files; an increased number of stele cells was observed in both *shr-2* and in plants with increased levels of *miR165* in the stele (Carlsbecker et al., 2010). Finally, the *lhw* mutant has roughly half as many vascular cells as wild-type plants, although the number of cells in the cortex and endodermis is unaltered (Ohashi-Ito and Bergmann, 2007); embryos of *lhw* plants have a reduced number of vascular initials (three instead of four) which fail to undergo subsequent divisions, indicating that the defect arises early in embryogenesis (Ohashi-Ito et al., 2013). Plants with the *lhw* mutation have only a single vascular pole, while the stele of *wol* plants is composed entirely of protoxylem; these observations are in keeping with earlier observations of a correlation between stele size and the number of vascular poles in dicots (Torrey, 1955).

Modelling Vascular Development

Modelling *per se* is hardly a novel practice in biology; pen-and-paper models abound in the literature. In recent years, however, computational modelling has emerged as a significant new approach complementing existing techniques in the toolbox of plant biologists. Simple linear models have proven inadequate to depict the ever-widening

cast of interacting players revealed by experiments; the resulting networks are often non-trivial and defy our intuition. Complex systems consisting of feedback loops, coupled networks, and other non-linear interactions exhibit non-intuitive behaviour that challenges our ability to make predictions about the stability of the system or the effect of manipulations. In these cases, theoretical and computational approaches are an indispensable tool to guide our interpretation of data and help formulate novel hypotheses. Computational modelling also enables us to strip away much of the complexity inherent to biological systems in the hope of identifying and understanding the principles underlying the particulars of a specific implementation. At its best, it allows us to abstract our results away from a particular system to other organs, organisms, or contexts. In principle, this practice is familiar to any biologist who has ever proposed a model or used one as the basis for a hypothesis; computational approaches simply extend this familiar framework beyond the limits imposed on pen-and-paper models by human intuition.

Recently, a combination of computational modelling and experiments demonstrated the existence of an inhibitory feedback loop between *CLV3* and cytokinin; this feedback loop acts to dynamically position the *WUS* domain at an appropriate distance from the shoot apex. Simulations also suggested that the regulatory network be further elaborated by *WUS* inhibition of cytokinin biosynthesis via the repression of *LOG4*; subsequent observations of *LOG4* expressions in the *clv3* mutant were consistent with this prediction (Chickarmane et al., 2012).

In the context of vascular development, work by Ibañes et al. (2009) used a combination of experiments and computational modelling to describe the interaction of auxin and brassinosteroids in establishing the vascular pattern of the *Arabidopsis* shoot. They constructed a model of auxin transport which was able to reproduce the observed periodic distribution of auxin signalling maxima and to predict the phenotype of the *pin1pin2* double mutant. Using this model, they showed that asymmetric auxin transport, rather than the auxin level, determined the number of auxin maxima and thus the number of vascular bundles, a result which was confirmed by experiments with auxin over-producing mutants. Previous work had already established a link between brassinosteroids and the number of vascular bundles; analysis of their model indicated that brassinosteroids could modulate the number of auxin maxima by altering either the period of the pattern or the total number of cells. Based on this insight, they measured the effect of brassinosteroids on both parameters and discovered that they affect the total cell number rather than the period of the auxin pattern.

Subcellular models incorporating the dynamics of transcription, translation, and turnover have also generated valuable insights. For example, Muraro et al. (2011) incorporated the effect of cytokinin signalling (and its cross-talk with auxin) into an earlier model of an AUX/IAA negative feedback loop developed by Middleton et al. (2010). Their model made testable predictions about the periodic dynamics of hormonal concentrations in the root apex; interestingly, they also showed that some elements of the network respond differently to changes in hormonal supply and genetic mutations. More recently, Muraro et al. (2013) constructed a one-dimensional model investigating the role of auxin-cytokinin crosstalk in root zonation, followed by a two-dimensional model of auxin-cytokinin crosstalk in vascular development (Muraro et al., 2014).

Studies such as these serve as excellent examples of the value of iteration between

theoretical and experimental approaches in guiding research. Computational and theoretical approaches have also been applied to great effect in a wide range of other contexts, from epidermal cell fate patterning (reviewed by Benítez et al. (2011)) to root stem cell niche development (reviewed by Azpeitia and Alvarez-Buylla (2012)), using a variety of modelling frameworks (reviewed by Grieneisen and Scheres (2009); De Vos et al. (2012)).

In addition to investigating specific systems, modelling can also guide our investigations by exploring the limits and possibilities of different mechanisms. For example, a mathematical model of auxin movement (Mitchison, 1980) revealed constraints on auxin velocity and other biophysical constants imposed by various proposed transport mechanisms, including the chemiosmotic theory. Grieneisen et al. (2012) examined the robustness of morphogen gradients produced by different mechanisms to evaluate their ability to generate positional information; focusing on auxin, they showed that a reflux loop, but not source-decay or unidirectional transport, could generate a positionally informative gradient. Band and King (2012) showed that cell length can exert a dominating effect on auxin transport due to the difference in the time scale of auxin distribution between and within cell files. They found that auxin flux is driven by the epidermis near the root tip, where cells are short, but depends on the distribution of auxin carriers in shootward cells which have elongated. A recent analysis of current models of polar auxin transport has shown that while existing models are capable of self-organizing to produce polarized auxin transport patterns, these are limited to unidirectional patterns (van Berkel et al., 2013); existing models cannot self-organize to produce the bidirectional "reverse fountain" patterns seen leaf primordia and the root meristem, respectively, in which auxin . The term "reverse fountain" has been used to describe different auxin flux patterns in leaf primordia and the root meristem. Grieneisen et al. (2007) use the term to describe auxin flux in the root meristem, where PINS direct auxin through the inner, vascular tissues towards a maximum in the QC and then laterally away and back shootwards in the outer layers; however, van Berkel et al. (2013) describe this pattern as a "fountain" and use "reverse fountain" for the reversed pattern in leaf primordia originally described by Reinhardt et al. (2003), in which PINs in the outer layers direct auxin towards a maximum from whence it is then transported into the inner tissues.

Chapter IV extends the work of Grieneisen et al. (2007), which modelled auxin diffusion and PIN-facilitated transport in a longitudinal root section. Using empirically-derived patterns of PIN expression and localization, they demonstrated that the site of auxin biosynthesis in the root does not affect the steady-state auxin distribution since the dynamics of auxin transport will quickly redistribute any synthesized auxin. Instead, the pattern is determined by the dynamics of the auxin reflux loop, which robustly self-organizes based on the pattern of PIN localization. The flow of auxin in the root was directed by the PIN efflux transporters in a "reverse fountain," travelling from the shoot rootward through the vascular cylinder and then outward through the columella to return shootward in the epidermis and cortex. The expression pattern of the PIN proteins and their localization on specific regions of the cell membrane was shown to be an important factor in determining the overall pattern of auxin distribution and flux, which generates and maintains an auxin-signalling maximum at the QC and acts as an "auxin capacitor" capable of maintaining high levels of auxin signalling in this domain even after transport

from the shoot has been disrupted.

These findings highlight the need to combine experimental and theoretical approaches to advance our understanding of the mechanistic basis by which polar auxin transport is organized. Computational models can reveal the short-comings of experimentally derived models and thus help to guide the direction of future work. By requiring a rigorous statement of assumptions and exploring their consequences, computational and mathematical models can also reveal constraints which limit the patterning mechanisms available in a given system.

Aims of the study

The suppression of cytokinin signalling at the protoxylem position seems to place *AHP6* at a crucial position in the regulation of vascular development in *Arabidopsis*, but the factors regulating *AHP6* remained unknown at the start of this study. We therefore set out to identify factors acting upstream of *AHP6* and understand the regulatory network controlling *AHP6* positioning and xylem specification. We discovered that auxin promotes *AHP6* expression, leading us to investigate its interaction with cytokinin during vascular development in the *Arabidopsis* root. The specific aims of this study were the following:

1. To identify the factor or factors upregulating AHP6 in vascular development.
2. To investigate the role of auxin in xylem development and its interaction with cytokinin.
3. To test whether the auxin-cytokinin loop we discovered is sufficient to establish the observed hormonal signalling domains in the root tip and maintain the pattern of the *Arabidopsis* stele.
4. To understand the dynamics of the auxin-cytokinin loop and its implications in vascular development and more generally.

Materials & Methods

The materials and methods used are described in detail in the individual publications. Table 1 lists the methods used in this study together with the publications in which they were used.

Method	Publication
Agrobacterium mediated transformation of *Arabidopsis*	II, III
Algorithmic generation of digital root cross sections	IV
Analysis of simulation results	IV
Anatomical analyses	(II), (III), IV
Aniline blue staining	(III)
Confocal microscopy	II, III, IV
DNA sequencing	II
Embedding and histological analysis of root cross sections	(II), (III), (IV)
Fuchsin staining of *Arabidopsis* roots	II, III
Genetic crosses of Arabidopsis	II, III
Immunolocalisation	(II)
Hormone induction assays	(II), (III)
Light microscopy	II, III, IV
Mass spectrometry	(III)
Plasmid construction	II, (III)
Polymerase Chain Reaction (PCR) analysis	II, III, (IV)
Programming and implementaion of computational simulations	IV
Quantification of intensity signal in confocal images	(IV)
Quantitative real-time PCR analysis	II, III, (IV)
Radiolabelled hormone transport assays	(III)
RNA extraction	II, III
Scintillation analysis	(III)
Segmentation of root cross section images	IV
Sequence analysis	II, III
Statistical analysis	II, III, IV

Table 1: The methods used in this study. Parentheses indicate methods performed by co-authors in the respective publication.

Results & Discussion

Vascular patterning

Auxin and cytokinin domains

Although it was known that mutual inhibition between cytokinin and *AHP6* position the protoxylem in the *Arabidopsis* root (Mähönen et al., 2006a), the positive regulator(s) of *AHP6* activity remained a mystery. Given cytokinin and auxin's well-known antagonism, auxin seemed likely to play a positive role in this process. We therefore used several different markers to investigate the status of auxin and cytokinin signalling in the *Arabidopsis* stele. We queried cytokinin signalling using two cytokinin-regulated promoters: the type A response regulator *ARR5* and the synthetic two-component signalling reporter *TCS*. In transverse sections, both reporters showed two domains of cytokinin signalling in the intervening procambium cells flanking the xylem axis. To our knowledge, this is the first report of the transverse pattern of cytokinin signalling in the *Arabidopsis* root meristem. Using the auxin-responsive promoters *IAA2* and *DR5rev* to examine the auxin response in the stele, we found a domain of high auxin signalling through the xylem axis with a stronger signal in the protoxylem cells. Previous work (Swarup et al., 2001) has reported *IAA2* activity in the protoxylem poles; however, the developmental implications of this finding were not explored further. Auxin and cytokinin signalling form complementary domains encompassing the entire *Arabidopsis* stele with the exception of the pericycle (although weaker auxin signalling was observed in the xylem-pole pericycle cells), suggesting that these domains may serve as positional cues during development (Figure 3).

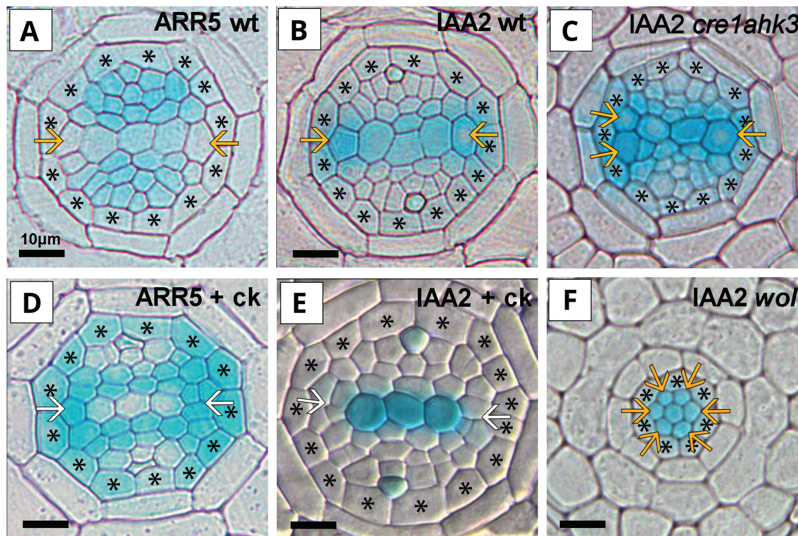

Figure 3: (A, B) Cytokinin and auxin signalling form complementary domains in the stele, shown by the markers ARR5 and IAA2, respectively. (C-F) The domains alter in response to cytokinin and in receptor mutants. Yellow arrows mark protoxylem; white arrows mark the position normally occupied by protoxylem; asterisks mark pericycle cells. ck=cytokinin; wt=wild-type. Modified from Bishopp et al. (2011).

Serial sections shootwards of the QC showed that the signalling domains of the two hormones change along the longitudinal axis. Near the QC, the domain of both hormones is broader, with cytokinin signalling throughout the stele and auxin signalling in the stele and endodermis. The domains become restricted to the complementary pattern reported above around $40\mu m$ from the QC. Refinement of the auxin domain continues in shootwards sections; by $100\mu m$ shootwards of the QC, the auxin pattern is restricted to the protoxylem cells, leaving the metaxylem devoid of either auxin or cytokinin signalling. The overlap of the high auxin signalling domain with the *AHP6* and *ATHB8* expression domains suggested that auxin might be promoting xylem specification via these genes.

Auxin specifies protoxylem

We used qRT-PCR to determine whether *AHP6* is auxin regulated. Following a two hour incubation with the auxin indole-3-acetic acid (IAA), we observed a 10-fold increase in the expression of *AHP6*, a response profile similar to that of the primary auxin response gene *IAA2*. A series of deletions of the *AHP6* promoter revealed that the 755bp upstream of the start codon were necessary and sufficient for consistent expression in the protoxylem position. This region contains five potential auxin response elements (TGTC). We created a version of the 755bp promoter in which these sites had been mutated to abolish ARF binding (TGTC→TG$\underline{\text{G}}$C) and observed no expression of GFP in the protoxylem position under this promoter. Auxin signalling therefore appears necessary for the correct expression of *AHP6* in the *Arabidopsis* stele.

In order to better understand the relationship between auxin, *AHP6*, and protoxylem development, we generated transgenic plants inducibly expressing *axr3-1*, a stabilized form of *AXR3/IAA17* which acts as an inhibitor of auxin signalling Rouse et al. (1998), throughout the stele. The *axr3-1* mutant has a radially symmetric stele which lacks protoxylem altogether in the root tip. Induction of *axr3-1* in non-mutant plants led to the loss of *AHP6* expression within 24hr, further supporting the positive relationship between auxin and *AHP6*. Taken together, these data suggest that auxin signalling in the protoxylem position activates expression of *AHP6* and the specification of protoxylem.

The PINs position auxin in the xylem axis

To assess the role of polar auxin transport in this process, we treated plants with the auxin transport inhibitor 1-naphthylphthalamic acid (NPA). We observed changes in the auxin signalling pattern following 5 days on NPA. Expression of *IAA2* and *DR5rev* disappeared from the xylem axis and was observed in the pericycle and throughout the outer cell layers. Likewise, *AHP6* was no longer expressed in the protoxylem position, although its expression spread circumferentially in the pericycle; induction of auxin biosynthesis under the *AHP6* promoter was able to restore expression in the protoxylem position. NPA treatment also resulted in a dose-dependent loss-of-protoxylem phenotype similar to that of the *ahp6* mutant. Prolonged NPA treatments led to a proliferation of cells within the stele; this was coupled with an increase in the number of poles of *AHP6* expression after 12 days and in the number of xylem poles after 21 days, in keeping with the previously

observed correlation between stele size and the number of vascular poles in dicots (Torrey, 1955).

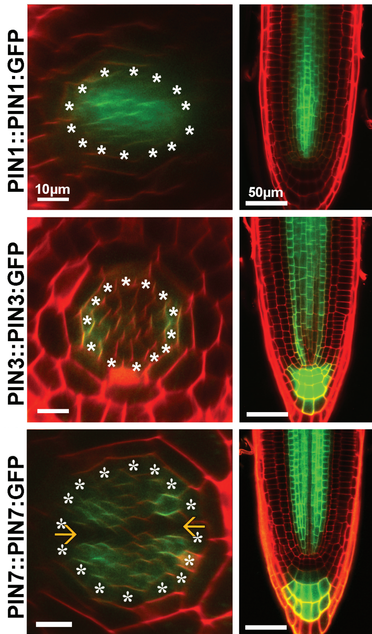

Figure 4: Location of PIN1, PIN3, and PIN7 near the root meristem. Asterisks mark pericycle cells; the xylem axis is indicated by yellow arrowheads. Modified from Bishopp et al. (2011).

Although the precise mechanism by which NPA accomplishes its effect remains unclear, it is known to act as an inhibitor of auxin efflux (Morgan, 1964). We therefore investigated the expression pattern of the PIN class of auxin efflux transporters in order to identify candidates which might be involved in establishing the transverse pattern of auxin distribution. The radially symmetric expression pattern of *PIN2* and *PIN4* makes them unlikely candidates to generate a radially asymmetric auxin pattern. By contrast, the expression patterns of *PIN1*, *PIN3* and *PIN7* are not radially symmetric (Figure 4). Although *PIN1* is expressed throughout the stele, immunolocalisation revealed it to be basally and laterally localized in the cells flanking the xylem axis but only basally localized in the xylem cells. The expression of *PIN3* varies along the longitudinal axis. Approximately 20μm shootwards of the QC it was observed in a bisymmetric pattern in the xylem-pole pericycle; expression then expanded to include the central xylem cells by around 50μm before becoming radially symmetric around 100μm shootwards of the QC. *PIN7* is expressed in two domains comprising the intervening procambium cells and the phloem poles in a pattern similar to *ARR5* expression. The expression patterns of *PIN1*, *PIN3*, and *PIN7* suggest a model in which enhanced transverse auxin transport in the procambium coupled with reduced rootwards efflux from the xylem cells leads to an accumulation of auxin within the xylem axis.

To test this hypothesis, we screened mutants of the PIN transporters for defects in vascular patterning. We observed abnormal protoxylem patterns in *pin1* but not in the other single mutants; however, the *pin3 pin7* double mutant showed abnormal expression of *AHP6* and *DR5,* as well as defects in protoxylem formation.

Cytokinin moulds the auxin domain

In the *ahp6* mutant or in plants treated with cytokinin, cytokinin signalling spreads to include the protoxylem position while auxin signalling is confined to the metaxylem cells; these plants also show inconsistent specification of protoxylem, with gaps or only a single protoxylem pole (Mähönen et al., 2006a). By contrast, mutants with reduced cytokinin expression such as *wol* or the double cytokinin receptor mutant *cre1 ahk3* show an expanded domain of auxin signalling concomitant with a reduction in the cytokinin domain and an increased number of protoxylem cells (Mähönen et al., 2006a). In order to better understand the relationship between auxin and cytokinin in this context, we treated plants which had altered cytokinin

signalling with NPA.

Inducing expression of the cytokinin degradation enzyme *CKX1* in the stele of plants grown on NPA led to an expansion of the domain of *AHP6* and *IAA2* along with the formation of many strands of ectopic protoxylem. Treating the *wol* mutant with NPA had no effect on the all-protoxylem phenotype or *AHP6* expression; although the *IAA2* expression pattern remained radially symmetric, it expanded to include the endodermis cells. Finally, plants treated with both cytokinin and NPA retained *AHP6* expression, suggesting that cytokinin repression of *AHP6* is normally mediated via changes in auxin efflux.

Cytokinin regulates the PINs

These results led us to speculate that cytokinin might affect vascular development by regulating the expression of the PIN efflux transporters or modulating their subcellular localization. We therefore looked for alterations in the distribution of the PINs in response to treatment with exogenous cytokinin and in plants with perturbed cytokinin signalling. Cytokinin treatment resulted in a shift of the *PIN3* domain to the central xylem cells, similar to the change in *IAA2* expression, and an expansion of *PIN7* expression into the protoxylem position, similar to the *ARR5* pattern. A similar change in *PIN7* expression was observed with the transcriptional reporter *pPIN7::GFP:GUS*, indicating that cytokinin regulation of *PIN7* occurs at the level of transcription. In *wol* plants, no *PIN7* expression was observed in the meristematic zone, while *PIN3* expands to include the entire stele, although the signal is faint. The expression pattern of *PIN1* was unaltered in *wol*, but the protein was found to be basally localized in all of the vascular cells, in contrast with wild-type, where it is also laterally localized in cells flanking the xylem axis. Finally, the PIN7 domain was reduced in *cre1 ahk3*, again following the expression of *ARR5*, which flanked an enlarged xylem axis. These data demonstrate that the pattern of PIN localization is sensitive to cytokinin signalling, a finding consistent with other research conducted around the same time, despite differences in the regulatory mechanisms uncovered in the studies (Dello Ioio et al., 2008; Laplaze et al., 2007; Růžička et al., 2009). Our results suggest that cytokinin upregulates *PIN7* transcription and *PIN1* lateralization in this context, while *PIN3* expression seems to closely follow (but not entirely correspond with) auxin signalling.

We next investigated the ordering of these events to confirm that the change in the expression of the markers preceded the anatomical changes rather than being a consequence of them. We therefore constructed transgenic plants expressing *IAA2*, *AHP6*, *PIN7*, or *DR5* in a background with inducible versions of the constitutive cytokinin signalling component *CKI* or the cytokinin degradation enzyme *CKX*. Changes in *PIN7* expression were observed 12 hours after induction of *CKI*; this was followed by changes in the *DR5* and *AHP6* pattern at 24 hours, while anatomical changes were not observed until 72 hours after induction. Likewise, induction of *CKX* resulted in changes in *IAA2* and *AHP6* expression after 24 hours, while anatomical changes were only observed after 48 hours. These experiments confirmed that the observed changes in hormonal signalling and gene expression take place upstream of changes in cell identity and anatomy.

Basipetal hormone transport in the phloem

Cytokinin has been detected in sap and leaf exudates (Takei et al., 2002, 2004a), indicating that it may be transported over long distances in the phloem. We therefore hypothesized that cytokinin transported from the shoot could affect PIN regulation and developmental processes in the root. We began by testing whether application of cytokinin to the aerial parts of the plant could elicit a response in the root. Three hours after application of cytokinin to the hypocotyl of 5-day old seedlings, we observed a spread of the *ARR5* domain in the meristem and an increase in its expression level. To determine that this was actually due to translocation of cytokinins, we developed an assay allowing us to visualize the migration of ^{14}C-labelled N^{6}-benzyladenine (BA) using an imaging plate as a radioactive energy sensor that was processed with a fluorescent image analyser. We observed a radioactive signal in the root meristem region four hours after applying radioactively labelled cytokinin to the hypocotyl of 5-day old plants, confirming that the labelled BA had been transported to the root tip. In order to determine whether this basipetal transport occurred via the phloem, we also conducted the assay in backgrounds with impaired phloem connectivity. In addition to the *apl* mutant, which lacks phloem (Bonke et al., 2003), we tested a transgenic line in which we could impair phloem transport by inducing phloem-specific expression of *cals3m*, which blocks symplastic connections via rapid callose biosynthesis (Vatén et al., 2011). Basipetal transport of labelled cytokinin was severely reduced in both lines, indicating that it occurs via symplastic connections in the phloem. Induction of *cals3m* expression also led to a reduction of the *ARR5* response in the root meristem, indicating that symplastic transport acts as a significant source of endogenous cytokinin to the root meristem.

We used the same assay to examine the basipetal transport of auxin. Following application of ^{14}C-labelled indole-3-acetic acid (IAA) to the hypocotyl, we observed a radioactive signal in the root tip; as with cytokinin, the basipetal transport of auxin was compromised by induction of *cals3m* in the phloem. However, we observed only minor changes in the auxin response in the root tip when phloem transport was blocked, suggesting that bulk auxin transport via the phloem is supplemented by other transport mechanisms or local biosynthesis. Finally, we tested the effect of NPA treatment on the basipetal transport of labelled auxin and cytokinin. While auxin transport was impaired by NPA, cytokinin transport was unaffected.

We next investigated the effect of reduced basipetal cytokinin transport on vascular patterning by examining the pattern of *PIN7*, *DR5*, and *AHP6* expression in these backgrounds. Impaired phloem transport resulted in altered expression of all three markers, often causing an expansion of the domain of *AHP6* expression and high auxin signalling, as well as defects in protoxylem formation. However, these results alone cannot distinguish whether the perturbed pattern is due to reduced cytokinin transport or an overall reduction in transport via the phloem. We therefore created *pAPL:XVE>>CKX1:YFP* plants in which we could inducibly degrade cytokinin in the phloem and examined the markers in this background. Again, the plants showed alterations in the expression pattern of all three markers together with defective protoxylem formation. To confirm that these non-cell-autonomous effects were due to a decrease of cytokinin in the phloem and not in the immature phloem of the root meristem, we also drove expression of *CKX1:YFP*

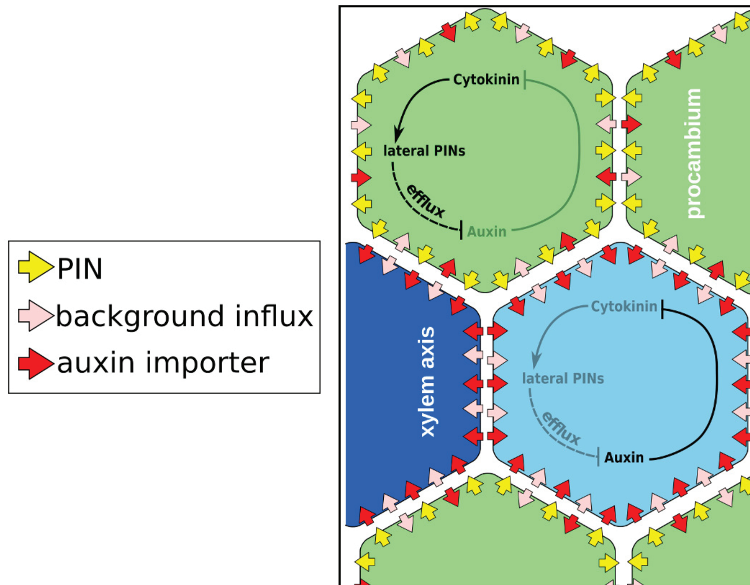

Figure 5: A schematic of the proposed model, showing active (dark) and downregulated (faint) elements in different cells. While the PINs (yellow arrows) were part of the experimentally-derived model, the role of the importers (red arrows) was revealed by our simulations. Modified from el-Showk et al. (2015).

under the *EARLY PHLOEM MARKER* (At2g37950, (Lee et al., 2006)) promoter but only seldom saw minor effects on protoxylem differentiation. Finally, to confirm that cytokinin was being transported from the shoot rather than from distal regions of the root, we grafted *35S::CKX2* shoots onto *AHP6::GFP* rootstocks and observed an expansion in the *AHP6* domain in the roots. Together, these results demonstrate that although the vascular pattern is initially specified during embryogenesis, shoot-to-root transport of cytokinin via the phloem is required for its maintenance throughout the life of the plant.

A model of vascular patterning

Based on these findings, we propose a model in which the vascular pattern of the *Arabidopsis* stele is regulated by a mutually inhibitory feedback loop between auxin and cytokinin mediated by *AHP6* and the PIN efflux transporters (Figure 5). High cytokinin signalling in the procambial cells generates a bisymmetric pattern of localization of the PIN class of auxin efflux transporters which directs the radial transport of auxin into the central axis; high auxin signalling promotes the expression of *AHP6*, which reciprocally restricts the domain of high cytokinin signalling. High auxin output in the xylem axis also promotes the expression of the xylem identity gene *ATHB8* and the specification of protoxylem. Manipulation of the transport or signalling of either hormone shifts the boundary between their domains. Thus, a feedback loop involving hormone transport dynamics and mutually inhibitory interactions provides a mechanism to generate positional information to pattern new organs.

Computational simulations

We tested whether our experimentally derived model was sufficient to explain the observed pattern using computational simulations, which also allowed us to better understand the dynamics of this system. To that end, we implemented a two-dimensional computational model of transverse auxin transport in two different root cross sections, a 'geometric' cross section generated by algorithmic subdivision of a circle and a 'realistic' section generated by segmentation of confocal cross sections. We positioned the auxin transporters *PIN1*, *PIN3* and *PIN7* according to experimental observations and included the hormonal regulation described above. In addition, our model included an auxin importer expressed in all stele cells and an unregulated exporter in the epidermis, cortex and endodermis to represent the reported expression of *PIN2* (Blilou et al., 2005), *PGP1,* and *PGP19* (Mravec et al., 2008) in these cells. Our model, which is an extension of the model developed by Grieneisen et al. (2007), takes into account the spatial structure of cells and cell walls as well as their organization in a tissue. Along with active transport of auxin, diffusion of both auxin and cytokinin was included in the model. For a mathematical description of the formalism, the reader is referred to the Materials & Methods in Chapter IV and to the description by Grieneisen et al. (2007). We used the model to simulate auxin flow in cross sections of wild-type, cytokinin-treated, and *wol* roots with *PIN1*, *PIN3* and *PIN7* placed according to experimental observations; in addition, we simulated *pin1* and *pin7* roots by disabling the appropriate PIN in the simulations.

'Static' simulations in which the transporters were correctly positioned but did not respond to hormonal regulation (and so were constantly at full strength) generated the experimentally observed auxin pattern in wild-type roots but not in *wol* roots or roots treated with cytokinin. Under these conditions, the expected auxin pattern was only generated in 'dynamic' simulations which included hormonal regulation of transporter expression levels. While these simulations indicate that the experimentally derived model is sufficient to explain the auxin pattern in the stele, they also demonstrate the necessity for hormonal regulation of the transporters; correct positioning of the PINs alone is not sufficient to consistently reproduce the observed pattern in all conditions.

In order to understand the contribution of hormonal regulation, we compared the auxin flux pattern in static and dynamic simulations of *wol* roots. In the static simulations, the exporters constantly act at full strength, which leads to higher auxin levels in the apoplast. Once in the apoplast, auxin can freely diffuse throughout the root cross-section without crossing any membranes, allowing it to 'leak' to the layers outside the stele. This 'leakage' may be obstructed *in planta* by the Casparian strip, which was not included in our model. Nevertheless, the difference between the static and dynamic simulations suggests that future experimental work should endeavour to measure the expression level of transporters as well as their spatial distribution pattern.

Cytokinin transport and gradients

Although experimental evidence indicates that cytokinin transport via the phloem is required to maintain the vascular pattern, our simulations demonstrate that a cytokinin gradient within the stele is not required; an even distribution of cytokinin

was sufficient to generate the expected auxin pattern in simulations. Furthermore, physical considerations impose constraints on the ability of cytokinin to form an informative gradient on the scale of the *Arabidopsis* stele via diffusion, particularly given the observation that cytokinin travels over long distances *in planta*. The gradient formed by a diffusing substance depends on the diffusion coefficient and the degradation rate; in order to form an informative gradient on the scale of the *Arabidopsis* root (50-100μm), these parameters need to be set to values that are unrealistic for cytokinin. When the cytokinin diffusion coefficient and degradation rate are similar to those of auxin, a gradient cannot form across the stele via diffusion since the scale of a diffusive gradient is much larger than that of the stele (blue line in Figure 6); in order to form a gradient via diffusion alone, one or both of these parameters must be changed by several orders of magnitude (e.g., red and green lines in Figure 6). This is true even in simulations with cytokinin biosynthesis localized to specific cells and cytokinin movement inhibited by cell membranes, since diffusion in the apoplast quickly evens out the cytokinin distribution (unless the diffusion coefficient is significantly lower in the apoplast). The challenge becomes yet more extreme in the developing embryo, since the distances involved are even smaller. Our analysis demonstrates that diffusion alone is not sufficient to form a patterned distribution on these scales; it must be supplemented by other dynamics, such as directed transport or a source-sink mechanism.

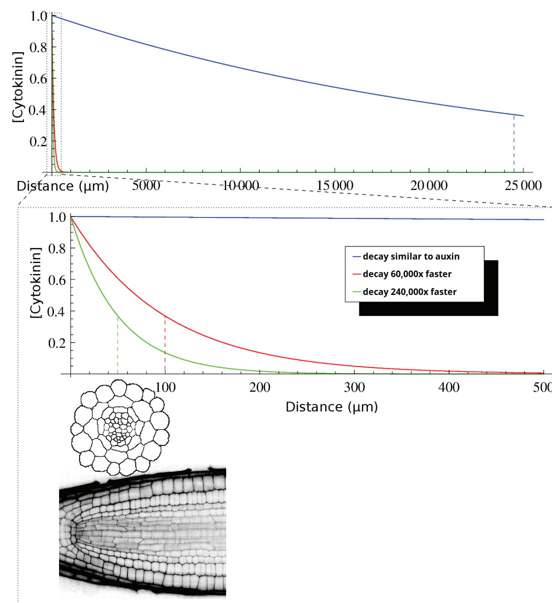

Figure 6: The steady-state concentration of cytokinin at a distance from a source in a diffusion-decay model. The lower box is zoomed in 50x, with root sections drawn to scale for comparison with the gradients. The dotted lines indicates the ‘characteristic length’, at which the concentration is about 35% of the source. Modified from el-Showk et al. (2015).

These findings directly conflict with a recent study of the same network which argued that local cytokinin biosynthesis forms a gradient essential for correct vascular patterning (De Rybel et al., 2014). However, the simulations by De Rybel et al. used a cytokinin degradation rate ten times greater than that of auxin and an extremely

low membrane permeability (equivalent to the lowest value we tested) coupled with the biologically implausible assumption of little or no cytokinin movement in the apoplast. Careful examination of the assumptions embedded in their simulations therefore reinforces our argument that it is challenging to form an informative cytokinin gradient on these scales via diffusion alone.

Nevertheless, spatially specific patterns of cytokinin signalling have been observed across the *Arabidopsis* stele; there appears to be a gradient between high cytokinin signalling in the procambium and lower signalling in the xylem axis. These observations may reflect an underlying cytokinin gradient which could be formed by directed cytokinin transport or spatially specific expression of cytokinin degradation genes; alternately, there may not be a cytokinin gradient underlying these observations, which might instead result from spatially specific expression of cytokinin perception genes on top of a homogeneous cytokinin distribution.

A directed cytokinin transport process might be self-patterning, as seems to be the case with auxin and the PINs. By contrast, patterned cytokinin perception or degradation seem likely to only push the pattern-formation question to another level. It may be that these patterns result from auxin regulation of genes involved in cytokinin perception (e.g., ARRs) or degradation (e.g., CKXs); another intriguing possibility is the notion that auxin might even regulate cytokinin transporters, if they exist. Regardless of the preferred scenario, our analysis raises questions and challenges regarding the mechanics and dynamics of cytokinin transport which must be addressed by experiments, thus guiding and constraining future work. Furthermore, these results highlight how proposals about local patterning mechanisms may have implications at the scale of the whole organism, stressing the importance of considering what constraints may be introduced by conflicting requirements at different scales.

A role for auxin import

Our simulations predicted no auxin maximum in the xylem axis (and therefore protoxylem defects) in *pin1* but not in *pin7*, consistent with empirical data. Furthermore, plotting the amount of auxin accumulated in the xylem axis in various simulations revealed that roots lacking active auxin import had lower auxin levels (Figure 7), suggesting that plants with mutations in one or more of the auxin influx transporters would have a protoxylem phenotype similar to *pin1* or perhaps even more severe. We therefore investigated the protoxylem phenotype of single and multiple mutants of *AUX1*, *LAX1*, and *LAX2*, the auxin importers expressed in the root meristem. While none of the single mutants showed a protoxylem phenotype, the *aux1 lax1 lax2* triple mutant showed a severe phenotype with defective protoxylem formation in more than 80% of the plants examined. We also found that protoxylem formation in the *aux1*

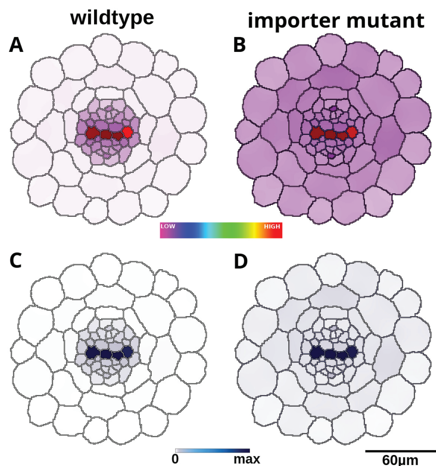

Figure 7: The auxin distribution in simulations of (A, C) wildtype and (B, D) importer mutant roots. The auxin concentration is shown in A & B, while C & D use a blue heatmap with a response curve similar to that of the auxin reporter *DR5*.

mutant is hypersensitive to inhibition by cytokinin, indicating that this mutation disrupts the stability of the auxin-cytokinin feedback loop. Previous work has shown that *LAX3* acts alongside *AUX1* in lateral root initiation (Péret et al., 2012) while *LAX1* and *LAX2* act with *AUX1* in regulating phyllotaxis (Bainbridge et al., 2008). Together with our findings and recent work showing that the LAX proteins are not correctly targeted to the membrane in the *AUX1* expression domain (Péret et al., 2012), this suggests that specific LAX genes may act in concert with *AUX1* to amplify or stabilize the auxin pattern in different developmental contexts.

A transverse auxin flux circuit

Our experimental data suggest that the lateral localization of PIN1 is polar in stele cells, but we cannot rule out the possibility of apolar localization. Fortunately, simulations enable us to evaluate the implications of these different scenarios despite the challenge of addressing them experimentally; we may therefore be able to generate hypotheses which may allow us to indirectly test the scenarios.

Apolar localization of PIN1 in the procambium resulted in lower auxin accumulation in the xylem axis, although a clear maximum remained. However, examination of the auxin flux patterns revealed a striking difference between simulations with polar and apolar PIN1. Polar auxin efflux within the procambium organized the auxin flux into a circuit towards the xylem axis and out through the protoxylem poles, while apolar localization resulted in disorganized auxin flux within the stele (Figure 8). The disorganized flux in simulations with apolar roots appears to be sensitive to the geometry of the root cross-section, although we did not test this rigorously.

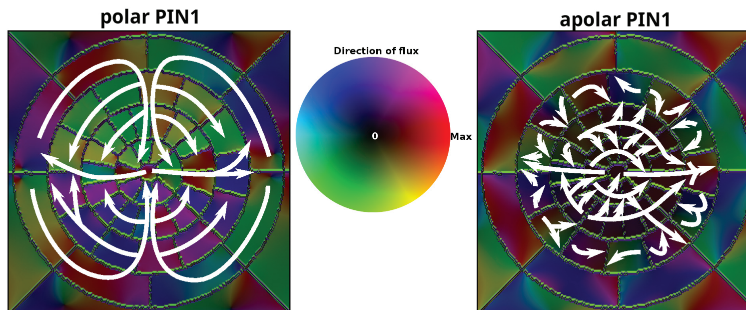

Figure 8: A heatmap of the auxin flux in the stele of simulated geometric cross sections with polar or apolar PIN1 localization; the overall flux pattern is depicted by the white arrows. Modified from el-Showk et al. (2015).

We investigated whether the auxin circuit stabilizes the auxin maximum in the xylem axis or otherwise affects the auxin pattern in the developing stele. In simulations with polar or apolar PIN1 & PIN7 in the procambium, we transiently activated *AUX1* expression in a single pericycle cell and observed whether this cell would destabilize the auxin maximum in the xylem axis and become a new auxin sink. In geometric cross-sections with apolar localization of the PINs, a 120 second activation of *AUX1* in any pericycle cell consistently led to auxin accumulation in a specific pericycle cell, suggesting that this cell was somehow favoured by the geometry of the cross-section. When PIN localization was polar, only the xylem-pole

pericycle cells could compete with the xylem axis and accumulate auxin; an *AUX1* pulse in other pericycle cells did not disrupt the auxin maximum in the xylem. In realistic cross-sections with apolar PINs, an *AUX1* pulse only led to auxin accumulation if it occurred in a subset of the xylem-pole pericycle cells, while polar localization enabled all of the xylem-pole pericycle cells to accumulate auxin. The auxin flux circuit generated by polar PIN localization therefore seems to stabilize the ability of the xylem-pole pericycle cells to compete with the xylem axis, while other factors (perhaps related to tissue geometry) seem to dominate in the absence of the circuit.

The potential developmental implications of auxin accumulation in xylem-pole pericycle cells are particularly intriguing since *AUX1* activation in these cells is one of the earliest steps in lateral root initiation (Laskowski et al., 2006). Given that lateral roots in *Arabidopsis* alternate between opposite xylem poles, we next conducted simulations testing the effect of PIN polarity when *AUX1* was simultaneously activated in opposing xylem-pole pericycle cells for 120 seconds. In these simulations, auxin always accumulated in only a single pericycle cell, following the same pattern as when *AUX1* was only activated in one pericycle cell (i.e., the same cell always accumulated auxin in apolar geometric simulations, but not in other conditions). We next introduced a delay between the *AUX1* pulse in the two pericycle cells in simulations with polar PINs in an effort to decide which xylem-pole pericycle cell would accumulate auxin. A short delay (5s) was sufficient to ensure that the first cell receiving *AUX1* accumulated auxin in realistic cross-sections, while a longer delay (100s) was necessary in geometric cross-sections.

Our simulations demonstrate that polar PIN localization in the procambium generates a preference for xylem-pole pericycle cells to accumulate auxin, offering the intriguing possibility that this regulatory network may be connected to lateral root initiation. This system is also bistable, ensuring that only one of the two poles can accumulate auxin. In addition to the implications for lateral root initiation, privileging the xylem-pole pericycle may have a wider-reaching impact on plant development. Passage cells, endodermal cells which do not form suberin lamellae, are also always positioned at the xylem poles (Geldner, 2013), and xylem-pole pericycle cells appear to be more pluripotent than other pericycle cells, since *in vitro* shoot regeneration is possible from xylem-pole but not phloem-pole pericycle cells (Atta et al., 2009). Furthermore, the pluripotent tissue callus seems to result from ectopic activation of the lateral root developmental program; callus expresses a xylem-pole pericycle marker and its formation requires *ABERRANT LATERAL ROOT FORMATION 4* (Sugimoto et al., 2010), a gene which acts downstream of auxin during lateral root initiation (Celenza et al., 1995). The auxin flux circuit identified here may thus serve to link patterning at several levels, connecting subcellular polarity to tissue-level organization.

Concluding Remarks

In this work we have described a novel mechanism regulating vascular patterning in the *Arabidopsis* root. Based on experimental data, we proposed a model in which mutual inhibition between auxin and cytokinin mediated by the regulation of signalling and transport establishes complementary signalling domains in the stele, providing the positional information needed to maintain the continuity of vascular

strands. Our experiments show that the hormone signalling domains are remarkably robust, showing only minor responses to a wide range of manipulations; major disruptions to either auxin or cytokinin signalling were required to significantly alter the pattern.

We then used computational simulations to confirm that the proposed network is sufficient to maintain the vascular pattern. Our simulations show that PIN patterning alone is not always sufficient to account for the observed auxin distribution; in several cases, hormonal regulation of PIN expression levels is also required. Furthermore, we identified a role for active auxin import in stabilizing the auxin pattern generated by the experimentally-derived model. Finally, we observed that polar PIN localization in the procambium results in an auxin flux circuit through the xylem axis which generated a preference for auxin accumulation in the xylem-pole pericycle cells, potentially linking this mechanism to later developmental events.

Our simulations also demonstrate the difficulty of forming an informative cytokinin gradient on the scale of the *Arabidopsis* root via diffusion. While a cytokinin gradient does not appear to be required in this patterning process, cytokinin is a central hormone in plant development, and a cytokinin gradient has been suggested to play an important role in process such as shoot meristem patterning (Chickarmane et al., 2012) and root gravitropism (Aloni et al., 2004). Despite the difficulty of forming a cytokinin gradient, observations of cytokinin signalling response show a clear pattern in many contexts, raising the question of how the patterns are formed. The basis of the observed patterns—whether they result from local, directed cytokinin transport or patterning of the cytokinin perception machinery—is therefore a pressing, open question.

Our model also highlights the importance of including the apoplast when considering hormone transport and patterning. In our 'static' simulations, the apoplast formed a conduit for auxin to reach the outer layers of the root after it was pumped out of stele cells by over-active exporters. Likewise, the formation of a cytokinin gradient via diffusion alone is limited by movement through the apoplast, which evens out the cytokinin distribution; similar dynamics constrain auxin gradients, which become more shallow when auxin moves freely in the apoplast in the absence of importers (Swarup et al., 2005). These findings underscore the importance of the apoplast and the potential pitfalls of overlooking it in our models, whether computational or pen-and-paper.

An understanding of *de novo* patterning is an important precursor to evaluating the applicability of our model to other plants. Ideally, the model would be able to account for the variation in vascular patterns found in different species, although this assumes that a common biological mechanism underlies these various patterns. How true this is remains to be seen; it is certainly plausible that vascular patterning is regulated differently in monocots than dicots. Modelling approaches will be invaluable in extending the model to other species, since it would be trivial to implement the network (or a modified version) in roots of different sizes and geometries and evaluate the resulting patterns. In addition, such an approach would directly test the utility of the underlying patterning mechanism rather than a specific implementation, thus allowing for the possibility of a similar network based on a different set of components. A combination of experimental and computational approaches thus informs our understanding of development in *Arabidopsis* and will help generalize these findings to other species.

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Since my last paper strayed outside of Ykä's area of expertise, he entrusted its supervision to others. Ari Pekka (AaPee), Verônica, and Stan, thank you for guiding me through that project. AaPee, thank you for all the discussions about my model and its implications, for helping flesh it out with experiments, for supporting me when the project got stuck and helping push it through to the end. Verônica and Stan, thank you for the inspirational course which got me started as a modeller, for welcoming me into your lab with friendship, for helping me turn my eagerness into a research project, and for the insightful discussions and technical help that brought it to fruition.

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