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Zusammenfassung

In höheren Pflanzen besteht die Vaskulatur aus drei Gewebearten. Das Phloem transportiert Assimilate, das Xylem transportiert Wasser von den Wurzeln zu den Blättern, das Kambium, oder Kambialmeristem, ist eine Stammzellpopulation. Im Stamm von *Arabidopsis thaliana* kann die vaskuläre Entwicklung in grob drei Phasen unterteilt werden. Im unreifen, oder primären Stamm besteht die primäre Vaskulatur aus separaten Bündeln. Jedes Leitbündel besteht aus Phloem auf der Innenseite, Xylem auf der Außenseite und dem faszikulären Kambium dazwischen. In der Übergangsphase beginnen sich das Kambium sowie Zellen im interfaszikularen Bereich zu teilen und führen zur Entstehung eines interfaszikulären Kambiums. Wenn das neue Kambium, welches das faszikuläre Kambium verbindet, sekundäres Phloem und Xylem produziert hat, ist die reife, oder sekundäre Phase erreicht. Diese Phase wird auch sekundäres Dickenwachstum genannt.

Das Phytohormon Auxin ist der initiiierende Faktor für sekundäres Dickenwachstum. Die Verteilung von Auxin in der Pflanze wird durch gerichteten Transport durch Zellen erreicht. Die verantwortlichen Transmembranproteine, welche polar in der Zellmembran verteilt sind, sind AUX1, verantwortlich für den Auxin-Influx, sowie die PIN und PGP Proteine für den Auxin-Efflux. Abseits dieser Transportwege findet auch ein passiver Transport durch Diffusion statt.

Cytokinin, ein anderes für die Entwicklung wichtiges Phytohormon, ist ebenfalls ungleichmäßig in der Pflanze verteilt. Die Signaltransduktion erfolgt über ein Phosphorelay-System, welches aus Cytokinin-Rezeptoren (AHK), cytosolischen Signaltransmittern (AHP) und nuklearen Reaktionsregulatoren (ARR) besteht.

In dieser Arbeit wurde die Rolle der *PIN* Gene und des Cytokinin Signaltransduktionswegs im sekundären Dickenwachstum durch die histologische Analyse von Einzel-Knockout Pflanzen untersucht. Diese Beobachtungen wurden durch Untersuchungen von Pflanzen mit induzierbaren künstlichen microRNAs gegen ausgewählte *PIN* Gene ergänzt. Mit demselben induzierbaren System wurde *CKX1*, eine Cytokinin Oxidase, überexprimiert, um den Cytokinin-Gehalt in der Pflanze zu reduzieren. Die Räumliche Verteilung der Expression der *PIN* Gene wurde durch Promoter-GUS Fusionen visualisiert. Zeitliche Expressions-Unterschiede wurden durch RT-PCR auf der Basis von RNA aus Stämmen aufgezeigt. Um den Effekt von Auxin, und verschiedenen anderen Molekülen, auf isolierte Stämme direkt beobachten zu können, wurde ein *in vitro* System zu diesem Zweck adaptiert.

Durch diese Ansätze konnte ermittelt werden, dass kein einzelnes *PIN* Gen alleine notwendig für die initiiierenden Bedingungen ist, aber *PIN1*, *PIN3* und *PIN4* wichtig für die normale Entwicklung sind. Besonders der Verlust von *PIN3* schien zu einem Verlust der Kontrolle über die Auxin-Verteilung zu führen, da derartige Mutanten wesentlich unkontrollierter auf externe Auxinquellen reagierten als der Wildtyp.

Mutationen im Cytokinin Signaltransduktionsweg hatten keinen wesentlichen Einfluss auf die Initiierung des sekundären Dickenwachstums. Dennoch waren die Verfügbarkeit von Cytokinin und die unversehrte Funktionsweise des Signaltransduktionswegs förderlich für gleichmäßige Zellteilungen und eine normale Rate der Zellproliferation.

Sowohl die Methode, um die Expression künstlicher microRNAs gegen ausgewählte Ziele zu induzieren, als auch das *in vitro* System konnten zufriedenstellend etabliert werden, und stehen nun als Werkzeug für künftige Untersuchungen bereit.

Abstract

In higher plants, the vasculature consists of three tissues. The phloem transports assimilates, the xylem transports water from roots to leaves, the cambium, or cambial meristem, is a stem cell population. In the stem of *Arabidopsis thaliana*, vascular development can be roughly separated into three phases. In the immature, or primary, stem, the primary vasculature consists of discrete vascular bundles. Each vascular bundle consists of phloem towards the outside of the stem, xylem towards the inside, and the fascicular cambium inbetween. In the intermediate phase, the cambium as well as cells in the interfascicular region start to divide, leading to an interfascicular cambium. When the new cambium, which connects the fascicular cambium, has produced secondary phloem and xylem, the mature, or secondary, stage is reached. This phase is also called secondary growth.

The phytohormone auxin is the initiating factor for secondary growth. Distribution of auxin across the plant is facilitated by polar transport through cells. The responsible transmembrane proteins, which are polarly distributed on the plasma membrane, are AUX1 for auxin influx, and the PIN proteins for auxin efflux.

Cytokinin, another phytohormone important for development, is also distributed nonpolarly across the plant. The signaling pathway is a phospho-relay system, which consists of cytokinin receptors (AHK), cytosolic signal transmitters (AHP) and response factors (ARR).

In this project, the role of the *PIN* genes and cytokinin signaling pathway in secondary growth was investigated by histological analysis of single knock-out stems. This analysis was complemented by the investigation of plants which could be induced by ethanol application to express artificial microRNAs against chosen *PIN* genes. With the same inducible system, *CKX1*, a cytokinin oxydase, was overexpressed to reduce cytokinin levels in the plant. Spatial distribution of *PIN* gene expression was visualized with promoter-GUS fusions. Temporal differences were shown with RT-PCR on stem RNA. In order to directly observe the effect of auxin, and eventually various other compounds, on isolated stems, an *in vitro* system was adapted to that effect.

Using these methods, it could be determined that no single *PIN* gene alone is required for induction, but *PIN1*, *PIN3*, and *PIN4* are important for normal development. Especially loss of *PIN3* seemed to result in a loss of the control of auxin distribution, since the mutant reacted in a more uncontrolled fashion to external auxin application than wild type.

Mutations in the cytokinin signaling pathway did not have a significant effect on the initiation of secondary growth. Still, availability of cytokinin and proper function of the signaling pathway were beneficial for regular cell divisions and an appropriate rate of cell proliferation.

Both the methods of inducing the expression of artificial microRNAs against chosen targets and the *in vitro* system could be shown to work reliably and offer a tool for future investigations.

Introduction

Higher plants possess specialized tissues to conduct transport throughout the organism: the vascular system. The vasculature consists of three types of tissue with dedicated functions: phloem, xylem and cambium (Fig. 1).

Phloem is responsible for the transport of organic compounds produced by photosynthesis and other processes. This transport takes place in the enucleated sieve tube cells, which are connected by porous cell walls, the sieve plates. These cells are aided through plasmodesmatal connections by companion cells, which still possess all of their organelles. Phloem further contains less specialized parenchyma cells. In addition to its transport function, phloem also serves as a signaling highway for the plant by transporting various signaling molecules.

Xylem transports water and inorganic nutrients from roots to the sink tissue of a plant. Transport takes place in the tracheary elements, which consist of tracheids and vessel elements. Both of these cell types undergo programmed cell death as they mature and differentiate. When these cells cease growth, heavily lignified secondary cell walls are formed. This strengthening adds to the stability of stems and roots. As in phloem, also xylem still contains parenchyma cells.

Both early (primary) phloem and xylem originate from procambium, a primary meristem which derives from the apical meristem. After differentiation of the primary vasculature, further secondary phloem and xylem tissue originates from the vascular cambium, a procambium-derived stem cell population situated in between the two transporting tissues (Fig. 1).

Vascular development in the stem is divided into two distinct phases (Fig. 2). The primary state of a plant is characterized by longitudinal growth of root and shoot, which is the result of the action of the apical meristems. In this state, the vasculature is separated into discrete bundles which run along the plant. The most common arrangement of these primary vascular bundles is of the collateral type: xylem is oriented towards the inside of the shoot, whereas phloem is oriented towards the outside. The two tissues are separated by the fascicular cambium. This pattern is widely distributed among gymnosperms and angiosperms.

As the plant matures, a new meristem is formed between the primary bundles: the interfascicular cambium. This new meristem will then proliferate and differentiate to secondary xylem towards the inside of the shoot, and secondary phloem towards the outside of the shoot. This process, which complements the primary lengthening of the plant by thickening of the stem, is called secondary growth. It is this process which leads to wood production and is therefore essential for all woody plants.

Economic interest in secondary growth is primarily oriented towards trees to control wood production. But trees are not the ideal system for genetic research. At the time of writing, *Populus trichocarpa* was the only tree with a fully sequenced genome. Trees have extraordinarily long life cycles, some as much as decades. This makes crossings almost impossible and observations on its development a long-term process.

Arabidopsis thaliana, a member of the *Brassicaceae*, is a dicot common throughout temperate zones. Though it is of no economical importance, it is the premier scientific model plant, owing to its rapid life cycle and fully sequenced genome, which extends across just five chromosomes. Being a short-

lived weedy plant, it is uncertain how important secondary growth is to its function, but occurs nonetheless. Therefore, it makes a good model to study secondary growth in comparison to trees. As the process of secondary growth itself is mostly identical to trees (Nieminen, Kauppinen 2004), the conclusions drawn from research on *Arabidopsis* might also apply to trees.

The primary, or immature, phase in *Arabidopsis* stem development is characterized by the presence of discrete vascular bundles (Fig. 1). These consist of a strip of (fascicular) cambium, phloem towards the outside of the stem and xylem towards the inside. The interfascicular space, though not xylemized yet, still lends the stem stability by the presence of lignified interfascicular fibers. Those are a common characteristic of the *Brassicaceae* (Metcalf & Chalk, 1950). The stem is protected towards the outside by the epidermis. This is followed by several layers of cortex cells. The innermost cortex cell layer, the starch sheath, contains starch granules which are responsible for gravity sensing (Wysocka-Diller et al., 2000). The inside of the stem is filled with parenchymatic cells, which are collectively named the pith.

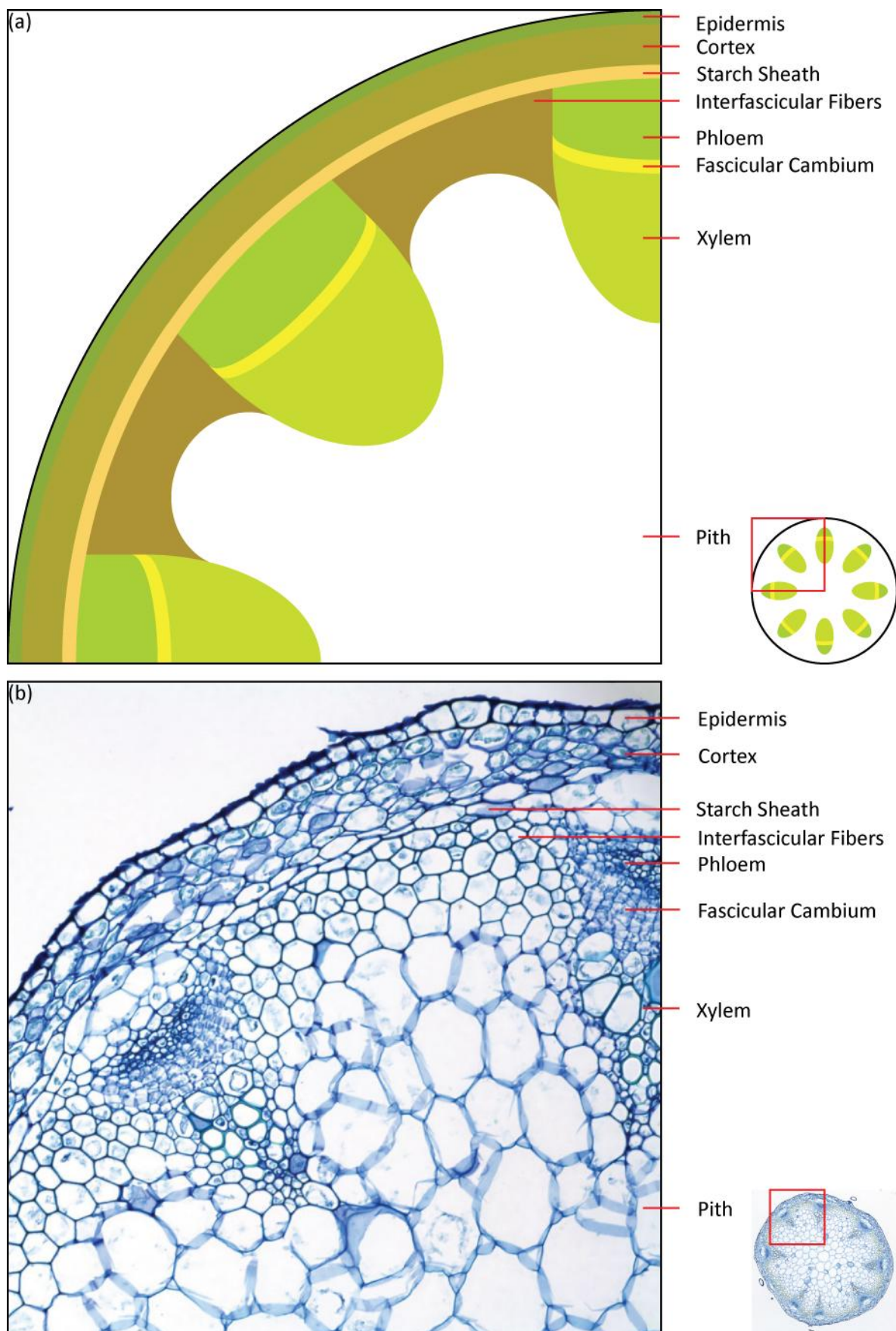


Figure 1: (a) Schematic and (b) 7 μm thick cross-section (stained with Toluidine blue) of an immature *Arabidopsis thaliana* stem, showing primary bundles and the interfascicular region.

Secondary Growth in *Arabidopsis thaliana*

During the initiation of secondary growth in *Arabidopsis*, or intermediate phase, a new layer of meristematic cells forms in the interfascicular region, the interfascicular cambium. The first periclinal cell divisions in interfascicular regions, which lead to the new cambium, are easily identified by light microscopy and serve as an indicator of the initiation of secondary growth (Fig. 2 h). The fascicular cambium is now connected by the new interfascicular cambium, both giving rise to secondary phloem towards the outside of the stem and secondary xylem towards the inside. In the mature stem, all three vascular tissues form a closed cylinder around the stem (Fig. 2 c, f). What factors account for this change of differentiated cell layers to a pluripotent stem cell layer is still unknown, though the phytohormone auxin is one essential initiating factor (Uggla, Moritz et al. 1996).

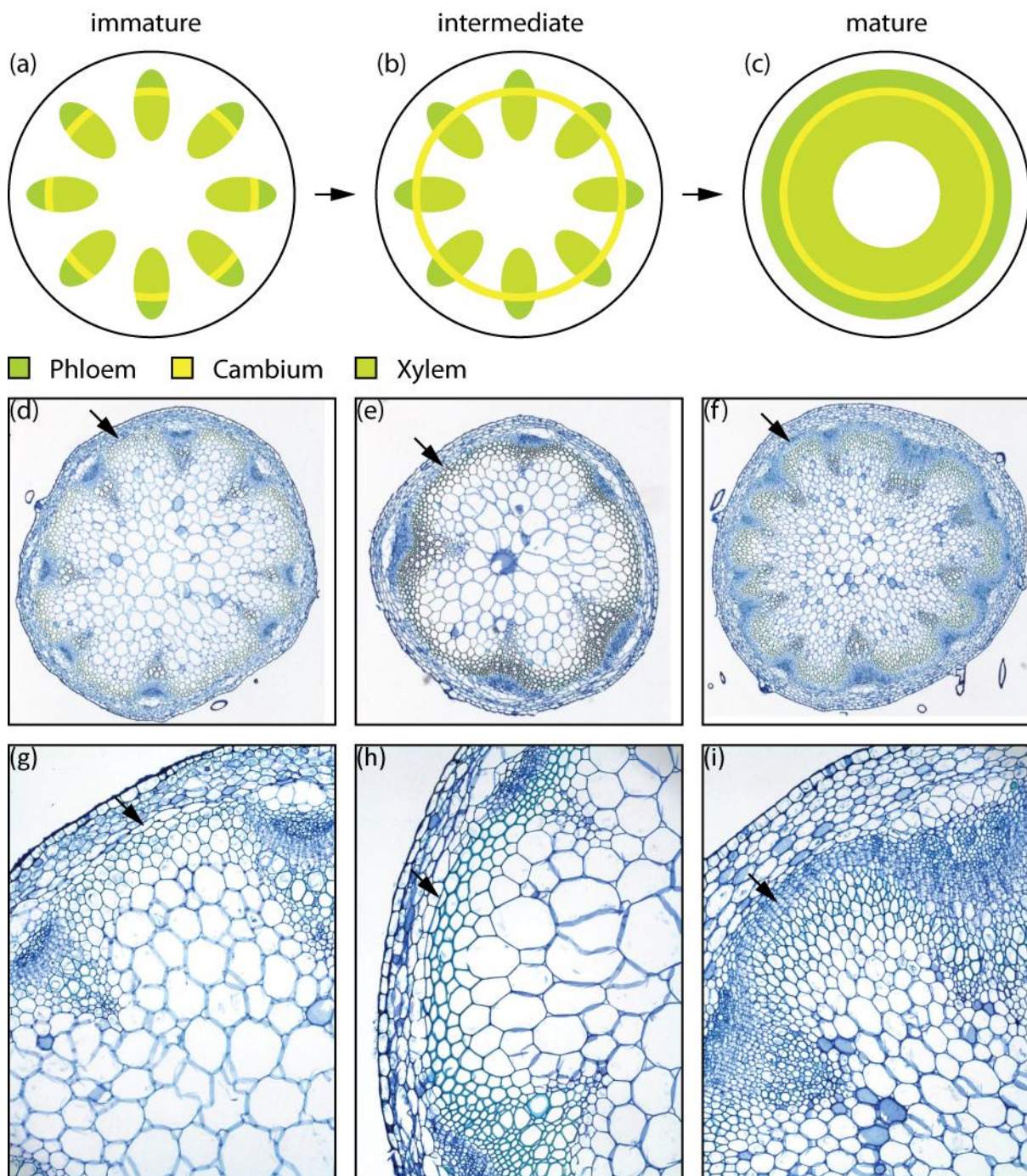


Figure 2: The process of secondary growth in the wild type Columbia stem, shown schematically (a-c) and in 7 μm thick cross-sections stained with Toluidine blue (d-i). Immature stems (a, d, g) contain discrete vascular bundles consisting of phloem, xylem, and fascicular cambium. The starch sheath in the interfascicular regions is discernible by its oblong cells (arrow) between the cortex to the outside and the interfascicular fibers and/or pith to the inside. During the initiation of secondary growth (intermediate stage; b, e, h), the starch sheath cells start to divide (arrow), and the patches of fascicular cambium are connected with new interfascicular cambium. In the mature stem (c, f, i), cell files of secondary phloem and xylem have formed, originating from the new cambium (arrow).

Auxin

Indole-3-acetic acid (IAA), a natural auxin, was the first plant hormone to be discovered in the early twentieth century, and early on linked to cambial activity (Snow et al., 1935). It forms a declining apical-basal gradient across the whole plant, ensuring apical dominance (Jacobs, Case 1965). It is one of the major phytohormones determining growth. Synthesis occurs mostly in the root and shoot tips, and on a lower level in leaves. (Benkova, Michniewicz et al. 2003). Auxin has numerous effects on a plant, most notably shoot apical dominance, side root growth, fruit growth, shoot branching and wounding response. Synthetic auxins such as 2,4-Dichlorophenoxy-acetic acid are commonly being used as herbicides, as plants lose control over auxin distribution and regular growth (Overbeek, Velez 1946).

It has been shown that auxin accumulates at sites of procambium formation, giving a strong hint that it might be responsible for vascular development (Scarpella, Francis et al. 2004). In *Pinus*, IAA levels peak at the cambial position (Uggla, Moritz et al. 1996). Several authors conducted decapitation experiments with subsequent auxin treatment. They have shown that secondary growth in the stem can be induced by external auxin application replacing the shoot apical meristem (Little, MacDonald et al., 2002).

Auxin distribution is controlled by an intricate transport system (Fig. 3). Though passive diffusion across the plasma membrane has been observed in cell culture for tobacco, most of the transport occurs via transmembrane proteins (Delbarre, Muller 1996). Auxin influx is facilitated by the polarly distributed auxin influx carrier AUX1 (Swarup, Kargul 2004). Phosphoglycoprotein transporters (PGP) are distributed nonpolarly across the plasma membrane and facilitate auxin efflux (Geisler, Blakeslee 2005). The PIN family members of auxin efflux carriers (Gälweiler, Palme 1998) are distributed in a polar fashion, though each individual PIN protein shows a different localization. All these factors contribute to a polar transport of auxin, thereby setting up a non-uniform distribution pattern.

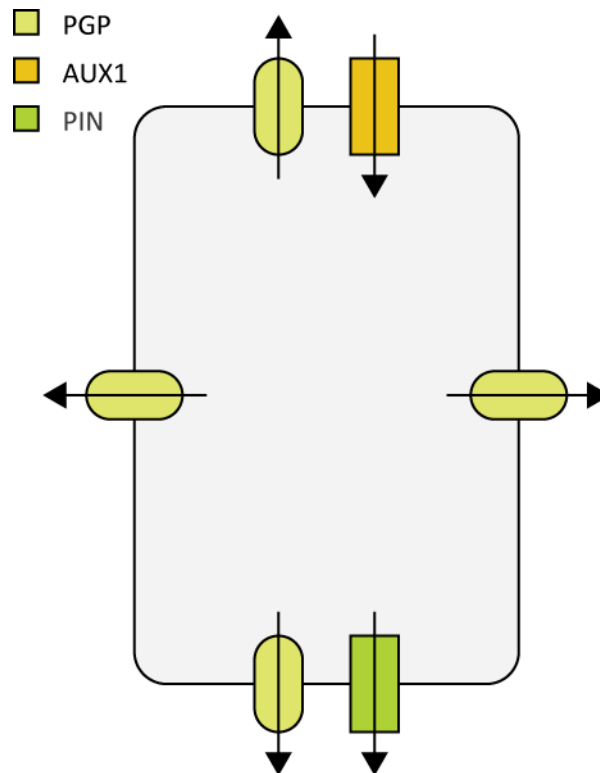


Figure 3: Schematic representation of a plant cell with auxin transporters. Arrows indicate the direction of auxin transport. Since AUX1 and PIN have a polar localization, overall auxin transport is directed.

These differences in auxin distribution across the plant contribute to the establishment of cell fates during development.

PIN Gene Family

It is known that auxin transport controls the development of vascular tissue, relying on the transmembrane auxin transporter PIN1. The *PIN* gene family consists of eight members. A phylogenetic comparison of the amino acid sequences show that PIN3, PIN4 and PIN7 form a closely related subgroup. Also, PIN5 and PIN8 form a branch distinct from the other PINs (Fig. 4).

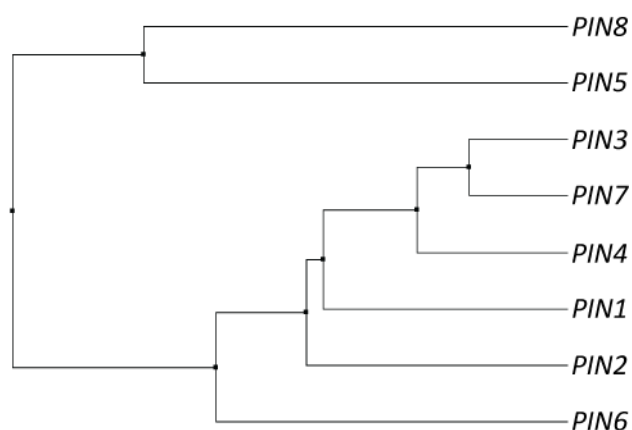


Figure 4: Phylogenetic tree of the *PIN* genes, based on their amino acid sequence. Made with ClustalW (Larkins, Higgins 2007)

Public expression data (Zimmerman, Gruissem 2004) show that *PIN1* and *PIN3* are the most strongly expressed members in the stem. *PIN3*, *PIN4* and *PIN7* also show significant, although lower, expression (Fig. 5).

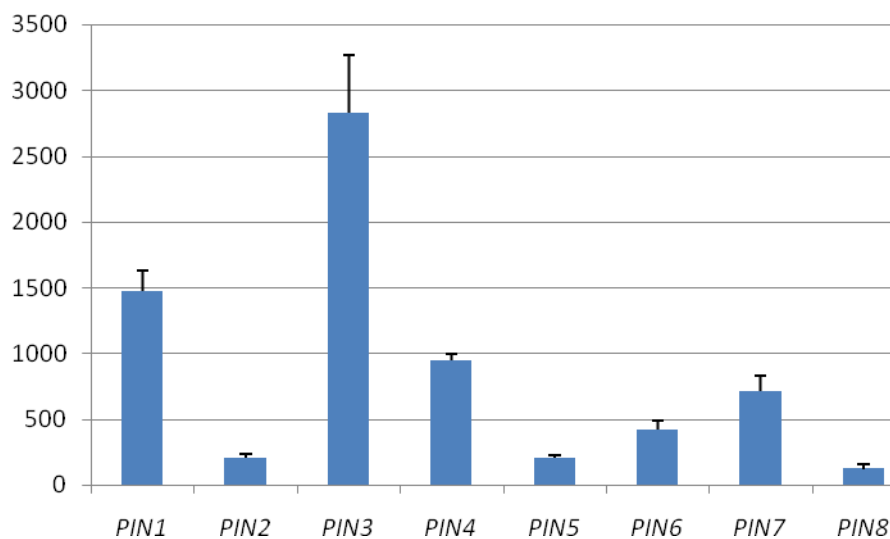


Figure 5: Expression levels of the PIN genes in stems based on public expression data.

PIN1 was the first *PIN* gene to be described (Gälweiler, Guan et al. 1998). The characteristic pin-like stem of its knockout allele gave the gene family its name. The protein is known to localize at the basal cell membranes in the root stele, suggesting auxin transport in the apical-basal axis (Benkova, Michniewicz et al. 2003). *PIN2*, which is essential for proper root development, is barely expressed in the stem at all and therefore unlikely to significantly affect stem development. In the immature stem, *PIN3* localizes to the starch sheath (Friml, Wisniewska et al. 2002). In the individual starch sheath cells, *PIN3* is found at the basal and abaxial plasma membrane. *PIN4* and *PIN7* play important roles during embryogenesis (Friml, Vieten et al. 2003), but little is known about their functions in vascularization. *PIN6* expression patterns correlate with those of *PIN1* (Vieten, Vanneste 2005). *PIN5* and *PIN8* are lacking significant domains of the other *PIN* proteins and are probably not bound to the plasma membrane (Friml, unpublished results).

Cytokinin

A second phytohormone which is essential for plant development is cytokinin. Like auxin, it is important for a number of processes in the plant, including cell cycle control (Wood, Braun 1969) and the differentiation of tracheary elements (Fukuda et al. 1992). An external cytokinin source has been shown to induce cambial activity in combination with auxin (Skoog, Miller 1957). Also, mutations in the cytokinin signaling pathway can lead to defects in vascular development (Mähönen, Bishopp et al. 2006).

Cytokinin signaling occurs via a two-component phosphorelay (Heyl, Schmölling 2003). Cytokinin molecules bind to their receptors, the AHK transmembrane proteins (Arabidopsis Histidine Kinases). Upon cytokinin binding, the AHKs autophosphorylate and transfer the phosphoryl group to the mobile component of the signaling pathway, the AHPs (Arabidopsis Histidine Phosphotransferases).

The AHPs migrate into the nucleus, where they transfer the phosphoryl group to the ARR transcription factors (Arabidopsis Response Regulator), thereby activating them (Fig. 6).

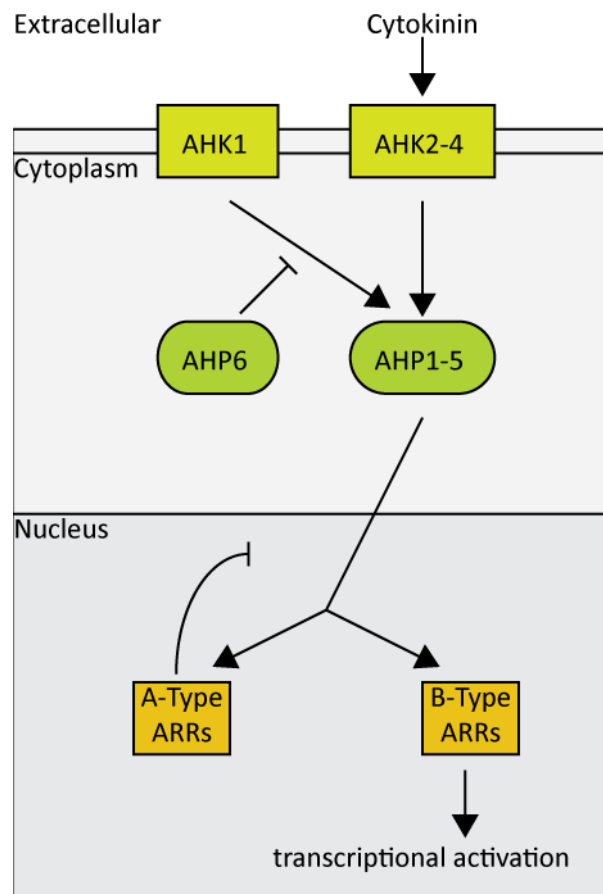


Figure 6: Schematic representation of the cytokinin signaling pathway.

For each of the three components, several genes exist, with differences in expression and function.

A total of four *AHK* genes exist (Fig. 7), though only the AHK2, AHK3, and AHK4/CRE1 proteins possess the CHASE domain for cytokinin sensing (Yamada et al. 2001; Mougél, Zhulin 2001). AHK1 can still transfer a phosphoryl group to the AHPs, but its activity does not depend on cytokinin (Tran, Urao 2007).

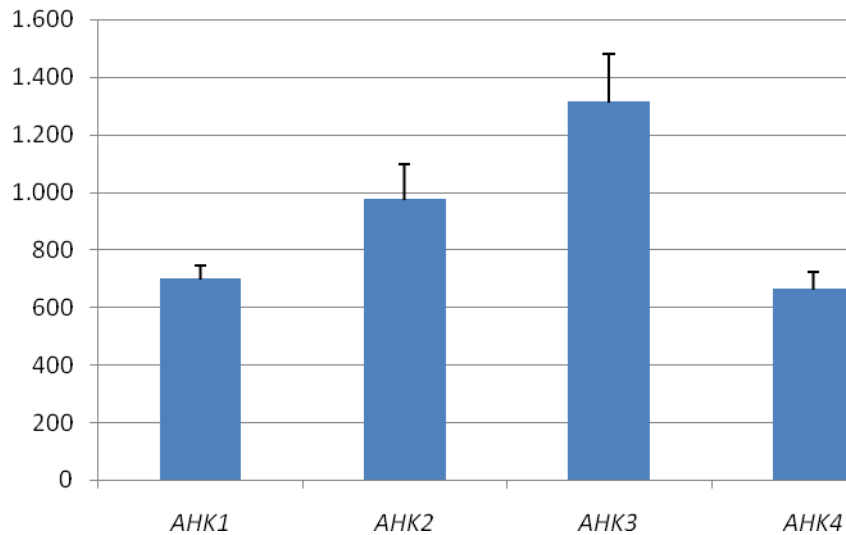


Figure 7: Expression levels of the *AHK* genes in stems, based on public expression data.

Six *AHP* genes are encoded in the *Arabidopsis thaliana* genome (Fig. 8). *AHP1*, *AHP2*, *AHP3*, *AHP4* and *AHP5* can transfer their phosphoryl group to the *ARRs* (Tanaka, Suzuki 2004). *AHP6* lacks the ability to transfer a phosphoryl group and acts as a competitive inhibitor to the other *AHPs* (Mähönen, Bishopp et al. 2006; Fig. 7). In roots, *AHP6* transcription is negatively regulated by cytokinin, and expression is restricted to specific cells.

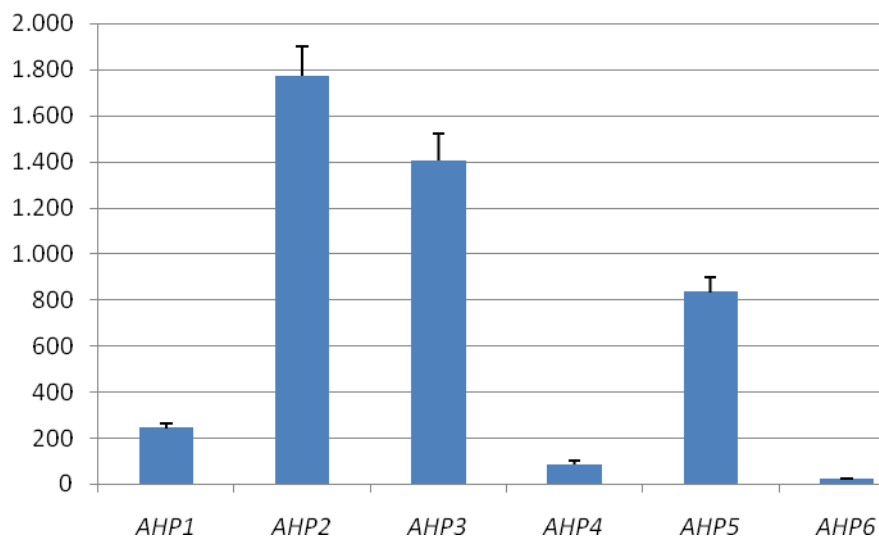


Figure 8: Expression levels of the *AHP* genes in stems, based on public expression data.

The large group of *ARRs* is further divided into two subgroups, A-Type *ARRs* and B-Type *ARRs* (Fig. 9). A-Type *ARRs* act as inhibitors of cytokinin signaling (Kiba, 2003), whereas B-Type *ARRs* bind to the DNA and activate transcription (Heyl, Schmulling 2003; Kakimoto 2003).

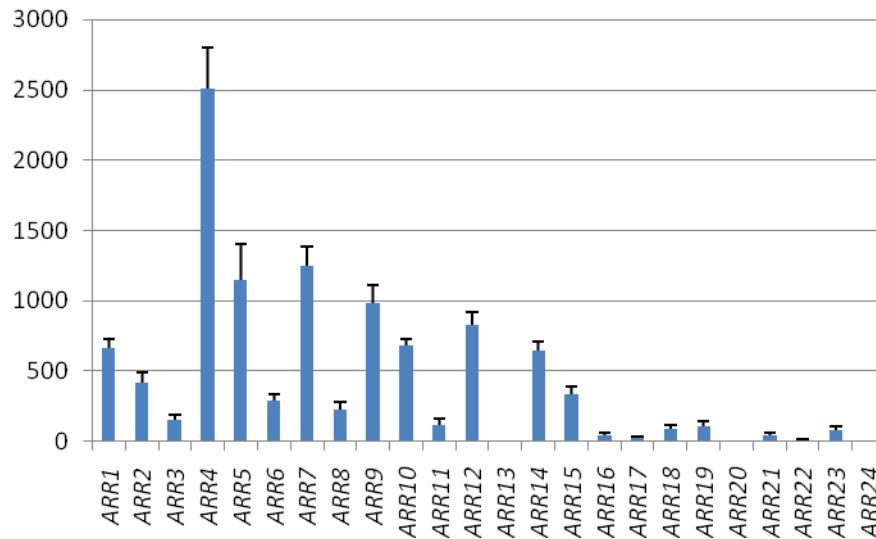


Figure 9: Expression levels of the *ARR* genes in stems, based on public expression data. No data was available for *ARR13*, *ARR20* and *ARR24*.

Thesis Goals

Although a lot of research has gone into both auxin and cytokinin signaling, little is still known about their roles in secondary growth.

Auxin is accepted as an inducing factor for secondary growth, but the auxin distribution pattern which is necessary for initiating secondary growth is still unknown. Likewise, the roles of the individual *PIN* genes, which establish the auxin distribution pattern, remains to be investigated. Here, analysis of mutants, both constitutive and inducible, of the *PIN* genes aimed at the investigation of the importance of individual *PIN* genes for the initiation of secondary growth. Knowledge was further complemented by spatial and temporal expression analysis.

Even though the connection between cytokinin and secondary growth is far less established than in the case of auxin, it has been shown that it is involved in meristem regulation. Since the formation and maintenance of new meristematic cells is a prerequisite for secondary growth, cytokinin signaling is likely to have an effect on secondary growth as well. Analysis of components in the cytokinin signaling pathway focused primarily on analysis of mutants, but also with inducible alteration of cytokinin levels. Since this pathway consists of a very large number of genes with wide-ranging redundancy, only a few genes could be selected for investigation. The selection was mostly based on the previously described impact of these mutants.

Furthermore, an experimental system to directly investigate the effects of hormones on stems was established.

Methods

Abbreviations

AHK	<i>Arabidopsis</i> histidine kinase
AHP	<i>Arabidopsis</i> histidine phosphotransfer protein
ARR	<i>Arabidopsis</i> Response Regulator
CHASE	cyclases/histidine-kinase-associated sensory extracellular
CKX1	Cytokinin Oxidase 1
CRE1	Cytokinin Response 1
EtBr	ethidium bromide
EtOH	ethanol
IAA	indole-3-acetic acid
FAA	formalin/acetic acid/alcohol
miRNA	microRNA
NAA	naphto-acetic acid
NPA	naphtyl-phtalamic acid
PIN	pin-formed
RT	room temperature
SAM	shoot apical meristem
SG	secondary growth
RPM	rounds per minute
WOL	Wooden Leg

PCR Programs

amiRNA (a), (b), (c)

95°C 2'

95°C 30"

55°C 30"

72°C 40"

24 cycles

72°C 7'

amiRNA (d)

95°C 2'

95°C 30"

55°C 30"

72°C 1'30"

-> 24 cycles

72°C 7'

Taq, Pfu

95°C 5'

95°C

58°C

72°C

72°C

Phusion

95°C 5'

95°C

58°C

72°C

72°C

RT-PCR

The RT-PCRs were carried out according to the Taq PCR protocol, using Taq polymerase and Taq buffer (both Fermentas).

If genomic DNA is still present in the sample, and the PCR product would be of the same size as the product from cDNA, the result would be ambiguous. Here, primers were chosen that bound to different exons, with one intron in between. In this way, PCR products from genomic DNA were longer than products from cDNA, and therefore easily distinguishable.

Initially, only a Tubulin fragment was amplified (30 cycles to avoid saturation), using 1 μ L cDNA of each sample as a template. After comparing signal intensities on an agarose gel (using KODAK software), the cDNA stocks were diluted so as to reach equal concentrations. Following verification of equal Tubulin signal intensity, PCRs with the *PIN* primer pairs were carried out (40 cycles).

Artificial microRNAs

Constructs for expression of artificial microRNAs were generated according to the protocol on <http://wmd2.weigelworld.org/> (Schwab, Ossowski et al. 2006). After target specification, four primers were suggested by the WMD2 program: I, II, III and IV. Primers A and B are common for all miRNAs based on miRNA319.

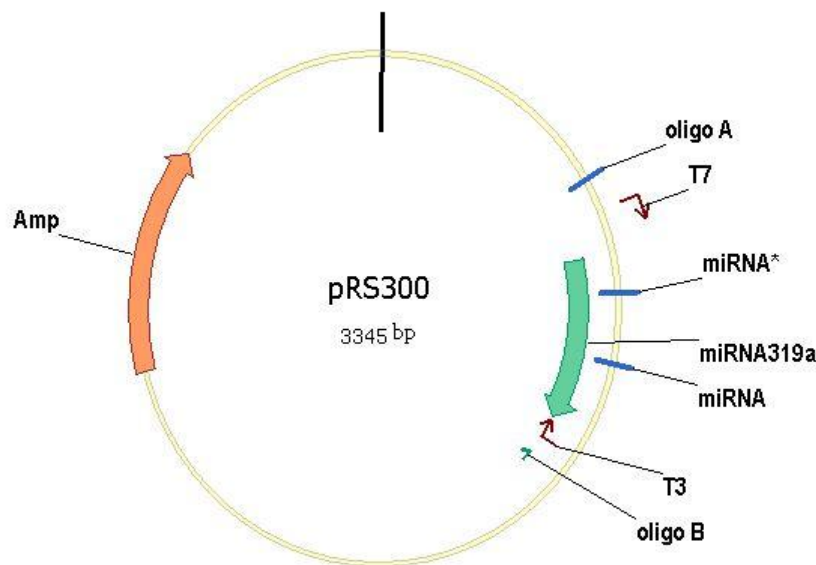


Figure 10: The pRS300 plasmid used as a template for the synthesis of artificial microRNAs.

Four PCR steps led to the final DNA for the amiRNA. Three PCRs used pRS300 (Fig. 11) as the template, with the primer pairs of (a) A and IV, (b) III and II and (c) I and B. These reactions were carried out with 5 μ L Pfu buffer (10x, with Mg⁺⁺, Fermentas), 5 μ L dNTPs (2mM), 2 μ L each oligo (10 μ M), 2 μ L pRS300 plasmid DNA (1:100) and 0,5 μ L Pfu polymerase (Fermentas). The reaction mix was filled to 50 μ L with 33,5 μ L H₂O. The three PCR products were loaded on 2% agarose gels and the desired bands cut and eluted in 20 μ L H₂O (Qiagen QIAquick gel extraction). The purified products were then used as the templates for the final PCR with the primer pair (d) A and B. This reaction was carried out with 5 μ L Pfu buffer (10x, with Mg⁺⁺, Fermentas), 5 μ L dNTPs (2 mM), 2 μ L each oligo (10 μ M), 0,5 μ L each PCR product (a), (b), (c), and 0,5 μ L Pfu polymerase (Fermentas). The reaction mix

was filled to 50 μL with 34,5 μL H_2O . The final product was applied to a 1% agarose gel, cut and eluted in 20 μL H_2O .

To clone the amiRNA into a pGreen vector, the PCR product was amplified with modified primers A and B, which contained restriction sites for the enzymes EcoRI and AatII. The resulting product was again purified.

DNA extraction

From a seedling, 2-3 leaves were put into an 1,5 mL microcentrifuge tube. After addition of 200 μL DNA extraction buffer (see Materials), the tissue sample was grinded. An additional 200 μL of DNA extraction buffer was added and the solution mixed. The sample was then centrifuged for 5 min at 13000 rpm. The supernatant was transferred to a fresh microcentrifuge tube. Following the addition of 1 volume isopropanol and mixing, precipitation was allowed to proceed for 10 min at RT. To compact the DNA into a pellet, the tube was centrifuged for 5 min at 13000 rpm. With the supernatant discarded, the pellet was washed by 300 μL 70% ethanol and centrifugation for 1 min at 13000 rpm. The dried pellet was resuspended in 50 μL buffer TE. If the pellet was difficult to dissolve, it was shaken for 10 min at 65°C. The DNA was stored at -20°C.

RNA extraction

Plant material was frozen with liquid N_2 and grinded with mortar and pestle. Of the homogenized tissue, 400 μL were transferred to an 1,5 mL microcentrifuge tube. Under the fume hood, 1 mL TRIZOL reagent (Invitrogen) was added, the tube well mixed and incubated for 5 min at RT. To separate the phases, the tube was centrifuged for 15 min at 13200 rpm, 4°C. Next, 900 μL of the supernatant was transferred to 1,5 mL microcentrifuge tubes containing 200 μL CHCl_3 . Following incubation for 5 min at RT, the tube was centrifuged again for 15 min at 13200 rpm, 4°C. For precipitation of the RNA, 400 μL of the aqueous layer was transferred to a fresh 1,5 mL microcentrifuge tube containing 500 μL isopropanol. After mixing, precipitation was allowed to proceed for 10 min at RT. To compact the RNA into a pellet, the tube was centrifuged for 10 min at 13200 rpm, 4°C. Discarding the supernatant, the pellet was washed by 1 mL 70% ethanol and centrifugation for 5 min at 10000 rpm, 4°C. The dried pellet was resuspended in 50 μL H_2O . If the pellet was difficult to dissolve, the tube was shaken for 10 min at 55°C. The RNA was stored at -80°C.

Purity of the extracted RNA was checked on a special agarose gel for RNA analysis.

Surface Sterilization of Seeds

To avoid contamination of the plant with bacteria or fungi, seeds had to be surface sterilized. In a microcentrifuge tube, about twenty seeds were sequentially treated with 70% EtOH, 50% bleach for 7 min, H_2O and finally suspended in sterile 0,1% agarose and plated.

***In vitro* system**

Plant material for *in vitro* work had to be surface sterilized. For this, plants with removed leaves and roots were transferred to ice water. To remove dirt, the plants with the ice water were put on a shaker for 5 min. The stem was then cut at the first node and its top dipped into liquid paraffin to coat the wound. Up to 25 such stems were put into 50 mL falcon tubes. In these tubes, the stems were first sterilized in 70% ethanol for 1 min on the rotor. In the second sterilization step, the ethanol was replaced by 50% bleach (+0,02% Tween-20) and the tubes put on the rotor for 15 min. The sterilized stems were then washed three times with sterile H₂O. Immediately before transferring them to the *in vitro* plates, 1 cm long stem fragments were cut out with sterile scalpels from 0,3-1,3 cm above the rosette.

To set up the hormone gradient, a petri dish with ½ MS was separated into two halves. For this, a 6mm wide strip was removed from the middle of each plate. The hormones could then be pipetted onto the media halves independently. The plates were stored upside-down for three days to ensure equal diffusion of the hormones throughout the medium. The upside-down orientation kept the medium-free strip dry and avoided contact of the media halves by water and disturbance of the gradient. Plates with a visible liquid connection between the media halves were discarded.

After three days, the sterile stem fragments were put between the media halves. Incubation took place in culture chambers for five days. The plates were placed horizontally and upside-down. While removing the stems from the plates, the apical-basal axis was preserved, as well as the upwards-downwards facing side of the stem during incubation.

GUS Staining

Stem samples for GUS staining were put in microcentrifuge tubes containing 1 mL GUS staining solution (see Materials). The samples were then vacuum-infiltrated for 15 min or until all air was extracted. The staining reaction was allowed to proceed overnight at 37°C in the absence of light.

After staining, the tissue had to be fixed. For this, the GUS staining solution was replaced by 1 mL FAA (see Materials) and again vacuum-infiltrated for 15 min. Fixation occurred over the course of at least one hour.

To store the fixed tissue, the FAA had to be replaced by ethanol. First, the FAA was replaced by 50% ethanol. After one hour, the 50% ethanol was replaced by 70% ethanol. From then on, the samples could be stored in ethanol at 4°C or be processed further for histology.

GUS histology

For histological analysis of GUS stained samples, the samples were embedded in paraffin by a Shandon HistoCentre 3 Embedding Centre. From this, slides with 7µm thick sections were prepared by a microtome (Leica). After drying of the slides, the wax was removed by putting the slides subsequently into two buckets of histoclear. Next, the slides were cleared of histoclear by washing

them two times with 100% ethanol. The dried slides were then mounted by Entellan (Merck) and sealed.

Toluidine Blue Staining

Histological analysis of the stem anatomy was aided by staining the cell walls with Toluidine blue. Dry slides with cut samples in paraffin were first stripped of wax by placing them into two buckets with HistoClear for 10 min each. Afterwards, the slides were slowly transferred to aqueous environment by putting them through a series of ethanol treatments with decreasing concentration. This consisted of washing, for a minute at each step, two times in 99,9% ethanol, followed by 96%, 85%, 50%, 30% and finally two times water. The slides were then stained in Toluidine blue solution for 4 min, and transferred to ethanol again, over a series of washes, each two times for one minutes, water, 96% and 99,9% ethanol. The dried slides were then mounted in Entellan and sealed with cover slips.

PCR Reactions

All PCR reactions, unless stated otherwise, were 50 μ L reactions prepared with 1x Buffer (5 μ L, type dependent on Polymerase), 0,1 mM each dNTP (0,2 μ L of 25 mM mix), 1,5 mM $MgCl_2$ (3 μ L of 50 mM stock), 0,2 μ M each Primer (1 μ L of 10 mM stock), template DNA and the appropriate Polymerase. The solutions were filled up with H_2O to or 50 μ L. Reactions were performed in a BioRad iCycler thermal cycler.

A-Tailing of PCR Products

Blunt-ended PCR products were extended with Adenosin tails by mixing 30 μ L purified PCR product (Qiagen QIAquick PCR purification), 5 μ L 10x Taq Buffer (Fermentas), 3 μ L $MgCl_2$, 0,2 μ L dATP and 0,2 μ L Taq (Fermentas). The volume was filled to 50 μ L by addition of 8,6 μ L H_2O .

Ethanol induction

To activate the ethanol switch in transformed plants, the trays were watered with 1 L 1% ethanol two times a week, starting with the onset of bolting. Watering with Ethanol- H_2O continued until plants were analysed.

Cloning into pGEM-T easy

Of the purified PCR product (A-tailed), 2 μ L were mixed with 5 μ L rapid ligation buffer (2x, T4 DNA ligase, Promega), 1 μ L pGEM-T easy vector (50 ng, linearized) and 1 μ L T4 DNA Ligase (3 u/ μ L, Promega). This was filled up to 10 μ L by addition of 1 μ L H_2O . Ligation proceeded overnight at 16°C.

Cloning into pGreen0229-AlcA

Both the vector and the insert had to be cut by the enzyme combinations giving compatible sticky ends. Here, AatII and EcoRI were used. The PCR product had to be amplified with linker primers containing recognition sites for these enzymes. Before ligation, both the purified PCR product and the vector were cut by AatII and EcoRI. The restriction products were again purified (Qiagen QIAquick PCR purification).

For the cloning of CKX1 into pGreen0229-AlcA (Hellens, Edwards 2000), MfeI was used instead of EcoRI, since the ORF of CKX1 already contains an EcoRI recognition site. MfeI has a different recognition site than EcoRI, but digestion results in identical overhangs.

Of the insert, 2 µL were mixed with 1 µL ligation buffer (10x, T4 DNA ligase, Fermentas), 1 µL pGreen0229-AlcA and 0,5 µL T4 DNA ligase (Fermentas). This was filled up to 10 µL by addition of 5,5 µL H₂O. Ligation proceeded overnight at 16°C.

Escherichia coli Transformation by Heat Shock

An aliquot of 50 µL frozen competent *E. coli* (DH5α strain) was thawed on ice. After addition of 5 µL ligation product and further 20 min on ice, the cells were heatshocked at 42°C for 90 sec. The heatshock was followed by cooling on ice for 2 min, addition of 350 µL LB (RT) and finally 1 h incubation at 37°C on a shaker. Of the transformed cells, 200 µL were plated on LB plates with appropriate antibiotics. The plates were incubated overnight at 37°C.

Agrobacterium tumefaciens Transformation by Heat Shock

An aliquot of 50 µL frozen competent *Agrobacterium tumefaciens* (C58C1), containing pSOUP, were thawed on ice. Of the plasmid, 5 µL were added. The bacteria were then kept on ice for 5 min, in liquid nitrogen for 5 min and heatshocked at 37°C for 5 min. After addition of 800 µL LB, the bacteria incubated at 28°C for 4 h on a shaker. Finally, 200 µL of the transformed cells were plated on LB plates with Kan (50 µg/mL), Rif (100 µg/mL) and Tet (5 µg/mL). Incubation at 28°C lasted for three days to produce colonies.

Arabidopsis thaliana Transformation by Floral Dip

A preculture of *Agrobacterium tumefaciens* carrying the DNA fragment to be transformed was grown overnight at 28°C in 5 mL YEB with Rif (50 µg/mL), Kan (25 µg/mL) and Tet (10 µg/mL). The next day, it was transferred to 400 mL YEB+Rif+Kan+Tet, which was grown at 28°C overnight. The suspension was centrifuged for 15 min at 5000 rpm, RT. The pellet was then resuspended in a solution containing 600 mL H₂O, 5% sucrose and 0,02% Silwet. Plant pots containing 16 flowering individuals were dipped into the suspension for 5 min. To ensure maximum transformation efficiency, the plants were then covered with plastic bags overnight.

Glycerol Stocks

For glycerol stocks of bacteria, 200 μ L glycerol was added to 800 μ L culture and frozen in liquid nitrogen. The stocks are kept at -80°C .

RNA Agarose Gel

To analyze total RNA on an agarose gel under denaturing conditions, 10-20 μ g RNA were mixed with 7,3 μ L formaldehyde (37%), 20 μ L formamide and 4 μ L MOPS buffer (10x). Also, 1 μ L EtBR (1 mg/mL) and 1 μ L loading dye were applied directly to the sample. This was filled up to 40 μ L by addition of 9 μ L H_2O . The sample was finally incubated at 65°C for 10 min.

For 100 mL agarose gel, 1,2 g agarose was dissolved in 83,3 mL H_2O using the microwave. The agarose was kept at 50°C in a waterbath. In the same waterbath, 5,8 mL formaldehyde (37%) together with 10 mL MOPS buffer (10x) were heated. When both solutions reached the same temperature, they were poured together and the gel was prepared in a fume hood. The gel was then run in 1x MOPS buffer.

Qiagen QIAprep Spin Miniprep Kit

Two times 2 mL of an overnight *E. coli* culture containing the desired plasmid were centrifuged at 9000 rpm for 3 min to pellet the bacteria. The miniprep then proceeded according to the manufacturer's instructions. The plasmids were eluted in 50 μ L buffer EB and stored at -20°C .

Qiagen QIAquick gel extraction

All centrifugation steps were done at 13.000 rpm in a tabletop centrifuge at RT. DNA was extracted from agarose gels according to the manufacturer's instructions. The DNA was eluted in 50 μ L buffer EB and stored at -20°C .

Qiagen QIAquick PCR purification

All centrifugation steps were done at 13.000 rpm in a tabletop centrifuge at RT. PCR products were purified according to the manufacturer's instructions. The DNA was eluted in 50 μ L buffer EB and stored at -20°C .

cDNA Synthesis with the RevertAid H Minus First Strand cDNA Synthesis Kit

In the first step, 1 μ g total RNA was mixed with 1 μ L oligo(dT)₁₈ primer (0,5 μ g/ μ L) and H_2O to a volume of 12 μ L. This was incubated at 70°C for 5 min and chilled on ice. Next, 4 μ L reaction buffer (5x), 1 μ L Ribolock ribonuclease inhibitor (20 u/ μ L) and 2 μ L dNTPs (10 mM) were added. The enzymatic reaction proceeded for at 37°C for 5 min. Finally, 1 μ L RevertAid H Minus M-MuLV RT (200 u/ μ L) was added. cDNA polymerization proceeded at 42°C for 60 min. The reaction was stopped by heating to 70°C for 10 min.

Nucleic Acid Measurements

Concentration measurements were carried out with an Ultrospec 3100 pro (Amersham Biosciences). DNA concentration was measured at 260nm UV light, RNA concentration also at 260nm.

Materials

DNA Extraction Buffer

200 mM	Tris, pH 7,5
250 mM	NaCl
0,5%	SDS
25 mM	EDTA

FAA Fixation Solution

50%	Ethanol
5%	Acetic Acid
3,7%	Formaldehyde
41,3%	H ₂ O

GUS Staining Solution

100 mM	phosphate buffer, pH 7
10 mM	EDTA, pH 8
0,1%	Triton X-100

Added before use:

0,5 mM	ferricyanide
0,5 mM	ferrocyanide
2 mM	X-Gluc

10x MOPS buffer

200 mM	MOPS, pH 7.0
80 mM	Sodium Acetate
10 mM	EDTA

50x TAE Buffer

2 M	Tris
1 M	acetic acid
50 mM	EDTA (pH8)

LB Bacterial Growth Media

800 mL	H ₂ O
10 g	Tryptone
5 g	yeast extract
10 g	NaCl
adjusted to pH 7,5 with NaOH	
filled to 1 L with H ₂ O	

LB Agar Bacterial Growth Media

800 mL	H ₂ O
10 g	Tryptone
5 g	yeast extract
10 g	NaCl
15 g	Agar
filled to 1 L with H ₂ O	
adjusted to pH 7,5 with NaOH	

½ MS (Murashige Skoog) Plant Growth Media

0,5 g	MES
10 g	Sucrose
2,15 g	MS salt
4 g	Plant Agar

filled to 1 L with H₂O

adjusted to pH 5,7-5,8 with 2M KOH

Enzymes

Restriction enzymes were obtained from Fermentas and New England Biolabs.

Polymerases were obtained from Fermentas (Taq, Pfu) and Finnzymes (Phusion, via New England Biolabs)

Mutant Lines

All mutants were in the Columbia background.

pin1-613

SALK T-DNA insertion, SALK_047613.36.05.x (NASC Nr N547613)

Donated by Joseph R. Ecker, The Salk Institute for Biological Studies (Alonso, Stepanova et al., 2003)

pin3-5

SALK T-DNA insertion, SALK_005544 (NASC Nr N505544)

Donated by Joseph R. Ecker, The Salk Institute for Biological Studies (Alonso, Stepanova et al., 2003)

pin4-3

EN-1 insertion (NASC Nr N9368)

Donated by Jiri Friml (Friml et al. 2002)

pin5

SALK T-DNA insertion, SALK_021738 (NASC Nr N521738)

Donated by Joseph R. Ecker, The Salk Institute for Biological Studies (Alonso, Stepanova et al., 2003)

pin6

SALK T-DNA insertion, SALK_046393 (NASC Nr N546393)

Donated by Joseph R. Ecker, The Salk Institute for Biological Studies (Alonso, Stepanova et al., 2003)

pin7-2

SALK T-DNA insertion, SALK_044687.21.30.x (NASC Nr N544687)

Donated by Joseph R. Ecker, The Salk Institute for Biological Studies (Alonso, Stepanova et al., 2003)

pin8

SALK T-DNA insertion, SALK_107965 (NASC Nr N607965)

Donated by Joseph R. Ecker, The Salk Institute for Biological Studies (Alonso, Stepanova et al., 2003)

ahk2-2tk

T-DNA insertion in exon 5. (Higuchi, Pischke 2004)

ahk3-3

SALK T-DNA insertion, SALK_069269 (NASC Nr N569269)

Donated by Joseph R. Ecker, The Salk Institute for Biological Studies (Alonso, Stepanova et al., 2003)

cre1-2

GABI_KAT_105E02

Donated by Bernd Weisshaar, Bielefeld University Fakultät für Biologie (Li, Rosso et al., 2003)

wol

Threonine at position 278 substituted by Isoleucine (T278I)

(Mähönen, Bonke 2000)

ahp6-2

Mutation in the first intron: G to A 5 base pairs from the 5' border of the AHP6b splice variant.

(Mähönen, Bischopp 2006)

arr15

Wisconsin T-DNA insertion, WiscDsLox334D02 (NASC Nr N851593)

Donated by Richard M. Amasino University of Wisconsin Department of Biochemistry, Patrick Krysan University of Wisconsin Horticulture Department, Michael Sussman University of Wisconsin-Madison Department of Biochemistry

arr3,4,5,6,8,9

T-DNA insertions (NASC Nr N25279)

Donated by: Luca Comai, Nigel Crawford, Gary Drews, Beth Krizek, George Eric Schaller, John M. Ward, Andreas Madlung, James Atland, Joe Kieber

(To, Haberer 2004)

Primers

RT-PCR

TUBfor2	ACCCCCAGCTTTGGTGATTG
TUBrev3	CCCTCACCTGTGTACCAATGCAAGA
pin1for2	TCACATGTTTGTGTGGAGCTCAAGT
pin1rev4	GGGATTACGAATAAGTTTCCTCCAAA
pin2for1	TGCAGATCGTGGTGCTTCAGAG
pin2rev1	AAGCTCGCGGCGTTATCATG
pin3for5	CCAGATCAATCTCACAACGGCG

pin3rev5	CGAGAGCCCAAATAAGTCCAATGAG
pin4for3	GGAGTTTTCAACCGGTACGGGT
pin4rev2	CAGCCCTGCTGTAGCTTTCTCTATCT
pin6for1	TCCGTCAACACTCCTAATAACCGT
pin6rev1	GGCACTGATCCATTGATATCTTT
pin7for1	GGCGGATATGTACTCTGTTTCAGTCC
pin7rev2	TCGGACCAGCTTTGTTTTCA

Artificial microRNAs

amiRNA_A	CTGCAAGGCGATTAAGTTGGGTAAC
amiRNA_B	GCGGATAACAATTTACACAGGAAACAG
miRfor1_AatII	ACTAGACGTCCAAACACACGCTCGGACG
miRrev1_EcoRI	AGGTGAATTCATGGCGATGCCTTAAATAAA

PIN3miR2-sl	GATAATCAACTAAGAGTTACCCCTCTCTCTTTTGTATTCC
PIN3miR2-all	GAGGGGTAACCTCTTAGTTGATTATCAAAGAGAATCAATGA
PIN3miR2*sIII	GAGGAGTAACCTCTTACTTGATTTTCACAGGTCGTGATATG
PIN3miR2*aIV	GAAAATCAAGTAAGAGTTACTCCTCTACATATATATTCCT
PIN1miR-sl	GATAACGTGGTAGAAGTCCCGCGTCTCTCTTTTGTATTCC
PIN1miR-all	GACGCGGGACTTCTACCACGTTATCAAAGAGAATCAATGA
PIN1miR*sIII	GACGAGGGACTTCTAGCACGTTTTTCACAGGTCGTGATATG
PIN1miR*aIV	GAAAACGTGCTAGAAGTCCCTCGTCTACATATATATTCCT

PIN3-4-7miR-sl	GATAACGGAGTACAAATCCGCCCTCTCTCTTTTGTATTCC
PIN3-4-7miR-all	GAGGGCGGATTTGTACTCCGTTATCAAAGAGAATCAATGA
PIN3-4-7miR*sIII	GAGAGCGGATTTGTTCTCCGTTATCACAGGTCGTGATATG
PIN3-4-7miR*aIV	GATAACGGAGAACAAATCCGCTCTCTACATATATATTCCT

CKX1

CKX1for3	ACTAGACGTCATGGGATTGACCTCATCCTTACGG	(AatII)
CKX1rev5	ACTACAATTGTTATACAGTTCTAGGTTTCGGC	(MfeI)

Results

Cytokinin Signaling Mutants

Mutants affected in the cytokinin signaling pathway were screened for deviations in secondary growth from wild type. In this way, the role of cytokinin signaling in secondary growth was assessed. Mutants were chosen which show defects in root vasculature (Mähönen, Bishopp et al. 2006).

The goal of the experiment was the observation of the mutant's capability to establish the interfascicular cambium and thereby undergo secondary growth.

The anatomy of the stem was investigated by transversal sections 3 mm above the rosette. After staining the cell walls with Toluidine Blue, conclusions were drawn by judging cell shape, number and cell wall thickness (a lighter shade of blue indicates lignified cell walls in Toluidine Blue staining). The extent, or rate of secondary growth could be assessed by the number of cells in the interfascicular cell files (Fig. 12 c) during the mature stage. More cell divisions led to larger cell files, amounting to an increase in secondary vasculature, and therefore secondary growth. All sections were 7 µm thick. Each developmental stage was investigated in 10 plants.

ahk2-2tk

ahk2-2tk showed no severe growth defects (Fig. 11 b), but the stems had a slightly smaller diameter and smaller primary vascular bundles than wild type stems. Secondary growth proceeded without problems, though the secondary vascular cells seemed slightly smaller and more numerous than in wild type (Fig. 12 d-f).

ahk3-3

ahk3-3 plants had a slightly reduced stem diameter and fewer side branches (Fig. 11 c). Secondary growth initiated as in wild type, but only a few cell divisions occurred in the interfascicular cambium. The newly formed cells towards the inside of the stem showed no thickening of the cell wall in Toluidine blue stainings. Therefore, those cells could not be classified as xylem. Also noteworthy are the comparatively irregular cell shapes of the secondary cells, suggesting further deficiencies in the cell cycle (Fig. 12 g-i).

cre1-2

Stem diameter was roughly equivalent to wild type (Fig. 11 d), but cell wall thickness of the xylem cells and interfascicular fibers seemed increased. Both the interfascicular fibers and secondary xylem seem to possess stronger cell walls (Fig. 12 j-l).

wol

Wooden leg (*wol*) is a gain-of-function mutant of AHK4, which is affected in its kinase activity but still retains its phosphorylation ability (Mähönen, Bonke 2000). It had the most drastic phenotype of the cytokinin signaling mutants in this project. Growth was severely stunted; with few of the plants exceeding heights of 15 cm. Side branches were limited to one or two per plant. Siliques were smaller and fewer than in wild type (Fig. 11 e). The stems were very thin in diameter, and primary vascular bundles seemed to be fewer, ranging from 3 to 5. Still, secondary growth was initiated and in some plants could proceed through several cell divisions (Fig. 12 m-o).

ahp6-1

Loss of the cytokinin signaling inhibitor AHP6 led to an increase in branches, as well as to larger, slightly curly axillary leaves (Fig. 11 f). Vascular development proceeded without any defects, though secondary growth seemed to be stronger than in the wild type (Fig. 12 p-r).

arr15

No deviations from wild type could be observed regarding vascularization and overall development in the *arr15* mutant (Fig. 11 g, Fig. 12 s-u).

arr3,4,5,6,8,9

This sextuple mutant of A-type *ARRs* showed no obvious growth phenotype (Fig. 11 h), but accomplished a larger degree of secondary growth during its development (Fig. 12 v-x).

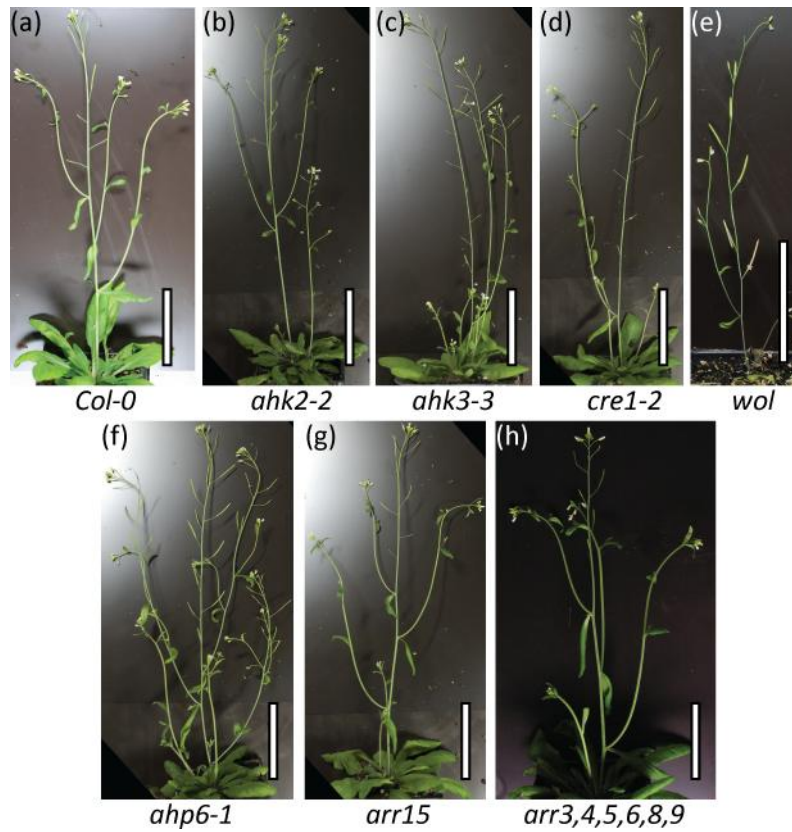
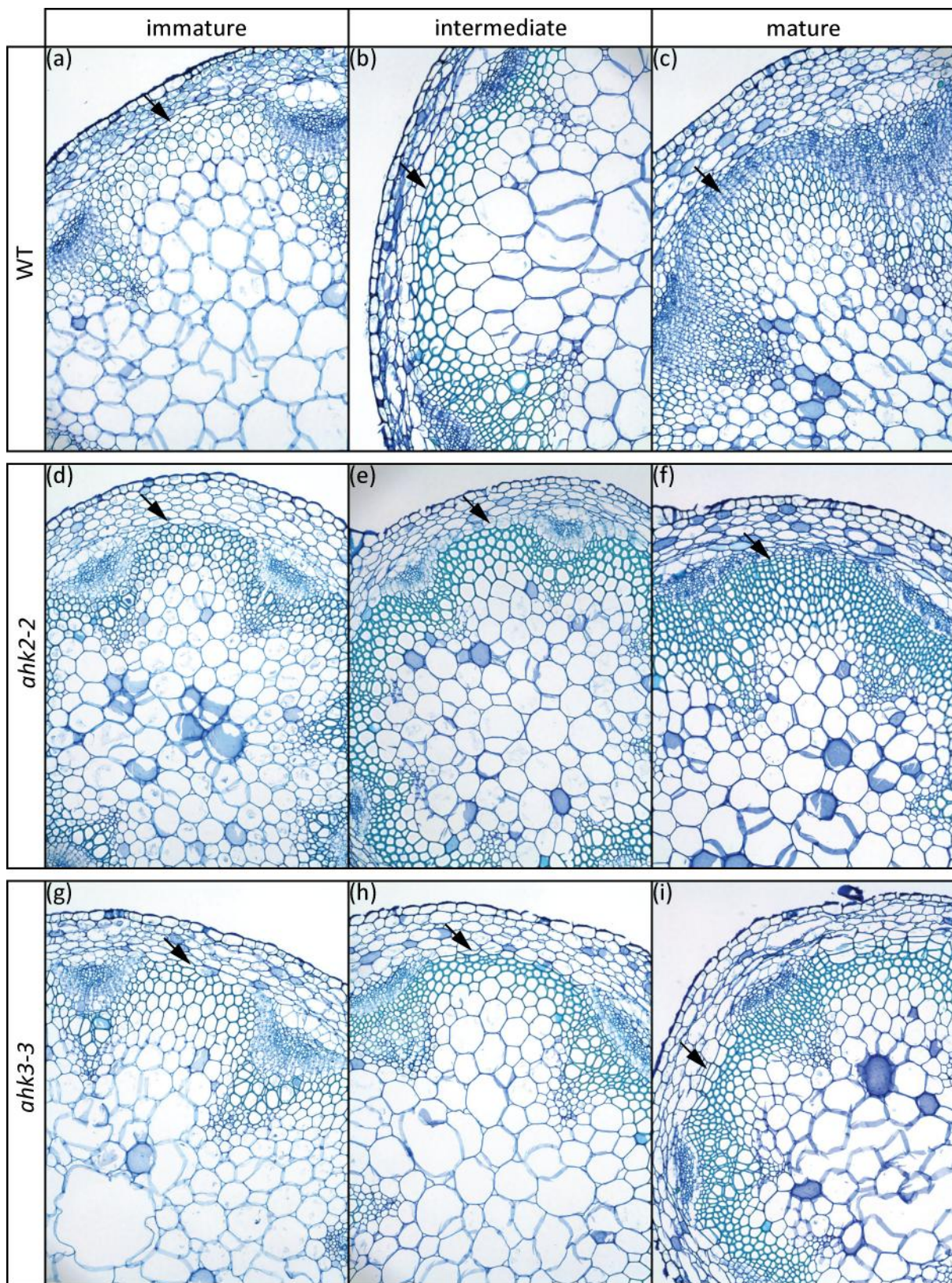
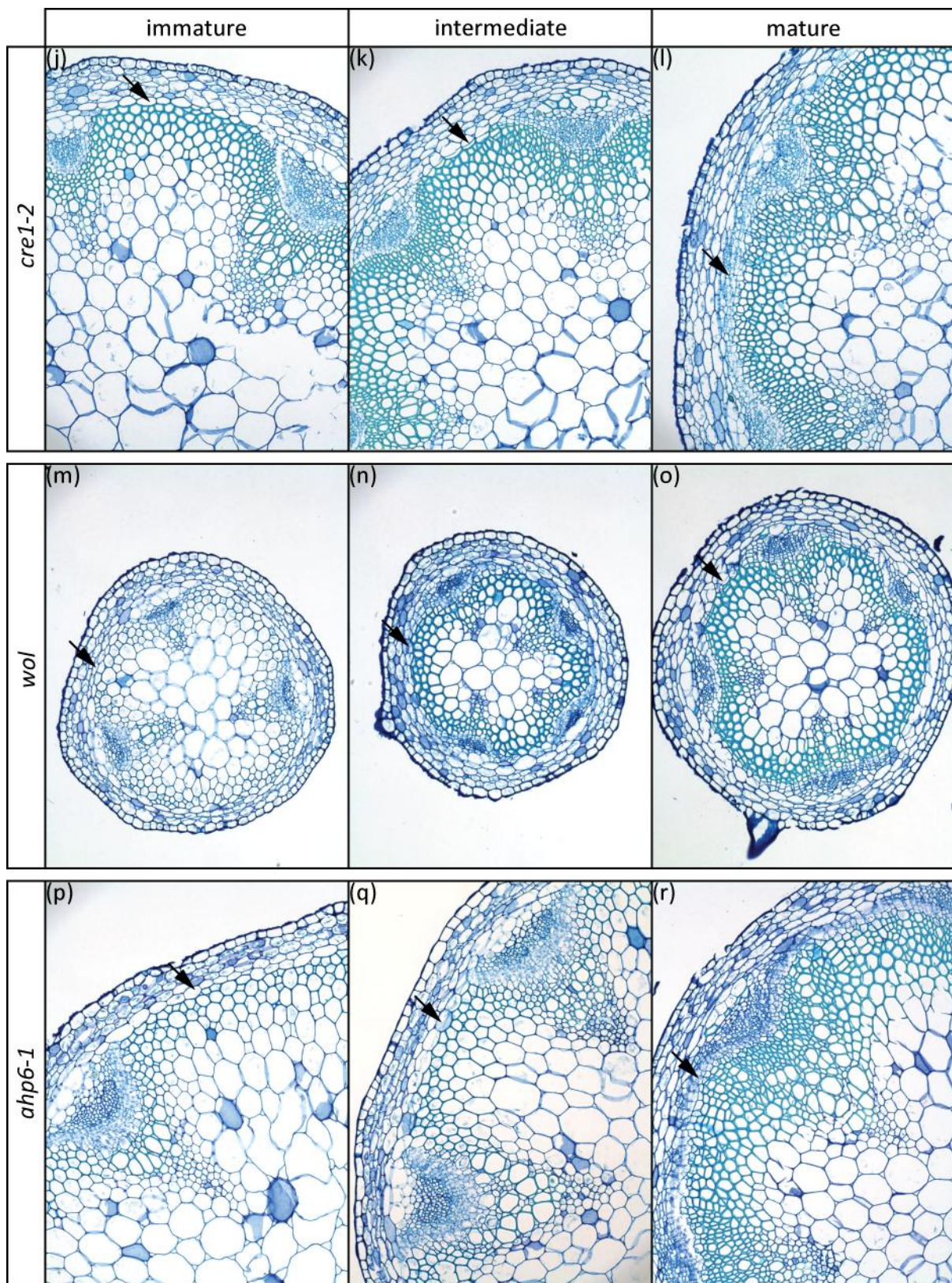


Figure 11: Mature *Arabidopsis* plants grown in long-day conditions. The cytokinin receptor mutants *ahk2-2* (b), *ahk3-3* (c) did not exhibit severe growth defects, though the stem diameter was on average smaller than in the wild type Columbia control (a). While the *AHK4* knockout allele *cre1-2* showed no growth aberrations from wild type, the *AHK4* gain-of-function allele *wol* (e) exhibited severely stunted growth, few or no side branches and early silique ripening. The knockout of the cytokinin signaling inhibitor *AHP6*, *ahp6-1* (f), had increased branching and slightly curled axillary leaves. Growth of the response regulator knockouts *arr15* (g) and *arr3,4,5,6,8,9* (h) did not deviate from the wild type (a). Scale bar = 5cm.





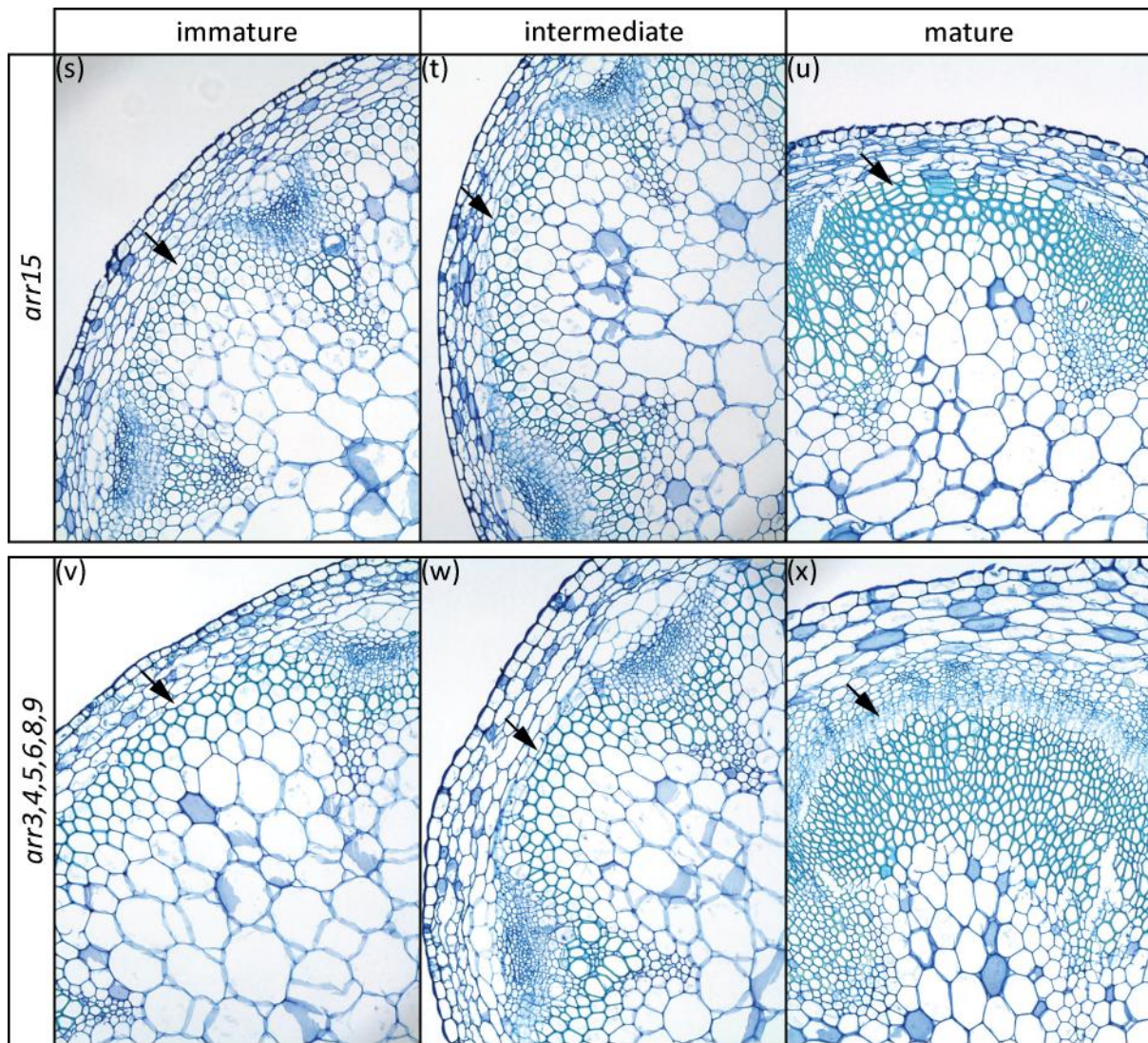


Figure 12: 7 μ m thick cross-sections of main stems 3 mm above the rosette, at the immature, intermediate and mature stages. Stained with Toluidine blue. Light blue indicates lignified cell walls. Arrows point the starch sheath (immature stages), the first cell division in the starch sheath during the initiation of secondary growth (intermediate stages), and the inter fascicular cambium (mature stages). In *ahk2-2*, already at the intermediate stage (e), the inter fascicular fibers possess stronger cell walls than in wild type (b). Secondary xylem cells in *ahk2-2* (f) appear more numerous as at the comparable wild type stage (c), and also exhibit slightly thicker cell walls. *ahk3-3* shows wild type-like development up to the intermediate stage (g, h, compared to WT a, b). In the mature stage (i), only few cell divisions have taken place in the inter fascicular cambium (arrow). As Toluidine blue staining shows no change in cell wall thickness, the new cells bordering the inter fascicular fibers can not be counted as secondary xylem. *cre1-2* development (j-l) overall follows wild type development, though the cell walls of xylem and the inter fascicular fibers appear stronger. In *wol* (m-o), only few vascular bundles are present in the very small stem (m). Though the initiation of secondary growth (n) does not differ from the wild type (b), only few cell divisions occur, with a lot of them diverting from the periclinal axis (o) seen in the wild type (c). Xylem and inter fascicular fibers have especially thick cell walls in *wol*, already from the intermediate stage on (n-o). Secondary growth in *ahp6-1* (p-r) proceeded as in wild type (a-c), but in the mature stage (r), secondary xylem proliferated further than in the wild type (c). *arr15* (s-u) shows no deviations from the wild type (b) in initiation of secondary growth (t), but accomplishes fewer cell divisions in the mature stage (u). Still, cell wall thickening in the putative secondary xylem occurs (u). The sextuple-knockout *arr3,4,5,6,8,9* follows wild type development (a-b) up the initiation of secondary growth (v-w), but the mature stage (x) shows the most extensive secondary growth of all the cytokinin signaling mutants. In addition to the large amount of secondary xylem, secondary phloem forms a thick, closed ring.

Inducible Expression of CKX1

CKX1

The cytokinin oxidase CKX1 decreases cytokinin levels in the plant (Werner, Motyka et al 2001). By cloning CKX1 into an ethanol inducible system (Deveaux, Peaucelle 2003), plants with conditionally lowered cytokinin levels were established (Fig. 13).

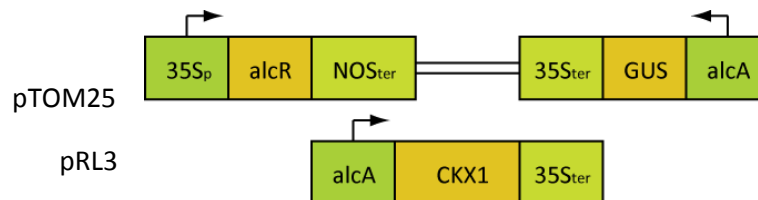


Figure 13: Schematic representation of the “ethanol switch” containing the *CKX1* gene and a *GUS* reporter.

Induced by ethanol treatment, the constitutively active 35S promoter drove expression of the alcR transcription factor. AlcR can bind to the alcA promoter and activate it. AlcA then drives expression of both a reporter gene (here GUS) and the gene of choice, here *CKX1*. This activation of alcA required the presence of ethanol, thereby enabling the regulation of gene expression by regulation of ethanol levels.

Using this system, the *CKX1* coding sequence was cloned into a plasmid with the alcA component (pRL3, based on pGreen0229-AlcA). To establish the transgenic plant lines, a two-step approach was utilized, as plants which contained the alcR and GUS components (pTOM25) were transformed with the plasmid pRL3.

Histology of 30 cm high stems showed that Ethanol treatment had no effect on the wild type, which underwent a large amount of secondary growth (Fig. 14 a-c).

After ethanol treatment, one part of the pRL3-transformed plants had a wild type-like phenotype, while the other part possessed more side shoots. Plants overexpressing CKX1 and showing a “bushy” phenotype still had a dominant stem which grew with the same speed to equal heights as the stem of the wild-type-like plants. Still, the main stem in the “bushy” phenotype had only a very small diameter. Though wild-type-like plants were capable of initiating secondary growth, only a few individual cell divisions occurred in the interfascicular region. In contrast, stems of the “bushy” plants showed a large extent of secondary growth, comparable to the wild type control plants. Even the secondary phloem, which differentiates only at late stages in development, was already very prominent (Fig. 14).

Since detection of CKX1 mRNA with RT-PCR could not be established in the timeframe of this project, it could not be verified whether the transgene was active.

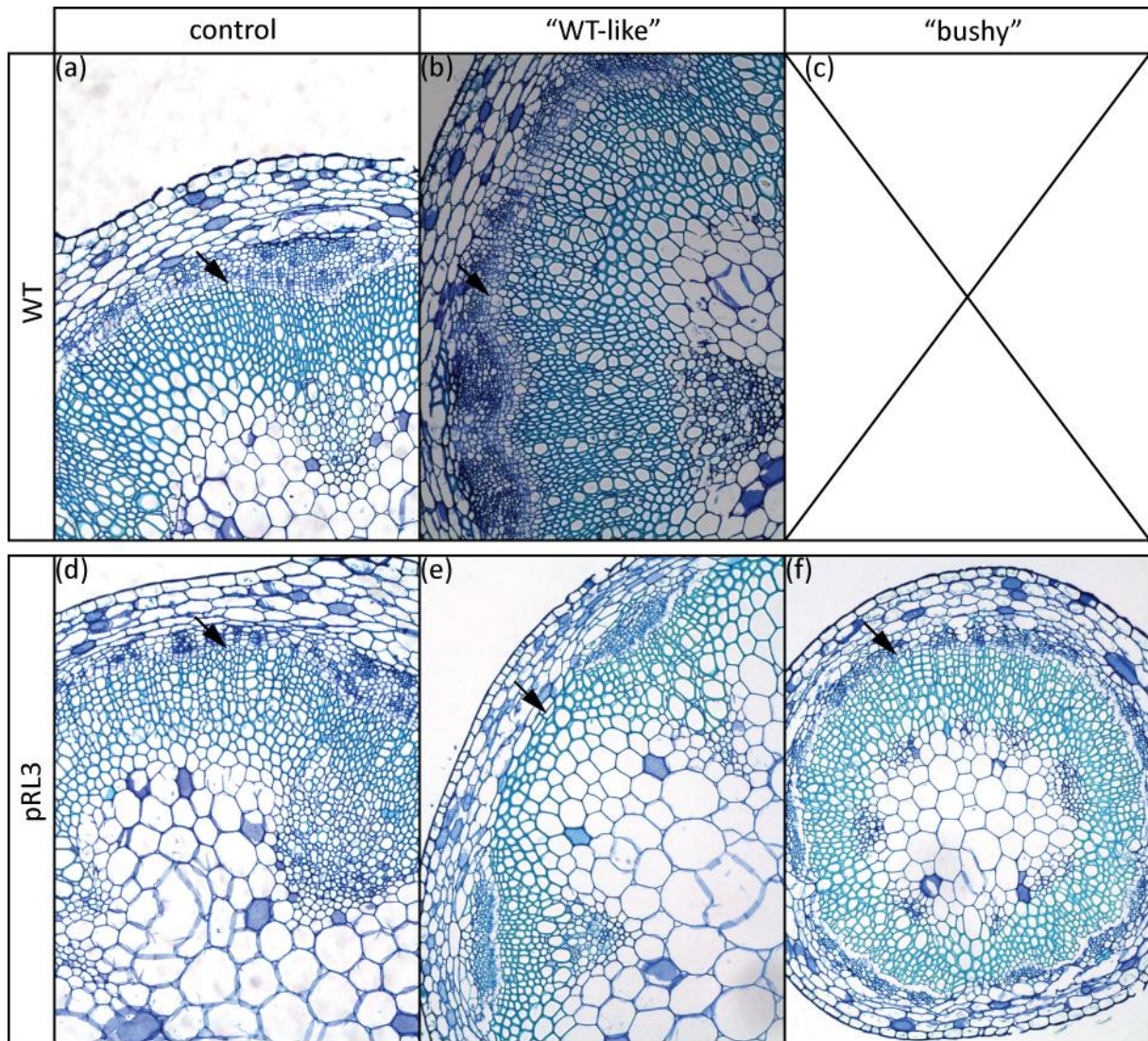


Figure 14: Cross-sections of mature stems 3 mm above the rosette in 30 cm high stems. Phenotypes after ethanol treatment were separated into "WT-like" for (b) WT and (e) pRL3; and "bushy" for (f) pRL3. The corresponding controls (a, d) were mature plants without ethanol treatment. Arrows point to the starch sheath and developing interfascicular cambium. In the wild type-like phenotype of the CKX1-overexpressing pRL3 line, only a few individual cell divisions occurred in the starch sheath (e). In contrast, the bushy phenotype exhibited extensive secondary growth (f). pRL3 plants without ethanol induction (d) developed like wild type (a), showing extensive secondary growth. Ethanol treatment on wild type plants did not show any effects in the vasculature (b).

Analysis of *PIN* knockouts

In order to get an understanding of the functions of the PIN proteins in secondary growth, respective single knockout lines were investigated. For each gene, a number of insertion lines were publicly available, so lines previously reported as being reliable knock-outs were chosen.

The experimental setup was the same as described above for cytokinin signaling mutants (p. 32)

pin1-613

Knockout mutants of *PIN1* showed a strong shoot phenotype (Fig. 15 b, as described before). After bolting, the stem was incapable of initiating lateral organs, instead pursuing only longitudinal growth. Homozygous plants were sterile, as no functional flowers were formed.

Immature stems contained overabundant primary vascular bundles. Distribution of these bundles was often unequal around the stem. In some plants, almost the whole stem was encompassed by primary vasculature, but this did not inhibit initiation and progress of secondary growth (Fig. 16 d-f).

pin2

No deviations from the wild type could be observed regarding vascularization (Fig. 16 g-i) and overall development (Fig. 15 c) in the *pin2* mutant.

pin3-5

The *pin3-5* insertion line showed no obvious growth differences from wild type (Fig. 15 d). Histological analysis showed no defects in the initiation of secondary growth (Fig. 16 j-l). However, in the maturing stem, the extent of secondary growth was reduced compared to wild type, and cell divisions were more irregular.

pin4-3

pin4-3 showed an environmentally dependent phenotype (Fig. 15 e-g). Under one set of growth conditions (near the center of the growth chamber), the mutant stems showed no differences from wild type plants. But under different conditions (near the wall of the growth chamber), the plants grew smaller and showed highly increased side branching. In the main stem, the first node contained three to four side branches initiating at almost exactly the same height. Also, this first node was significantly lower on the main stem compared to the wild type. All plants showed curled leaves.

While the stems of the wild type-like phenotype showed no differences to wild type plants (Fig. 16 m-o), the stems of the second, “bushy”, phenotype consistently showed decreased diameter. While initiation of secondary growth and maturing of the stem did not divert from the wild type, plants with immature stems could not be isolated (Fig. 16 p-r). Already at a height of 5cm, first cell divisions in the starch sheath could be observed, though this might also be due to the proximity of the first node.

pin5

No deviations from the wild type could be observed regarding vascularization (Fig. 16 s-u) and overall development (Fig. 15 h) in the *pin5* mutant.

pin6

Overall, *pin6* developed like wild type (Fig. 15 i). Secondary growth in the mature stage seemed slightly reduced, but all secondary tissues were present (Fig. 16 v-x).

pin7-2

The first siliques did not develop properly in *pin7-2*, but later siliques on the same inflorescence stem showed no defects, all plants were fertile (Fig. 15 j). Otherwise, development and vascularization (Fig. 16 y-a1) proceeded as in wild-type.

pin8

No deviations from the wild type could be observed regarding vascularization (Fig. 16 b1-d1) and overall development (Fig. 15 k) in the *pin8* mutant.

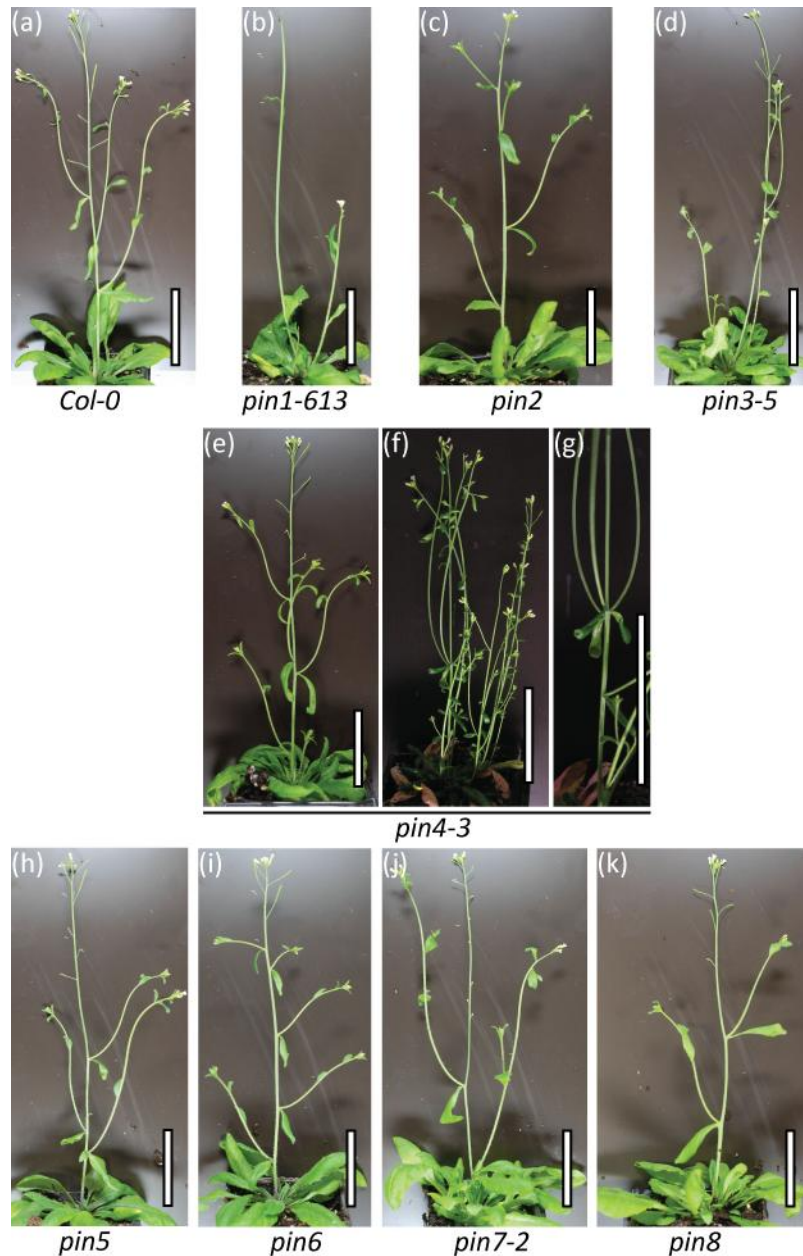
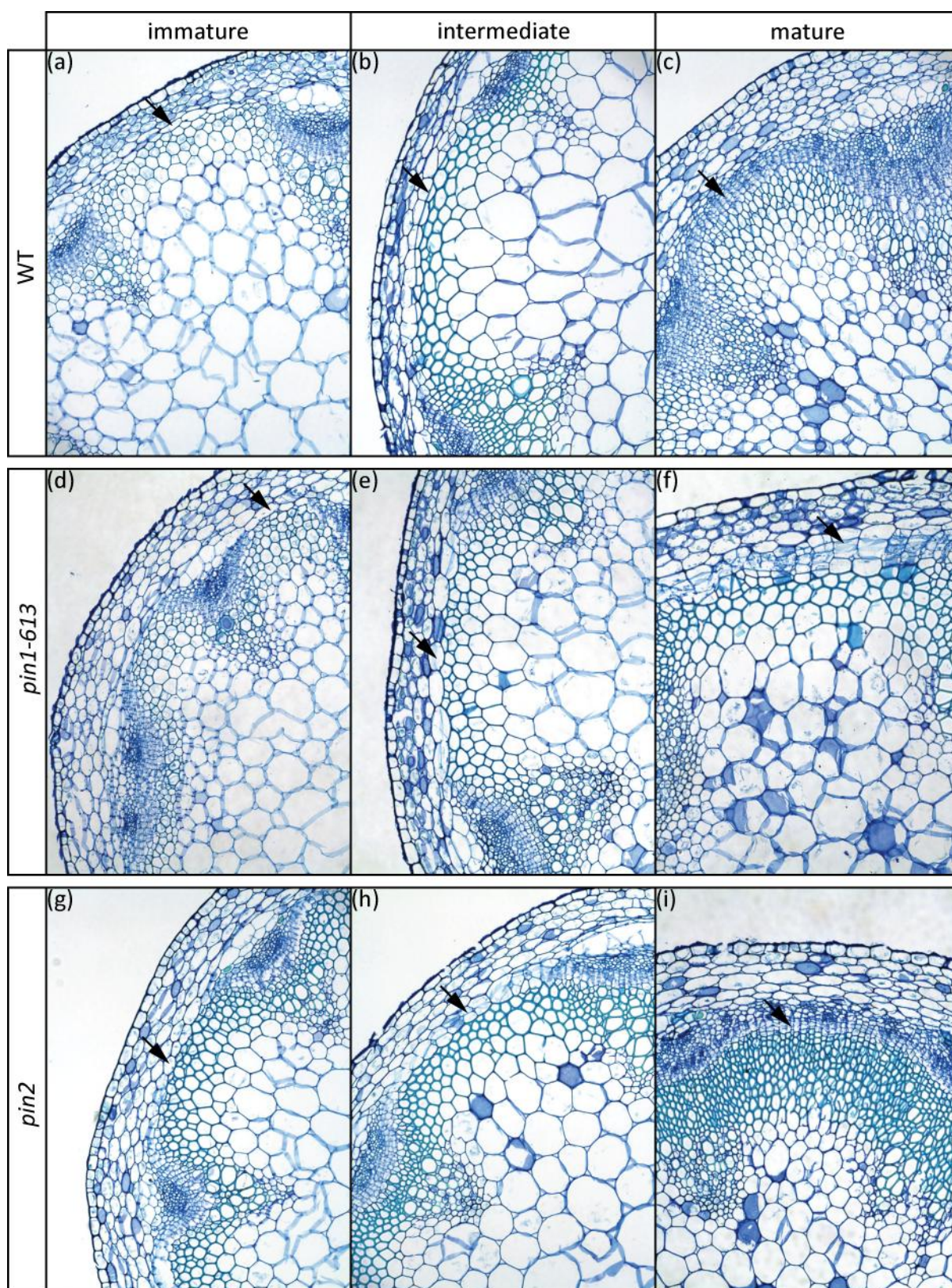
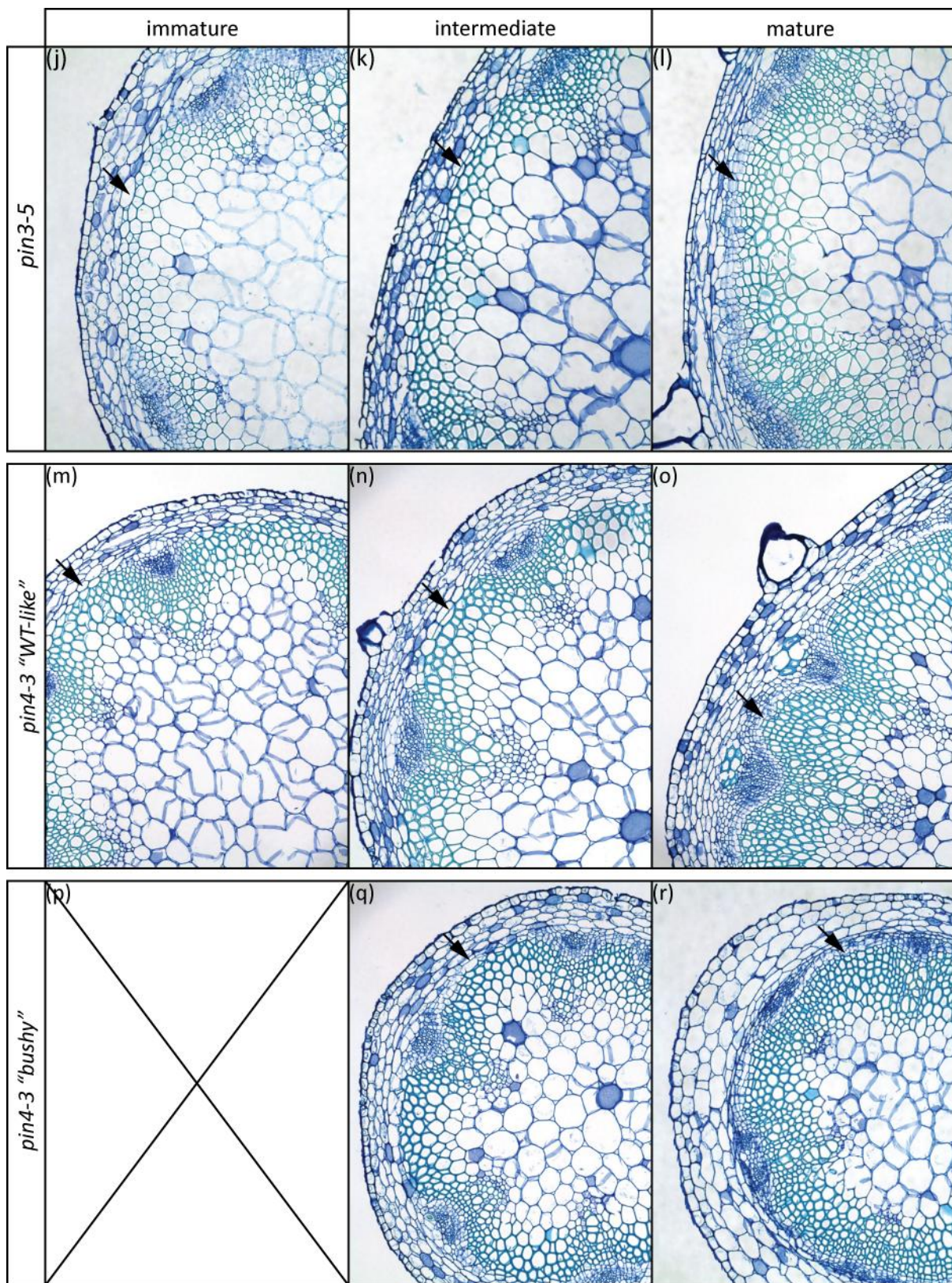
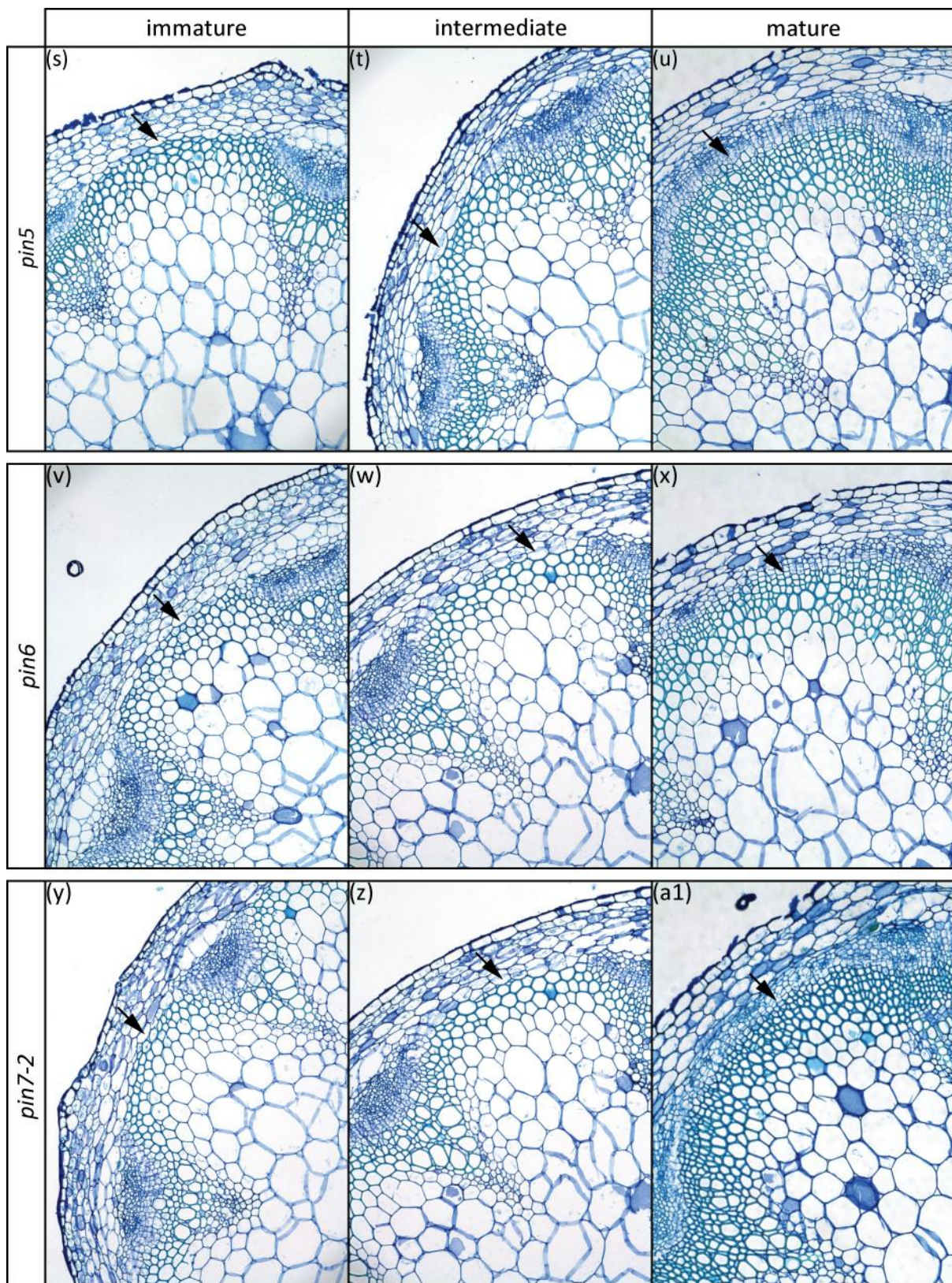


Figure 15: Mature *Arabidopsis* plants grown in long-day conditions. *pin1-613* (b) exhibits the namesake pin-like stem, with no side branches or flowers, due to the defective apical meristem. In contrast, *pin2* (c), *pin3-5* (d), *pin5* (h), *pin6* (i) and *pin8* (k) did not exhibit any growth deviations from wild type (a). Two distinct phenotypes could be observed in *pin4-3* (e-g). Plants grown at the wall of the growth chamber were almost wild type-like (e), with the exception of slightly curled rosette and axillary leaves. The second, „bushy“, phenotype (f), which occurred in plants placed near the center of the growth chamber, showed a sharp increase in the amount of side branches and secondary shoots, as well as branching events in which three side branches come out of a single node (g). Also, the first node on the main stem occurred significantly closer to the rosette (f) than in wild type (a). The growth of *pin7-2* (j) was overall wild type-like, but the first siliques aborted at early stages.







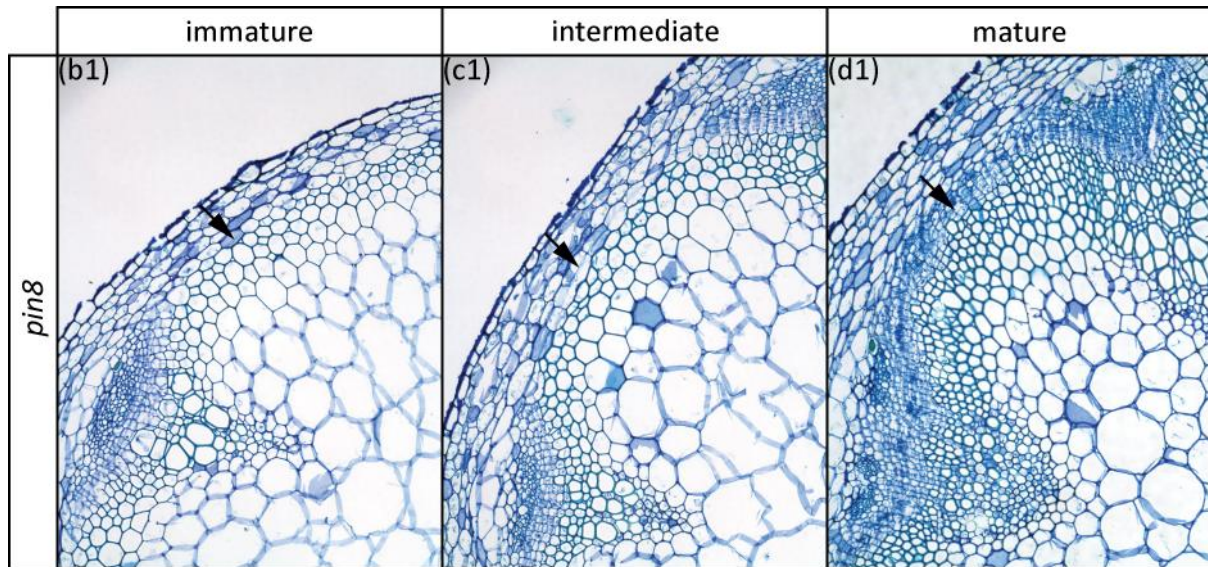


Figure 16: 7 μm thick cross-sections of main stems 3 mm above the rosette, at the immature, intermediate and mature stages. Stained with Toluidine blue. Light blue indicates a lignified cell wall. Arrows point the starch sheath (immature stages), the first cell division in the starch sheath during the initiation of secondary growth (intermediate stages), and the interfascicular cambium (mature stages). The primary vascular bundles in pin1-613 (d-f) were distributed irregularly, as illustrated by the two almost joined bundles in the immature stage (d). Regardless, initiation of secondary growth (e) occurred as in wild type (b), though fewer cell divisions occurred in the mature stage (f) in comparison to the wild type (c). No deviations from wild type (a-c) could be observed in pin2 (g-i), pin5 (s-u), pin7-2 (y-a1) and pin8 (b1-d1). Initiation of secondary growth occurred in the starch sheath at the intermediate stage (h, t, z, c1), followed by proliferation of secondary phloem and xylem (i, u, a1, d1) in a magnitude comparable to wild type (c). Though initiation of secondary growth in pin3-5 (k) occurred as in wild type (b), cell proliferation lagged behind in the mature stage (l). Also, not all cell divisions were strictly periclinal, leading to a more disturbed pattern in the cell files (l, arrow). While the „WT-like“ phenotype of pin4-3 (m-o) did not show any deviations from wild type in secondary growth, the „bushy“ phenotype (p-r) had strong aberrations. The stem diameter was severely reduced (q-r), though the number of primary vascular bundles did not decrease significantly. No immature stage plant could be found (p), as all individuals had initiated secondary growth in the starch sheath already. In the mature stage (r), secondary growth proceeded as in wildtype (c), but the phloem got slightly crushed between cortex and xylem, including the primary phloem in vascular bundles. pin6 (v-x) initiated secondary growth successfully (w), but slightly fewer cell divisions occurred in the mature stage (x) compared to wild type (c).

Expression studies

RT-PCR

Temporal changes in *PIN* expression levels in stems were investigated with RT-PCR (Fig. 17). Total RNA was extracted from 1 cm long stem segments cut at 0,3 and 1,3 cm above the rosette. The cDNA synthesized from these extracts was used as the template for PCR reactions with primer pairs specific for each *PIN* cDNA. Concentrations were equalized with the Tubulin positive control.

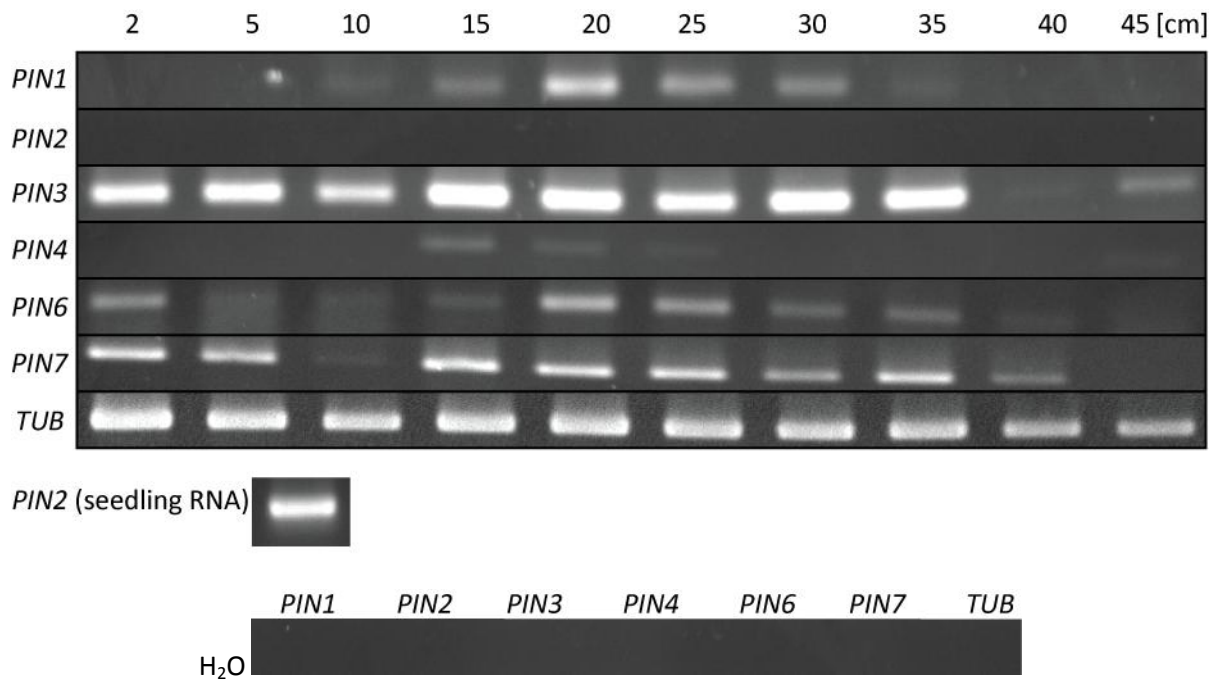


Figure 17: PCR products based on cDNA from 1 cm long sections of bottom stems. The X-axis denotes the total stem height. The product for tubulin serves as a loading control. *PIN2* cDNA was additionally amplified on seedling RNA to show the functionality of the primers. PCR reactions with H_2O instead of cDNA served as negative controls.

Analysis of expression levels at the bottom of the stem at different stages of development divided the *PIN* genes into two groups. *PIN3* is expressed at constant levels during the whole lifespan. *PIN7* is expressed at constant levels except for minima at 10 cm and 30 cm. For both *PIN3* and *PIN7*, severe downregulation occurs only in plants as high as 40 cm, which marks the end of the lifespan in the Columbia ecotype.

PIN1, *PIN4* and *PIN6* show non-uniform expression levels. They rise and are downregulated again. In the case of *PIN4*, the peak occurs in plants with 15 cm tall stems, which is classified as the intermediate stage and when secondary growth is just beginning. *PIN1* and *PIN6* peak slightly later, in plants with 20 cm tall stems. At this stage, plants are just on the brink of the mature stage, with secondary growth already having progressed for a few cell divisions.

PIN2, as predicted by public expression data, could not be detected in stems. Seedling RNA was used as a positive control.

GUS Staining

Spatial *PIN* expression patterns were visualized with a GUS assay. The *PIN* promoters drove expression of the beta-D-glucuronidase gene from *E. coli*. The expressed beta-D-glucuronidase could then be stained with X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide), showing where the promoters were active (Jefferson, Kavanagh et al., 1987).

GUS activity in the stem was found in *PIN1:GUS*, *PIN3:GUS* and *PIN6:GUS* lines (Fig. 18). Though the *PIN4:GUS* and *PIN7:DS-GUS* lines showed signals in seedlings (results not shown), no GUS signal could be detected in stems.

PIN1:GUS

PIN1 expression was not clearly restricted to one particular cell type (Fig. 18 a-c). In an immature stem, GUS localization was ubiquitous inside the cortex. This distribution was restricted slightly in intermediate stems, where GUS was localized mainly in and around the fascicular and interfascicular cambium. This pattern remained unchanged in mature stems.

PIN3:GUS

Localization of *PIN3* expression was mostly similar to *PIN1* (Fig. 18 d-f). Starting in the primary cambium and the interfascicular regions with some spreading in the cortex and pith, GUS staining localized more specifically in and around the fascicular and interfascicular cambium in intermediate stems. Expression stays equally strong throughout the plant's life, up until to the mature stages.

PIN6:GUS

In immature stems, *PIN6* was expressed almost exclusively in the fascicular cambium (Fig. 18 g-i). Only after the first cell divisions of the interfascicular cambium did expression spread into the interfascicular region. This pattern of GUS localization in the fascicular and interfascicular cambium remained also in the mature stems.

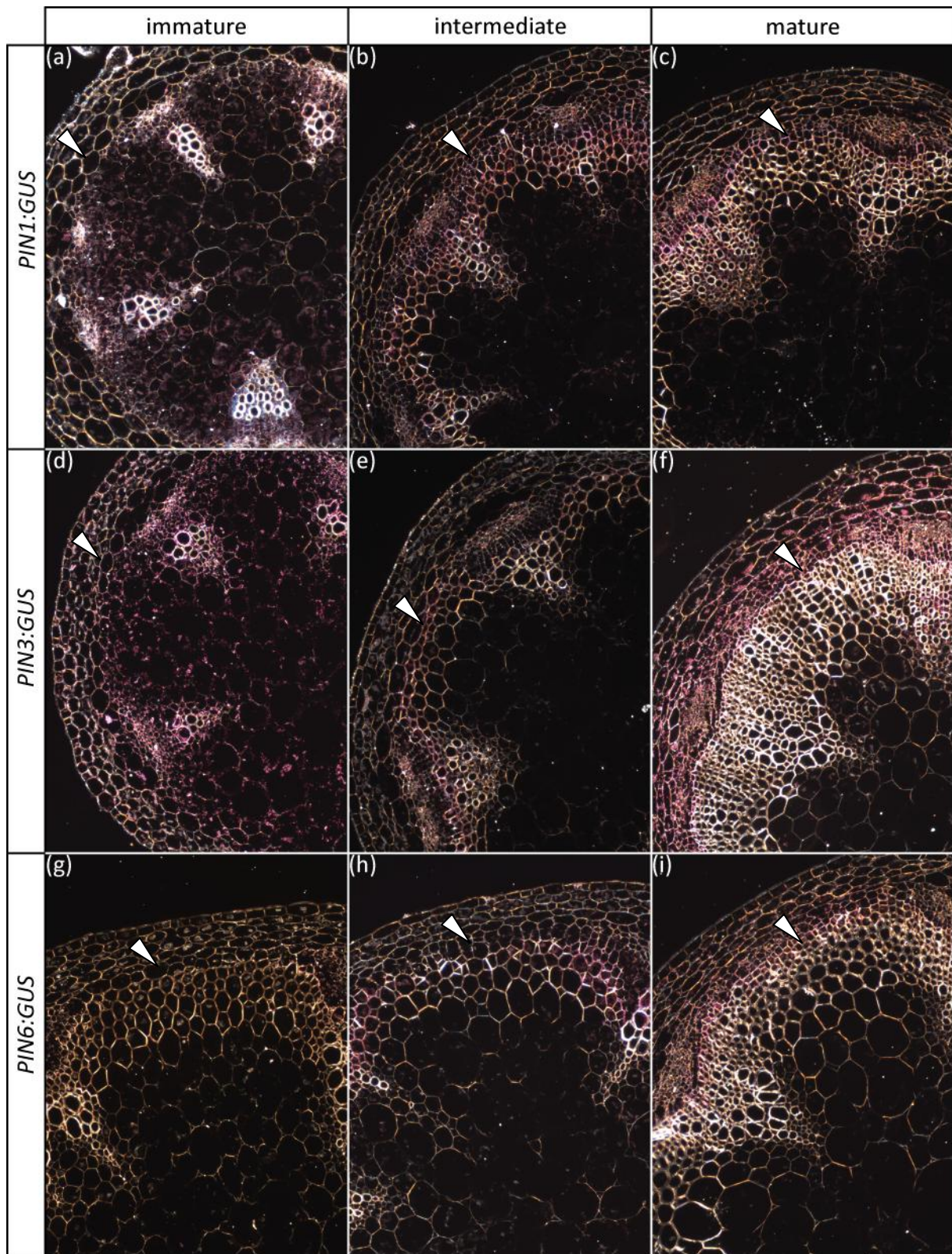


Figure 18: Stainings of promoter:GUS constructs, shown in 7 μ m thick sections, darkfield microscopy (GUS in pink). Arrows point the starch sheath (immature stages), the first cell division in the starch sheath during the initiation of secondary growth (intermediate stages), and the interfascicular cambium (mature stages). Both *PIN1:GUS* (a-c) and *PIN3:GUS* (d-f) signals were uniformly distributed across the pith and vasculature in the immature stage (a, d), and spread slightly into the cortex. During the initiation of secondary growth (b, e), the signal was localized in the starch sheath and adjacent cells, the region of the newly forming cambium. This distribution stayed stable in the mature phase (c, f), though *PIN3:GUS* (f) showed a clearer border against secondary xylem than *PIN1:GUS* (c), which spread slightly into it. *PIN6:GUS* (g-i) was restricted to the fascicular cambium in the immature stage (g), but spread into the interfascicular region during the onset of secondary growth (h). In the mature stage (i), the signal remained localized at the now closed cambium ring.

Inducible Expression of Artificial microRNAs

Knockout mutants (ideally) lack any expression of the gene of interest. But this efficiency can also lead to several problems. In the worst case, the mutations can be lethal at early stages, for example embryogenesis. But also if the plants themselves are viable, they are often sterile, as is the case with the *pin1* knockout. Lines can then be kept only heterozygously, with selection necessary during each experiment. Also, the phenotype can be so strong that it remains unclear whether the observed aberrations are the direct result of the lacking gene expression or secondary effects because of the strongly affected plant. These problems are amplified when establishing and maintaining lines with multiple gene knockouts. Also, establishing lines with several mutations can be very time-consuming due to the long generation time of plants. But when working with gene families with several homologous and potentially redundant members, study of just single knockout mutants will possibly not lead to any observable phenotypic changes.

One alternative for knockout mutants are conditional knockouts, where the experimenter can alter gene expression at any time point, while still working with wild type-like phenotypes. Genes can then be silenced by artificial microRNAs (Schwab, Ossowski et al. 2006) under induction conditions.

MicroRNAs are small single-stranded RNA molecules with a length of 21-23 nucleotides (Lagos-Quintana, Rauhut, et al. 2001; Lau, Lim, et al. 2001; Lee, Ambros, et al. 2001). They are derived from double-stranded pre-miRNAs with a stem-loop structure, which is cut by the endonuclease Dicer (Bernstein, Caudy, et al. 2001). One of the two resulting single-stranded miRNAs is integrated in the RNA-induced silencing complex, RISC (Hammond, Bernstein, et al. 2000). The RISC then binds to mRNA complementary to its miRNA. This leads to inhibition of translation or mRNA degradation. In this way, gene expression is silenced (Reinhardt, Weinstein et al. 2002).

Because of their short sequence, miRNAs can pair with conserved domains in different mRNAs. This makes them very useful for knocking out several members of a gene family with high sequence homology, as is the case for the *PIN*s. In this way, mutants with multiple knockout mutations, which would lead to the experimental problems described above, can be avoided.

In this study, expression of *PIN3*, *PIN4* and *PIN7* was knocked down at the transcription level, using artificial microRNAs (Fig. 19). These were cloned into the “ethanol switch” system described above (P. 38).

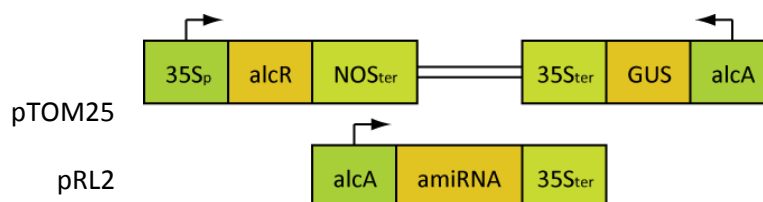


Figure 19: Schematic representation of the “ethanol switch” containing a region for artificial microRNA synthesis and a *GUS* reporter.

Using this system, an artificial miRNA targeting the mRNAs of *PIN3*, *PIN4* and *PIN7* was designed in the WeigelWorld amiRNA tool, and the template cloned into a plasmid with the alcaA component (pRL2, based on pGreen0229-Alca). To establish the transgenic plant lines, a two-step approach was utilized, as plants which contained the alcaR and GUS components were transformed with the plasmid pRL2 containing the alcaA and microRNA component.

Preliminary Results

Ethanol treatment of pRL2 plants led to two distinct phenotypes in the T1 generation: about one half of the plants retained a wild type-like phenotype. The other half, however, grew very slowly and possessed a large number of side shoots and branches. When the wild type-like phenotype reached a height of 40 cm and started to die, the second, “bushy”, phenotype reached a height about 20 cm. Of each phenotype, 10 T1 generation plants were chosen for anatomical analysis. Wild type Col-0 plants treated with ethanol in the same manner were used as a control. Wild type and “wild type-like” pRL2 plants were analyzed when the main stem reached a height of 30 cm. “Bushy” pRL2 plants were analyzed when the main stem reached a height of 20 cm.

Histology showed that Ethanol treatment had no effect on the wild type, which underwent a large amount of secondary growth (Fig. 20 a-c, as described above).

“Wild type-like” pRL2 plants were able to initiate secondary growth, though not to a large extent. The main stem of the “bushy” phenotype never initiated secondary growth and showed a very small diameter (Fig. 20 d-f).

Due to time and experimental constraints, it could not be satisfactorily verified how severely the artificial microRNA affected expression of *PIN3*, *PIN4* and *PIN7*.

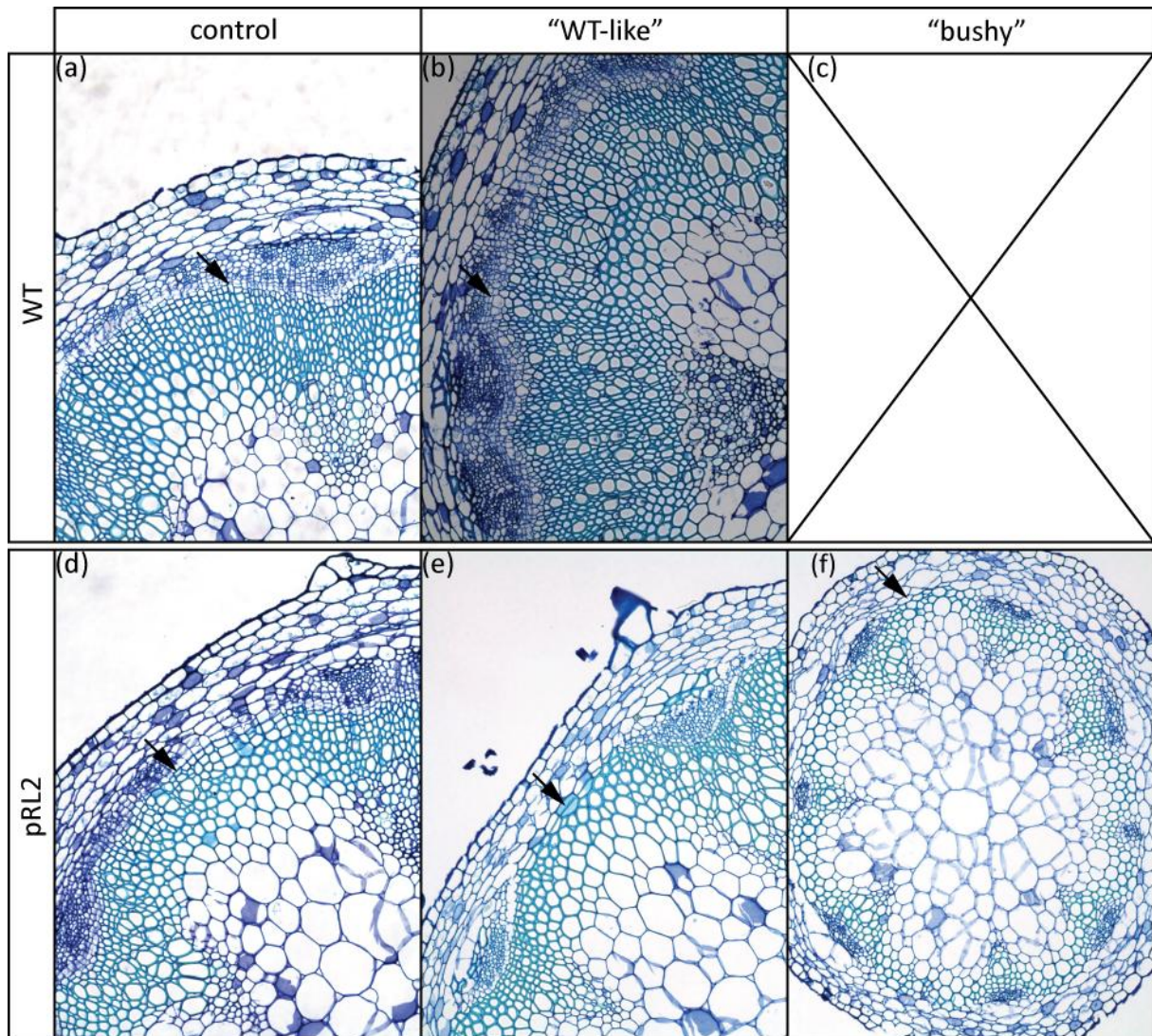


Figure 20: Cross-sections of mature stems 3mm above the rosette of 30 cm high stems. Phenotypes after ethanol treatment were separated into "WT-like" for (b) WT and (e) pRL2; and "bushy" for (f) pRL2. The corresponding controls (a, d) were mature plants without ethanol treatment. Arrows point to the starch sheath and developing interfascicular cambium. pRL2 plants with a wild type-like phenotype were able to initiate secondary growth (e), but did not proceed for more than one cell division. pRL2 plants with the "bushy" phenotype were not able to initiate secondary growth at all, as no cell divisions could be observed in the interfascicular region (f). pRL2 plants without ethanol induction (d) developed like wild type (a), showing extensive secondary growth. Ethanol treatment on wild type plants did not show any effects in the vasculature (b).

In vitro System

While the analysis of mutants can give insight into the function of genes involved in hormone signaling, the effects of different hormone concentrations and polarity remain elusive in this approach. On numerous occasions, decapitation and subsequent auxin treatment of *Arabidopsis* have been successfully used to induce secondary growth in an immature stem (Lev-Yadun, 1994). But this approach answers only a very specific question – is an apical source of auxin needed to induce secondary growth?

Still, the lower parts of the plant remain intact, and it is therefore not possible to say whether the observed induction of secondary growth is the direct result of auxin action in the stem, or might derive from subsequent effects in other parts of the plant. Also, hormone concentrations on the lower part of the stem might not be altered.

A more direct approach to testing the effects of phytohormones on secondary growth was needed. Based on the work of Chatfield and Leyser (Chatfield, Stirnberg et al. 2000), an *in vitro* system was to be established to provide a direct and reproducible approach (Fig. 21).

This method was based on exposing isolated stem pieces to an artificial hormone gradient. Isolated stem pieces were incubated for 5 days, touching media halves with independent hormone concentrations.

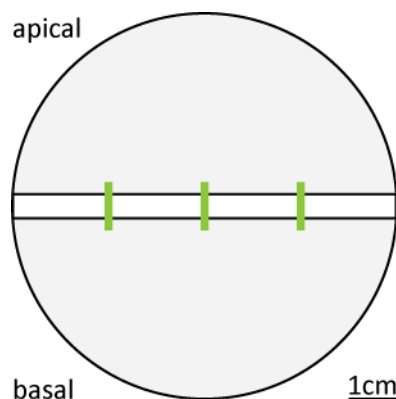


Figure 21: Schematic representation of the *in vitro* system, with three stem pieces bridging the two media halves.

Wild type Columbia

Wild type stem segments were subjected to external auxin sources in the *in vitro* system to verify that the developmental response would be comparable to that of stems in whole plants. Addition of the competitive auxin transport inhibitor NPA inhibits any effects that are due to auxin transport. To investigate a potential correlation between secondary growth and auxin dosage, several auxin concentrations along a gradient were used.

Stem segments of 1 cm length, cut 5 mm above the rosette, were used for analysis. When the apical media half contained 1 $\mu\text{g/mL}$ NAA, noticeable callus growth could be observed both at the apical and basal end of the stems. After 5 days incubation, previously immature stems showed strong secondary growth (Fig. 22 a). NPA addition (1 $\mu\text{g/mL}$) to both media halves inhibited both basal callus

growth and secondary growth along the entire stem (Fig. 22 c). NPA treatment alone had no effect on the stem (Fig. 22 e).

Basally applied 1 $\mu\text{g/mL}$ NAA led to basal callus formation only, and failed to induce secondary growth (Fig. 22 b). Basal and apical NPA addition of 1 $\mu\text{g/mL}$ showed no further change of the effect (Fig. 22 c).

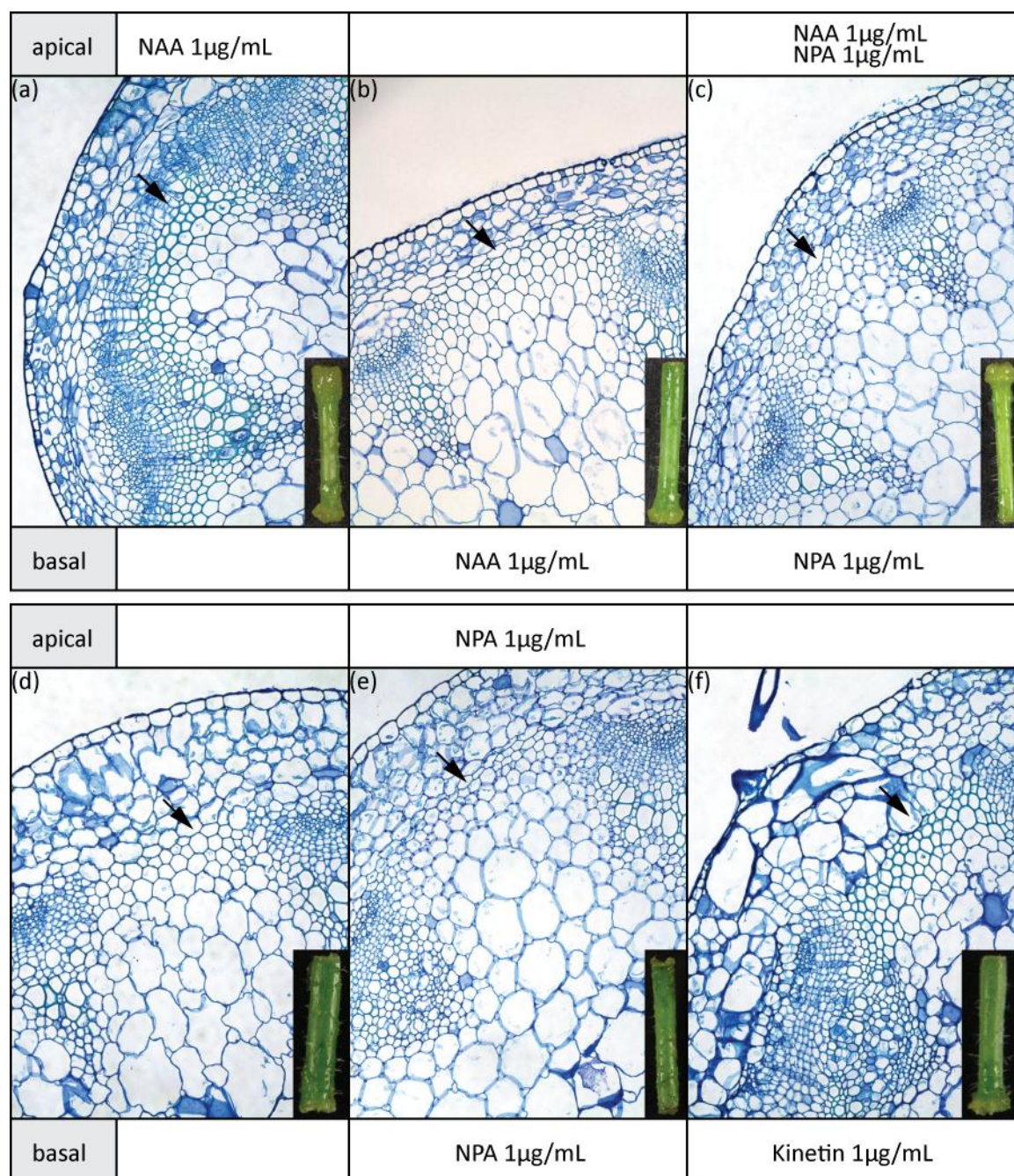


Figure 22: WT Columbia stems after 5 days in the *in vitro* system. The 7 μm thick cross-sections from the bottom of the stem (the first part which did not touch the growth media) were stained with Toluidine blue. Arrows point to the starch sheath in immature stems, as well as to the developing interfascicular cambium. NAA was applied apical (a), (c) or basal (b). NPA prohibited NAA transport (c). No hormone treatment (d) and NPA treatment (e) served as negative controls. Basal cytokinin application (f) effects were also shown. Arrows point to the starch sheath in immature stems, as well as to the developing interfascicular cambium. Apical NAA (1 $\mu\text{g/mL}$) was able to induce secondary growth, as seen by several rounds of cell division in the interfascicular region (a). Basal NAA (1 $\mu\text{g/mL}$) did not have any visible effect on the stem, which remained in its immature phase (b). A background of NPA (1 $\mu\text{g/mL}$) completely negated the effects of apical NAA (1 $\mu\text{g/mL}$), since not a single cell division occurred in the interfascicular region. Both the absence of external

hormone sources (d) and treatment with NPA (1 $\mu\text{g/mL}$) (e) did not have any visible effect on an immature stem. Basal cytokinin treatment with Kinetin (1 $\mu\text{g/mL}$) led to uncontrolled cell enlargement in the cortex, including the starch sheath (f), but did not induce cell divisions necessary for secondary growth.

In the presence of an apical auxin source (1 $\mu\text{g/mL}$ NAA), secondary growth proceeded uniformly along the stem segments (Fig. 23 a-c). A basal auxin source (1 $\mu\text{g/mL}$ NAA) failed to initiate secondary growth at any region of the stem segments (Fig. 23 d-f).

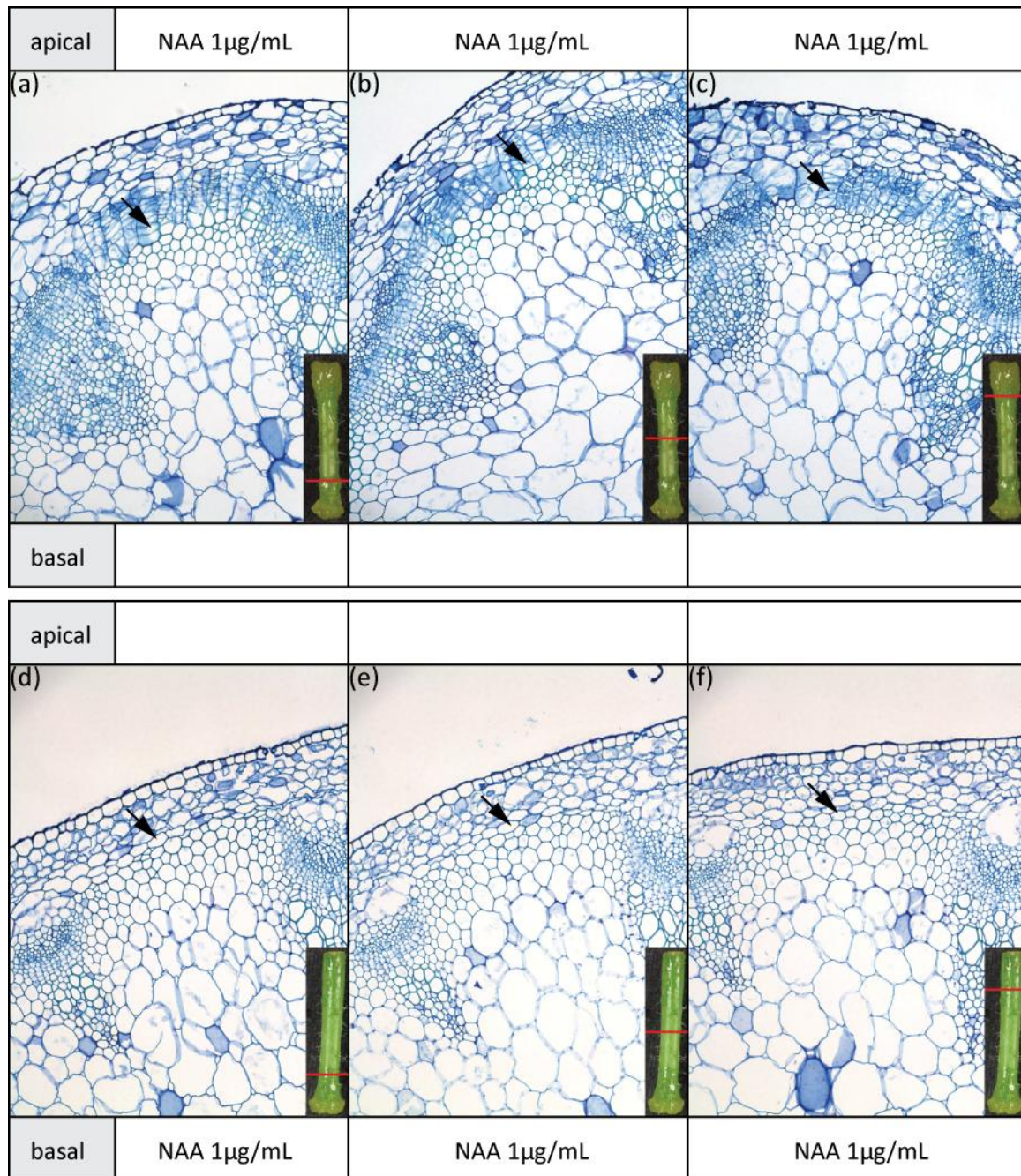


Figure 23: Basal (a,d), middle (b,e) and apical (c,f) sections through WT stems after 5 days in the *in vitro* system. The 7 μm thick cross-sections were stained with Toluidine blue. Red bars indicate the cross-section. NAA was applied apically (a-c) and basally (d-f). Arrows point to the starch sheath in immature stems, as well as to the developing interfascicular cambium. Stems in which secondary growth has been induced by treatment with apical NAA (1 $\mu\text{g/mL}$) showed the same extent of secondary development in sections taken from the bottom (a), middle (b) and apical (c) region of the same stem. Likewise, absence of induction of secondary growth by treatment with NAA (1 $\mu\text{g/mL}$) stayed stable in the basal (d), middle (e) and apical (f) region of the same stem.

The apical NAA concentration could be as low as 0,03 $\mu\text{g/mL}$ and still initiate secondary growth, though no more than one to two cell divisions occurred per cell file. Continually increasing apical NAA concentration over the series of 0,03, 0,1, 0,3 and 1 $\mu\text{g/mL}$ correlated with increasing cell division rates during secondary growth as well as callus size (Fig. 24 a-f). Stems treated with 3 $\mu\text{g/mL}$ apical NAA did not exhibit an increase in either callus growth or the cell division rate during secondary growth compared to treatment with 1 $\mu\text{g/mL}$ apical NAA.

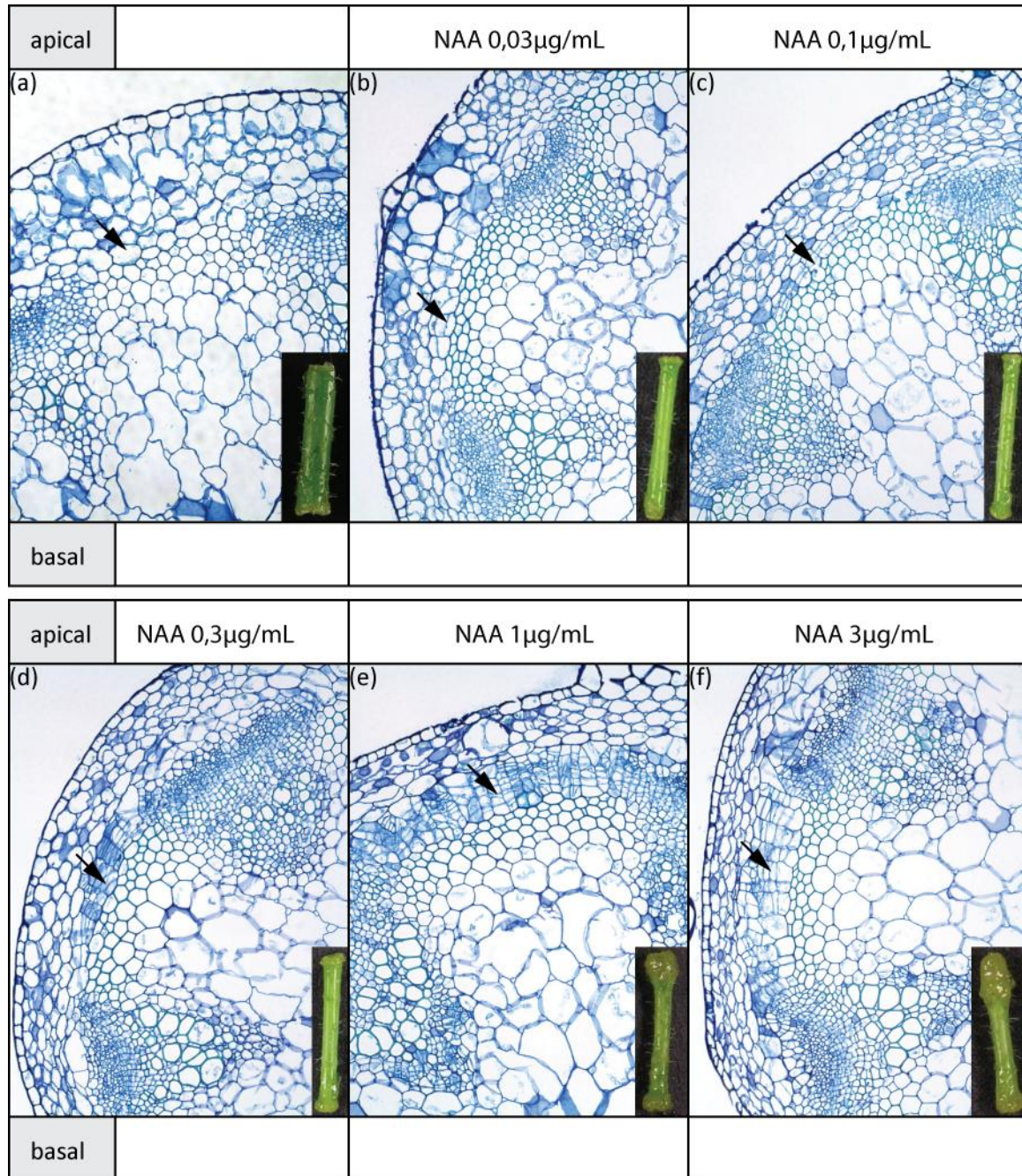


Figure 24: Increasing apical NAA concentrations and their effect on secondary growth in WT stems after 5 days in the *in vitro* system. The 7 μm thick cross-sections from the bottom of the stem (the first part which did not touch the growth media) were stained with Toluidine blue. Arrows point to the starch sheath in immature stems, as well as to the developing interfascicular cambium. The extent of secondary growth scaled well from 0,03 $\mu\text{g/mL}$ apical NAA to 1 $\mu\text{g/mL}$ apical NAA (b-e), ranging from single cell divisions (b) to large cell files (e). A further increase to 3 $\mu\text{g/mL}$ apical NAA did not cause further increase in secondary growth (f), instead staying in the same range as treatment with 1 $\mu\text{g/mL}$ apical NAA (e).

An apical NAA source can induce secondary growth. The Cell division rate correlates with auxin dosage. Because addition of the auxin transport inhibitor NPA inhibits this induction, and a basal NAA source does not induce secondary growth, it can be concluded that apical-basal auxin transport is necessary for the induction of secondary growth.

Cytokinin

Because cytokinin is involved in vascular development, it is possible that cytokinin might also be involved in the initiation of secondary growth.

Basally applied Kinetin (1 µg/mL) induced callus growth, but did not induce secondary growth in immature stems. Cortex cells were enlarged (Fig. 22).

External cytokinin sources are not sufficient to induce secondary growth.

pin3-5

Because of its strong expression in the stem and localization to the starch sheath, PIN3 was identified as one of the most likely factors of establishing an auxin pattern necessary for secondary growth. When this factor is missing, as in the knockout line *pin3-5*, external auxin sources should have growth effects different from wild type.

Apical treatment of immature *pin3-5* stems with 0,3 µg/mL NAA led to almost no callus formation, but stronger secondary growth (Fig. 25 a) compared to the wild type (Fig. 24 c). Also at a lower concentration of 0,1 µg/mL apical NAA induced stronger secondary growth (Fig. 25 b) than in the wild type (Fig. 24 d). Apical and basal 1 µg/mL NPA enhanced apical callus formation and reduced secondary growth significantly, but a few cell divisions still occurred (Fig. 24 d).

Basal application of 0,3 µg/mL NAA led to basal callus formation and secondary growth induction, though only at low cell division rates (Fig. 25 c). In the *pin3-5* background, controls without added NAA did not survive the 5 days of incubation (Fig. 25 e).

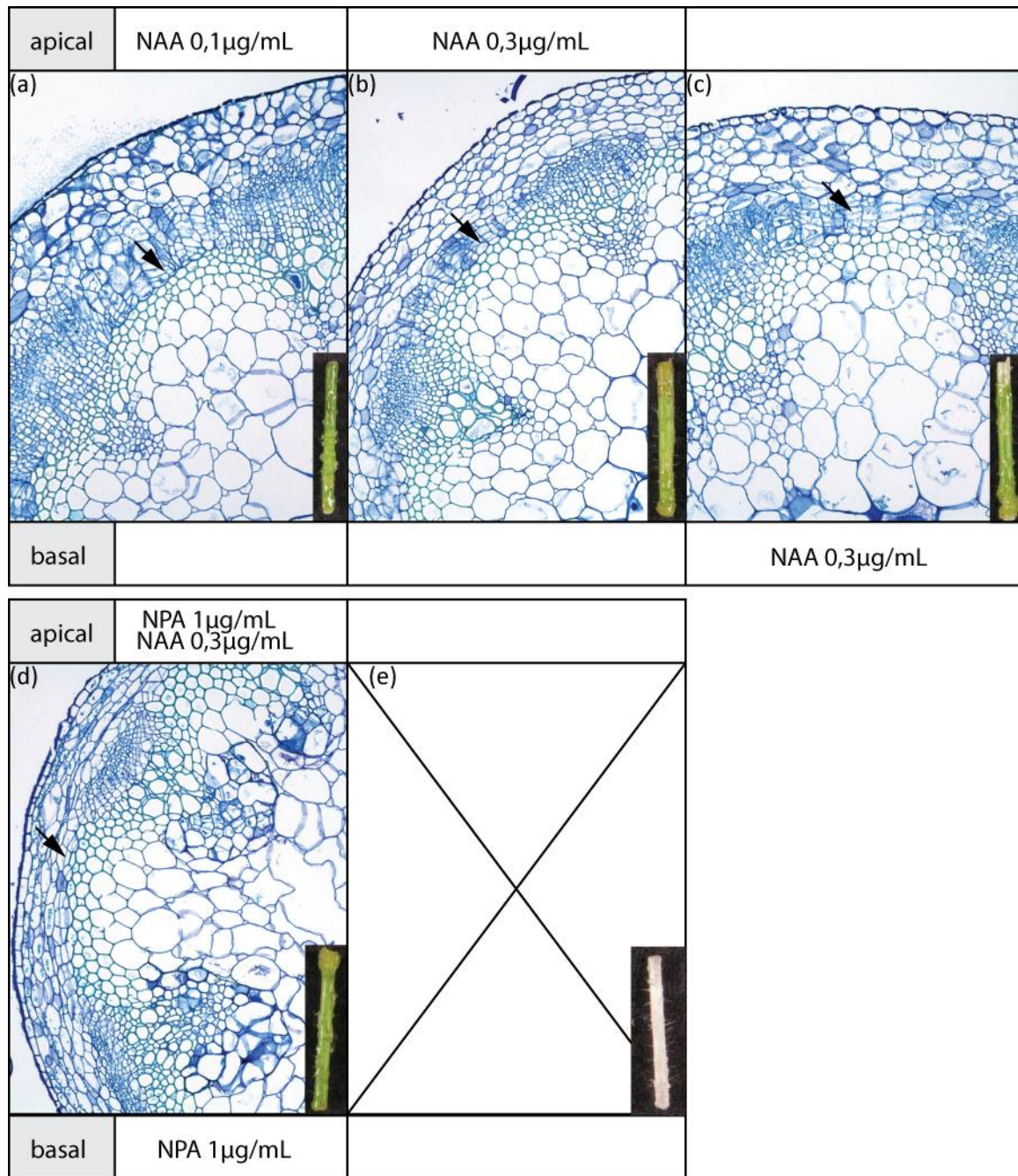


Figure 25: The effects of apical (a,b) and basal (c) NAA application on *pin3-5* stems after 5 days in the *in vitro* system. The 7 µm thick cross-sections from the bottom of the stem (the first part which did not touch the growth media) were stained with Toluidine blue. Arrows point to the starch sheath in immature stems, as well as to the developing interfascicular cambium. Treatment with 0,1 µg/mL apical NAA (a) resulted in significantly stronger secondary growth than in wild type (Fig. 24 b). The extent of secondary growth did not increase with the increase of apical NAA to 0,3 µg/mL (b). Treatment with 0,3 µg/mL basal NAA (c) caused induction of secondary growth to the same extent as apical induction (b), which is in stark contrast to the total absence of secondary growth induction in wild type with an even stronger NAA concentration (Fig. 22 b). The effects of treatment with 0,3 µg/mL apical NAA were slightly lessened in a background of 1 µg/mL NPA (d). Induction of secondary growth still occurred, but proceeded to a lesser extent than without NPA (b). In wild type, secondary growth is not induced at all in a NPA background (Fig. 22 c) with a higher NAA concentration. Stem fragments without any external hormone source did not survive conditions on the plate (e). Wild type survived the same conditions (Fig. 22 d).

Discussion

To investigate the effects of auxin signaling on secondary growth, the anatomy of knockout mutants of the PIN auxin efflux carriers was analysed. This effort was complemented by a line expressing an artificial microRNA against *PIN3*, *PIN4* and *PIN7*. Expression profiling was accomplished both by RT-PCR and promoter-GUS reporters. The effects of IAA on wild type and *pin3-5* were further assessed in an *in vitro* system.

The connection between secondary growth and cytokinin signaling was investigated by the analysis of mutants of the signal transduction pathway and a line overexpressing the cytokinin oxidase *CKX1*. Furthermore, the effects of cytokinin on the wild type were investigated in an *in vitro* system.

Utilizing the *in vitro* system, it could be shown that an external apical auxin source is necessary and sufficient to induce secondary growth in isolated immature Arabidopsis stems. The cambial activity has been shown to correlate with the auxin concentration, until reaching a maximum at 1µg/mL NAA.

Since the induction of secondary growth can be prevented with the addition of the competitive auxin transport inhibitor NPA it can be concluded that active transport is essential for it. Furthermore, the transport must occur from apical to basal, since basally applied NAA did not show any effect on cambial activity. NAA bypasses import by AUX1 and diffuses passively into the cell (Delbarre, Muller et al. 1996), so it is likely that the necessary apical-basal transport involves the polarly distributed PINs. If transport through the non-polarly distributed PGP were sufficient, basal application of NAA would have induced secondary growth as well. Judging from the single knock-out mutants of *PIN* genes, not one of them alone is responsible for the necessary auxin transport. Even a heavily impaired mutant like *pin1-613* was still capable of activating the interfascicular cambium and proceeding with at least a few cell divisions.

Instead, if *PIN* genes are important for secondary growth, it is probably a subset of the gene family. As shown with RT-PCRs, *PIN1*, *PIN4* and *PIN6* show an expression pattern peaking during or directly after the onset of secondary growth. Since *PIN1*, *PIN3* and *PIN4* are induced by auxin (Vieten, Vanneste et al. 2005), the expression peaks of *PIN1* and *PIN4* could be explained by heightened auxin levels during the initiation of secondary growth. But since *PIN3*, which would in this case also be induced, does not show any change in expression levels, auxin likely has no significant effect on PIN expression under these conditions.

Of this selection, *PIN4* expression peaks earliest, just in plants entering the intermediate stage. Besides *pin1-613*, *pin4-3* was the only mutant with a changed growth habitus.

My preliminary results using an artificial microRNA against *PIN3*, *PIN4* and *PIN6* support the results of the *pin4* knockout mutant: an increase in secondary growth in very small stems, with an increased number of side shoots and branches. The proximity of the first node to the rosette might also be responsible for an observed increase in secondary growth, because an axillary shoot apex provides a new auxin source (Ko, Han et al. 2004). But cell proliferation was uniform across the stem. If the secondary vasculature would have been caused by the nearby branch, it would have been more polarly distributed towards the axillary branch. Therefore, the lack of *PIN4* seems to promote secondary growth. This would probably be due to misdistribution of auxin and thereby shifting of auxin maxima. A shift of auxin maxima is possible if it accumulates in regions from which it would

normally be exported by PIN4 in wild type. While auxin efflux is not facilitated by the PIN proteins alone, a quantitative difference in the efflux rate or loss of directional efflux might elevate auxin concentration over a threshold, triggering new developmental processes.

Though knocking out *PIN6* results in no vascular phenotype, the GUS expression studies show an interesting pattern. As opposed to *PIN1* and *PIN3*, it does not occupy the interfascicular fibers, but only spreads into the interfascicular regions when the interfascicular cambium has already been activated. This hints at a role in maintaining secondary growth.

PIN3 has the highest expression of the PIN family in stems and the protein localizes to the starch sheath (Friml, Wisniewska 2003). This makes it a likely factor in the initiation of secondary growth. Still, the role of *PIN3* has proven to be the most elusive to determine. Results from the transcription studies do not correlate with the behavior of the actual protein (E. M. Sehr, unpublished results). Both RT-PCR and *GUS* studies show *PIN3* expression lasting from the earliest observed time point (2 cm stem) up to the last before senescence (35 cm). In contrast, the PIN3 protein disappears during the initiation of secondary growth (E. M. Sehr, unpublished results). Though the GUS protein is known to be long-lived, the effect is much too significant to be explained by this factor. Instead, post-transcriptional regulation through an, as of yet, undiscovered mechanism seems likely. Spatially, though the PIN3 protein localizes exclusively to the starch sheath, transcription shows no such restriction. Again, the GUS method has a methodological problem: the protein can diffuse and thus blur the signal. It is known that auxin promotes the expression of *PIN1*, so a similar mechanism might be at work here. While transcription would be held at high levels by the presence of auxin, distribution of PIN3 or the removal of the protein would have to be accomplished post-transcriptional.

Knocking out *PIN3* did not impair secondary growth significantly, though a reduced zone of secondary growth is observed (this study; Sehr, Greb, unpublished results). The *pin3* mutant did not differ significantly from wild type at a height of 3 mm above the rosette regarding secondary growth, but less secondary growth could be observed at 5 mm height. When the mutant was treated with apical auxin in the *in vitro* system, it reacted more strongly than the wild type, also at low concentrations. Local auxin induction in the form of callus growth was severely affected, insofar as the terminal calli were reduced, but numerous outgrowths occurred along the whole length of the stem. Unlike in the wild type, induction could not be stopped completely with either addition of NPA or shifting of the auxin source to the basal end. All this points to a yet to be specified loss of control of auxin distribution.

A possible explanation lies in the rate of auxin transport and the absolute auxin concentration at any time in the stem. In wild type, PIN3 efficiently carries auxin from apical to basal, thereby keeping the concentration at any time rather low. But when PIN3 is missing, as in the mutant, apical auxin has to be transported by other PINs and passive diffusion alone, probably at a slower rate. Thus, the auxin concentration at any given time in the stem might be significantly higher than in wild type, leading to induction of secondary growth already at lower levels of external auxin. Since auxin transport does not rely so much on PIN3 anymore, a competitive transport inhibitor like NPA, which relies on the same transport mechanisms, could lose some efficiency. As transport of auxin out of the stem at the basal end would be affected, a basal auxin source could spread much farther into the stem in the *pin3* mutant than in wild type, where it would immediately be transported out by PIN3 again.

In a wild type plant, the auxin transport velocity would also be lowered in the absence of PIN3. But while auxin accumulates in a reservoir at the basal end of the stem in the *in vitro* system, in the whole plant there are still tissues present which transport auxin further, thereby draining the basal auxin reservoir. The reduction of secondary growth in a intact *pin3-5* plant could be the result of cytokinin action or other plant hormones resulting from the presence of the root. While this would be counteracted by auxin in the wild type, the altered auxin pattern in *pin3-5* might lack this ability.

PIN7 was the only *PIN* gene which showed a similar expression pattern to *PIN3* in the RT-PCR experiment. The *PIN7::GUS* construct proved insufficient to detect transcription. Concluding from the RT-PCR data and homology to *PIN3*, *PIN7* could act redundantly to *PIN3*, but remains at low expression levels in wild type.

According to the *in vitro* experiment, cytokinin alone was not capable of initiating secondary growth. Also, none of the single knock-outs of members of the cytokinin signaling pathway showed an inability of initiating secondary growth. The most distinct phenotypes were observed in the knock-outs of the *AHK* cytokinin receptors. As only three *AHK* genes with cytokinin recognition ability exist, knockouts of just one of the genes can be expected to inhibit cytokinin signaling stronger than a mutant in the other more redundant components of the cytokinin pathway. The only gain-of-function allele in the experiment, *wol*, showed the most severe phenotype, but was still capable of interfascicular cambial activity. Still, all of the knock-out mutants were capable of initiating secondary growth, though at a different extent. Cytokinins are known to play an important role in the cell cycle, so mutations which primarily affect cytokinin signaling could also have consequences in the cell cycle and ultimately the rate and which secondary vasculature is forming from a newly established cambium. Also, it has been shown that cytokinin directly regulates cambial activity (Matsumoto-Kitano, Kusumoto et al. 2008) and that cytokinin regulates root meristem size by controlling cell differentiation (Dello Ioio, Linhares et al., 2007). This could explain the observed differences in cell file size of the various cytokinin signaling mutants. Mutants which showed severely reduced secondary growth (*ahk3-3*, *arr15*) could also have halted their secondary development entirely, as their interfascicular cambium might have differentiated and lost its pluripotent character. Especially *ahk3-3* did not develop clearly distinguishable secondary xylem and no secondary phloem at all. Also this would be an effect of premature differentiation of the stem cell population, as the daughter cells always stay in the region which would still be part of the cambium in wild type.

The sextuple mutant of the A-Type *ARR* cytokinin signaling inhibitors showed an increase in secondary growth, which might correlate with an increase in cytokinin signaling. While this result would not be in accordance with the inducible CKX1 overexpression, which shows an increase in secondary growth as well, it is not yet proven if the CKX1 transgene is active.

Still, these conclusions rely on inferring aberrations in the cell cycle by comparing the cell count in secondary vascular tissue between mutants and wild type. Differentiation is determined by the grade of cell wall lignification, which is easily observed in Toluodine blue stainings. It is difficult to reach conclusions about the cell stage with these methods. For more in-depth research into both cell cycle and cell differentiation aberrations in various mutants, it would be necessary to introduce reporters for visualizing cell cycle stages and cell types into those mutants.

At the bottom line, an apical auxin source is necessary for secondary growth, but the mechanism of its distribution remains to be investigated further. Especially PIN3 and PIN4 seem to play an important role.

Cytokinin has an influence on cell division, which is possibly due to its role in the cell cycle. This effect might go hand in hand with an influence on stem cell maintenance: an impaired cytokinin signaling pathway might not be able to maintain the pluripotent characteristic of the cambium and lead to premature differentiation, thereby halting secondary development.

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