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DISSERTATION

Titel der Dissertation

Molecular control of secondary growth initiation
in the *Arabidopsis* stem

angestrebter akademischer Grad

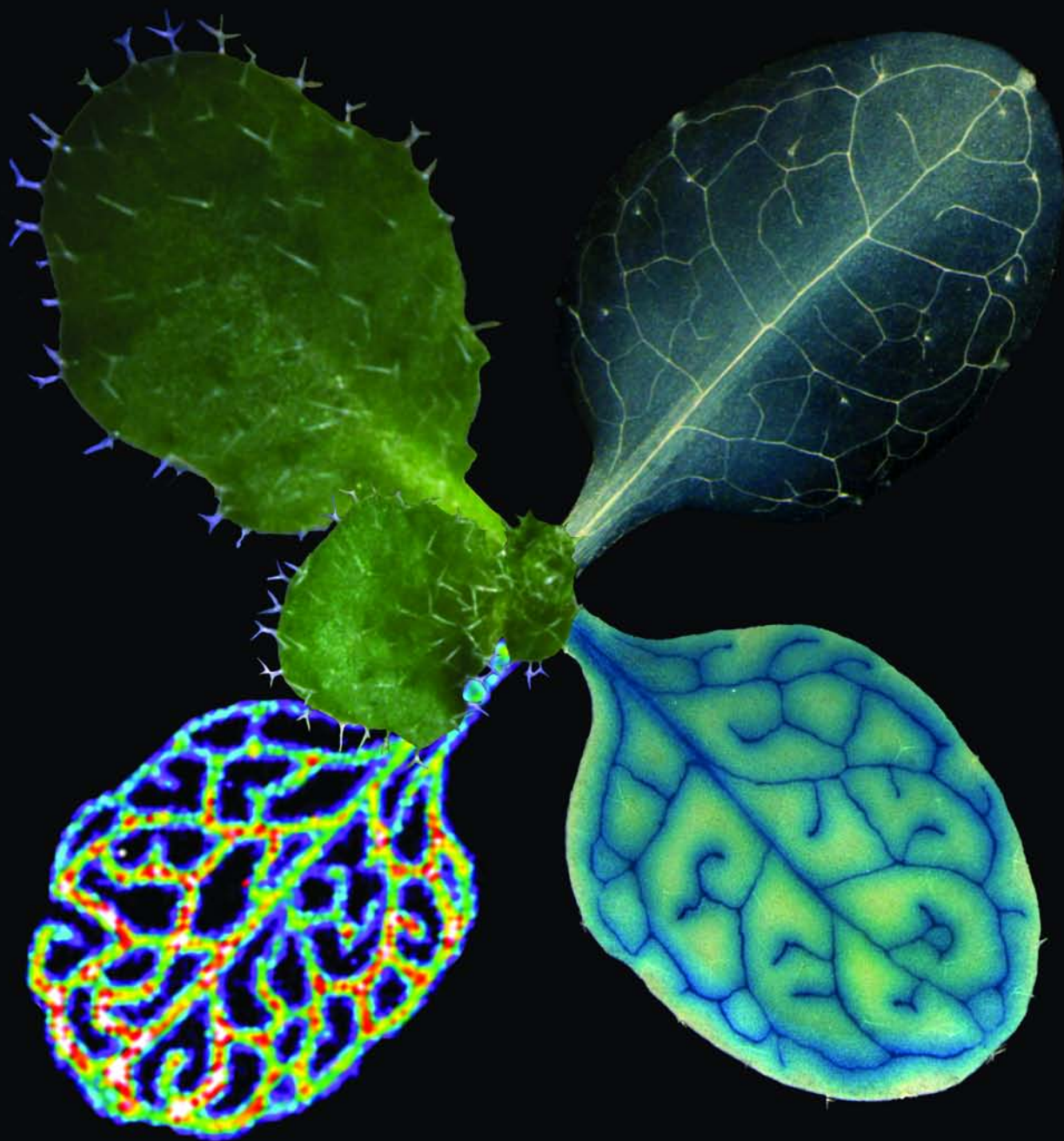
Doktorin der Naturwissenschaften (Dr. rer. nat.)

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Dissertationsgebiet (lt. Studienblatt): Dr.-Studium d. Naturwissenschaften: Biologie-Botanik (A091 438)

Betreuer: Dr. Thomas Greb

Wien, am 17. September 2010



Phantasie ist wichtiger als Wissen, denn Wissen ist begrenzt.

Albert Einstein (1879-1955)

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Abbreviations

BFA	Brefeldin A
dH ₂ O	distilled water
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ET	Ethylene
EtBr	Ethidium bromide
EtOH	Ethanol
FC	Fascicular cambium
HCl	Hydrochloric acid
IAA	Indole 3-acetic acid
IC	Interfascicular cambium
ICD	Interfascicular cambium derived zone
IFs	Interfascicular fibres
JA	Jasmonic acid, jasmonate
JA-Ile	Jasmonic acid conjugated with L-isoleucine
KCl	Potassium chloride
LD	Long day
meJA	Methyl jasmonate
MG132	Z-Leu-Leu-Leu-al
MgCl ₂	Magnesium chloride
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NPA	N (1-naphthyl) phthalamic acid
RAM	Root apical meristem
RT	Room temperature
SAM	Shoot apical meristem
SD	Short day
SDS	Sodium dodecyl sulphate
SE	Standard error of means
SG	Secondary growth
TRIS	Tris (hydroxymethyl) aminomethane
WT	Wild type

Deutsche Zusammenfassung

Das Wachstum von Pflanzen wird in zwei unterschiedliche Prozesse eingeteilt, einerseits in das Längenwachstum und andererseits in das Dickenwachstum. Die Voraussetzung für das Längenwachstum ist durch die Aktivität der Spitzenmeristeme der Spross- und Wurzelspitzen gegeben. Diese führt zur Bildung sogenannter primärer Spross- und Wurzelachsen. Neben diesem primären Wachstum findet in vielen Pflanzenarten, vor allem aber bei Nadelhölzern und ausdauernden Dikotyledonen, das sekundäre Dickenwachstum (SD) statt. Dadurch kommt es zu einer Vergrößerung des Stamm- und Wurzeldurchmessers aufgrund der Bildung von sekundärem Leitgewebe. Initiiert wird das SD durch die Ausbildung eines geschlossenen Kambiumrings, einem sekundären Lateralmeristem, welches nach außen (abaxial) und nach innen (adaxial) hin neu gebildete Zellen des Phloems und Xylems (also Teile des Leitgewebes) abgibt, ergo dipleurisch arbeitet. In krautigen Dikotyledonen, wie z.B. in der Modellpflanze *Arabidopsis thaliana* (der Acker-Schmalwand aus der Familie der Kreuzblütler), beginnt die Bildung des Kambiumrings mit der Ausbildung eines sogenannten interfazikulären Kambiums (IK) aus schon ausdifferenzierten Zellen von Gewebetypen, die zwischen den primären Leitbündeln liegen, nämlich des Kortex (inklusive seiner innersten Zellschicht, der Stärkescheide) und des Marks. Das IK fusioniert mit dem faszikulären Kambium (FK) der primären Leitbündel und beide bilden so einen geschlossenen Kambiumring der neben sekundärem Phloem abaxial hauptsächlich sekundäres Xylem adaxial produziert. Dies resultiert in der Akkumulation von Biomasse in Form von Holz, also sekundärem Xylem. Trotz der essentiellen Rolle des SD in der Produktion von Holz, welches eine der wichtigsten erneuerbaren Energieressourcen darstellt, ist über die molekulare und genetische Regulierung der Initiierung und Aktivität des Kambiums noch sehr wenig bekannt.

In dieser Studie wurden histologische, molekulare, genetische und auf Phytohormonen basierende Versuchsansätze miteinander kombiniert um die Initiierung und Aktivität des IKs an der Basis des Hauptstammes von *Arabidopsis* zu untersuchen. Auxin, welches als eines der am besten untersuchten Hormone der Pflanzenwelt gilt, ist schon bekannt für seine stimulierende Wirkung auf die Bildung des IKs und, in weiterer Folge, auf die kambiale Aktivität. Ebenso bekannt ist, dass ein Teil des Auxins aktiv in polarer Art und Weise von seiner Bildungsstätte zu seinem Bestimmungsort in basipetaler Richtung durch den Pflanzenkörper transportiert wird. Dieser sogenannte polare Auxin-Transport (PAT) basiert auf der sehr speziellen asymmetrischen Lokalisation von Proteinen (Mitglieder der AUX1/LAX und PIN Protein Familien) an der Plasmamembran ausgewählter Zellen und der Regulierung dieser Protein-Lokalisation durch Protein-Kinasen (PID und D6PKs). Innerhalb der vorliegenden Arbeit deutet die Untersuchung

von Mutanten, in denen die Aktivität dieser Transportproteine gestört ist, auf einen Einfluss des PAT auf die Initiierung und Aktivität des IKs hin.

Um weitere regulierende Komponenten des SD zu finden wurden Ergebnisse eines sogenannten „genomweiten transkriptionellen Profilings“ verwertet, welches Stammstücke ohne SD und Stammstücke mit SD miteinander vergleicht. Hierdurch wurden im Stammstück mit SD hochregulierte Gene identifiziert, die vor allem mit Stress in Zusammenhang gebracht werden können und des Weiteren durch Berührung (mechanischen Stress) induzierbar sind. Zu dieser Gruppe werden unter anderem Mitglieder des Jasmonsäure- (JA) und des Ethylen-Signalwegs (ET) gezählt. Einzelne Komponenten aus beiden Signalwegen, nämlich COI1, MYC2, JAZ7, das durch mechanischen Stress induzierbare JAZ10 protein und ERF104, wurden hier mit der Stimulierung und Regulierung des Kambiums in Zusammenhang gebracht. Des Weiteren bewirkte die Applikation von JA eine höhere kambiale Aktivität, wodurch die Hypothese einer stimulierenden Wirkung des JA-Signalweges auf das SD unterstützt wird.

Schlussfolgernd wird ein positiver Einfluss von mechanischem Stress, der durch den JA Signalweg vermittelt wird, auf die Bildung des IKs vorgeschlagen. Möglicherweise wird dieser mechanische Stress durch die Aktivität des FK der primären Leitbündel hervorgerufen. Aus dieser Schlussfolgerung ergeben sich neuartige Erkenntnisse bezüglich des SD und seiner Regulierung via eines komplexen Zusammenspiels von Hormonen und deren Regulationsmechanismen.

Abstract

Once the main primary body plan has been formed by the activity of the apical meristems, many plant species undergo secondary growth (SG). SG represents an increase in the diameter of shoots and roots through the formation of secondary vascular tissues and thus provides stability and rigidity to the growing plant. The process of SG is based on the formation of the vascular cambium, a secondary lateral meristem working in a dipoleuric fashion. In herbaceous dicotyledons, like *Arabidopsis thaliana*, the formation of the vascular cambium depends on the establishment of a so called interfascicular cambium (IC) between primary vascular bundles out of already differentiated cells of both tissues, the cortex (with its innermost layer the starch sheath) and the pith. The IC fuses with the fascicular cambium (FC) of the primary bundles to the vascular cambium from which predominantly secondary xylem emanates resulting in the accumulation of biomass in form of wood. In spite of its essential role in the production of wood, one of the most important renewable energy sources, knowledge about the molecular regulation of the initiation and activity of the vascular cambium is only poorly understood.

Here, histological, molecular, genetic and hormonal approaches were combined to characterize IC initiation and activity at the base of the *Arabidopsis* inflorescence stem. Auxin, one of the best-known phytohormones is already described to trigger IC initiation and to stimulate cambial activity. In this study, the analysis of the auxin transporters of the AUX1/LAX and PIN protein families and their regulators (the protein kinases PID and D6PKs) reveals an impact of polar auxin transport (PAT) on IC initiation and activity.

Exploiting results obtained from a genome-wide transcriptional profiling, it is shown that stress-related and touch-inducible genes are up-regulated in stem fragments where SG takes place. In particular, components of the jasmonic acid (JA) and ethylene (ET) signalling pathways are identified. Elements of both, the JA and ET signalling pathway, namely COI1, MYC2, JAZ7, the touch-inducible JAZ10, and ERF104, are shown to be cambium regulators. The positive effect of JA application on cambium activity confirms a stimulatory role of JA in SG.

In conclusion, these results support the hypothesis of a stress-stimulated IC formation which might depend on mechanical forces generated by the activity of the FC in primary bundles. With this, a novel insight into the process of SG and its regulation via a complex hormonal crosstalk is given.

Introduction

Secondary growth (SG) is the scientific synonym for the process of wood production which is characteristic to many land plants, especially woody, arborescent taxa among conifers and dicotyledons. This process exhibits remarkable variation in nature but is always based on the function of the vascular cambium, a lateral meristem that works in a dipoleuric fashion by producing secondary phloem (bark) towards the periphery (abaxially) and secondary xylem (wood) towards the centre of the growth axis (adaxially). This form of plant growth leads to an increase of the diameter of stems and roots, an increase of long-distance transport capacities and the newly formed tissues keep the plant firm due to their rigidity and stability.

Wood is a very important part in the list of renewable energy sources providing timber, fibres and energy. In 2008 about 1.6 billion m³ of industrial roundwood were produced worldwide. Records from the Food and Agriculture Organisation of the United Nations (FAO, www.fao.org) from 1961 onwards support the fact that wood production and thus its consumption as raw material increased worldwide (Fig. 1B). By contrast, the area of forested land decreased due to its conversion to non-forested land (deforestation) by human activities. Nevertheless, the annual net loss of forest area is decreasing due to tree planting and natural expansion of forests in some countries. Importantly, in light of the widespread depletion of fossil energy resources, wood has become a potential source of ligno-cellulose-based bio fuels (Demura and Ye 2010).

Therefore, wood production represents a process of increasing importance and a detailed understanding of its biological regulation is highly desirable.



Figure 1: Production of industrial roundwood. The picture of a pile of roundwood (A) was taken from <http://www.krappen.de/gfx/bg1.jpg>. The production of roundwood increased since 1961 worldwide to about 1.6 billion m³ in 2008 (B). Data were taken from www.fao.org.

Arabidopsis thaliana as model system for studying SG

Using *Arabidopsis* instead of a tree species as model organism for studying wood formation has many advantages. This herbaceous dicotyledon is characterised by a short generation time, a small plant size enabling growth in growth chambers, self-pollination and prolific seed

production, and a small fully sequenced genome (Meinke et al. 1998; Koornneef and Meinke 2010). Furthermore, a remarkable number of mutants and marker lines as well as molecular and computational tools are available for this unimposing herb. Especially the establishment of mutant and marker lines is still a bottleneck for genetic analyses in tree species. Even though wood formation is an evident characteristic of trees, *Arabidopsis* already has been described to possess a vascular cambium and to undergo SG, which has been shown especially for the root and hypocotyl (Lev-Yadun 1994; Dolan and Roberts 1995; Busse and Evert 1999; Altamura et al. 2001; Chaffey et al. 2002). Although SG and wood formation in *Arabidopsis* is spatially very limited and is not subject to seasonal variation, this process still displays striking similarities to that in trees (Chaffey, Cholewa et al. 2002; Nieminen et al. 2004). Thus, *Arabidopsis* can be used to address basic questions about the genetic control of cambial growth and secondary xylem development, including in-depth analyses of cell-to-cell communication and cell fate regulation. This genetic and molecular knowledge of SG obtained by studying *Arabidopsis* could be transferred to tree species, e.g. *Populus*, to further investigate SG in detail.

Primary growth

Before dealing with SG, one has to understand the process of primary growth. Primary growth is the result of the activity of the apical meristems, namely the shoot apical meristem (SAM) and root apical meristem (RAM), producing new cells which differentiate into organs and tissues of the primary plant body (primary root, shoot and vascular system). This process results in an increase in the length of the growth axes and, to a lesser extent, predominantly due to cell expansion, an increase in the thickness (diameter) of the growth axes. Among these primary tissues produced is the primary vascular system, which is essential for plants because it functions as a long-distance transport system for water, nutrients, assimilates, and signalling molecules, and, which is of utmost importance in the context of this work, it serves as the inception of SG. Additionally, the primary vasculature provides stability and strength to the plant axes due to its differentiated cells and special cellular architecture. All these facts already led to many studies regarding the development, anatomy, organisation and patterning of the (primary) vasculature in many plant species, one of them *Arabidopsis* (Kaussmann and Schiewer 1989; Altamura, Possenti et al. 2001; Ye 2002; Ye et al. 2002; Scarpella et al. 2006; Turner and Sieburth 2009; Caño-Delgado et al. 2010).

Primary vascular patterning

Already in developing embryos vascular tissue is established in that way that in the lower tier of the embryo during the globular stage elongated cells are generated. Those cells will become procambium cells, which are precursors of cells of the vascular tissues. The accurateness of this process relies on auxin and cytokinin signalling and on a correct specification of adaxial/abaxial patterning during embryogenesis (Capron et al. 2009; Turner and Sieburth 2009). Differentiation of the procambium only proceeds when growth continues following seed germination (Turner and Sieburth 2009). In the developing primary shoot the procambial strands differentiate into primary vascular bundles. Vascular bundles vary in their morphology largely on the basis of the topology of xylem and phloem. The most abundant type is the collateral bundle in which the xylem and phloem are arranged side by side, the first adaxially and the latter abaxially (Fig. 2). In collateral closed bundles, xylem and phloem are directly adjacent to each other but in collateral open bundles xylem and phloem are separated by the fascicular cambium (Beck 2005).

In *Arabidopsis* around 8 vascular bundles are organised in a tube-like domain at the periphery in the internodal parts of the primary stem. These bundles are typical collateral open vascular bundles (Fig. 2A, B) (Kaussmann and Schiewer 1989; Busse and Evert 1999; Altamura, Possenti et al. 2001; Ye, Freshour et al. 2002; Caño-Delgado, Lee et al. 2010). Furthermore, the primary stem is composed of the epidermis, the cortex with its innermost cell layer, the starch sheath, and the pith (Fig. 2A, B).

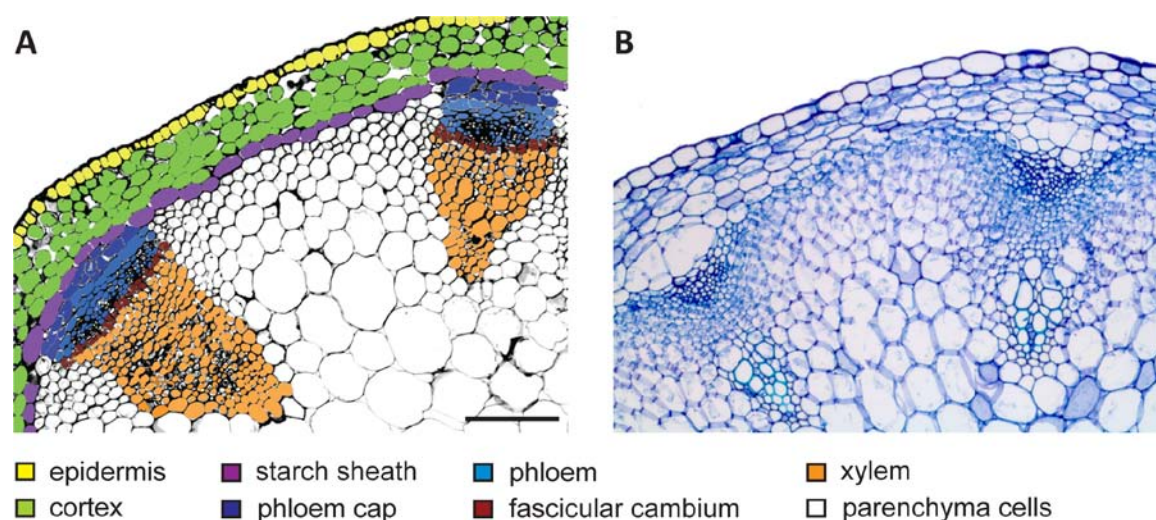


Figure 2: The *Arabidopsis* primary stem organisation. A detail of a cross-sectioned primary stem of *Arabidopsis* is shown in A and B. In the scheme (A), the various tissues are characterised by various colours. Embedded into the pith at the periphery of the stem are the collateral open vascular bundles consisting of phloem and its cap (blue, dark blue), the fascicular cambium (dark red) and xylem (orange). The actual cross section is stained with toluidine blue (B). Scale bar: 100 μ m.

Fascicular cambium

The fascicular cambium (FC) is characterised as a lateral meristem and is supposed to be the remainder of the procambium between the primary xylem and primary phloem. During primary growth the FC is inactive but regains its meristematic activity at the onset of SG (Beck 2005). This leads to the initiation of periclinal cell divisions in a dipleuric manner, thereby emitting cell derivatives alternately towards the centre and towards the periphery of the growth axis (Kaussmann and Schiewer 1989; Ye 2002; Ye, Freshour et al. 2002; Evert 2006). The FC also serves as one of the main routes of auxin transport from the shoot towards the root apex (Goldsmith 1977).

Xylem

The xylem is the major water-conducting tissue in a vascular plant. It transports water and soluble mineral nutrients from the roots to the upper ground part of the plant. The xylem tissue is composed of tracheary elements, xylem parenchyma cells and xylem fibres. The water conducting components are the tracheary elements, namely tracheids and vessel elements. Both cell types are elongated and are characterised by a lignified secondary cell wall which is interrupted by pits and are nonliving at maturity. At least two vessel elements fuse to form a vessel in which an efficient barrier-less water transport is given (Kaussmann and Schiewer 1989; Evert 2006; Turner et al. 2007).

Phloem

The phloem is in addition to xylem the second major part of the vasculature. It is important for long distance transport and allocation of nutrients, assimilates and signalling molecules including auxin through the plant body (Petrasek and Friml 2009; Dinant and Lemoine 2010). Even small RNAs and mRNAs were found in the phloem sap leading to the designation of this tissue as a “signalling super highway” (Kehr and Buhtz 2008). The phloem tissue is composed of highly differentiated sieve tube members, phloem parenchyma cells and phloem fibres. The most important cells of the phloem are the sieve elements, which are divided into companion cells and sieve tube elements. The latter are assembled in a longitudinal series into a sieve tube. In contrast to the vessel elements the sieve elements are living cells, although their nucleus is degraded. However, companion cells are intimately connected with their associated sieve tube elements via plasmodesmata and serve as the steering entity of the latter (Kaussmann and Schiewer 1989; Evert 2006).

Secondary growth

Once the main body has been formed, the plant may proceed with SG, which takes place in a more developed zone in a certain distance from the apical meristems. The prerequisite for SG is the establishment of the vascular cambium, which appears as a ring-like, meristematic zone in cross sections (cf. Fig. 4). Spatially, the vascular cambium is organised in a tube-like domain pervading all plant axes which are in the secondary stage (Lachaud et al. 1999).

Development of the vascular cambium in the stem

For SG, the establishment of the vascular cambium is of utmost importance. Due to the fact that SG exhibits remarkable variation in nature, the establishment of the vascular cambium can be divided into 3 main types (Kaussmann and Schiewer 1989):

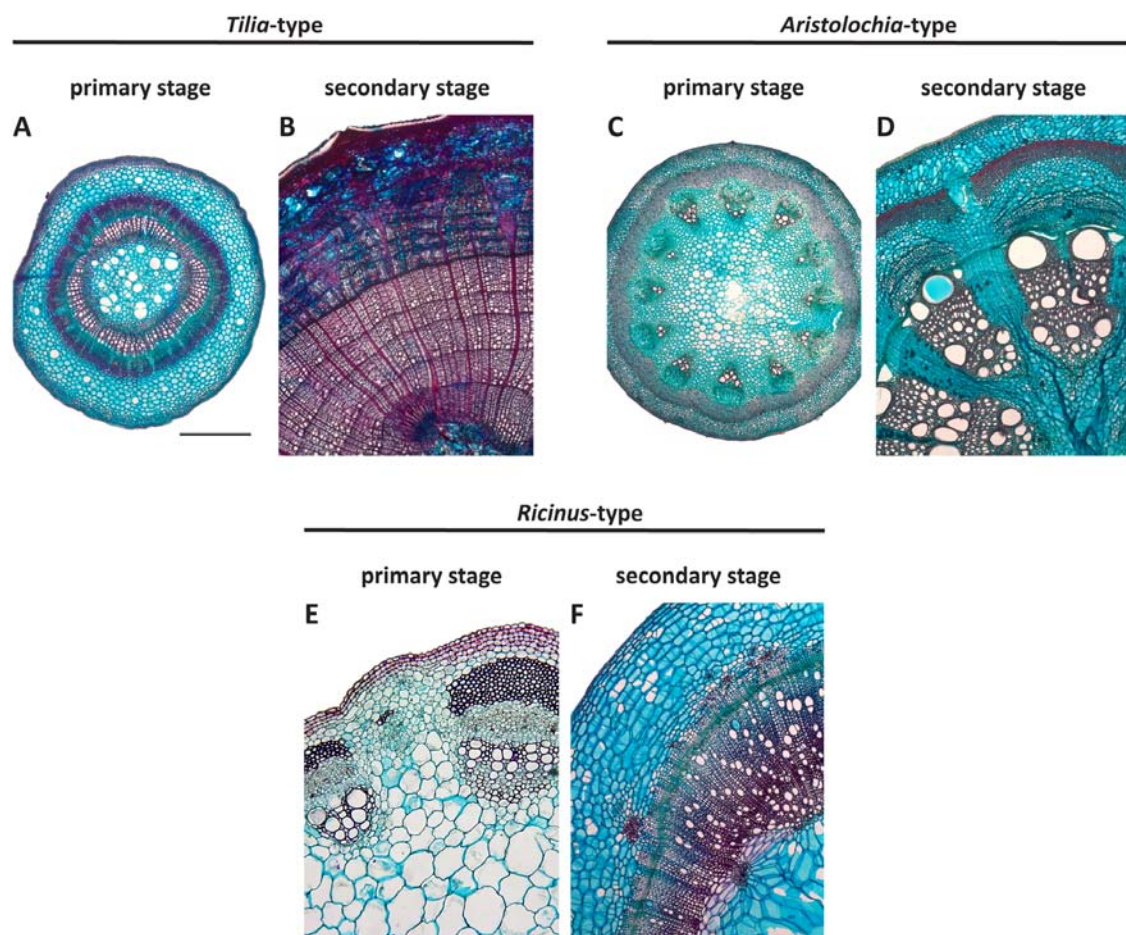


Figure 3: Tilia-, Aristolochia- and Ricinus-type of SG. The Tilia-type is represented by *Tilia cordata*. Already in the primary stage a closed cambial ring is visible in the cross section (A). The secondary stage shows massive xylem production (B). The Aristolochia-type is represented by *Aristolochia macrophylla*, which shows distinct bundles in the primary stage (C). Although a cambium is formed, no secondary vasculature is formed in the interfascicular region but only secondary parenchyma (D). The Ricinus-type, represented by *Helianthus annuus*, is similar to the Aristolochia-type. However, in this case secondary vasculature is formed in the interfascicular region in the secondary stage (F). Picture of the young *Helianthus* stem (E) was taken from <http://plantphys.info/organismal/labaid/stemvb.jpg>. Scale bar (except for E): 500 μ m.

- 1) *Tilia*-type: the procambium is already established as a closed cambial ring which then differentiates into the vascular cambium in the secondary stage (Fig. 3A, B). This type is common for gymnosperms and woody dicots.
- 2) *Aristolochia*-type: the primary stage is characterised by single vascular bundles, which are then connected by the formation of the interfascicular cambium (IC) leading to a closed cambial ring in the secondary stage, but the IC produces only secondary parenchymatic tissue instead of new secondary vasculature (Fig. 3C, D). This type is typical for lianas.
- 3) *Ricinus*-type: this type resembles the *Aristolochia*-type, but the IC produces secondary vascular tissue (Fig. 3E, F). This type is common for herbaceous dicots like *Arabidopsis*.

To simplify matters I will focus on the description of the *Ricinus*-type, to which *Arabidopsis* is belonging, the species which is studied here.

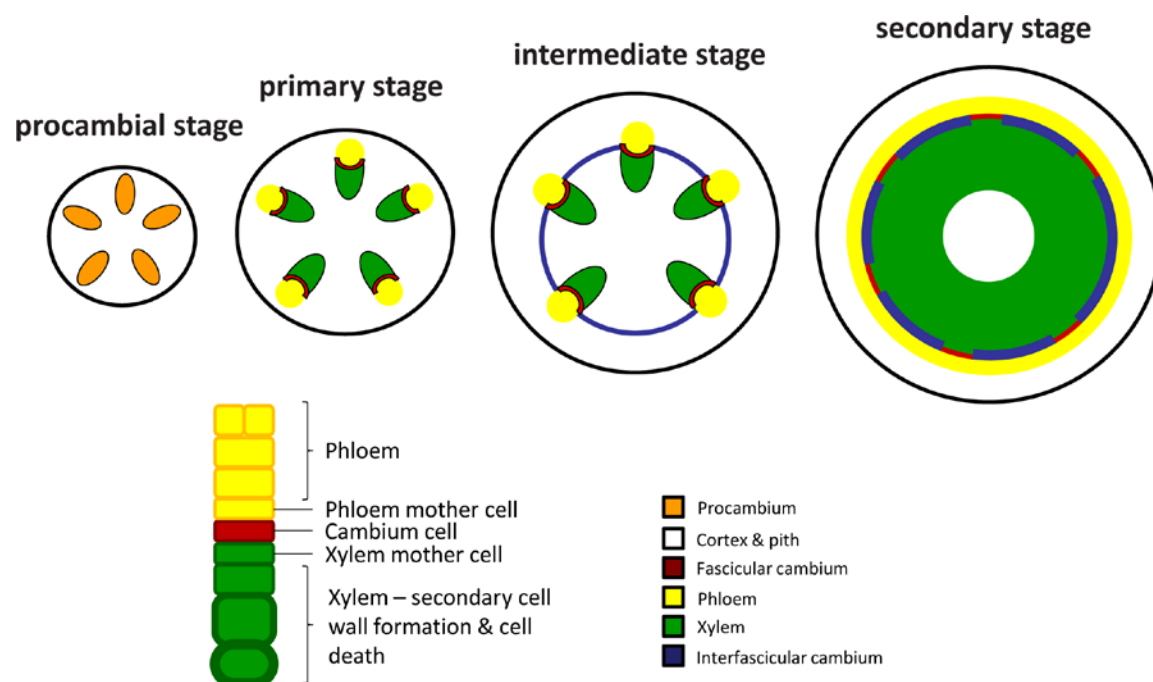


Figure 4: Vascular development from the procambial to the secondary stage. The procambial strands (orange) differentiate into vascular bundles during plant growth. In the primary stage, the collateral open vascular bundles are organised in phloem abaxially (yellow) and xylem adaxially (green), both separated by the remainder of the procambium, the fascicular cambium (FC, red). In an intermediate stage towards secondary growth the interfascicular cambium (IC, blue) forms in the region between the primary bundles. When a closed cambial ring is established, namely the vascular cambium (red-blue), the production of secondary tissues is found in both, the fascicular and interfascicular region. The one-cell layer-wide cambium works in a dipleuric fashion and produces alternately a phloem and a xylem mother cell, which then divides further and differentiates into proper phloem and proper xylem, respectively.

As already described above, the procambial strands differentiate into primary vascular bundles and in many plant species the fascicular cambium stays as a remainder of the procambium, e.g. in the case of collateral open bundles (Fig. 4, procambial, primary stage). Before the

differentiation of the IC occurs, the FC becomes active, producing some secondary xylem and phloem within the primary vascular bundles. This means that cambial activity begins initially in the vascular bundles. In most of the cases, which is true for the *Ricinus*- and *Aristolochia*-type, this event is then followed by the formation of the IC between the bundles (Fig. 4, intermediate stage) (Beck 2005). The already differentiated parenchyma cells serve as the precursors for the interfascicular cambium (Swamy and Krishnamurthy 1980). After the IC has connected to the FC, the production of secondary xylem towards the centre and secondary phloem towards the periphery starts in the interfascicular region (Fig. 4, secondary stage). The main characteristic of secondarily produced vascular tissue is its specific organisation in radial cell files due to constant periclinal cell divisions of the cambium cell (Fig. 4, scheme of a cell file). This applies especially for secondary xylem (see *Tilia*-type in its secondary stage, Fig. 3B).

The functionality of the vascular cambium differs extremely within the plant kingdom. In arborescent forms it functions throughout the life of the plants in comparison to annuals, where the cambium persists only during the growth phase of the plant and may cease its activity and differentiates into phloem and xylem before the plant dies. In some of the herbaceous dicotyledons, most monocotyledons, and most pteridophytes no vascular cambium and thus no SG occurs at all (Beck 2005).

Secondary growth in *Arabidopsis thaliana*

In *Arabidopsis* the process of SG has already been described for the root and hypocotyl (Dolan and Roberts 1995; Chaffey, Cholewa et al. 2002). However, less and still no complete data are available for the formation of the interfascicular cambium and SG in the inflorescence stem (Lev-Yadun 1994; Altamura, Possenti et al. 2001; Little et al. 2002; Elo et al. 2009).

The actual state of knowledge is that the IC is only established at the very base of the stem and that the rest of the stem is devoid of a vascular cambium (Altamura, Possenti et al. 2001; Little, MacDonald et al. 2002). Different origins of the IC at the base of the stem have been reported: histological analyses reveal that a so called internodal IC (which refers to the upper part of the zone of IC initiation) is produced next to the primary bundle by transforming parenchymatic cells, whereas far from the bundle, it is produced by starch sheath cells only. A so called nodal IC (which refers to the lower part of the zone of IC initiation adjacent to the rosette) is produced proximate to the starch sheath in non-lignified parenchyma cells. In the latter case the starch sheath is not involved in the process of cambial formation (Altamura, Possenti et al. 2001; Ye, Freshour et al. 2002). Furthermore, at the stage of IC initiation cambial activity in the primary vascular bundles is visible. The established IC produces then secondary xylem parenchyma,

which differentiates into thin lignified cells, and a few sieve elements. The formation of the first secondary vascular elements in the interfascicular region is preceded by the transformation of parenchyma cells into interfascicular fibres. In parallel, phloem fibre-sclereids are differentiated adaxially to the primary phloem, which are known as the phloem cap (Altamura, Possenti et al. 2001).

Hormonal control of secondary growth

SG is a very complex process with underlying signalling and transduction pathways that are still incompletely understood. That SG is, at least partly, controlled by different hormones, is already state-of-the-art. In particular, auxin, cytokinin, ethylene and gibberellins are known to regulate cambial development and activity (Elo, Immanen et al. 2009). However, knowledge of how the hormonal signalling pathways regulate SG is still scarce. In the course of this study I focused on regulatory effects of auxin and jasmonic acid on IC initiation and activity.

Auxins

Auxins are plant hormones playing an essential role in coordinating many growth processes. The most important member and the most potent native auxin is indole-3-acetic acid (IAA). Other naturally occurring auxins are 4-chloro-indoleacetic acid, phenylacetic acid and indole-3-butyric acid (IBA), whereas 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) belong to the synthetic auxin analogs (Davies 1995). Applied auxin has been shown to reactivate cambium in decapitated shoots in classic hormone treatment experiments (Savidge 1988; Little, MacDonald et al. 2002). Furthermore, auxin concentration has been shown to peak in cambial cells and to decrease towards the differentiating tissues in both, *Populus* and *Pinus* trees (Tuominen et al. 1997; Uggla et al. 1998). This gradient is assumed to be formed through the transport of apex-derived auxin along the shoot and its lateral diffusion/transport.

Auxin is generally transported in two distinct ways. In addition to a passive auxin flow, an actively regulated, carrier-mediated directional cell-to-cell transport moves auxin via the vascular cambium and xylem tissue, and, additionally, also mediates short-range auxin movement within different tissues. This cell-to-cell transport of auxin is also referred to as polar auxin transport (PAT) (Goldsmith 1977; Petrasek and Friml 2009; Vanneste and Friml 2009). The direction of PAT and/or the local accumulation of auxin in cells correlates well with the polarity of auxin transport proteins at the plasma membrane, such as members of the PIN (PIN-FORMED) and AUX1/LAX (AUXIN RESISTANT1/LIKE AUX1) protein family, suggesting that this protein polarity determines the direction of intercellular auxin flow (Fig. 5A) (Parry et al. 2001; Tanaka et

al. 2006; Vieten et al. 2007; Krecek et al. 2009; Grunewald and Friml 2010). PIN proteins are well studied by now and are known to be the primary auxin efflux facilitators (Krecek, Skupa et al. 2009). This very directional but still flexible auxin transport is based on the constitutive intracellular movement of the PINs mediated by clathrin-dependent endocytosis and translocation (Dhonukshe et al. 2007; Grunewald and Friml 2010). PIN recruitment to the different trafficking pathways is related to its phosphorylation status regulated by the activity of protein kinases and phosphatases (e.g. PINOID and PP2A). This determines the polar targeting of PIN proteins and thus the directionality of the auxin flow in various tissues and organs (Titapiwatanakun and Murphy 2009; Zhang et al. 2010). The steady-state levels of PIN proteins are actively regulated by ubiquitin-mediated protein degradation via the targeting of the proteins to the vacuole (Kleine-Vehn et al. 2008). Due to their specific role in many developmental processes, PINs show a highly specific expression in distinct organs and tissues (Fig. 5B) (Krecek, Skupa et al. 2009).

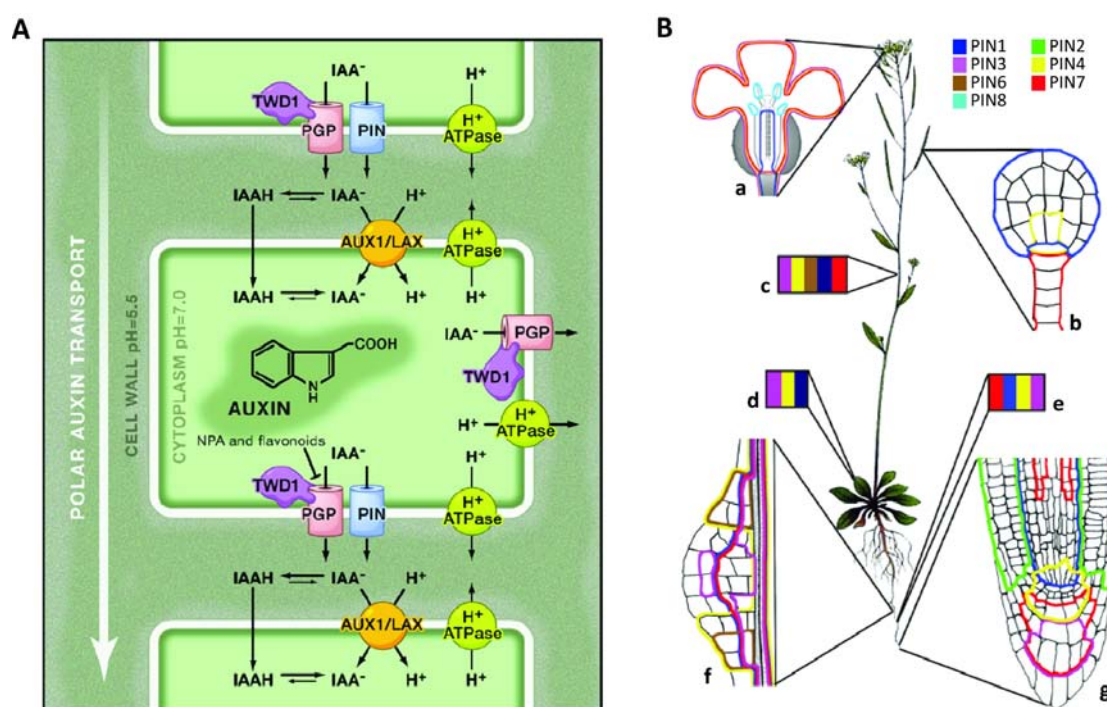


Figure 5: Polar auxin transport and PIN localisation. In the acidic environment of the apoplast (cell wall), a fraction of the weak acid IAA becomes protonated ($\text{IAA}^- \rightarrow \text{IAAH}$), thus it is more lipophilic and can diffuse freely through the plasma membrane into the cell (A). Besides this passive diffusion, auxin is actively taken up from the apoplast by H^+/IAA^- symport mediated by AUX1/LAX influx carriers. Once inside the pH neutral cytosol, auxin is deprotonated and becomes trapped inside the cell. Auxin can leave the cell by efflux carriers such as PIN and PGP proteins. The polar subcellular localisation of PINs determines the direction of auxin flow out of the cell and thus the unidirectional net flow of auxin through tissues. Picture A reprinted from Vanneste et al. (2009). Each PIN expression and protein localisation (depicted by various colours with exception of PIN5) is very distinct for each organ (B), namely the flower (a), embryo (b), stem (c), rosette leaf (d), primary root (e), lateral root primordium (f), and root tip (g). Picture B reprinted from Krecek et al. (2009).

Especially this carrier-dependent PAT is required for the generation and maintenance of local auxin maxima and gradients, which are in turn essential positive stimuli for cambium initiation and activity through auxin-responsive gene expression (Fig. 6) (Snow 1935; Shininger 1971; Little, MacDonald et al. 2002; Aloni 2004; Nilsson et al. 2008).

Jasmonates

Jasmonates include jasmonic acid (JA) and its esters, such as methyl jasmonate (meJA), which are derived from fatty acids. The most important endogenous biologically active member of the jasmonates is JA conjugated to L-isoleucine (JA-Ile). It is known, that JA synthesis can be induced very rapidly in response to various stresses. Stress responses that depend on jasmonate signalling include defence response against pathogens and insects but also responses to ozone, UV light, wounding, and other abiotic stresses (Wasternack 2007). Additionally, jasmonates have been implicated in plant thigmomorphogenetic responses upon mechanical stimulation (like touch) (Jaffe 1973; Braam 2005; Tretner et al. 2008; Chehab et al. 2009).

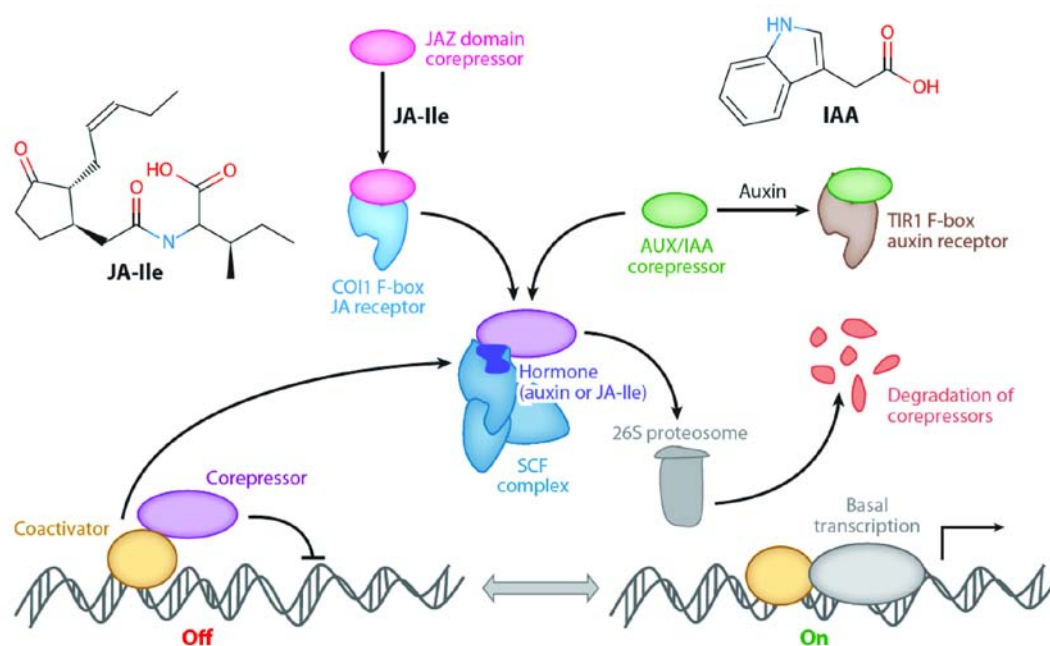


Figure 6: Auxin and jasmonate signalling. In the absence of the hormone (IAA or JA), corepressors bind and inhibit transcription. In the case of JA, the corepressors are the JAZ proteins, whereas for auxin the corepressors are the AUX/IAA proteins. Binding of each hormone to its particular F-box receptor targets corepressors for ubiquitin-mediated proteolysis via the SCF complex, which is for JA-Ile-mediated degradation the SCF^{COI1} complex and for IAA-mediated degradation the SCF^{TIR1} complex. This degradation of the corepressors results in rapid activation of gene expression by the release of transcriptional coactivators. Reprinted from Lumba et al. (2010).

Regarding SG, no interconnections with jasmonates have been described so far, although jasmonates have been reported to regulate developmental processes like reproductive

development, trichome patterning and senescence (Chung et al. 2009). The jasmonate signalling pathway is comparable with the auxin signalling pathway (Fig. 6). The accumulation of JA promotes the activation of the transcriptional regulator MYC2 and the resulting JA-dependent gene expression via the degradation of the JAZ repressor proteins (Fig. 6). The rapid MYC2 dependent induction of JAZ genes themselves contributes to a balanced JA response (Chini et al. 2007; Pauwels et al. 2009).

Hormonal crosstalk

Plant growth and development is coordinated through complex interactions of hormone signalling systems. It is known, that both auxin and cytokinin can synergistically induce ethylene biosynthesis (Vogel et al. 1998), and vice versa, ethylene can induce PIN protein expression, auxin transport and auxin biosynthesis in the root (Ruzicka et al. 2007; Stepanova et al. 2007; Swarup et al. 2007), as well as inhibit auxin transport in the stem (Suttle 1988; Chilley et al. 2006), which places auxin signalling downstream of ethylene signalling. Furthermore, ethylene and JA pathways converge in the activation of ERF1, a member of the ETHYLENE RESPONSE FACTOR family that regulates the expression of pathogen response genes (Lorenzo et al. 2003). On the other hand, JA stimulates auxin biosynthesis via ASA1 (ANTHRANILATE SYNTHASE α 1) and additionally represses PIN-mediated auxin transport in the root (Sun et al. 2009). Overall, it seems that auxin is a main player of this hormonal crosstalk and is used to coordinate hormone action across tissues and organs (Jaillais and Chory 2010). This complexity of the signalling systems leads to serious challenges to understand how cells respond to multiple hormones and how developmental processes like the initiation of the IC can arise out of this. It has been already described that cambial development and activity is regulated individually by several hormones (Elo, Immanen et al. 2009). Whether those hormones act in concert via a hormonal crosstalk in regulating these processes is still elusive.

Aims of the study

Although its importance in plant development and wood formation, cambial formation and activity have been scarcely analysed in the past due to the lack of a defined model system for which extensive molecular and genetic tools are available. The model plant *Arabidopsis* has recently been shown to undergo secondary growth in the root and hypocotyl and to possess a vascular cambium similar to that of trees (Chaffey, Cholewa et al. 2002).

The general aim of this study was to accurately examine cambial development, in particular, the establishment of the interfascicular cambium (IC) in the inflorescence stem of *Arabidopsis* by histological analyses. IC formation represents an attractive target for addressing various aspects of cambium regulation due to its traceability and its *de novo* initiation out of differentiated cell types. In order to evaluate and characterise this developmental process, mutants were studied to answer the question whether different cell identities contribute to IC initiation and, furthermore, marker lines were analysed visualising the cell identity of the IC-derived vascular tissues.

That auxin is a key factor in the regulation of secondary growth (SG) has been described earlier (Little, MacDonald et al. 2002). To correlate SG and auxin accumulation/signalling at the base of the stem on the cellular level, the *DR5rev:GFP* marker line was investigated. To clarify the question how polar auxin transport (PAT) is involved in the establishment of the IC, mutants impaired in the regulation of PAT, namely members of the AUX1/LAX, PIN, D6PK and PINOID families were analysed.

To find additional factors involved in SG, results from a transcriptional profiling performed by Javier Agustí, were processed. This analysis led to the identification of a role of jasmonates and ethylene in the regulation of SG and resulted into experiments addressing the influence of (mechanical) stress on IC formation. Mutants of members of the jasmonate (JA) signalling pathway, COI1, MYC2, JAZ10 and JAZ7, as well as ERF104, an ethylene response factor, were further analysed for that reason. To address the issue whether mechanical stimuli serve as the trigger of IC initiation via JA signalling, mechanical stress-related experiments (e.g. weight treatment, touching) were performed.

Materials

Plant material

Arabidopsis thaliana (L.) Heynh. plants of the accessions Columbia0 (Col0), Wassilewskija (Ws) and Landsberg *erecta* (Ler) were used as wild type lines. Additional analysed plant lines (marker lines and mutants) are listed in alphabetical order.

Genotype	Mutation/Construct (NASC ID)	Reference
Mutant lines		
<i>aux1 lax1 lax2 lax3</i>	A823 deleted in AUX1, dSpm insertions in LAX	Bainbridge et al. 2008
<i>cad-c cad-d</i>	Versailles T-DNA insertions	Sibout et al. 2005
<i>coi1-1</i>	Point mutation G1401 to A	Xie et al. 1998
<i>d6pk d6pk1 d6pk2</i>	SALK_061847, SALK_056618, SALK_086127	Zourelidou et al. 2009
<i>erf104</i>	SALK_057720 (N557720)	Bethke et al. 2009
<i>jaz7-1</i>	WiscDsLox7H11 (N849196)	This study
<i>jaz10-1</i>	SAIL_92_D08 (N872819)	This study
<i>jaz10-2</i>	GK_421G12 (N371009)	This study
<i>myc2-3</i>	SALK_061267 (N656547)	This study
<i>nst1-1 nst3-1</i>	SALK_120337, SALK_149909	Mitsuda et al. 2007
<i>pid-3</i> (Ler)	EMS mutagenesis (N8064)	Bennett et al. 1995
<i>pin1-613/+</i>	SALK_047613 (N547613)	Gälweiler et al. 1998
<i>pin3-5</i>	SALK_005544	Friml et al. 2002
<i>pin6-1</i>	SALK_046393 (N546393)	This study
<i>pin7-2</i>	SALK_044687	Friml et al. 2003
<i>rev-5</i>	Point mutation, C779T	Otsuga et al. 2001
<i>scr-3</i> (<i>sgr1-1</i>)	Mutation W490 to stop (N3997)	Fukaki et al. 1998
<i>shr-2</i> (<i>sgr7-1</i>)	10bp deletion & 431bp insertion (N2972)	Helariutta et al. 2000
Marker lines		
WT	<i>APL:GUS</i> (<i>pTOM13</i>)	Sehr et al. 2010
WT	<i>DR5rev:GFP</i>	Benkova et al. 2003
WT (C24)	<i>GAL4</i> enhancer trap line J1721	Haseloff 1999, Sawchuk et al. 2007
WT	<i>JAZ10:GUS</i>	Sehr et al. 2010
WT	<i>JAZ10 OE 4A</i>	Yan et al. 2007
WT	<i>JAZ10 OE 4B</i>	Yan et al. 2007
WT	<i>JAZ10 RNAi #7</i>	Yan et al. 2007
WT	<i>JAZ10 RNAi #9</i>	Yan et al. 2007
WT	<i>PIN3:PIN3-GFP</i>	Jiří Friml, unpublished
WT	<i>SCR:AlcR</i> (<i>pTOM36</i>)	Thomas Greb, unpublished
WT	<i>SCR:GFP</i>	Wysocka-Diller et al. 2000

Table 1: Plant material used in this study. Mutant lines are listed in alphabetical order as well as the marker lines based on their constructs inserted.

Bacterial strains

The competent *Escherichia coli* strain DH5 α was used in cloning procedures and the competent *Agrobacterium tumefaciens* strain C58C1 was used for plant transformation.

Buffers and solutions

Toluidine blue staining solution (200 ml)

100 mg of Toluidine blue (Applichem) was dissolved in 200 ml dH₂O

Phloroglucinol staining solution

2 g phloroglucinol

80 ml 20% EtOH

20 ml 37% HCl

GUS staining buffer

100 mM phosphate buffer, pH 7

10 mM EDTA, pH 8

0.1% Triton-X 100

Autoclave

10x PBS

1.4 M NaCl

27 mM KCl

100 mM Na₂HPO₄

18 mM NaH₂PO₄

Adjust pH to 7.3 with HCl and autoclave

50x TAE

242 g TRIS

100 ml 0.5M EDTA

57.1 ml Acetic acid

Fill up to 1 l with water, adjust pH to 7.6 with acetic acid and autoclave

DNA extraction buffer

200 mM TRIS, pH 7.5

250 mM NaCl

0.5% SDS

25 mM EDTA

Autoclave

10x TE buffer

100 mM TRIS, pH 8

10 mM EDTA, pH 8

Autoclave

CTAB buffer

140 mM Sorbitol

220 mM TRIS-HCl, pH 8

22 mM EDTA, pH 8

800 mM NaCl

1% Sarkosyl

0.8% CTAB (Cetyltrimethylammonium bromide)

Check pH to 8 and autoclave

Denaturation buffer

1.5 M NaCl

0.5 M NaOH

Autoclave

Neutralisation buffer

1.5 M NaCl

0.5 M TRIS

Adjust pH with HCl to 7 and autoclave

20x SSC

175.3 g NaCl

88.2 g Sodium citrate

Fill up with water to 1 l, adjust pH to 7 and autoclave

1 M Sodium phosphate buffer (100 ml)

68.4 ml 1M Na₂HPO₄

31.6 ml 1 M NaH₂PO₄

Autoclave

Hybridisation buffer (100 ml)

35 ml 20% SDS

50 ml 1 M Na₂PO₄ buffer, pH 7.2

1 g BSA

200 µl 0.5 M EDTA

5x Oligonucleotide labelling buffer (OLB)

250 mM TRIS, pH 8

25 mM MgCl₂

5 mM Mercaptoethanol

2 mM each dATP, dGTP and dTTP

1 M Hepes, pH 6.6

Keep aliquots at -20°C

Loading dye

0.25% xylene cyanole (XC)

0.25% bromophenol blue (BPB)

50% glycerol

10 mM Tris, pH 8

1 mM EDTA, pH 8

LB medium (for plates, 1 l)

1% Peptone

0.5% Yeast extract

1% NaCl

1.5% Bacto agar

Autoclave

YEB medium (for plates, 1 l)

0.5% Meat extract

0.5% Peptone

0.1% Yeast extract

0.5% NaCl

0.5% Sucrose

2 mM MgSO₄

1% Bacto agar

Autoclave

½ MS medium (for plates)

2,21 g/l Murashige & Skoog medium including B5 vitamins

10 g Sucrose

6 g Plant agar

Fill up to 1 l with water, adjust pH to 5.8 and autoclave

Primers

All primers used in this study were designed either using Vector NTI 10.1.1. (Invitrogen) or the CLC Main Workbench (CLC bio) and were purchased at Sigma-Aldrich.

Primer name	Sequence	Usage
Primers for vector design		
PIN3mutagenic1	ggaggaaactcatcagaaacccaacactGCcttagtctcattgg	Site-directed mutagenesis
PIN3mutagenic2	ccaatgagactagagGCagtgttgggttctgatgagttcctcc	Site-directed mutagenesis
PIN3mutagenic3	ggaggaaactcatcagaGCCcaaacacttactctagtctcattgg	Site-directed mutagenesis
PIN3mutagenic4	ccaatgagactagagtaagtgttgggGCtctgatgagttcctcc	Site-directed mutagenesis
PIN3mutagenic5	ggaggaaactcatcagaGCCcaaacactGCcttagtctcattgg	Site-directed mutagenesis
PIN3mutagenic6	ccaatgagactagagGCagtgttgggGCtctgatgagttcctcc	Site-directed mutagenesis
PIN3for3	atgatctcatggcagcactc	Design of pEMS16 & pEMS19
PIN3rev-MfeI	actaCAATTGttataacccgagtagaatgtag	Design of pEMS16 & pEMS19
T3	AATTAACCCCTCACTAAAGGG	Verify pEMS16 & pEMS19
T7	GTAATACGACTCACTATAGGGC	Verify pEMS16 & pEMS19
Primers for Southern blot probes		
PIN3for3	atgatctcatggcagcactc	<i>PIN3</i> probe for Southern blot
PIN3rev7	ccttggtcagatctccgccttga	<i>PIN3</i> probe for Southern blot
Primers for genotyping		
SALK_047613-LP	CAAAAACACCCCCAAAATTC	Genotyping of <i>pin1-613/+</i>
SALK_047613-RP	TTTTTCATCGATAAGGGGGTC	Genotyping of <i>pin1-613/+</i>
SALK_005544-LP	CCCATCCCCAAAAGTAGAGTG	Genotyping of <i>pin3-5</i>
SALK_005544-RP	GGAAGTGTGGAGAGGGAAAAG	Genotyping of <i>pin3-5</i>
SALK_046393-LP	TTGTAAACGGAGATTAGGCG	Genotyping of <i>pin6-1</i>
SALK_046393-RP	TCTCCTTCTCTGGCACTGATC	Genotyping of <i>pin6-1</i>
SALK_044687_LP	CTCTTTTGCAAACACAAACGG	Genotyping of <i>pin7-2</i>
SALK_044687_RP	GGTAAAGGAAGTGCCTAACGG	Genotyping of <i>pin7-2</i>
WiscDsLox7H11-LP	GGTACACCGCGGATTAATC	Genotyping of <i>jaz7</i>
WiscDsLox7H11-RP	ACCCATTTTAGGAGACCGTTG	Genotyping of <i>jaz7</i>
SAIL_92_D08-LP	CTTCTCGAGAAAACGTTGCAG	Genotyping of <i>jaz10-1</i>
SAIL_92_D08-RP	TCACATGAGAAATCAGAATCCG	Genotyping of <i>jaz10-1</i>
GABI_421G12-LP	AGGGAAGATCAGCCTCAGATC	Genotyping of <i>jaz10-2</i>
GABI_421G12-RP	AACATGGATGTCTTCCGATTG	Genotyping of <i>jaz10-2</i>
SALK_061267-LP	CTCGAGCTGGTTCTTGATTG	Genotyping of <i>myc2</i>

Primer name	Sequence	Usage
SALK_061267-RP	TGGTTTTCTTGGTTTCGATG	Genotyping of <i>myc2</i>
SALK_057720.40-LP	CCTCTGCTCAGTTGCTTGAC	Genotyping of <i>erf104</i>
SALK_057720.40-RP	GAAACAGGCCCTAGAACCATC	Genotyping of <i>erf104</i>
SALK_LBb1.3	ATTTTGCCGATTTCGGAAC	Left border primer
WiscDsLox-LB-p745	AACGTCCGCAATGTGTTATTAAGTTGTC	Left border primer
SAIL_LB3	TAGCATCTGAATTTCAACCAATCTCGATACAC	Left border primer
GABI-Kat	TCTCCATATTGACCATCATACTCATT	Left border primer
coi1-1_Vspl-for	CTACATCGGACAGTACAGTCCAAACGTGAGATT	dCAPS
coi1-1_rev2	CGGTCTAGCCATCTGCATTAG	dCAPS
Primers for (q)RT-PCR		
TUBfor3	TTCGTTCTCGTGCAGTGCTCA	Internal control for RT-PCR
TUBrev2	AAGGTAAGCATCATGCGATCTGGGT	Internal control for RT-PCR
PIN1for2	TCACATGTTTGTGTGGAGCTCAAGT	RT-PCR
PIN1rev4	GGGATTACGAATAAGTTCTCCAAA	RT-PCR
PIN2for1	TGCAGATCGTGGTGCTTCAGAG	RT-PCR
PIN2rev1	AAGCTCGCGCGTTATCATG	RT-PCR
PIN3for5	CCAGATCAATCTCACACGGCG	RT-PCR
PIN3rev5	CGAGAGCCCAAATAAGTCCAATGAG	RT-PCR
PIN4for3	GGAGTTTTCAACCGGTACGGGT	RT-PCR
PIN4rev2	CAGCCCTGCTGTAGCTTTCTCTATCT	RT-PCR
PIN6for1	TCCGTCAACACTCCTAATAACCGT	RT-PCR
PIN6rev1	GGCACTGATCCATTGATATCTTT	RT-PCR
PIN7for1	GGCGGATATGTACTCTGTTCACTCC	RT-PCR
PIN7rev2	TCGGACCAGCTTTGTTTTCA	RT-PCR
EIF4A1for	ATCCAAGTTGGTGTGTTCTCC	Internal control for qRT-PCR
EIF4A1rev	GAGTGCTCTGAGCTTCCACTC	Internal control for qRT-PCR
IAA5_for	ATCAAAGATGGCGAATGAG	qRT-PCR
IAA5_rev	TAAGGCTCACTCACATTACA	qRT-PCR
MYC2_for	TCCGGCGAGATATTAAAC	RT-PCR
MYC2_rev2	GATTCACTTCCAACCTCA	RT-PCR
MYC2_for3	GGGGAGATGGTTATTACA	RT-PCR
MYC2_rev3	GAATTCGGATTCTGGGT	RT-PCR
JAZ10_for_ATG	ATGTCGAAAGCTACCATAGAACTCG	(q)RT-PCR
JAZ10_rev_Exon2	GCCGATGAATCGGAATTGTTCCAGTGG	(q)RT-PCR
JAZ10_rev_Exon4	CTCTCTTGCGCTTCTCGAGAAAACG	RT-PCR
JAZ10_rev	CGATGTCGGATAGTAAGGAGATG	RT-PCR
JAZ7_for2	CATCATCATCAAAAACCTGCG	RT-PCR
JAZ7_rev4	GTAACGGTGGTAAGGGGA	RT-PCR
JAZ7_rev5	AGAGCTGCTTGATTCTGTC	RT-PCR

Table 2: Primers used in this study.

Enzymes

All enzymes used in this study were purchased either from Fermentas or from New England Biolabs (NEB) and were used according to the manufacturer's instructions.

Hormones and other substances

All hormones and substances used in this thesis, their stock concentration and solvent are listed.

Substance	Company	Stock
Z-Leu-Leu-Leu-al (MG132)	Sigma Aldrich	5mM (dissolved in DMSO)
N-(1-naphthyl) phthalamic acid (NPA)	Duchefa	5mM (dissolved in DMSO)
Brefeldin A (BFA)	Invitrogen	5mM (dissolved in DMSO)
Methyl jasmonate (meJA)	Sigma Aldrich	
Indole 3-acetic acid (IAA)	Duchefa	20mM (dissolved in 1M NaOH)
Kinetin	Duchefa	20mg/ml (dissolved in 1M NaOH)
Jasmonic acid (JA)	Duchefa	1M (dissolved in EtOH)
Propidium Iodide	Calbiochem	25mg/ml (dissolved in DMSO)

Table 3: Hormones and other substances used in this study.

Cloning vectors

As templates for cloning the vectors pGreen0229 (Hellens et al. 2000) and pGreen0229-AlcA (Deveaux et al. 2003) were used (Fig. 7). Resistance to kanamycin is conferred by a bacterial gene encoding the enzyme neomycin phosphotransferase (NPTI). Resistance to glufosinate ammonium (BASTA) is conferred by the bacterial bialaphos resistance gene (nos-bar) encoding the enzyme phosphinotricin acetyl transferase (PAT).

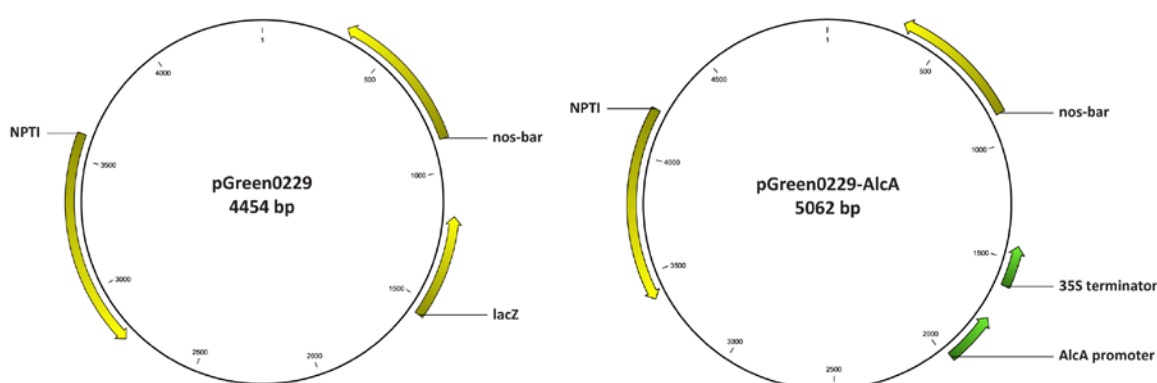


Figure 7: Vectors. In this study the vectors pGreen0229 and pGreen0229-AlcA were used.

Methods

Plant growth

On soil

After disseminating on soil seeds were stratified for 3 days (4°C, 24 h dark) and, subsequently, transferred to short day (SD) conditions (22°C, 8 h light, 16 h dark). After 3 weeks, plantlets were singularised into single pots (6 x 6 cm) filled with a soil-perlite mixture (4:1) and transferred to long day (LD) conditions (22°C, 16 h light, 8 h dark). Plants were sprayed once in a while with 2.5 g/l sulphur against mildew and treated every 2nd week with nematodes against the larvae of black flies.

On plates

Seeds were surface sterilised utilising an Eppendorf tube by incubating them successively in 70% EtOH for 1 min, 50% conventional bleach (Danklorix) for 5 min, and 3 times in sterile H₂O for each about 3 min. Under sterile conditions (laminar flow) the seeds were pipetted onto a ½ MS plate and spread using a small spoon. For selection of drug-resistant plants the plates were supplemented either with 50 µg/ml kanamycin or 5-30 µg/ml hygromycin. Plates were sealed with Leukopor and placed into the plant culture room (21°C, 16 h light, 8 h dark).

Ethanol induction

Plants carrying ethanol-inducible constructs (Deveaux, Peaucelle et al. 2003) were grown as described above. When plants reached a height of about 15 cm, pure ethanol was filled into 2 ml Eppendorf tubes which were placed in the soil next to the plants (about 1 tube per 4 plants). The plants were covered with a plastic hood so that an ethanol atmosphere could be generated. After an overnight incubation ethanol-induced plants were harvested for further analysis.

Weight treatment

Weight treatment was performed by attaching paperclips (around 0.16 g each) to the main shoot. 1, 2, 3, and 6 paperclips were consecutively added to 2, 5, 10, and 15 cm high plants, respectively (Fig. 8A). Stems were harvested at 15 cm height carrying a weight of 0.95 ± 0.13 g and at 30 cm carrying 1.81 ± 0.23 g of paperclips.

Hormone treatments

Lanolin

Lanolin paste (Sigma Aldrich) was heated until it liquefied and mixed in a plastic weighing pan with the following reagents: MG132 (final concentration 100 μ M), NPA (final concentration 100 μ M), or BFA (final concentration 30 μ M).

Plants of a height of 15-20 cm were used for the lanolin treatment. If not stated otherwise, lanolin paste was applied onto the 1st internode of the inflorescence stem about 2 cm above the rosette utilising a small spoon (Fig. 8B, arrow). After 1 week of incubation, plants were harvested and processed for further analyses.

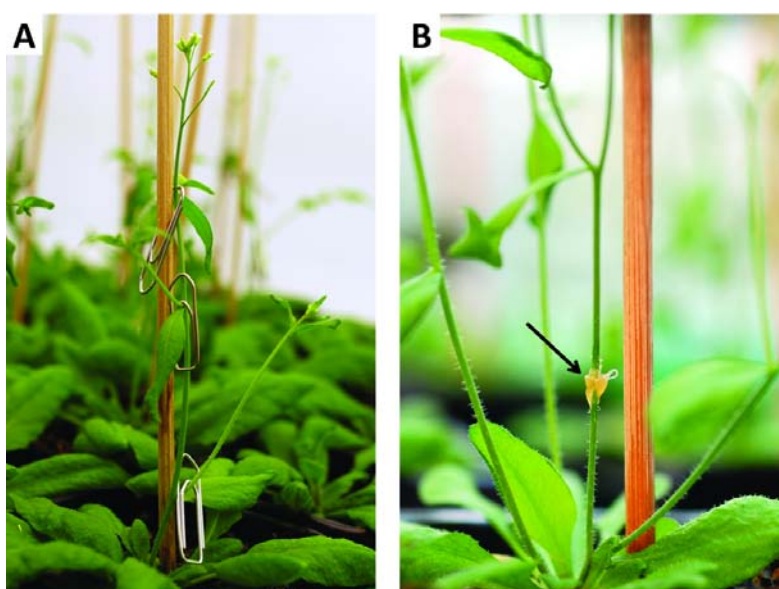


Figure 8: Weight and hormonal treatments. For the weight treatment paperclips were put onto the main inflorescence stem (A). A stick was provided for stability. Lanolin mixed with the corresponding hormones was applied onto the first internode (B).

Spraying

3 weeks old soil-grown seedlings were sprayed either with mock (tap water), 50 μ M meJA, 20 μ M IAA or 185 μ M kinetin. Seedlings were harvested after 2 and 4 hours for RNA extraction.

Watering

Plants were grown on soil under the conditions described above. After singularising the plantlets they were exclusively watered either with mock (tap water) or with 0.5 mM jasmonic acid (dissolved in tap water) during the whole growth period. 15 cm and 30 cm high plants were harvested for further analysis.

Histology

Fixation

If not stated otherwise, fresh plant material was collected and fixed by 70% EtOH, vacuum infiltrated for about 10 min and incubated over night at room temperature (RT).

Embedding

The fixed plant material was then transferred into embedding cassettes (Sanowa) and the cassettes were put into a tissue processing machine (Thermo Shandon Tissue Excelsior or Sakura Tissue-Tek VIP) following a standard embedding protocol over night. The cassettes were infiltrated by paraffin, transferred to the embedding centre (Thermo Shandon or Sakura Tissue-Tek TEC) and the samples were manually embedded into specific molds (Tissue-Tek). After about 30 min on the cooling platform, the molds were peeled off and the samples are kept at 4°C until sectioning.

Sectioning

For sectioning, the wax blocks were prepared by trimming the excess wax. If not stated otherwise, 10 µm thick sections were produced using a rotary microtome (Microm). The sections (wax ribbons) were placed on a preheated glass slide (Superfrost, Menzel) covered with dH₂O to let them expand. After about 5 min the water was removed with a pipette and the sections on the glass slides were dried at 42°C on a heating bank over night. If not continued by dewaxing and staining on the next day, the slides were kept at 4°C.

Dewaxing & staining

The slides were dewaxed and stained after the following incubation procedure:

XEM 200 (Xylolersatz)	10 min
XEM 200	10 min
100% EtOH	1 min
100% EtOH	1 min
95% EtOH	1 min
85% EtOH	1 min
50% EtOH	1 min
35% EtOH	1 min
dH ₂ O	1 min

dH ₂ O	1 min
Toluidine blue	5 min
dH ₂ O	30 sec
dH ₂ O	30 sec
dH ₂ O	30 sec
95% EtOH	30 sec
100% EtOH	30 sec

The slides were air dried and the cover slips (24 x 50 mm, VWR) were mounted and sealed with Entellan (Merck).

GUS staining

A standard GUS staining solution was prepared freshly by mixing 42 mg X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide, final concentration 2 mM) dissolved in 2 ml DMF (dimethylformamide) with each 210 µl ferro- and ferricyanide (final concentration 1 mM each) and filled up to 40 ml with GUS staining buffer. For gene expression analyses, samples of marker lines were collected and directly put into the GUS staining solution. After vacuum infiltration for about 10 min samples were incubated at 37°C over night. Subsequently, the GUS staining solution was replaced by 70% EtOH for clearing the sample at least over night at RT.

Paraffin embedding & sectioning utilising a rotary microtome

Stained samples were embedded as described above for sectioning with the exception that after the dewaxing steps (2x XEM followed by 2x 100% EtOH) toluidine blue staining was not performed. Sectioning utilising the rotary microtome were done as described above.

Technovit embedding & sectioning utilising a rotary microtome

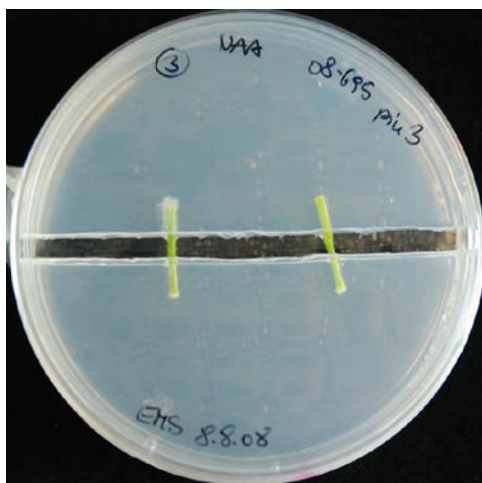
For thinner sections, GUS stained samples were embedded into Technovit 7100 (Kulzer). For this procedure, samples were transferred from the clearing step (70% EtOH, see above) to 90% EtOH for 1 h and finally washed twice in 100% EtOH each for 1 h. Subsequently, Technovit embedding was performed as described in the manufacturer's protocol utilising PCR tubes as embedding molds. After hardening, the tubes were peeled off; the samples were trimmed and glued onto specific plastic blocks for histology (Kulzer). On the rotary microtome 3 µm thick sections were produced and placed on a preheated glass slide covered with dH₂O mixed with a few drops of 100% EtOH (Igersheim and Cichocki 1996). The slides were air dried and sealed with Entellan as described above.

Agarose embedding & sectioning utilising a vibratome

To prevent the tissue of going through many clearing and washing steps, freshly GUS stained material was embedded in low melting agarose type XI (Sigma Aldrich, A3038) after the following procedure: The stained samples were washed twice in 1x PBS for about 10 min each. Tissues were embedded in 5% agarose into disposable plastic molds and allowed to cool for solidifying. The agarose block containing the tissue was trimmed, and glued onto a specific metal block holder with commercial superglue and was allowed to dry before sectioning utilising a vibratome (HM430, Microm). Sections (about 20-30 μm thick) were placed onto dH_2O droplets on a glass slide and covered with a cover slip which was sealed with rubber cement (FixoGum, Marabuwerke).

In vitro system

Preparation of split-plates



A petri dish with $\frac{1}{2}$ MS medium was separated under sterile conditions (laminar flow) into two halves by removing a 6 mm wide strip from the middle of the plate utilising two scalpels (Fig. 9). The substances and hormones of choice were pipetted if necessary independently from each other onto a specific half of the separated plate. The plates were sealed by Nescofilm (Roth) and stored upside-down for 3 days (4°C, dark) to ensure diffusion of the substances throughout the medium half.

Figure 9: Split-plate *in vitro* system. See text for further details.

Preparation of plant samples

The collected plant material had to be surface sterilised for the *in vitro* system. For this, a stem fragment of about 4-5 cm including the region of interest (about 2 cm above the rosette and 2 cm away from the next node) were excised. During all following steps the original orientation of the stem fragment was considered. Fragment ends were sealed by dipping them into preheated liquid paraffin and the collected stem fragments were then put into sterile dH_2O in a 50 ml falcon tube. For surface sterilisation, the water was replaced by 70% EtOH and the stems were incubated for about 30 sec (incubation time must not exceed 1 min!). The ethanol was

immediately replaced by 50% conventional bleach (DanKlorix) and after 5 min of incubation, 3 washing steps, 5 min each, with sterile dH₂O under sterile conditions (laminar flow) followed. With a sterile set of instruments (scalpels and forceps) a 1.5 cm long stem fragment was excised and put into the split-plate considering the original apical-basal orientation. The plates were placed upside-down into a culture chamber for 1 week. The stem fragments were collected and processed as described above.

Photography and microscopy

Digital photography

All photographs were taken using the digital camera Nikon D80 carrying the objectives AF Micro Nikkor 60mm (1:2.8 D), AF Nikkor 35mm (1:2 D) or Tamron AF 17 50mm (1:2.8 IF).

Stereo microscopy

Samples were analysed using the LEICA MZ16FA binocular and photographed with the attached LEICA DFC300FX colour camera. Pictures were processed with the Leica Application Suite.

Light microscopy

Toluidine blue stained cross sections were analysed by differential interference contrast settings (DIC) whereas GUS stained cross sections were analysed using dark field optics. For both techniques, the ZEISS Axio Imager M1 upright microscope was used. Pictures were taken using a colour camera from Visitron Systems by the help of the SPOT Advanced software version 4.6.

Confocal microscopy

Sample preparation

For the preparation of hand sections, stem fragments were sandwiched between two pieces of insulating material and sections were produced using a razor blade. The freshly cut sections were counterstained by 5 µg/ml propidium iodide for 1 min, put on a glass slide with tap water and covered with a cover slip.

Settings

All imaging was done using a LEICA TCS-NT confocal microscope utilising the following filters: LP665 (barrier FW #4), RSP660 (detection beam splitter FW #3) and RSP500 (excitation beam splitter FW). GFP and propidium iodide were excited at 488 nm and detected at 500-520 nm and 610-630 nm respectively. The pinhole was set to 1.00 airy units (AU) and the voltage of the

photomultiplier tube (PMT) was adjusted between 800 and 1000 V depending on the samples. The samples were scanned with 8 frame averages.

Image processing

All pictures were processed using the application software Adobe Photoshop CS4 and Adobe Illustrator CS4.

Quantitative analysis of cambial development

The dynamics of cambial development was analysed using 2, 5, 15 and 30 cm high plants for all measurements. For each measurement between 5 and 10 individuals were scored. To see to what extent cambial activity influences lateral growth, the stem diameter was measured at the very stem base immediately above the uppermost rosette leaf (Fig. 10A, arrow). IC activity itself was analysed by measuring the interfascicular cambium derived (ICD) zone (Fig. 10B, arrow) which was quantified at 3 different sites per individual section. The zone of ICD included secondary phloem (densely packed cells at the abaxial side of the cambium, red asterisk), the interfascicular cambium, and the secondary xylem which is organised in typical cell files (black asterisk marking the last cell within the cell file adjacent to the cells of the interfascicular fibres). SG along the stem was quantified by measuring the longitudinal extension of IC initiation on 10 μm thick sections starting at the very stem base immediately above the uppermost rosette leaf along the stem until the position where no closed vascular cambium was detected anymore (Fig. 10C, arrow). Distal to this position, IC initiation between individual bundles, but no closed vascular cylinder, was detectable.

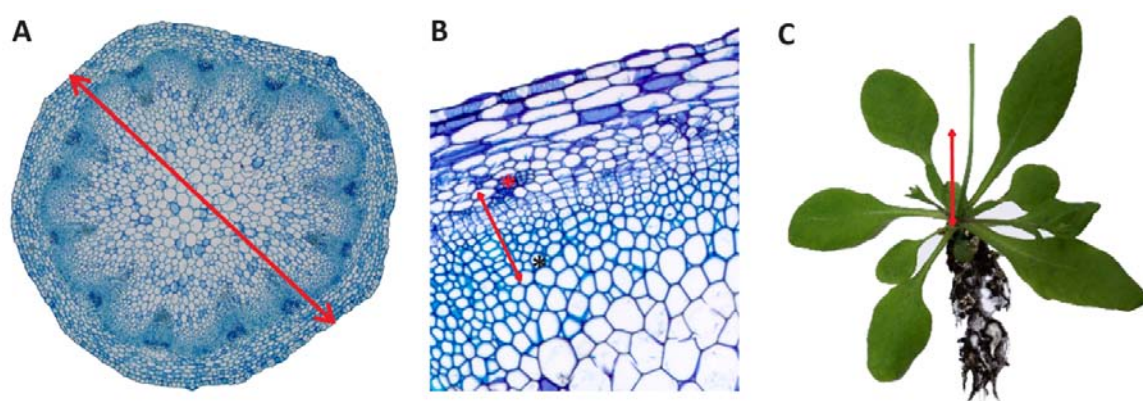


Figure 10: Quantitative analysis of cambial activity in the stem. Measurements (indicated by arrows) were taken of the stem diameter at the very base (A), the interfascicular cambium derived zone (ICD) (B) and the longitudinal extension of secondary growth (C).

Statistical analyses

Means were compared utilising the software SPSS statistics 7.0. An independent *t*-test was performed with the confidence interval set to 95%. If the p-value was less than 0.05 the compared means were not significantly different (n.s.), if the p-value ≤ 0.05 , the compared means were significantly different (*), if the p-value ≤ 0.01 , the compared means were highly significantly different (**), and if the p-value ≤ 0.001 , the compared means were most significantly different (***).

Molecular techniques

DNA extraction

“Quick and dirty”

Small pieces of a leaves were harvested into PCR tubes. 50 μ l 0.25 M NaOH were added and the plant material was ground using a pipette tip. The PCR tubes were placed for 30 sec at 95°C into the PCR machine, subsequently 50 μ l 0.25 M HCl and 30 μ l 0.5M TRIS (pH 8, incl. 0.25% IGEPAL) were added. The PCR tubes were placed a second time into the PCR machine for 2 min 30 sec at 95°C. 2 μ l of this plant suspension were used for PCR reactions.

“Quick and clean”

1-3 leaves were harvested into an Eppendorf tube, 200 μ l of extraction buffer were added and the plant material was ground utilising a blue drill. Another 200 μ l of extraction buffer were added and the tubes were centrifuged for 5 min at maximum speed (14000 rpm). The supernatant was pipetted into a new Eppendorf tube containing 350 μ l isopropanol and after an incubation of 10 min at room temperature the samples were centrifuged for 5 min at maximum speed. The supernatant was decanted, 700 μ l 70% EtOH was added to wash the pellet and the tubes were centrifuged for 1 min at maximum speed. The ethanol was removed; the pellets were dried by putting them for 10 min at 30°C into the Eppendorf concentrator 5301 and subsequently were suspended in 50 μ l TE buffer for 10 min at 65°C using a thermo mixer (Eppendorf). Finally the samples were stored at -20°C.

CTAB

Fresh plant material was harvested into a 1.5 ml Eppendorf tube and immediately frozen in liquid nitrogen. Frozen plant material was ground using a mortar and pestle; frozen pulverised plant material was put into an Eppendorf tube containing 1 ml CTAB buffer. Samples were vortexed and heated to 65°C for 15 min. 400 μ l chilled chloroform were added and samples

were centrifuged at maximum speed for 10 min. After transferring the aqueous phase (around 800 µl) into a new Eppendorf tube supplied with 700 µl ice-cold isopropanol, the samples were incubated at -20°C for a minimum of 30 min. To compact the DNA to a pellet, the tubes were centrifuged for 10 min at 4°C at maximum speed. The supernatant was decanted and the pellets were dried utilising the Eppendorf concentrator 5301 for 10 min at 30°C. The pellets were dissolved in 200 µl TE buffer at 65°C; 2 µl RNase A (DNase and protease-free, Fermentas) was added and the tubes were incubated at 37°C for 20 min. 200 µl of the lower phase of phenol:chloroform:isoamyl alcohol (25:24:1, AppliChem) was added and the tubes were centrifuged at maximum speed for 10 min. The upper phase (around 200 µl) was transferred into new Eppendorf tubes supplied with 20 µl 3 M NaAc (pH 5.2) and 400 µl EtOH absolute. After the tubes had been incubated at -20°C for at least 1 hour, they were centrifuged for 10 min at 4°C at maximum speed. The supernatant was decanted, 1 ml of 70% EtOH was added and the tubes were centrifuged for 5 min at maximum speed. The supernatant was discarded and the pellet was dried as described above. The pellets were dissolved in 50 µl TE buffer by incubating them at 65°C with agitation. DNA concentration was determined using the NanoDrop Spectrophotometer ND-1000.

RNA extraction

Plant material was harvested into an Eppendorf tube and frozen in liquid nitrogen. The frozen tissue was ground with a mortar and pestle and about 400 µl of the fine powder was put into a 1.5 ml Eppendorf tube to which 1 ml of chilled TRIzol reagent (Invitrogen) was added. After the reagent was melted, the samples were incubated 5 min at RT and subsequently centrifuged at 4°C at maximum speed for 5 min. The supernatant was transferred into a new tube containing 200 µl of chloroform. After incubation for 5 min at RT, the tubes were centrifuged at maximum speed at 4°C for 15 min. The upper aqueous phase was pipetted into tubes supplied with 500 µl chilled isopropanol and the tubes were incubated at -20°C at least 20 min (frequently over night) to allow precipitation of the RNA. To compact the RNA into a pellet, the tubes were centrifuged for 10 min at maximum speed at 4°C. The supernatant was discarded and the pellets were washed by 1 ml 70% EtOH (in DEPC water) by centrifuging the tubes for 5 min at 4°C at 10000 rpm. The supernatant was removed and the pellets were dried by putting them for 10 min at 30°C into the Eppendorf concentrator 5301. The pellets were suspended in 50 µl DEPC water by incubating them for 10 min at 55°C.

The DNase digest of the RNA was performed by adding 1 µl DNase I (RNase-free, Fermentas), 0.5 µl RiboLock RNase inhibitor (Fermentas), 10 µl 10x DNase buffer (Fermentas) and 33.5 µl DEPC

water and incubating at 37°C for 30 min. For RNA precipitation 10 µl 3M NaAc (pH 5.2, in DEPC water) and 250 µl 96% EtOH (in DEPC water) were added and the tubes were incubated at least 20 min (frequently over night) at -20°C. To pellet the RNA the tubes were centrifuged at 4°C at maximum speed for 15 min. The pellet was washed, dried and suspended as described above. RNA concentration was determined using the NanoDrop Spectrophotometer ND-1000.

Complementary DNA (cDNA) generation

cDNA was generated using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) by following the manufacturer's protocol.

Polymerase chain reaction (PCR)

Standard PCR

The standard PCR reaction was as following:

- 2.5 µl 10x Buffer (homemade)
- 0.25 µl 10 mM dNTP (Fermentas)
- 1.5 µl 25 mM MgCl₂ (homemade)
- 0.5 µl 10 µM forward primer
- 0.5 µl 10 µM reverse primer
- 0.5 µl Taq DNA polymerase (1:50, homemade)
- + template (100 ng DNA or 1 ng plasmid)
- filled up with dH₂O to a final volume of 25 µl

PCRs were performed utilising either a Bio-Rad iCycler or a Biometra T3000 Thermocycler after the following standard PCR programme:

- 95°C – 3 min
- 32x: 94°C – 20 sec
- 58°C – 20 sec
- 72°C – 2 min
- 72°C – 6 min

PCR products were analysed with standard gel electrophoresis as described below.

Reverse transcriptase (RT) PCR

RT-PCR was performed similarly to a standard PCR with the exception that cDNA (generated with oligo (dT)₁₈ primer) was used as template. As internal control, the tubulin beta chain 2 (*TUB2*,

At5g62690) was used. To prevent saturation of the PCR the number of cycles was adapted individually to each primer combination. The resulting PCR products were analysed with standard gel electrophoresis as described below.

Quantitative real-time (qRT) PCR

qRT-PCR was performed as described in (Nolan et al. 2006). As template for qRT-PCR, cDNA (generated with oligo (dT)₁₈ primer) was used which was diluted 1:5.

Primer analysis

The primer pairs used for qRT-PCR were designed in that way that the amplified fragment had a size of 150-200 bp and was located near the 3' end of the cDNA. The primer pairs had to be tested in advance to determine their correct melting temperature and to detect possible primer dimer formation. For this, a standard cDNA dilution series was used: the original diluted (1:5) cDNA was used as starting solution, from this 1:10, 1:100 and 1:1000 dilutions were done. For each primer pair 3 technical replicates were prepared after the following master mix with a total volume of 15 µl:

- 7.5 µl 2x SensiMix SYBR (Peqlab)
- 0.4 µl 10 µM forward primer
- 0.4 µl 10 µM reverse primer
- 1.7 µl dH₂O
- 5 µl cDNA template

The primers were tested utilising the IQTM5 Multicolor Real-time PCR Detection System (Bio-Rad) mounted on an iCycler PCR machine (Bio-Rad) and the following protocol:

- 95°C – 10 min
- 35x: 95°C – 10 sec
- 60°C – 30 sec
- 72°C – 20 sec
- 78°C – 11 sec
- 80°C – 11 sec
- 72°C – 1 min

After the run, the melting curve chart and the melting peak chart were analysed with the IQTM5 Optical System software and the melting temperature was determined.

qRT-PCR

As internal control, the *Arabidopsis* eukaryotic translation initiation factor 4A1 (*EIF4A1*, At3g13920, see Metz et al., 1992) was used. For each gene of interest (GOI), qRT-PCR reactions were performed in technical triplicates with the master mix described above following a standard qRT-PCR protocol with the melting temperature of 73°C, which was modified for each GOI:

95°C – 10 min

35x: 95°C – 10 sec

60°C – 30 sec

72°C – 30 sec

73°C – 11 sec

72°C – 30 sec

Ct values for each GOI were determined by the IQTM5 Optical System software and exported into a MS Excel Workbook (Microsoft Inc.) for further analysis.

Agarose gel electrophoresis

For the preparation of a standard agarose gel, 1% peqGOLD universal agarose (Pqqlab) in 1x TAE buffer was prepared, heated in the microwave and poured into a gel tray carrying a suitable comb. Prior to loading PCR products were mixed with 0.8% loading dye. If needed, 7.5 µl of DNA ladder (Gene Ruler, 100 bp or 1 kb, Fermentas) were loaded in parallel. Standard electrophoresis was done under 120 V for about 30-40 min. For DNA staining, the gel was incubated in an ethidium bromide staining bath (50 µl EtBr [10 mg/ml] in 500 ml 1x TAE buffer) for about 20-30 min and subsequently photographed and analysed utilising the Gel Logic 2000 Imaging System (Kodak).

Vector design

All plasmids designed in this study were created according to the following procedures:

Amplification of the sequence of interest

Primer with specific restriction sites were used for amplifying the sequence of interest (see Tab. 2). A PCR was performed either using cDNA obtained from seedlings carrying the *PIN3:PIN3-GFP* construct (for pEMS16 and pEMS19) or using the plasmids pEMS16 and pEMS19 (for pEMS23-pEMS28) as template. Either Phusion High Fidelity DNA Polymerase (New England Biolabs) or Pfu DNA Polymerase (Fermentas) was used following the manufacturer's protocols. The PCR product

was purified using the QIAGEN QIAquick PCR purification kit following the manufacturer's protocol.

Site-directed mutagenesis

To induce point mutations in the internalisation motif of *PIN3* (Fig. 11, black square), QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer's protocol. For the creation of pEMS23 and pEMS26 the tyrosine within the internalisation motif (NPXXY) was changed into an alanine by mutating the TAC to a GCC of pEMS19 and pEMS16, respectively. For the creation of pEMS24 and pEMS27 the asparagine (NPXXY) of the internalisation motif was changed into an alanine by mutating the AAC to a GCC of pEMS19 and pEMS16, respectively. pEMS25 and pEMS28 contain both mutations.

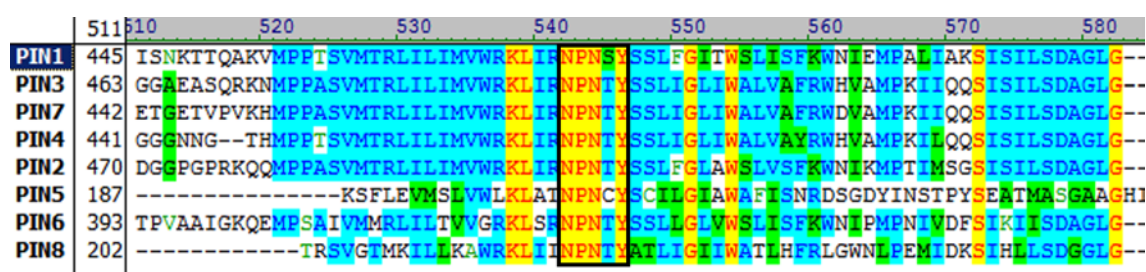


Figure 11: Internalisation motif. Alignment of a selected part of the protein sequences of all PIN proteins with their specific internalisation motif (NPXXY, enclosed by the black square).

Restriction enzyme digestion

The purified PCR product was double digested by the specific enzymes given by the restriction site of the primers used. In parallel, the target vector was digested with the same restriction enzymes as the insert following the manufacturer's protocol. The digested products were cleaned with the QIAGEN QIAquick PCR purification kit following the manufacturer's protocol.

Ligation

The digested and purified (QIAGEN QIAquick PCR purification kit) insert was ligated to the target vector either for 1 hour at RT or over night at 16°C utilising T4 ligase (Fermentas) following the manufacturer's protocol.

Heat shock transformation of *E. coli*

After thawing of the cells, to 200 µl of competent cells 10 µl of the ligation reaction were added and incubated on ice for about 20 min. Subsequently, for the uptake of the plasmids, the cells were exposed to a heat shock (42°C) for 90 sec and immediately put back on ice. 800 µl of LB

medium were added and the suspension was incubated at 37°C under agitation to enforce cell proliferation. After 1 h about 160 µl of bacterial suspension were plated onto LB plates containing a selective medium (mostly kanamycin). An overnight incubation at 37°C followed.

Plasmid preparation

Colonies of pEMS16 and pEMS19 were tested by PCR for their insert with the primer pair T3 + T7. Positive colonies or non-tested colonies (as it was the case for pEMS23-pEMS28) were picked with a sterile toothpick which was put into a glass tube containing 5 ml of a selective LB medium. Colonies were grown under agitation at 37°C over night. To isolate the plasmids, the QIAGEN QIAprep Spin Miniprep Kit was used following the manufacturer's protocol. DNA concentration was measured using the NanoDrop Spectrophotometer ND-1000.

Sequencing

Cloned inserts were sequenced using specific primers (see Tab. 2). For that, a master mix of a total volume of 7 µl was prepared containing about 150 ng of plasmid DNA, 1 µl 10 µM primers and water. These reactions were brought to the in-house sequencing facility. The AB1-files were processed and analysed utilising the CLC Main Workbench (CLC bio).

Transformation of *Arabidopsis thaliana*

Transformation of *Agrobacterium tumefaciens*

After thawing 200 µl of the cell suspension on ice, 500 ng of the plasmid of interest was added and incubated on ice for 5 min. According to the freeze-thaw method (Hoefgen and Willmitzer 1988), the tube was frozen for 5 min in liquid nitrogen and subsequently incubated at 37°C for another 5 min. 800 µl LB medium was added followed by an incubation at 28°C for 2-4 h. About 160 µl of this cell suspension was plated on selective medium containing 50 µg/ml rifampicin (selects for *Agrobacterium* itself), 5 µg/ml tetracycline (selects for the helper plasmid pSoup) and the specific antibiotic for the plasmid of interest (mostly 50 µg/ml kanamycin). The plates were incubated at 28°C up to 3 days.

Floral dip transformation of *Arabidopsis thaliana*

A colony picked from the plates described before was incubated in a 5 ml YEB preparatory culture containing the selective antibiotics over night at 28°C under agitation. For the preparation of a glycerol stock, 800 µl of this cell suspension were mixed with 200 µl glycerol in a cryo tube. Subsequently the cryo tube was frozen in liquid nitrogen and stored at -80°C.

For the floral dip transformation (Clough and Bent 1998), a 2 l Erlenmeyer flask containing 400 ml YEB with 50 µg/ml kanamycin was inoculated with the rest of the preparatory culture and incubated over night under agitation at 28°C. This cell culture was transferred into 500 ml plastic centrifugation tubes and centrifuged for 15 min at 5000 rpm at RT utilising an Avanti J-26 XP centrifuge (Beckman Coulter™). The supernatant was removed and the bacterial pellet was suspended in 500 ml 5% sucrose solution containing 0.02% Silwet L-77 (Lehle Seeds). Inflorescences of plants (per pot about 16 plants, per construct about 5 pots) were dipped into the bacterial solution for about 5 min. The dipped plants were covered using a plastic cover until the next day. Seeds of the transformed plants were harvested for further analysis.

Selection of transformed plants

T1 seeds (seeds of the transformed plants) carrying a kanamycin resistance were laid out on ½ MS plates with 50 mg/l kanamycin and were placed into the plant culture room. Surviving plantlets were transferred to soil and raised in the growth chamber for further analysis. T1 seeds carrying a BASTA resistance were directly laid out on soil. After germination, seedlings were treated every 2nd to 3rd day by spraying with 200-300 µM BASTA (glufosinate ammonium). Here again, healthy green plantlets were transferred to new pots for further analysis.

Southern hybridisation

Southern blots (Southern 1975) were performed using the upward capillary transfer of DNA fragments according to Sambrook and Russel (2001) in order to check how many copies of the foreign plasmid have been inserted into the genome of the T1 generation of transformed plants.

Crossings with *Arabidopsis thaliana*

“Female” parent flower buds were opened with forceps and all floral organs besides the gynoecium were removed. A “male” parent flower showing mature stamens were cut off the inflorescence and the stamens were brought into contact with the pistil of the dissected “female” flower. The siliques were collected after ripening (Weigel and Glazebrook 2002).

Genotyping of *Arabidopsis thaliana*

Genotyping of insertion lines

To test whether plant lines ordered from seed stock centres (NASC, INRA) are homozygous genotyping had to be performed. For this analysis, seeds were laid out and plants were grown

under normal conditions. DNA was prepared according to the “quick and dirty” method. Primers for PCR were designed and PCR was performed as described on the SIGnAL homepage (SIGnAL T-DNA verification primer design, <http://signal.salk.edu/tdnaprimers.2.html>). PCR products were analysed by standard gel electrophoresis. Seeds from homozygous plants were harvested for further analysis.

Genotyping with CAPS/dCAPS marker

To test whether mutant lines carrying a point mutation are homozygous, CAPS (cleaved amplified polymorphic sequences) or dCAPS (derived CAPS) marker were used (Konieczny and Ausubel 1993). Primers for this method were generated utilising the dCAPS Finder 2.0 program (Neff et al. 2002). The resulting dCAPS-PCR products were separated by gel electrophoresis utilising a 4% agarose gel to find homozygous mutant lines from which seeds were harvested.

Results – Part I – Secondary growth

Histological characterisation of the process of secondary growth initiation

SG is a well described process in many plant species. However, in *A. thaliana* this process didn't attract much attention so far. Previous studies only touched on the process of secondary growth, thus, by performing histological analyses of the main inflorescence stem, SG was analysed here in detail.

Cambial activity at the base of the inflorescence stem

Main inflorescence stems in 4 different stages of development, to wit 2 cm, 5 cm, 15 cm and 30 cm high stems were subjected to histological analysis and the process of SG was examined at the cellular level in its lateral and longitudinal extension.

2 cm high plants showed SG activity at the very base of the inflorescence stem (Fig. 12A). In the interfascicular region between the vascular bundles (VB) a continuous zone of periclinally dividing cells was present producing radial cell files which are characteristic for the vascular cambium. The interfascicular cambium derived zone (ICD), which includes the interfascicular cambium (IC) and the tissues derived from it, consisted of 3-4 cells in radial orientation with an extension of 0.036 ± 0.002 mm and was located 3-5 cells proximal to the cortex (Fig. 12A, Fig. 13A). Cells between the cortex and the ICD were classified as pith parenchyma cells based on their shape and their apparent unordered organisation. In this 2 cm high stems, the closed cambial cylinder with interfascicular cambial cell divisions (which refers to SG) was identified up to a position of 2.37 ± 0.15 mm above the uppermost rosette leaf (Fig. 12C, Fig. 13A). The ICD was found to be closer to the cortex in more apical positions than in basal positions, and was directly adjoined to it from a position of approximately 0.6 mm above the rosette.

At the very base of 5 cm high stems, the ICD extended laterally to 7-8 cells (0.063 ± 0.005 mm, Fig. 12D, Fig. 13A). Here the formation of secondary phloem in form of cell clusters distally to the IC was observed (Fig. 12D, red arrows). SG, characterised by a closed cambial cylinder, can be found up to a height of 3.48 ± 0.19 mm above the rosette (Fig. 13A). In more apical parts the IC was located closer to the cortex/starch sheath than in basal parts (Fig. 12D-F).

SG had extended up to 5.30 ± 0.19 mm above the rosette in 15 cm high plants (Fig. 13A) and islands of phloem tissue could clearly be identified at the stem base (Fig. 12G, red arrows). At this stage, the differentiation of pith parenchyma cells into interfascicular fibres (IFs) became

evident due to secondary cell wall formation proximal to the ICD along the whole stem segment analysed (Fig. 12G). Additionally, cell divisions in interfascicular regions were not only observed in pith cells but also in the starch sheath at the upper margin of the IC-initiating stem segment (Fig. 12I).

In 30 cm high stems, the longitudinal extension of SG had progressed to 7.25 ± 0.21 mm (Fig. 13A). At this stage, cell divisions in the starch sheath were found in a region from about 4.5 to 7.1 mm above the rosette. At this stage cambial cells at the distal margin of the SG initiating zone seem to differentiate based on the presence of secondary cell wall formation (Fig. 12L). Furthermore, the IFs became much more prominent due to thicker secondary cell walls.

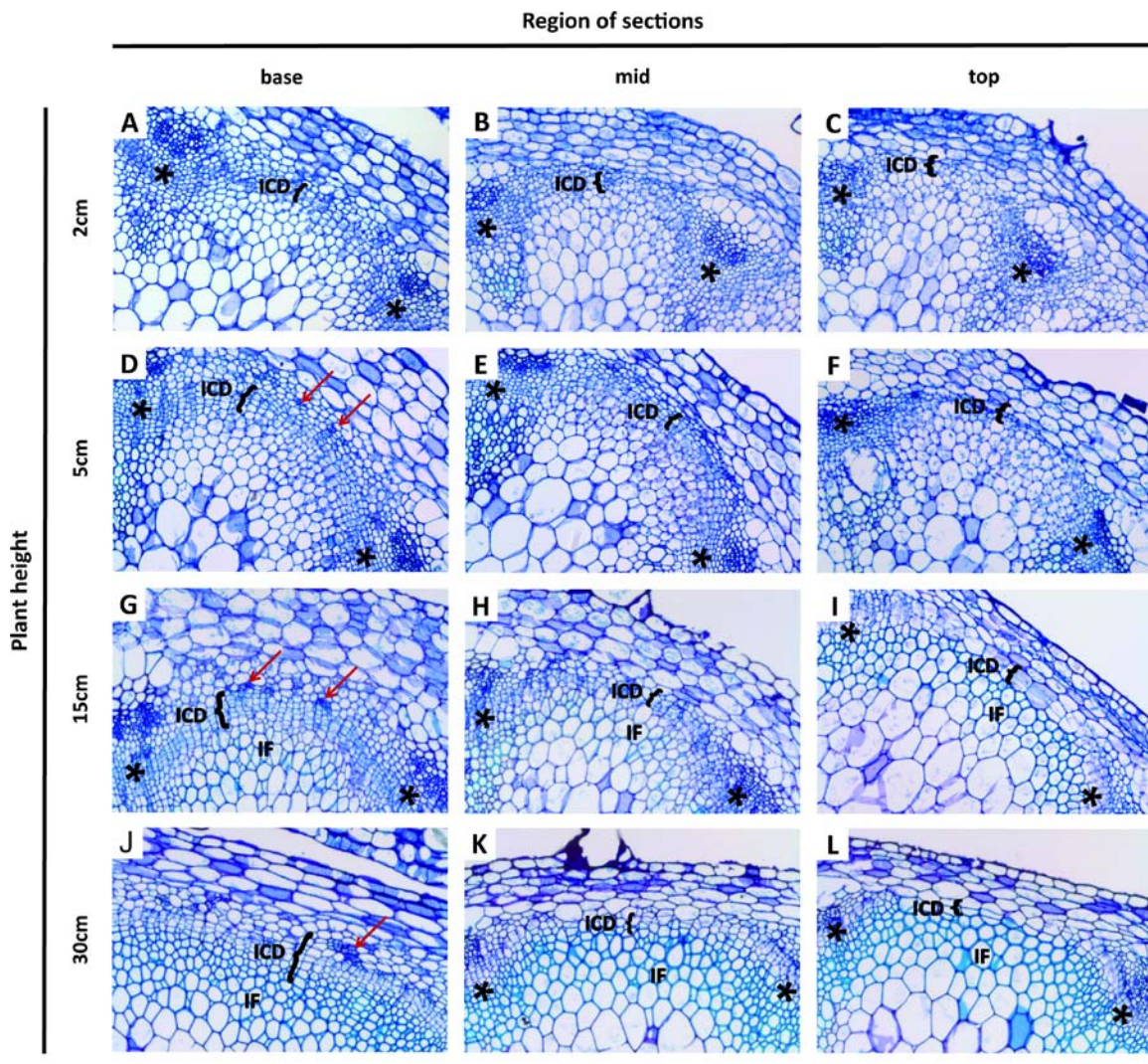


Figure 12: Histological analysis of IC development at the basis of the inflorescence stem. 2 cm high stems (A-C) were sectioned at the base (A), 1 mm (B) and 2 mm (C) above the rosette. At this stage of stem development, SG was observed from the base up to a height of around 2.4 mm. 5 cm high stems (D-F) were analysed at the base (D), at 1.8 mm (E) and 3.5 mm (F) above the rosette. At this stage, the lateral extension of ICD is much higher in comparison to 2 cm high stems. Furthermore, secondary phloem can be seen in the interfascicular region. A closed cambial cylinder can be found in longitudinal orientation until around 3.5 mm above the rosette. 15 cm high stems (G-I) were sectioned at the base (G), 2 mm (H) and 5.2 mm (I) above the rosette. SG is much more advanced at this stage and the formation of IFs has started. 30 cm high stems (J-L) were analysed at the base (J), 2.7 mm (K) and 7.1 mm (L) above

the rosette. Here, SG reached a position of about 7.3 mm above the rosette and the cell walls of the IFs became thicker. ICD: interfascicular cambium derived zone. IF: interfascicular fibres. Vascular bundles are indicated by asterisks. Red arrows point to secondary phloem in interfascicular regions. Scale bar: 100 μ m.

Taken together, the analysis of IC development in different stages of plant growth showed an acropetal progression of IC initiation (Fig. 13A) starting from 2 cm until 30 cm high plants. During its acropetal progression, the position of IC initiation is gradually shifted towards the cortex and in later growth stages the starch sheath was the origin of the IC.

Stem thickening during secondary growth

To reveal the contribution of SG to stem thickening, the stem diameter was measured at the same positions as where the lateral extension of IC-derived tissues was determined (see above, Fig. 12). This analysis showed that there is constant stem thickening even in regions where hardly any IC activity is observed (Fig. 13B), suggesting that in addition to SG, also general cell expansion contributes to lateral shoot growth in *Arabidopsis*.

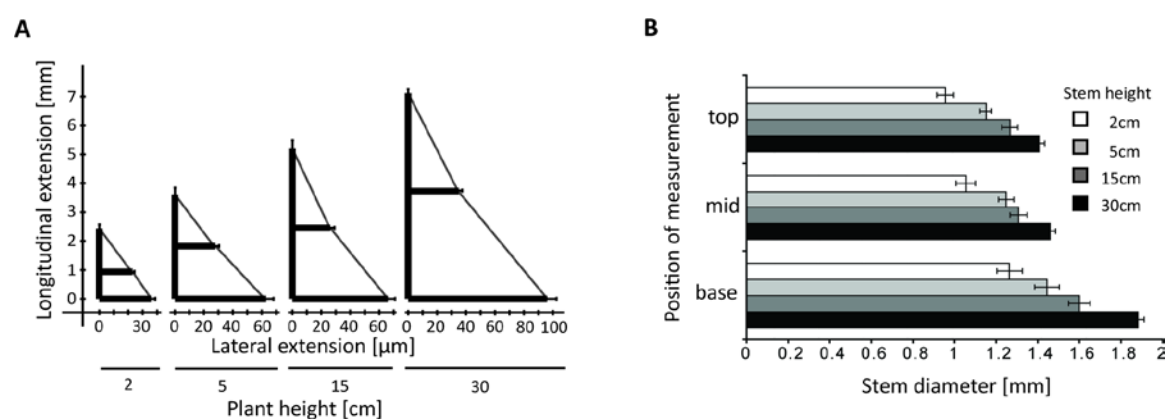


Figure 13: Quantification of the lateral and longitudinal extension of SG and its impact on the increase of shoot diameter. Longitudinal extension of SG and lateral extension of cambial activity in wild type Columbia 0 plant stems of different heights (2 cm, 5 cm, 15 cm and 30 cm) (A). For more details see text. The stem diameter measured at the very base of the inflorescence stem, at the middle of the longitudinal extension zone of secondary growth and at its top (B). Error bars: standard error of means (SE).

Impact of various short day lengths on cambial activity

To analyse the impact of an extended life cycle on cambium activity, plants grown for 0, 3 and 7 weeks under SD conditions before stem elongation was induced by shifting plants to long days were compared. Histological analyses of the base of the inflorescence stem showed that the longitudinal extension of the zone with IC initiation in plants grown solely in long days was less than in plants grown for three weeks under short day conditions at all developmental stages. Plants grown for 3 weeks in SD conditions, established less IC than plants grown for seven weeks under short day conditions (Fig. 14D). Furthermore, the stem diameter, the number of vascular

bundles and the cell wall thickness of the IFs increased the longer the plants were grown under SD conditions (Fig. 14A-C).

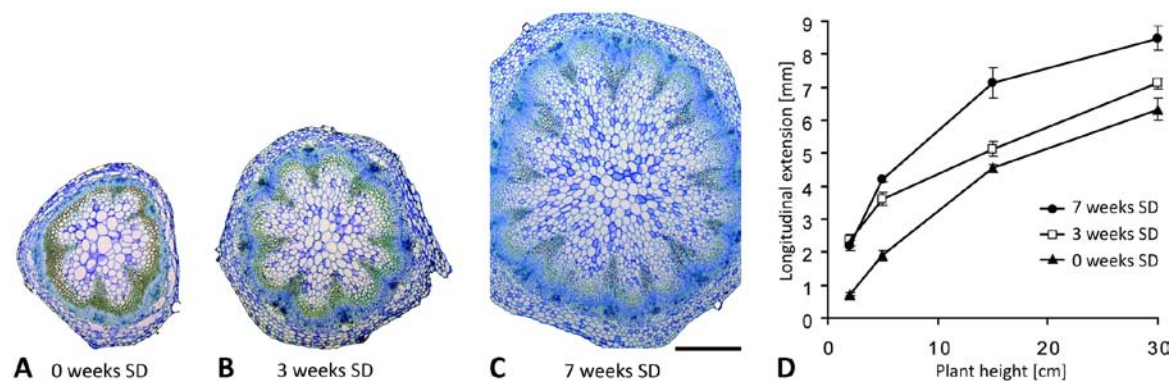


Figure 14: Various short day lengths are influencing SG. Plants grown for 3 weeks in SD conditions before they were shifted to LD conditions (B) showed more vascular bundles, a thicker stem diameter and thicker cell walls of the IFs than plants grown only in LD conditions (A). On the other hand, those plants grown for 3 weeks in SD conditions developed much less vascular bundles, a thinner stem diameter and thinner cell walls of the IFs than plants grown for 7 weeks in SD conditions (C). Furthermore, the longitudinal extension of SG increased the longer the plants were grown in SD conditions (D). Scale bar: 500 µm. Error bars: standard error of means (SE).

In conclusion, the initiation of the vascular cambium along the stem measured by the longitudinal extension of SG is positively influenced by extended periods of plant growth in *Arabidopsis*. However, the ability to initiate the IC and to undergo SG in general was present under all growth conditions.

Cambial activity at nodes of the inflorescence stem

Whether and how cambial activity occurs in nodal parts of the *Arabidopsis* stem is still not clear. To study IC initiation at nodal parts, nodes of the main inflorescence stem of around 20 cm high plants were subjected to histological analysis (Fig. 15).

Below the node (Fig. 15A) no cambial cell divisions in the interfascicular regions were observed. At this position, the stem shape was oval and two distinct vascular bundles (black asterisks) were branching. One branch of the bundle was connected to the bract/side shoot and the other branch remained part of the main shoot. Additionally, the vascular bundle between the branching bundles (red asterisk) differed from all the others in having a broader shape, less tracheary elements and thus more parenchyma cells within the xylem tissue. At the base of the node, cambial cell divisions were detected in the interfascicular regions of the bract/side shoot (Fig. 15B, arrows). Further apically, the formation of the interfascicular cambium (Fig. 15C, arrows) as well as the migration of the branched bundles towards the bract/side shoot became more evident. These bundles indicated the branch traces which are the starting point of the side shoot vasculature, whereas the dominant bundle (red asterisk) displayed the leaf trace. A few

sections further apically, cambial activity was observed in the side shoot (Fig. 15D, E, arrows). At the top of the node the two vascular systems of the main and side shoot disconnected and the latter started to separate from the main shoot (Fig. 15F, G). Above the branching point (Fig. 15H) no cambial cell divisions in the interfascicular region where the side shoot emerged were observed. The outgoing leaf trace of the main stem (Fig. 15A, red asterisk) left a so called leaf gap behind (Fig. 15H, red arrow).

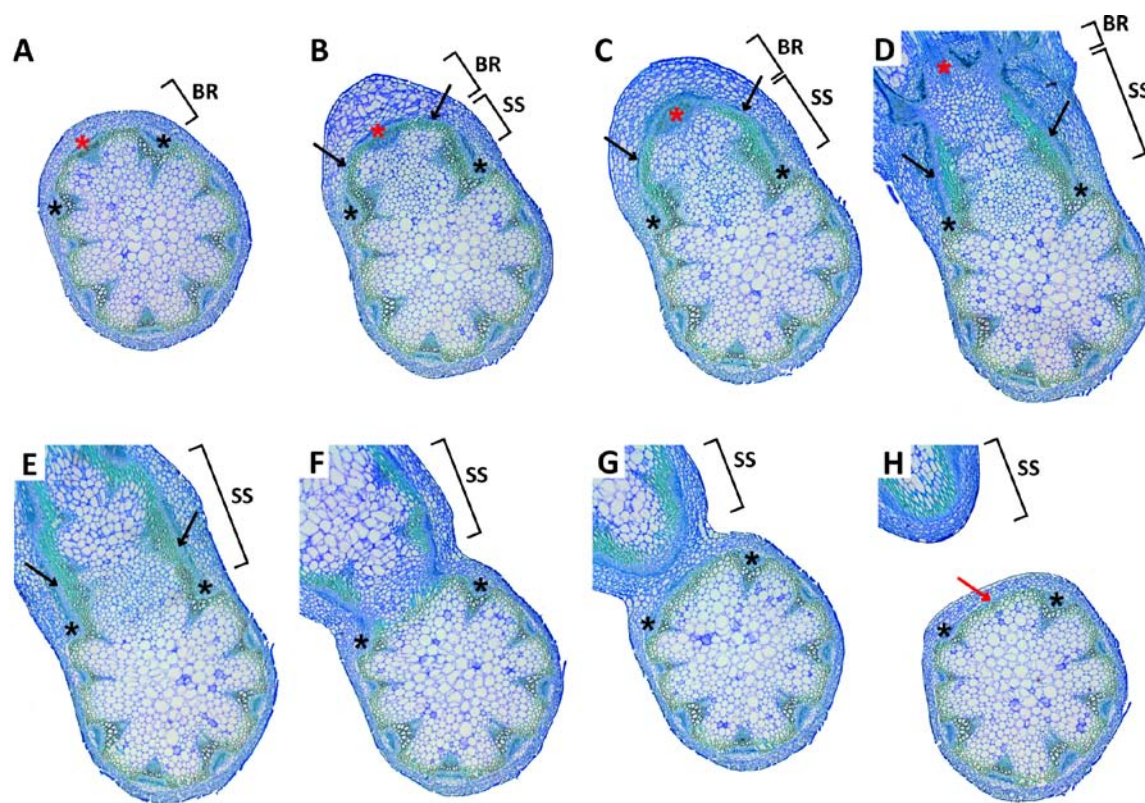


Figure 15: Histological analysis of a node. A series of sections was made through a node starting below the node (A), within the node (B-G) and above the node (H). Black asterisks mark the fate of the vascular bundles of the main shoot which were branching into side shoot traces. The red asterisk marks the fate of the leaf trace. BR: bract, SS: side shoot. Scale bar: 500 μ m.

In summary, the analysis of nodes showed that cambial cell divisions were present only in the bract/side shoot. No IC formation was observed in the nodal region of the main stem. Therefore, SG in *Arabidopsis* shoots seems to be restricted to their basis.

***Arabidopsis* produces secondary vascular tissues in interfascicular regions**

That *Arabidopsis* undergoes SG has been shown already for the root and hypocotyl (Chaffey, Cholewa et al. 2002), however, for the stem evidence for the production of secondary vascular

tissues as it is the case for trees is missing. To address this aspect, marker lines visualising either the phloem or the xylem were analysed.

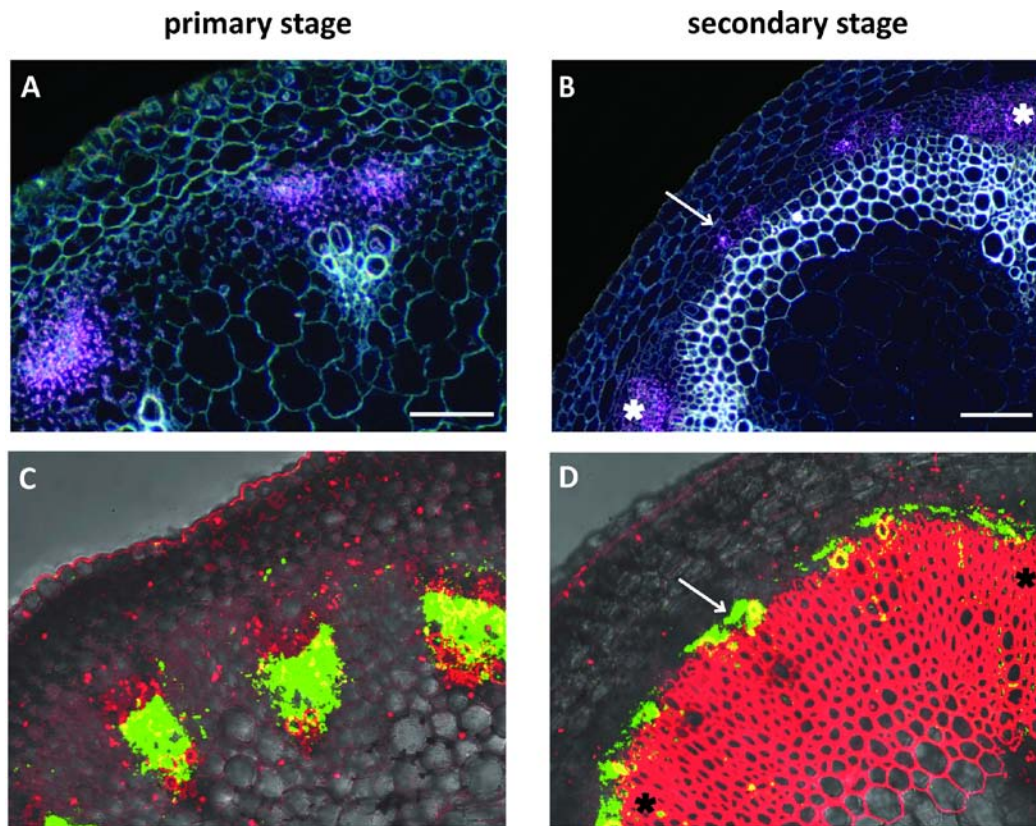


Figure 16: Analysis of cell identity of IC-derived tissue. By analysing the *APL:GUS* marker line (**A, B**) it was shown, that secondary phloem was produced in interfascicular regions, which can be seen by the violet GUS stained cell clusters in the interfascicular region (**B, arrow**). Samples were taken from 15 cm (**A**) and 25 cm (**B**) tall stems at a height of approximately 0.5 mm above the rosette. The same was true for the marker line J1721 (**C, D**). At the secondary stage secondary xylem marked by GFP (green signal) was observed in the interfascicular region (**D**). Sections were taken from 5 cm (**C**) and 30 cm (**D**) high plants and were counterstained with propidium iodide (red signal) White asterisks mark vascular bundles. Scale bars: 100 µm.

Cells with phloem identity are marked by the *APL:GUS* reporter (Bonke et al. 2003; Sehr et al. 2010) whereas xylem identity is labelled in the GAL4 enhancer trap line J1721 (Sawchuk et al. 2007). By comparing the primary and secondary stage of the *APL:GUS* marker line (Fig. 16A, B), secondary phloem was found to cluster in the interfascicular region of a stem in the secondary stage (Fig. 16B, arrow). When J1721 was analysed, less clustered GFP signal was detected proximal to the IC. Whereas in the primary stage the GFP signal was present only in the primary xylem of the vascular bundles (Fig. 16C), in the secondary stage xylem tissue could be detected in the interfascicular region (Fig. 16D, arrow).

To clearly distinguish between fibres and secondary vessel elements of the xylem tissue in the interfascicular region, the *nst1-1 nst3-1* double mutant was analysed, in which secondary cell

wall formation in fibres but not in vessel elements is effected (Mitsuda et al. 2007). In this double mutant at the stem base proximal to the IC, groups of vessel elements organised in typical tangential cell files were observed (Fig. 17B, arrows). This observation indicates the formation of secondary xylem in interfascicular regions. However, vessel elements were not produced in a continuous domain adjacent to the vascular cambium but, like secondary phloem, in distinct cell clusters.

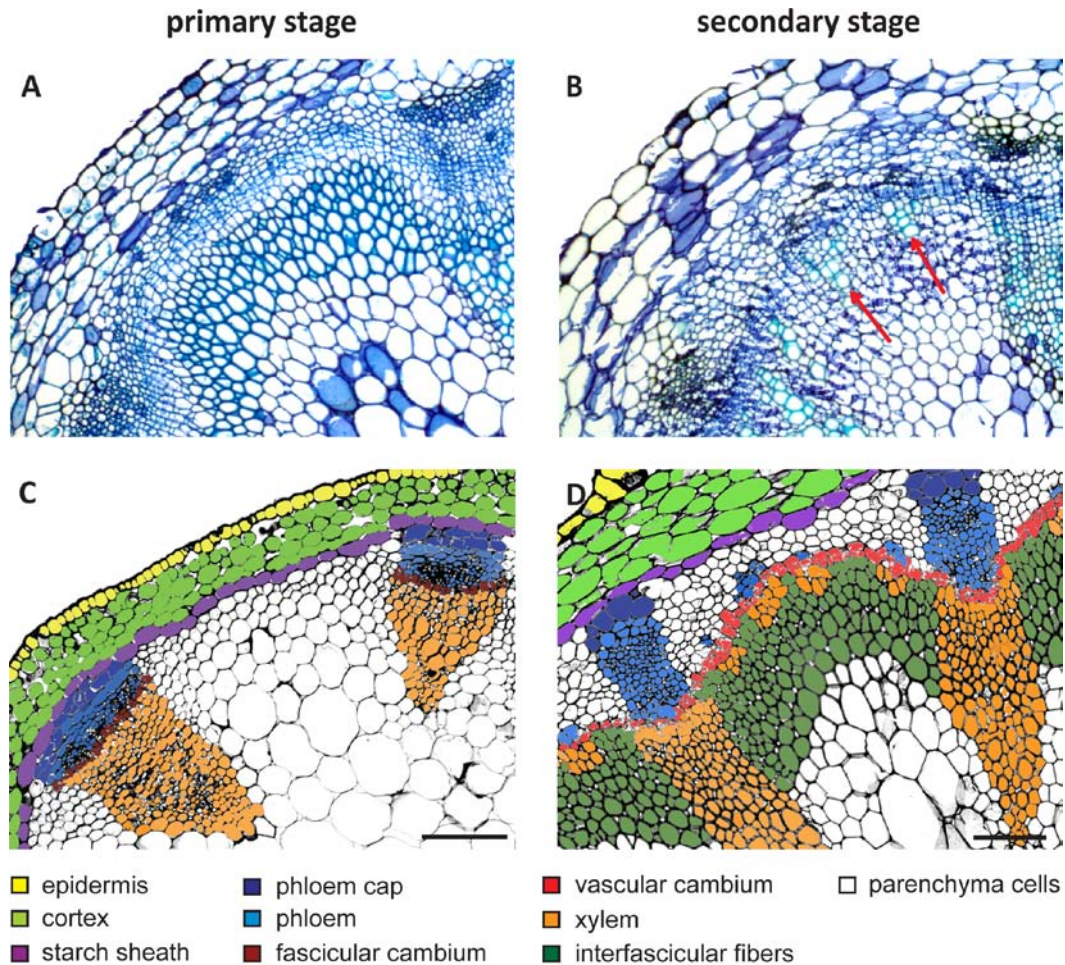


Figure 17: Formation of vessel elements during SG. Only a few groups of vessel elements were detected in the interfascicular region of the *nst1-1 nst3-1* stem base (**B**, arrows) in comparison to the overall lignified wild type situation at this stage (**A**). The schemes depict the tissue composition of the primary and secondary stem (**C**, **D**). The primary stem is composed of epidermis, cortex with its innermost cell layer, the starch sheath, the pith and the primary vascular bundles (**C**). The secondary stage is characterised by the tube-like vascular cambium, which produces secondary phloem and xylem in the fascicular as well as in the interfascicular region (**D**). Additionally, interfascicular fibres have developed between vascular bundles. Scale bar: 100 μ m.

In summary, secondary phloem and xylem tissue was formed in the interfascicular region at the base of the *Arabidopsis* inflorescence stem (Fig. 17D). However, no continuous zone of both tissues was established as it is for example the case in trees.

Does the tissue composition in interfascicular regions influence IC formation?

The question was raised whether the identity of a particular tissue type in the interfascicular region is necessary to initiate IC formation and whether cells in this area are predetermined for this process. As described before, the main tissue types between the vascular bundles are cortex, starch sheath and IFs. To address this aspect, a starch sheath-specific marker line and mutants lacking different tissue types were analysed.

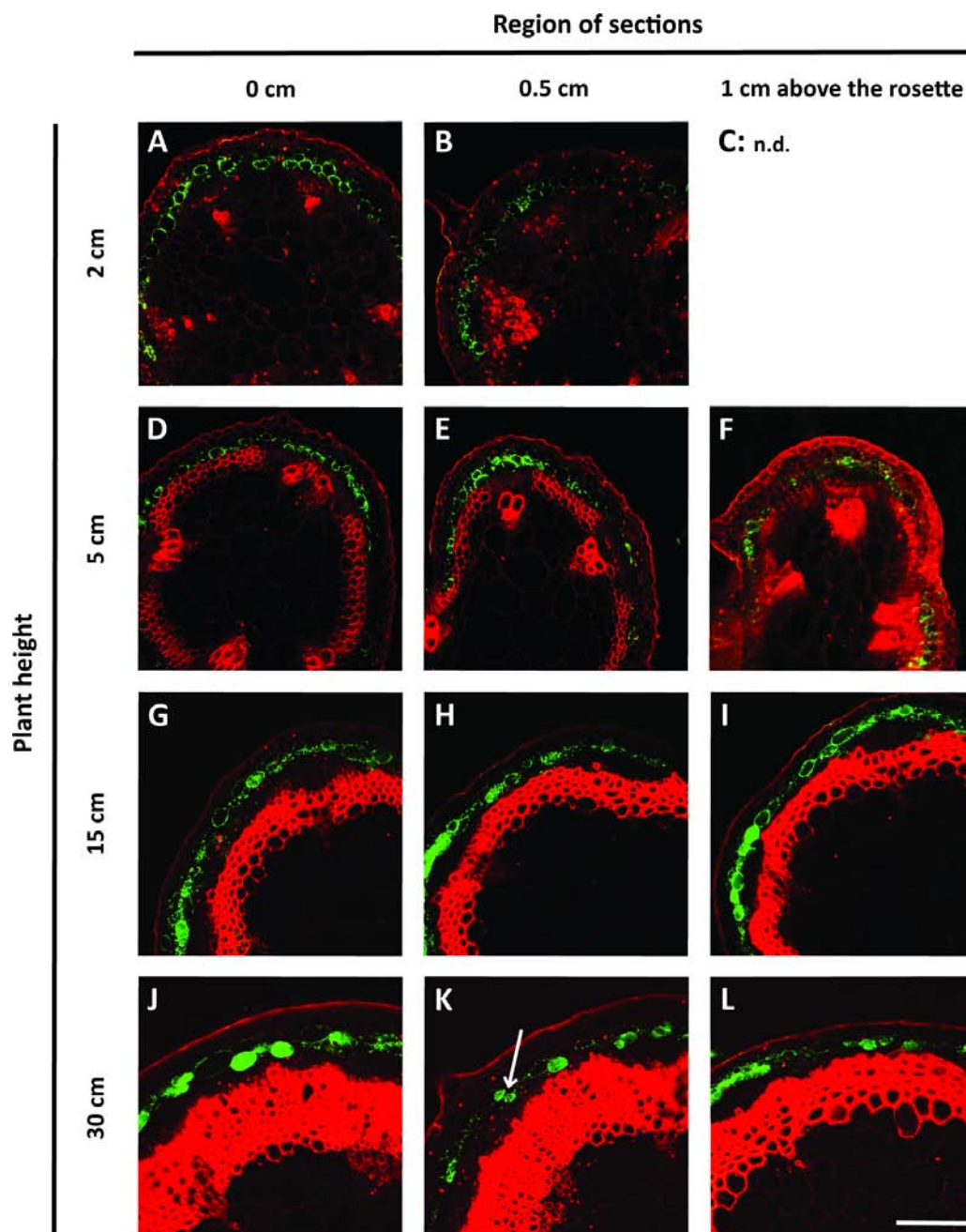


Figure 18: Fate of starch sheath identity during IC initiation. The *SCR:GFP* marker line (Wassilewskija background) was analysed during plant development at the base of 2 (**A, B**), 5 (**D-F**), 15 (**G-I**) and 30 cm (**J-L**) tall stems which were sectioned directly at the base (0 cm above the rosette), in the mid (0.5 cm

above the rosette) and in the top region of secondary growth (1 cm above the rosette). In all positions at all developmental stages a clear GFP signal in the starch sheath was visible. Sections were counterstained with propidium iodide (red signal). Scale bar: 100 μ m.

In previous studies, the starch sheath is described to be the origin of the IC (Altamura, Possenti et al. 2001; Ye, Freshour et al. 2002), which was confirmed by the results described above, where it is shown, that the starch sheath is partially and predominantly in advanced growth stages involved in the formation of the IC. To elucidate the dynamics of starch sheath identity during IC formation the marker line *SCR:GFP* was analysed (Wysocka-Diller et al. 2000). The GFP signal was present in the starch sheath in all developmental stages analysed as well as along the whole region with IC initiation (Fig. 18). This suggests that the identity of the starch sheath is maintained during IC initiation. The GFP signal appeared not only in a single row of cells, but frequently in two cell layers (Fig. 18K, arrow). Thus, with this marker line, it was not possible to confirm that cell divisions happen in the starch sheath.

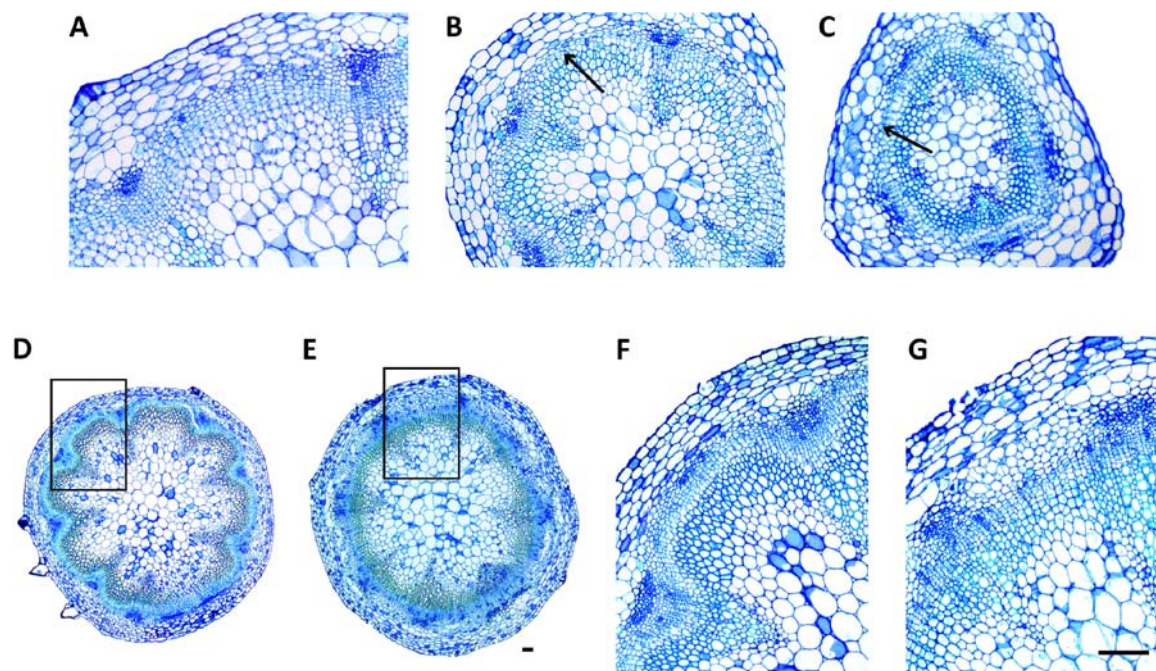


Figure 19: IC formation in *shr-2*, *scr-3* and *ifl1/rev-5*. In the starch sheath-less mutant *scr-3* (B) and *shr-2* (C) the IC was formed similarly to the wild type situation (A). In both mutants a clear interfascicular cambium with its distinct cell divisions was visible (arrows). The same was true for *ifl1/rev-5* (E, G), an interfascicular fibre-less mutant, in which the IC was established like in wild type but shifted towards the centre of the stem (D, F). Sections were taken at the base of 5 cm high stems (A-C) and 15 cm high stems (D-G). Scale bars: 200 μ m.

To elucidate the necessity of the starch sheath during IC initiation, *shr-2* and *scr-3*, two mutants lacking the starch sheath and thus having gravitropic defects in roots and shoots (Fukaki et al. 1998) were analysed histologically. In both lines the IC was established similarly to wild type proving that starch sheath identity is not crucial for the formation of the IC (Fig. 19B, C, arrows).

The other main tissue type in the interfascicular region is represented by the IFs. To elucidate the importance of this specific tissue type during IC formation, the *ifl1/rev-5* mutant line (Zhong and Ye 1999; Otsuga et al. 2001), which lacks the IFs, was analysed. Again, in this mutant the IC was established like in wild type (Fig. 19D-G) suggesting that the identity of the IFs is not important for the process of IC formation. However, in *ifl1/rev-5* the establishment of the IC was shifted towards the centre of the stem, thus, giving the vascular cambium a ring-like pattern (Fig. 19E), whereas in wild type plants the vascular cambium is patterned like a circular sine curve (Fig. 19D). This suggests that the presence of the IFs determines the position of the IC.

Whether this shifted position of the IC in the *ifl1/rev-5* mutant is due to the lack of lignified secondary cell walls in interfascicular regions was clarified by analysing the *cad-c cad-d* double mutant in which the lignification of cell walls is dramatically reduced (Sibout et al. 2005). Histological analyses revealed no change in the position of the IC in this double mutant (Fig. 20B) arguing against a major impact of cell wall lignification on IC position. Taking also into account the wild type-like position of the IC in the *nst1 nst3* double mutant in which the formation of secondary cell walls is severely impaired (Fig. 17), IF-specific cell wall properties do not seem to be a reason for an impact of IF formation on IC position.

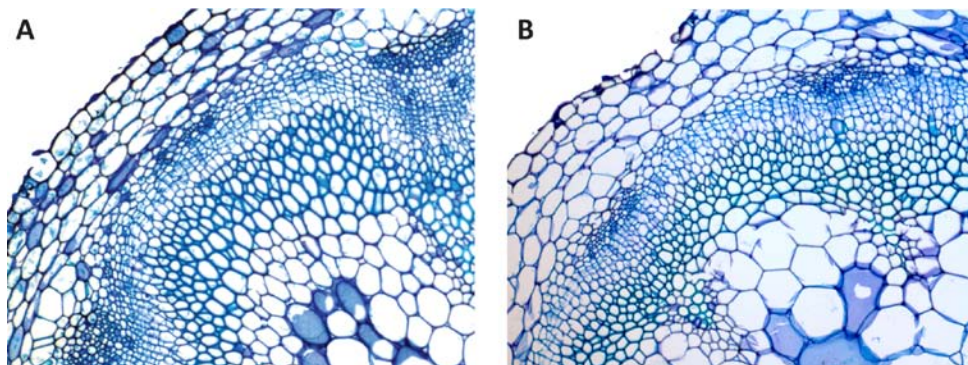


Figure 20: IC formation in *cad-c cad-d*. The IC was formed similarly to the wild type situation (A) in the *cad-c cad-d* double mutant (B). No shift of the IC towards the centre was observed as described in the *ifl1/rev-5* mutant (see Fig. 19).

Taken together, these results suggest that not the IC initiation per se but its position is influenced by tissue characteristics, in particular by IF characteristics, present in the interfascicular region.

Results – Part II – Hormonal control of SG: Auxin

Auxin accumulation at the stem base

Auxin plays a crucial role in many processes of plant development. More importantly in the context of this work, it has been shown to be a key regulator of secondary growth (Tuominen, Puech et al. 1997; Wang et al. 1997; Little, MacDonald et al. 2002). With the following experiments I intended to see whether the process of IC initiation is positively correlated with auxin accumulation/signalling especially at the base of the inflorescence stem and whether auxin itself could function as a stimulus for IC initiation.

Analysis of the *DR5rev:GFP* marker line

DR5rev is a synthetic promoter consisting of several repeats of a specific 11 bp long composite auxin response element (AuxRE) (Ulmasov et al. 1997). *DR5*-driven reporters are reported to show high auxin responsiveness and thus serve as useful tools for studying the amount of endogenous IAA levels in living cells (Sabatini et al. 1999; Benkova et al. 2003; Bai and Demason 2008). However, one has to take into consideration that indications have accumulated, that the *DR5* promoter is not only activated upon auxin, but also upon other hormones like brassinolide (Nakamura et al. 2003) and thus may not reflect the natural auxin distribution pattern and its subsequent signalling but may be “contaminated” by other hormone-induced pathways. Furthermore, *DR5* activation does not directly depend on auxin, but reports auxin response. Thus, interpretation of data must take into consideration that *DR5* activity represents merely auxin concentration as well as the presence of auxin signalling/response components (Chandler 2009). Additionally, a distinct threshold of expression is necessary for visualisation, below which expression may be undetected. Nevertheless, the *DR5* promoter is the so far most applicatory tool for visualising auxin accumulation/signalling in living plant cells.

...in intact plants

To visualise auxin signalling/accumulation at the stem base in interfascicular regions the *DR5rev:GFP* marker line (Benkova, Michniewicz et al. 2003) was analysed during plant development (Fig. 21). 2, 5, 15 and 30 cm high plant stems were hand-sectioned at the very base, at 0.5 cm and at 1 cm above the rosette and analysed by confocal microscopy.

At the base of 2 cm high plants a clear signal in the primary vascular bundles was observed. However, in the interfascicular region only a few cells proximal to the starch sheath showed auxin signalling activity (Fig. 21A, arrow). At 0.5 and 1 cm, no GFP signal was found in the interfascicular region (Fig. 21B, C).

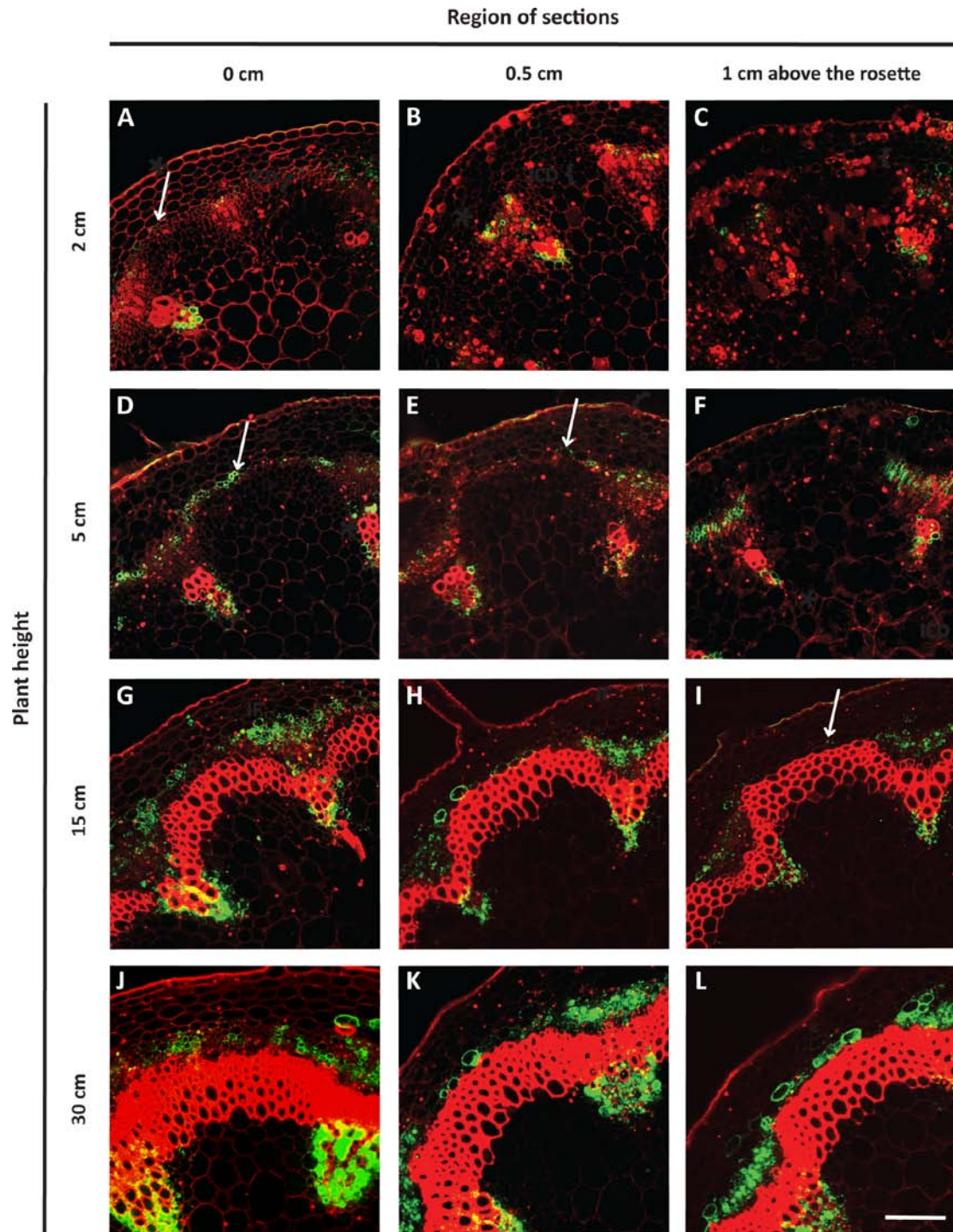


Figure 21: Auxin signalling/accumulation at the stem base. In 2 cm high stems (**A-C**) a signal in the primary vascular bundles can be seen both in xylem and phloem. In the interfascicular region of the base section only a few cells adjacent to the starch sheath displayed a GFP signal (**A**). Auxin signalling proximal to the starch sheath in the interfascicular region was detected at the base of 5 cm tall stems (**D**). 0.5 cm above the rosette a weak signal was detected in the starch sheath (**E**). In 15 cm high plants high auxin signalling appeared at the base in the vascular bundles as well as in the interfascicular region proximal to the starch sheath (**G**). 0.5 cm above the rosette, GFP signal appeared to be exclusively in the starch sheath (**H**). 1 cm above the rosette only a few starch sheath cells displayed a GFP signal (**I**, **arrow**). In 30 cm tall stems auxin signalling was visible up to 1 mm above the rosette (**J-L**). At the base auxin signalling occurred in the interfascicular region proximal to the starch sheath (**J**), whereas at the positions 0.5 and 1 cm above the rosette the starch sheath itself showed high auxin signalling (**K**, **L**). Hand sections were counterstained with propidium iodide. Scale bar: 100 μ m.

In 5 cm high stems auxin signalling was clearly present in the interfascicular region from the base up to 0.5 cm above the rosette (Fig. 21D, E). At the very base, the GFP signal appeared in cells proximal to the starch sheath where also SG takes place, whereas 0.5 cm above the rosette the starch sheath itself carried GFP (Fig. 21D, E, arrows). The same was true for 15 cm high stems. However, a few starch sheath cells with GFP signal were already visible 1 cm above the rosette (Fig. 21I, arrow). In 30 cm high stems the region of auxin signalling extended up to 1 cm above the rosette (Fig. 21J-L). Be reminded that, according to histological analyses, a closed cambial cylinder can be found only up to a height of around 7 mm at that stage of development (see Fig. 13A). Here as well, GFP signalling was detected adjacent to the starch sheath and within the starch sheath in more apical positions.

Taken together, auxin signalling visualised by the *DR5rev:GFP* marker line is specifically found in cambium initiating cells even before those cells show any sign of cambium activity.

...in vitro

The results above demonstrate that auxin accumulation/signalling positively correlate with IC initiation. With this, the question arose whether auxin has the potential to trigger IC formation. It has been already shown that synthetic auxin (NAA) utilising the split plate *in vitro* system (see p. 43) induces IC in the interfascicular region of stem pieces in which no SG takes place under natural conditions (Lichtenberger 2010).

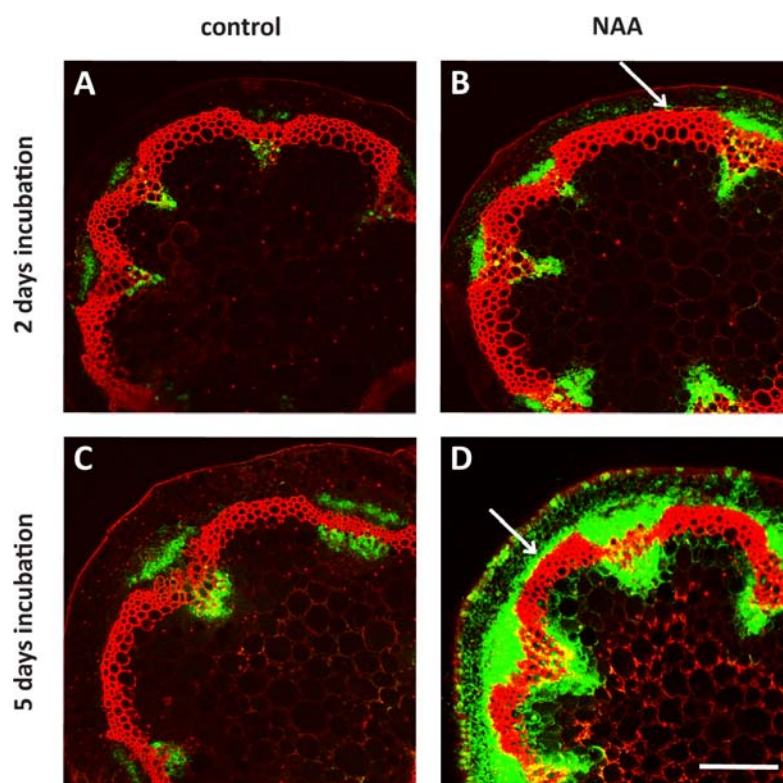


Figure 22: Auxin signalling/accumulation utilising the *in vitro* system. After a 2 day incubation of a stem piece in a split plate with apically applied NAA (1 µg/ml) GFP signal and thus auxin signalling/accumulation appeared to be in a few cells in the interfascicular region (**B, arrow**) in contrast to the control without any hormone application (**A**). The GFP signal itself was overall enhanced. After a 5 day incubation the GFP signal became much stronger and was clearly located in the starch sheath in the interfascicular region (**D, arrow**) although diffusion of the signal into the cortex, epidermis and pith occurred presumably due to the hormone overdose. Scale bar: 100µm.

In this study the marker line *DR5rev:GFP* was used in the *in vitro* system to study auxin signalling upon NAA treatment. As a result, not only the IC can be induced in the interfascicular region but also auxin signalling/accumulation visualised by the GFP signal was induced very locally in the starch sheath of the interfascicular region slightly after a 2 day and heavily after a 5 day incubation (Fig. 22).

In conclusion, these results from the *in planta* and *ex planta* experiments suggest that high auxin signalling/accumulation promotes the process of IC formation cell-autonomously and that it represents a very early event during this process.

Is polar auxin transport important for IC initiation?

In addition to a passive mass flow of auxin, the hormone is transported actively via cell-to-cell. This polar auxin transport (PAT) brings auxin from its site of biosynthesis, e.g. in young lateral organs, well-directed to its site of action (Goldsmith 1977; Zazimalova et al. 2007; Delker et al. 2008). Since the results above suggest that auxin is a local trigger for IC initiation, the question arose, whether PAT is necessary for the auxin accumulation/signalling during IC formation. In *Arabidopsis*, members of the *PIN*, the *AUX1/LAX* and the *ABCB/PGP* gene families are known to be active auxin transporters and thus main mediators of PAT (Parry, Marchant et al. 2001; Geisler and Murphy 2006; Zazimalova, Krecek et al. 2007; Mravec et al. 2008; Krecek, Skupa et al. 2009). Additionally, PAT is known to be regulated by the protein kinase PINOID (PID) and its antagonist the PP2A phosphatase, and the D6 protein kinases (Benjamins et al. 2001; Michniewicz et al. 2007; Zourelidou et al. 2009; Zhang, Nodzynski et al. 2010). These connections are good reasons to further investigate PIN, AUX1/LAX, D6PKs and PID, since all are known to interfere directly or indirectly with PAT.

PIN gene family

The *PIN-FORMED (PIN)* gene family is classified as auxin efflux facilitators and consists of 8 members of transmembrane proteins, from which PIN1, 2, 3, 4 and 7 are localised at the plasma membrane in a polar fashion and PIN5, 6 and 8 were recently classified as being localised at the

endoplasmatic reticulum membrane (Krecek, Skupa et al. 2009; Mravec et al. 2009). Furthermore, *PIN5* and *PIN8* are sharing similar sequences and forming together with *PIN6* the “endoplasmatic reticulum clade” which is opposed to the “plasma membrane clade” consisting of *PIN2*, *PIN1*, *PIN4* and the most derived branch, *PIN3* and *PIN7* (Mravec, Skupa et al. 2009) (Fig. 23A). Utilising the AtGenExpress Visualisation Tool (Schmid et al. 2005) the expression profiles of the stem tissues of each *PIN* were analysed (Fig. 23B). A high level of transcript accumulation in the stem tissue was observed especially for *PIN3* and *PIN1*, followed by *PIN7*, 4 and 6. Based on these data the most prominent *PINs* regarding their expression levels in the stem, namely *PIN1*, *PIN3*, *PIN6* and *PIN7*, were chosen for further investigation using mutant lines.

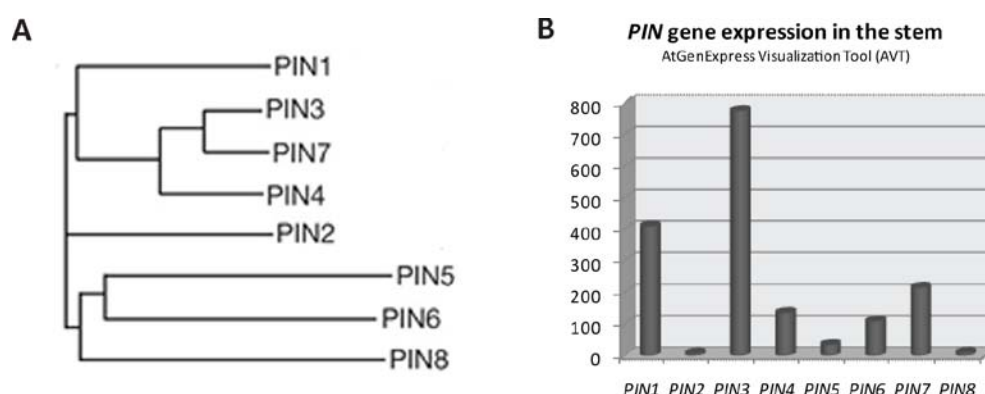


Figure 23: *PIN* gene family. The phylogenetic tree, taken from Mravec et al. (2009), supports the separation into an “endoplasmatic reticulum clade”, which includes *PIN5*, *PIN8* and *PIN6*, and a “plasma membrane clade” including the remaining *PIN1-4* and *PIN7* (A). For the comparison of the *PIN* expression in the stem, the expression intensities of the stem tissues (hypocotyl, first node, second internode) were averaged (B).

pin1

The *pin-formed 1* mutant is characterised by naked, pin-forming inflorescences, abnormal vein branching in leaves, fasciated inflorescences and a poor basipetal auxin transport (Okada et al. 1991; Gälweiler et al. 1998). In this study the SALK insertion line *pin1-613/+* was analysed as described above. In homozygous mutants, a closed cambial cylinder and thus IC formation were formed like in wild type (Fig. 24B, C), however, the patterning of the vasculature (shape of bundles, cell size, and abundance of IFs) and the stem diameter were highly variable within the samples analysed. When compared to wild type, the longitudinal extension of SG was significantly less in the mutant with 0.6 ± 0 mm in 2 cm, 2.58 ± 0.35 mm in 5 cm, 4.8 ± 0.11 mm in 15 cm, and 5.17 ± 0.12 mm in 30 cm high plants (Fig. 24K).

pin3

Mutants defective in *PIN3* show reduced gravitropic and phototropic responses as well as reduced apical hook maintenance (Friml et al. 2002). Here, the line *pin3-5* obtained from the

SALK collection was analysed. Besides having no aberrant patterning of the stem and its vasculature, a wild type-like formation of the cambium and a normal secondary growth was observed (Fig. 24E, F). However, the longitudinal extension of SG was significantly decreased in comparison to the wild type, with 1.43 ± 0.25 mm in 2 cm, 2.37 ± 0.32 mm in 5 cm, 4.34 ± 0.22 mm in 15 cm, and 5.95 ± 0.05 mm in 30 cm tall stems (Fig. 24K).

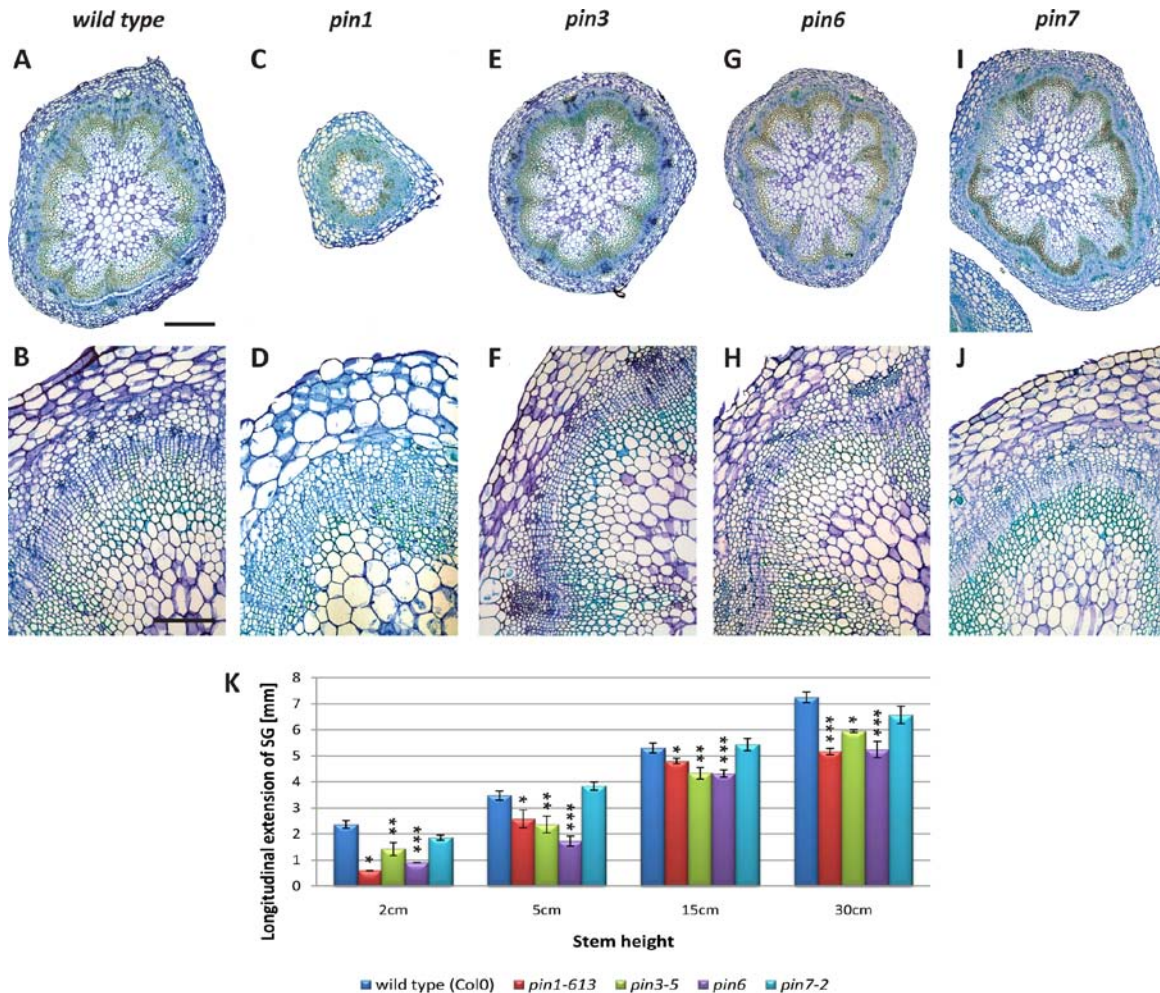


Figure 24: Histological analysis of *PIN* mutant lines. Cross sections of the base of 30 cm high plant stems are shown for wild type (A, B), *pin1-613* (C, D), *pin3-5* (E, F), *pin6-1* (G, H) and *pin7-2* (I, J). *pin3*, *pin6* and *pin7* are comparable to wild type in its vascular patterning and secondary growth. *pin1* initiates secondary growth, but shows high variability in the stem architecture among individuals. The longitudinal extension of SG is significantly less in *pin1*, *pin3* and *pin6* in comparison to the wild type whereas *pin7* is not significantly different (K). Scale bar: 500 μ m (A, C, E, G, I) and 100 μ m (B, D, F, H, J). Error bars: standard error of means (SE).

pin6

The *pin6* mutant has not been characterised so far. For this study the insertion line SALK_046393 for *PIN6* was used and designated as *pin6-1*. The overall growth habit of this mutant line was wild type-like, as well as the stem diameter, the vascular patterning and the formation of the cambium (Fig. 24G, H). The longitudinal extension of SG in *pin6-1* was highly significant less than

in wild type. 2 cm high plants showed an extension of 0.9 ± 0 mm, 5 cm high plants 1.73 ± 0.2 mm, 15 cm high plants 4.32 ± 0.14 mm and 30 cm high plants 5.24 ± 0.31 mm (Fig. 24K).

pin7

PIN7 is important for the establishment of the apical-basal auxin gradient during embryogenesis. However, due to the high redundancy within the *PIN* gene family, the *pin7* mutant recovers and develops like wild type in subsequent growth stages (Friml et al. 2003). Here, the line *pin7-2* obtained from the SALK collection was used. This mutant line showed in all aspects a wild type-like phenotype (Fig. 24I, J, K), even the longitudinal extension of SG was not significantly different from wild type with 1.86 ± 0.1 mm in 2 cm, 3.84 ± 0.15 mm in 5 cm, 5.44 ± 0.23 mm in 15 cm, and 6.57 ± 0.33 mm in 30 cm tall stems.

In conclusion, all analysed loss-of-function mutants except *pin7* showed a reduced longitudinal extension of SG which implies less ability to initiate cambial activity along the stem. This suggests that PAT sustained by single PIN proteins contributes quantitatively to IC initiation.

AUX/LAX gene family

The *AUXIN RESISTANT 1 (AUX1)* gene family of auxin influx carriers consists of *AUX1* (Bennett et al. 1996), *LAX1*, *LAX2* and *LAX3 (LIKE AUX1)* and belongs to the ATF (amino acid transporter) family of proteins (Parry, Marchant et al. 2001). It has been shown for the *AUX1* and *LAX3* protein that they are plasma membrane-localised and actively transport auxin into cells. More specifically, *AUX1* is required for both polar- and phloem-based auxin transport pathways in shoot and root tissues (Swarup et al. 2004; Yang et al. 2006; Swarup et al. 2008). For the stem, no expression data are available so far. However, *AUX1* is expressed in a subset of columella, lateral root cap and stele tissues in the root. Reduced basipetal auxin transport has been reported for the *aux1* root apical tissues (Swarup et al. 2001). Since no aerial phenotype has been previously described for *aux1* or for the *lax* single mutants, the already available quadruple mutant was further investigated.

aux1-21 lax1 lax2 lax3

The quadruple mutant shows a highly irregular phylotactic pattern in the rosette as well as in the inflorescence, and an aberrant distribution of auxin and the loss of PIN polarisation at the SAM (Bainbridge et al. 2008). By analysing cross sections, no differences between the wild type and the quadruple mutant regarding vasculature and its pattern were observed. However, the quadruple mutant showed a broader zone of IFs than the wild type (Fig. 25A, B). The longitudinal extension of SG was significantly less in the mutant than in wild type with the values of $0.95 \pm$

0.1 mm in 2 cm, 1.71 ± 0.18 mm in 5 cm, 4.33 ± 0.25 mm in 15 cm, and 5.5 ± 0.25 mm in 30 cm tall stems (Fig. 25C).

D6PKs

The D6 PROTEIN KINASE (D6PK) belongs to a subfamily of the AGCVIII subgroup of the *Arabidopsis* AGC kinase family. Within this subfamily three close homologs D6PK-LIKE1–3 (D6PKL1–3) are found. It has been shown that PIN proteins are substrates of D6PKs but PIN polarity is unaltered in *d6pk* mutants. D6PKs show expression in the vasculature of the cotyledons and, partly, in the hypocotyl as well as broadly in the root (Zourelidou, Muller et al. 2009). No expression data are available for adult plants, and especially for stems. Single mutant lines do not display any aberrant phenotype, but multiple mutant lines show several developmental defects (deficient in lateral root formation, twisted leaves, fewer axillary shoots, partially infertile, mildly agravitropic). Furthermore, reduced auxin transport was detected in *d6pk* mutants (Zourelidou, Muller et al. 2009). In this study, the triple mutant *d6pk d6pk1 d6pk2* was used for analysing the dynamics of IC initiation. The anatomy of cross sections was wild type-like (Fig. 25D, E), but the longitudinal extension of SG in this mutant was significantly less than in wild type, namely 1.24 ± 0.17 mm in 2 cm tall stems, 2.46 ± 0.15 mm in 5 cm, 4.05 ± 0.23 mm in 15 cm, and 5.04 ± 0.39 mm in 30 cm high plants (Fig. 25F).

PINOID

The *PID* gene belongs likewise to the AGCVIII subgroup of the *Arabidopsis* AGC kinase family and encodes a protein-serine/threonine kinase and positively regulates polar auxin transport via the control of PIN polarity (Zhang, Nodzynski et al. 2010). *PID* itself is auxin responsive, directly phosphorylates PIN proteins and is expressed inter alia in the vasculature. However, no vascular defects were detected in the loss-of-function mutants (Benjamins, Quint et al. 2001; Zhang, Nodzynski et al. 2010). The overall phenotype of *pid* mutants closely resembles that of *pin1* mutant plants, but less severe. *pid* mutants show a reduction of PAT, especially in older stems (Bennett et al. 1995). Here, the allele *pid-3* (Landsberg *erecta* background) was further analysed. The habit of *pid-3* was similar to the wild type Landsberg *erecta*, although the mutants grew slightly higher than the wild type plants. Regarding SG, the mutant line showed a significant higher longitudinal extension during plant development than the wild type control. 2 cm high plants of *pid-3* showed 1.26 ± 0.2 mm, 5 cm 1.62 ± 0.26 mm, 15 cm 2.83 ± 0.14 mm and 30 cm tall stems 4.52 ± 0.49 mm (Fig. 25I). In comparison to that, the longitudinal extension of SG in wild type plants of the background Landsberg *erecta* was 0.28 ± 0.25 mm in 2 cm, 0.34 ± 0.10 mm in 5 cm, 0.5 ± 0.12 mm in 15 cm and 2.44 ± 0.13 mm in 30 cm tall stems (Fig. 25I).

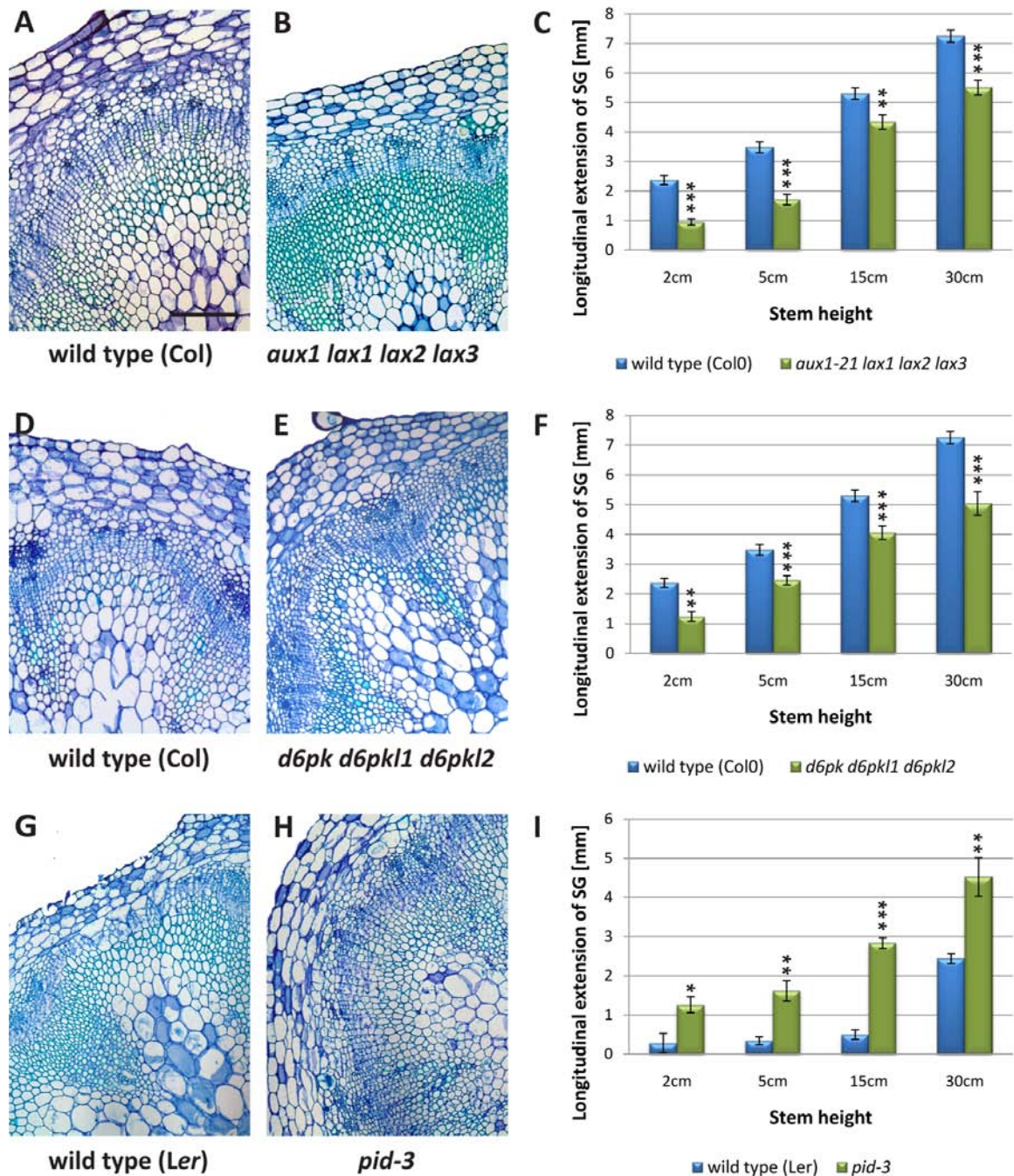


Figure 25: Histological analysis of *AUX/LAX*, *PID* and *D6PK* mutant lines. Cross sections of the base of 30 cm high plant stems are shown for wild type (A) and *aux1 lax1 lax2 lax3* (B). The quadruple mutant is comparable to wild type in its vascular patterning and IC initiation dynamics. The longitudinal extension of SG is significantly less in the quadruple mutant in comparison to the wild type (C). Cross sections of the base of 15 cm high plant stems are shown for wild type (D) and the *d6pk* triple mutant (E). In comparison to the wild type, the longitudinal extension of SG is significantly less in the mutant (F). For the Landsberg *erecta* wild type (G) and *pid-3* (H) cross sections of 15 cm tall stems are shown. At all stages analysed, the longitudinal extension of SG is significantly increased in the mutant in comparison to the wild type (I). Scale bar: 100 μ m. Error bars: standard error of means (SE).

In summary, all the mutants analysed were able to initiate the IC in general. No abnormalities regarding vasculature and stem architecture were found. However, except for *pid-3*, which showed a significantly increased ability of IC initiation along the stem, all the other mutant lines

had a significantly reduced longitudinal extension of SG along the stem in comparison to wild type plants.

Analysis of *PIN* gene expression

The results above show that no PIN protein alone is essential to establish the IC, but that several PINs contribute quantitatively to regulate IC activity. To explore the site of action of the *PIN* genes along the stem, *PIN:GUS* marker lines were analysed and RT-PCR was performed with focus on the stem base.

GUS marker lines

PIN1 is expressed in the vasculature predominantly in cambial and xylem tissue (Gälweiler, Guan et al. 1998; Lichtenberger 2010). During IC initiation, the *PIN1:GUS* signal spreads into the starch sheath and adjacent cells (Lichtenberger 2010). In this study, GUS-staining of vascular strands was observed with a homogenous reporter gene activity along the stem (Fig. 26A-C). However, GUS signal was elevated at the base of side shoots (Fig. 26B, arrow) and floral branching points, and the receptacle (Fig. 26A, arrow).

For *PIN3*, a vascular-specific expression pattern has been shown for seedlings (Friml, Wisniewska et al. 2002). The *PIN3:GUS* analysis showed that the reporter behaved similarly to the *PIN1:GUS* reporter, although the signal was not clearly restricted to the vascular strands, but was more diffuse (Fig. 26D-F). A peak of expression at branching points and at the receptacle were visible here as well. During IC initiation, the GUS signal spreads into the interfascicular region into the starch sheath and adjacent cells (Lichtenberger 2010).

The GUS signal in the vasculature of the *PIN6:GUS* marker line was weaker when compared to *PIN1:GUS*, but *PIN6* appeared to be highly expressed at the base of the stem exactly in that region where SG occurs (Fig. 26I, arrow). Furthermore, *PIN6* is clearly expressed at the base of side shoots (Fig. 26H, arrow) and at the receptacle (Fig. 26G, arrow). It has been shown, that at the primary stage *PIN6* is exclusively expressed in the fascicular cambium and during IC initiation the expression pattern spreads into the interfascicular cambium (Lichtenberger 2010).

PIN7:GUS resembles *PIN3:GUS* in its activity pattern, although a gradient was observed starting with a low activity at the base of the stem and increasing towards the shoot tip (Fig. 26J-L).

Among the available *PIN:GUS* marker lines, *PIN4:GUS* did not show any GUS signal at all (data not shown).

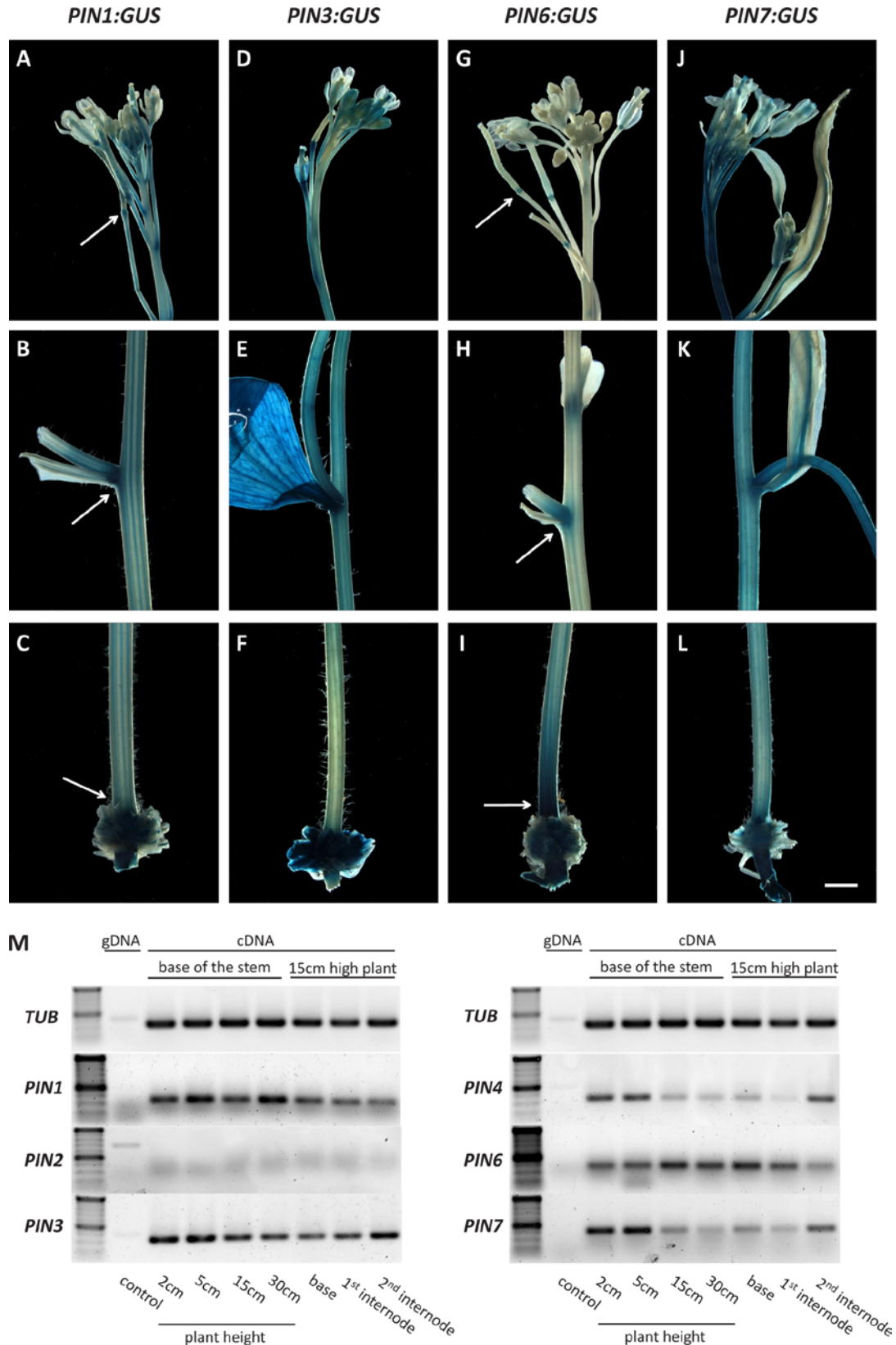


Figure 26: Analysis of *PIN* promoter activity using *GUS* marker lines and RT-PCR. 15-20 cm high plants were *GUS* stained and analysed utilising a binocular. *PIN1:GUS* (A-C) gave a clear expression in the vascular strands. Furthermore, higher expression levels were detected at the base of side shoots (B, arrow) and receptacles (A, arrow). Slightly higher expression at the stem base could be detected (C, arrow). *PIN3:GUS* (D-F) was expressed in the vascular strands, but the signal was more diffuse. Higher expression levels were detected at the base of side shoots and receptacles. *PIN6:GUS* (G-I) was weaker expressed in the

vasculature, but strongly at the stem base (**I, arrow**), at the base of side shoots (**H, arrow**) and at the receptacle (**G, arrow**). *PIN7:GUS* (**J-L**) was expressed in vascular strands, with higher expression at the top of the plant (**J**). Scale bar: 1 mm. For RT-PCR (**M**) cDNA samples of the base of wild type stems of 2 cm, 5 cm, 15 cm and 30 cm height were used. Furthermore, samples of 15 cm high plants were taken from the base, the 1st and the 2nd internode. Genomic DNA was used as control. Primers used for *TUB* and the *PIN*s are listed in the primer table.

RT-PCR analysis

Transcript levels of all *PIN*s relevant for this study were tested by RT-PCR (Fig. 26M). *PIN2* served as a negative control, since it is known to be expressed in the root only (Muller et al. 1998). *TUBULIN* served as a positive control and internal standard.

PIN1 seemed to be transcribed continuously at the same level at the base of the stem during plant development as well as along the stem axis. *PIN3* mRNA levels decreased at the base of the stem during plant growth and increased from base to top along the stem axis. The same was true for *PIN4* mRNA levels, although it was generally weaker than *PIN3*. In contrast to *PIN3*, *PIN6* showed an increase of mRNA accumulation during plant development at the stem base and a decrease from base to top along the stem axis. Transcript accumulation of *PIN7* resembles the one of *PIN4* with a decrease during plant development at the stem base and an increase along the stem axis from base to top.

Taken together, these results suggest activity of *PIN1*, 3, 6 and 7 predominantly in the vascular system. Furthermore, *PIN3*, *PIN4* and *PIN7* mRNA accumulation was higher at the top of the stem than at the base. In contrast, *PIN6* and *PIN1* showed increased promoter activity at the stem base.

Analysis of PIN protein localisation

PAT involves polar localisation of PIN proteins at the plasma membrane. This enables the creation of an auxin flow and concentration gradients which is fundamental for many processes in plant development (Vanneste and Friml 2009). To detect where along and within the stem the PIN proteins are localised, and whether a correlation of protein localisation and IC initiation is detectable, available PIN-GFP protein fusion marker lines for the PINs of interest were analysed utilising confocal microscopy (Fig. 27).

The distribution of *PIN1* within the stem is restricted to the vasculature (Gälweiler, Guan et al. 1998). This is supported by the results obtained here, where *PIN1* localisation along the stem was detected predominantly in tissues of the vascular bundles (Fig. 27A-C). At the base of the stem, the *PIN1*-GFP protein accumulation spread into the interfascicular region starting at the

stage of 5 cm plant height. However, PIN1-GFP accumulation was only observed after the IC was formed localising predominantly to the IC itself (Fig. 27J-M).

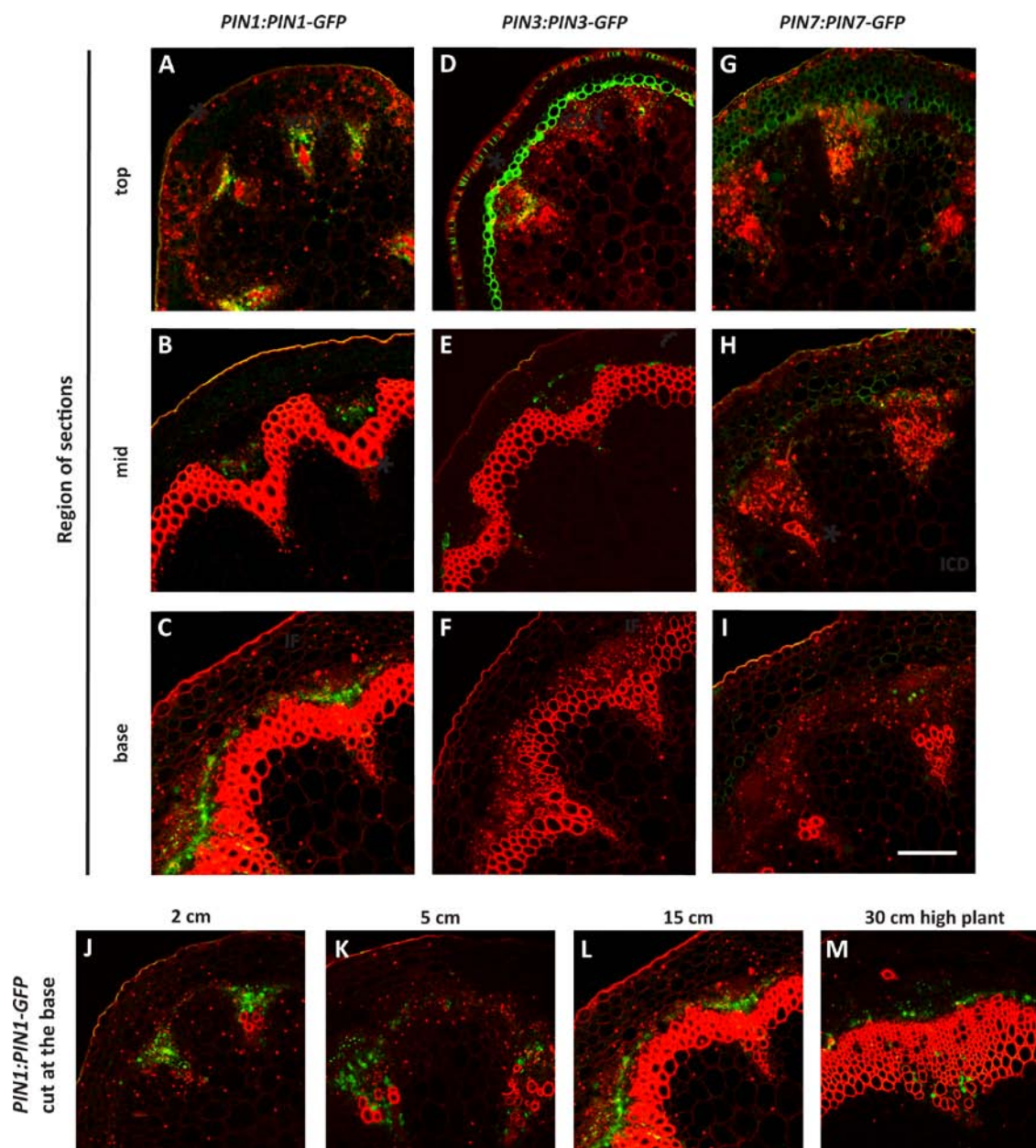


Figure 27: PIN protein localisation. To explore the PIN-GFP protein localisation along the stem, 15-20 cm high plants were cross sectioned at the base, at the middle of the plant (mid) and within the inflorescence shortly below the apex (top). Sections were counterstained with propidium iodide (red signal) and analysed utilising confocal microscopy. The marker lines *PIN1:PIN1-GFP* (A-C), *PIN3:PIN3-GFP* (D-F) and *PIN7:PIN7-GFP* (G-I) were studied to observe specific PIN protein localisation. To explore the dynamics of PIN1-GFP accumulation during IC initiation and SG at the base of the stem, plants of a height of 2, 5, 15 and 30 cm were cross sectioned (J-M). For more details see text. Scale bar: 100 μ m.

In the stem, PIN3 is known to be localised exclusively in the starch sheath (Friml, Wisniewska et al. 2002). In this study, a gradient of PIN3-GFP abundance was detected with a high abundance in the starch sheath at the apical half of the stem (Fig. 27D), a lower abundance in the middle

part (Fig. 27E), and no protein at the basal half of the stem (Fig. 27F). This gradient of PIN3-GFP protein level is in line with the gradient of transcript accumulation of *PIN3* along the stem which could be detected by RT-PCR (Fig. 26M), but not by the analysis of the *PIN3:GUS* marker line (Fig. 26D-F). Furthermore, the PIN3 protein was localised in the epidermis and partly in the vascular bundles as well, but only at the top of the stem (Fig. 27D).

PIN7-GFP protein accumulation showed a similar apical-basal gradient as PIN3-GFP, however, PIN7-GFP is localised in a much broader domain including the cortex (and starch sheath) and the vascular bundles (Fig. 27G-I). Here as well, such a gradient was detected in the RT-PCR analysis (Fig. 26M) but was not found when the *PIN7:GUS* reporter was analysed (Fig. 26J-L).

In conclusion, in addition to accumulate in the vasculature all along the stem, the PIN1-GFP protein accumulated at later stages of development in the IC after the IC has already been initiated. Thus, PIN1-GFP protein accumulation does not correlate with IC initiation but with an active IC. On the other hand, PIN3-GFP was never localised to the IC, but exclusively in the starch sheath in regions where no secondary growth takes place. Here, I hypothesise that the absence of the PIN3 protein at the base of the stem leads to reduced auxin flow in comparison to more apical parts and, thus, auxin accumulation which contributes to IC formation. The same could be true for PIN7, which showed a similar, but broader pattern of promoter activity and, according to the PIN7-GFP reporter, a similar but broader pattern of protein accumulation than PIN3.

Heterologous promoter/Ethanol-inducible system

To test whether a differential accumulation of the PIN3-GFP protein along the stem was due to transcriptional or post-transcriptional regulation, I took advantage of the heterologous *SCR* promoter which is active homogenously in the starch sheath along the stem. Furthermore, I aimed to induce PIN3-GFP from a certain time point onwards in the starch sheath to avoid an undesired ectopic function of PIN3 during embryogenesis and thus during the establishment of the primary vascular system. For this reason, the ethanol switch system was used to induce tissue-specific gene expression (Deveaux, Peaucelle et al. 2003). For this, two constructs are necessary. The first construct contains a tissue specific promoter which drives the transcription factor *AlcR*. The second construct is build up by the *AlcA* promoter driving the gene of interest. *AlcR* is produced constantly, but only upon ethanol treatment, *AlcR* can bind to the *AlcA* promoter and drive the expression of the gene of interest in the tissue of interest (Deveaux, Peaucelle et al. 2003).

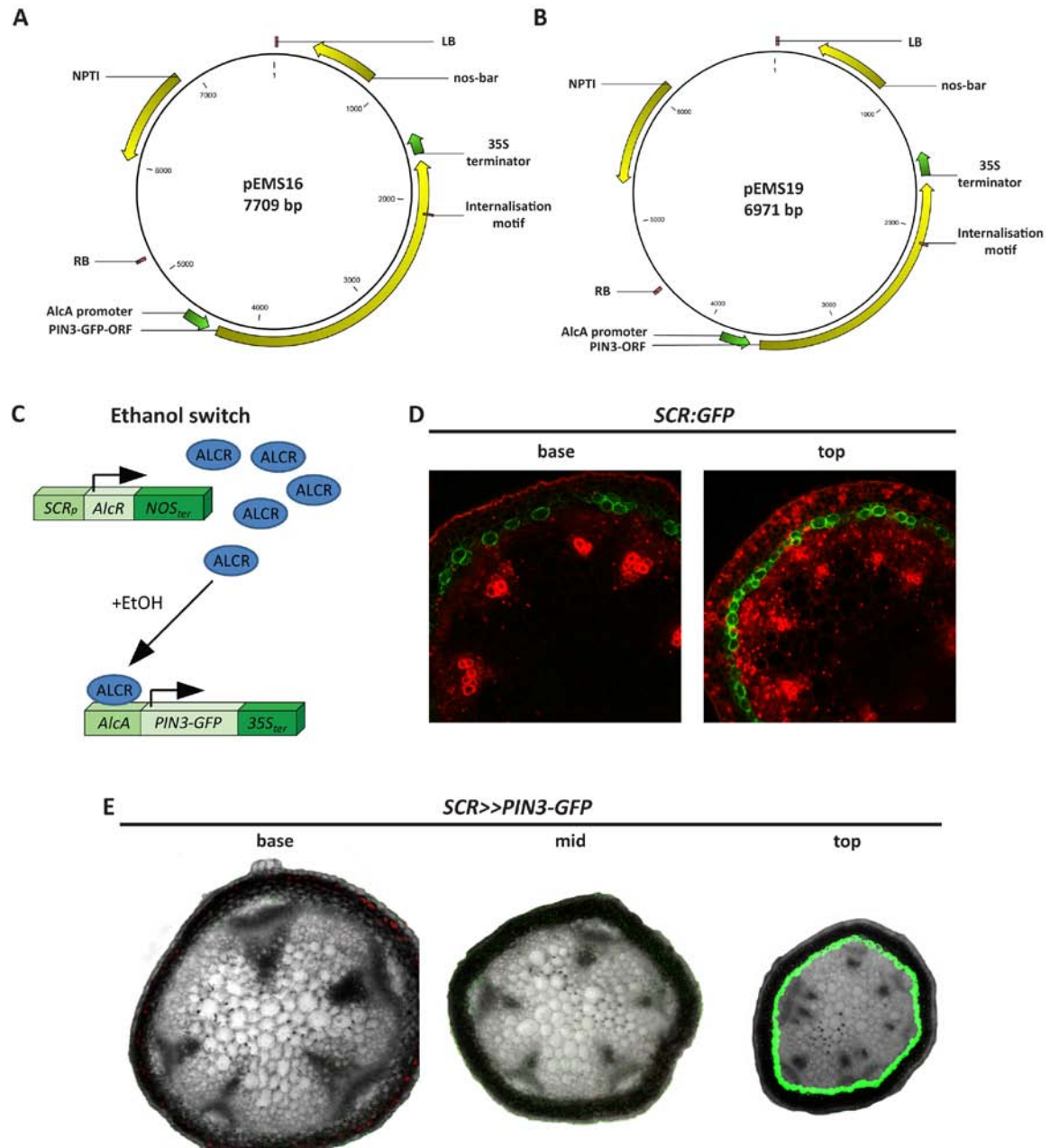


Figure 28: PIN3-GFP expression under the starch sheath-specific *SCR* promoter. The designed construct pEMS16 (A) is composed of the *AlcA* promoter driving the *PIN3* open reading frame (ORF) and in pEMS19 (B) the *AlcA* promoter drives the *PIN3-GFP* ORF. Both constructs carry a resistance to kanamycin (NPTI) and to glufosinate ammonium (nos-bar). LB (left border) and RB (right border) indicate the endings of the part of the construct which is inserted into the plant genome. The scheme of the ethanol switch (C) demonstrates that the constantly expressed *AlcR* under the *SCR* promoter binds upon ethanol treatment to the *AlcA* promoter and induces hereby the expression of *PIN3-GFP* only in the starch sheath. GFP signal was detected in the *SCR:GFP* marker line along the whole stem (D). The *SCR:AlcR AlcA:PIN3-GFP* plants showed after ethanol induction a GFP signal within the starch sheath only at the top part of the stem. No signal was visible at the base and at the mid part of the stem (E).

For this, the constructs *AlcA:PIN3* (pEMS19) and *AlcA:PIN3-GFP* (pEMS16) were generated (Fig. 28A, B). To ensure starch sheath tissue-specific expression of PIN3 along the whole stem axis, plants carrying already the *SCR:AlcR* (pTOM36) construct were transformed either with pEMS16 or pEMS19. After BASTA selection and the selection of lines carrying only one copy of the insert

using Southern blot analysis, plants of the second generation (T2) carrying pTOM36 and pEMS16 were further analysed. Although the *SCR* promoter is functional in the starch sheath along the stem from base (shortly above the last rosette leaf) to top (shortly below the tip of the inflorescence) (Fig. 28D), the ethanol-induced plants showed a PIN3-GFP signal in the starch sheath only at the top part of the plant stem (Fig. 28E), which actually reflected the pattern of PIN3-GFP accumulation when expressed under the control of the *PIN3* promoter (Fig. 28D). At the base and at the mid part of the plant stem no signal at all was detected (Fig. 28E).

Based on these observations, I conclude that the absence of PIN3-GFP expressed under the *SCR* promoter is due to a post-transcriptional regulation.

Influence of endocytic trafficking inhibitor substances on PIN protein abundance in the stem

PIN1 and PIN2 protein levels are known to be post-translationally regulated by a diverse set of mechanisms, namely protein phosphorylation and ubiquitination, and endocytic trafficking, thus enabling rapid changes in PIN polarity at the plasma membrane (Titapiwatanakun and Murphy 2009). Furthermore, it has been shown that internalisation of the proteins happens predominantly in a clathrin-dependent manner (Dhonukshe, Aniento et al. 2007). To test whether an endocytosis-based PIN3 protein degradation explains the discrepancy between promoter activity and protein accumulation, the following substances were applied on the *PIN3:PIN3-GFP* marker line: Brefeldin A and MG132.

Brefeldin A

Brefeldin A (BFA) is a fungal toxin and has been widely used as a reversible inhibitor of vesicle trafficking in yeast, mammalian, and plant cells. It inhibits the activation of ARF proteins by binding to an ARF-GDP/ARF-GEF complex and blocks thus Golgi-mediated transport and processing of proteins targeted to the plasma membrane (Robinson 1993; Morris and Robinson 1998; Geldner et al. 2003). To test whether PIN3 gets internalised and cycles via vesicle trafficking at the basal region of the stem where no PIN3-GFP was detected, BFA was applied onto this part of the stem of the *PIN3:PIN3-GFP* marker line mixed with lanolin as a carrier and incubated. After 3 days the GFP signal occurred in the section of the treatment zone, however not only in the starch sheath (arrow) but also in the xylem tissue of primary vascular bundles (Fig. 29D-F). Above the treatment zone the signal in the vascular bundles was much more intense than within and below the treatment zone. No signal was found in the starch sheath above and below the treatment zone.

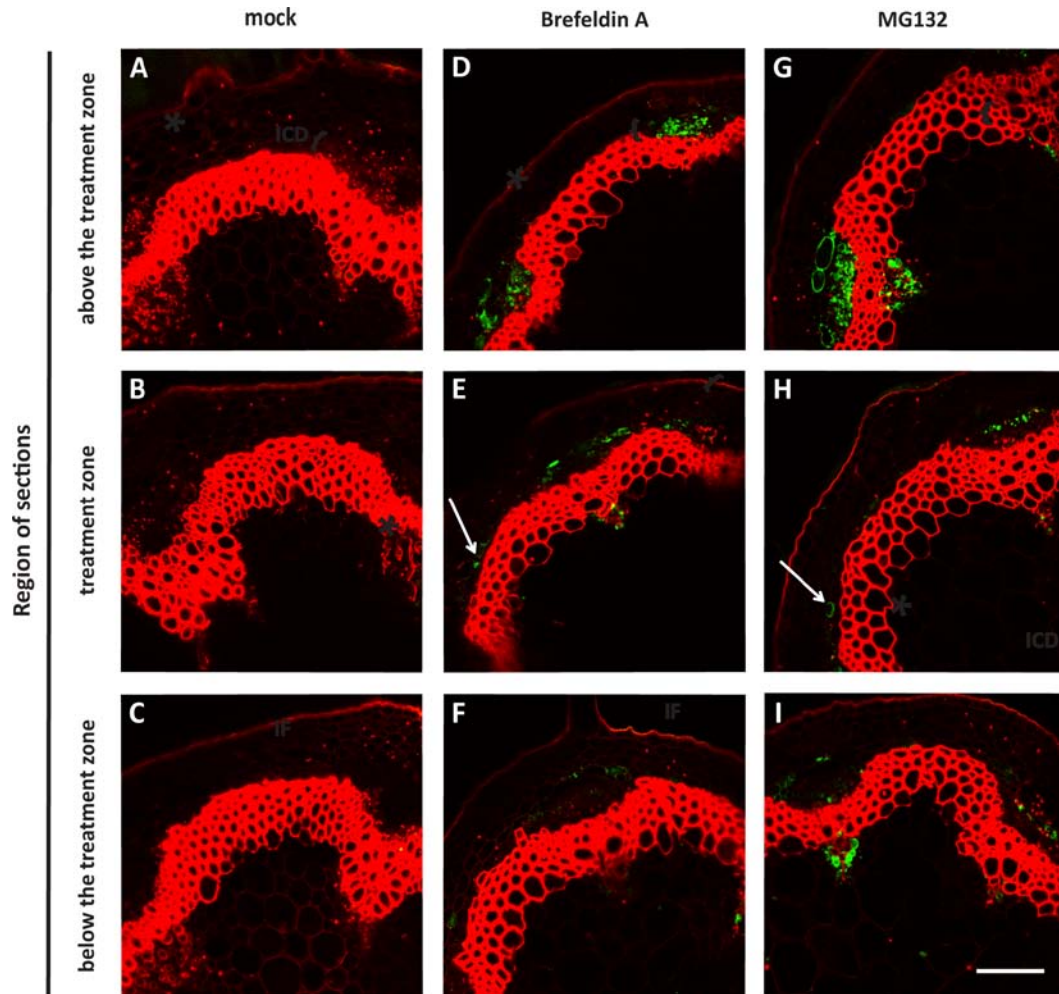


Figure 29: Post-translational regulation of PIN3. 15-20 cm high plants of the marker line *PIN3:PIN3-GFP* were mock-treated (**A-C**), with Brefeldin A (**D-F**) or with MG132 (**G-I**). After 3 days of incubation stems were sectioned 5 mm above the treatment zone (1st row), within the treatment zone (2nd row) and 5 mm below the treatment zone (3rd row). Upon BFA treatment GFP signal appeared in the vascular bundles in all sections (**D-F**). Furthermore, the sections taken within the treatment zone showed GFP signal also in the starch sheath (**E**, **arrow**). Upon MG132 treatment GFP signal appeared as well predominantly in the vascular bundles in all sections (**G-I**), showing a higher intensity above the treatment zone (**G**). GFP signal in the starch sheath could be detected only in sections taken within the treatment zone (**H**, **arrow**). Scale bar: 100 μm.

MG132

MG132 is an inhibitor of the 26S proteasome, which is involved in the turnover of plant proteins. It has been demonstrated that MG132 treatment results in the accumulation of ubiquitinated PIN2 and interferes with the internalisation of PIN2 in gravi-stimulated roots (Abas et al. 2006). Furthermore, the ubiquitination is proposed to serve as a signal to influence degradation of PIN2 in a proteasome-dependent manner (Abas, Benjamins et al. 2006). To see whether MG132 has the same effects on PIN3-GFP accumulation, the marker line *PIN3:PIN3-GFP* was treated with MG132 mixed with lanolin and incubated for 3 days. The mock treated plants did not show any GFP signal (Fig. 29A-C). The MG132 treated plants showed GFP signal within the vascular

bundles in the phloem and xylem (Fig. 29G-I) and also in the starch sheath (Fig. 29H, arrow). Above the treatment zone the signal in the vascular bundles was much more intense than within and below the treatment zone. No signal was found in the starch sheath above and below the treatment zone.

In conclusion, these results suggest that PIN3 protein accumulation is regulated post-translationally by endocytosis and degradation via the ubiquitin-dependent 26S proteasome pathway in the more proximal part of the stem.

PIN3 protein stabilisation

To reveal an influence of PIN3 protein degradation on IC initiation, I aim to stabilise the protein at the basal part of the stem. My strategy is to destroy the functionality of the internalisation motif and thus to stabilise the PIN3 protein at the plasma membrane. To achieve this, site-directed mutagenesis was performed using the constructs pEMS16 and pEMS19 (Fig. 28A, B, Tab. 4) as starting plasmids. After the mutagenesis, plants already carrying *SCR:AlcR* (pTOM36) were transformed with one of these constructs, namely pEMS23 to pEMS28 (Fig. 30A-F, Tab. 4). This procedure was carried out at the time of writing. After harvesting the transformed plants, the seeds of the first generation (T1) will be selected for plants carrying the desired insertion using BASTA. A successful mutagenesis of the internalisation motif of PIN3 is expected to lead to a stabilisation of the PIN3 protein within the starch sheath (due to the *SCR* promoter) along the stem, which will be visualised by confocal microscopy as soon as single copy lines are identified by southern blotting. The question whether the presence of the stabilised PIN3 protein at the base of the stem influences IC formation is still open and will be clarified by analysing these plants by histological means.

Plasmid	Backbone	Insert
pEMS16	pGreen0229-AlcA	<i>AlcA:PIN3-GFP</i>
pEMS19	pGreen0229-AlcA	<i>AlcA:PIN3</i>
pEMS23	pEMS19	<i>AlcA:PIN3</i> (Y498A)
pEMS24	pEMS19	<i>AlcA:PIN3</i> (N494A)
pEMS25	pEMS19	<i>AlcA:PIN3</i> (Y498A & N494A)
pEMS26	pEMS16	<i>AlcA:PIN3-GFP</i> (Y498A)
pEMS27	pEMS16	<i>AlcA:PIN3-GFP</i> (N494A)
pEMS28	pEMS16	<i>AlcA:PIN3-GFP</i> (Y498A & N494A)

Table 4: Designed plasmids regarding PIN3 stabilisation. All plasmids are characterised by a bacterial resistance to kanamycin and the transformed plants are resistant to BASTA.

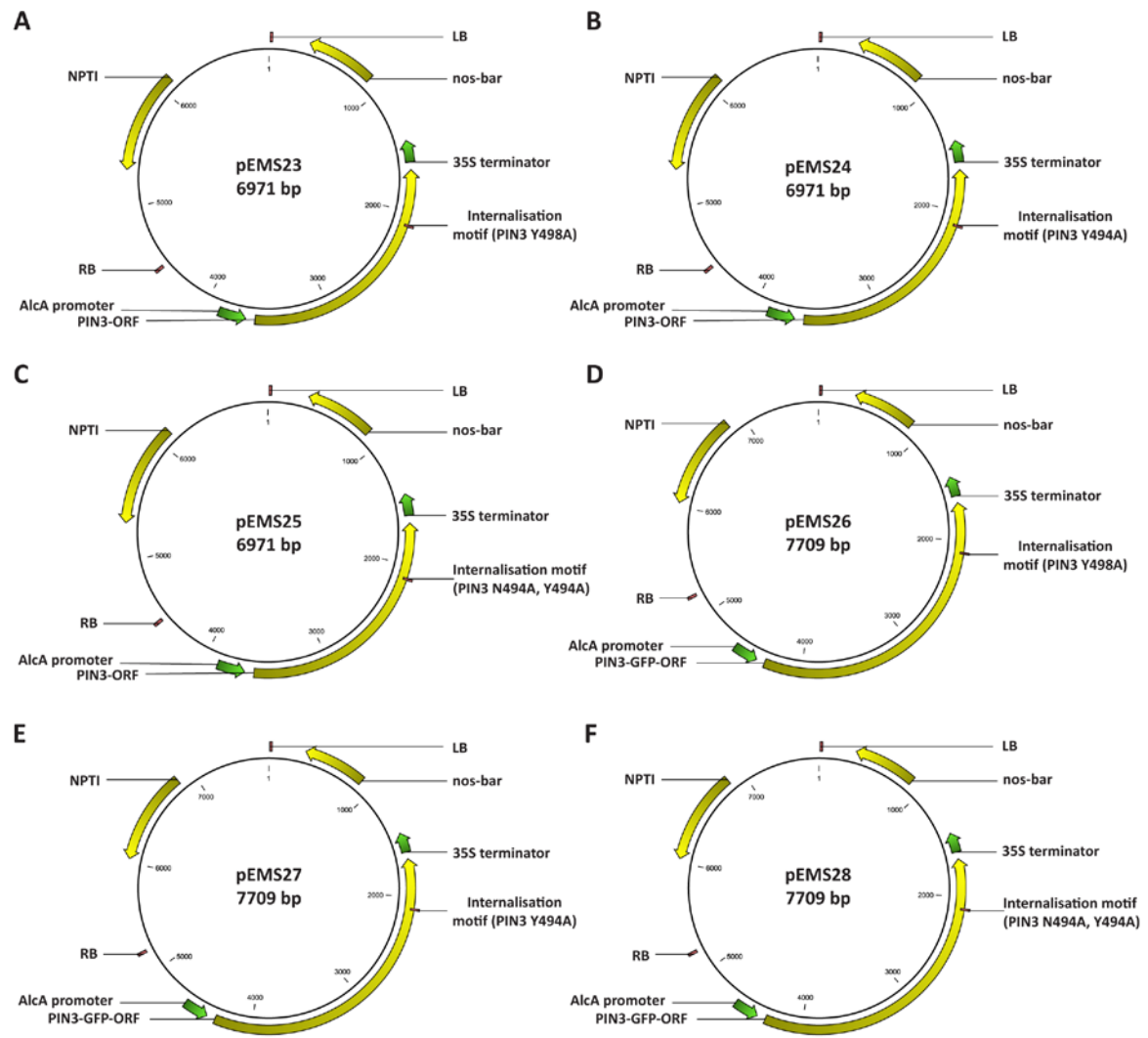


Figure 30: Maps of the vectors pEMS23-pEMS28.

Results – Part III – Hormonal control of SG: Jasmonic acid and ethylene

No major regulator of the auxin transport, in particular, no single PIN protein was identified in regulating IC initiation and activity alone, suggesting that the translation of basipetal auxin transport into IC initiation is rather complex. It has been already shown that vascular development is not only regulated by auxin, but acts in concert with other hormones, e.g. cytokinin, gibberellin and ethylene (Shininger 1971; Savidge 1988; Dettmer et al. 2009; Caño-Delgado, Lee et al. 2010). To find factors involved in IC initiation in an unbiased manner, transcriptional profiling was performed in the lab by Javier Agustí (Sehr, Agustí et al. 2010).

Transcriptional profiling

The first 0.5 cm of the stem base (with SG) was compared with a 0.5 cm segment from the 1st internode (no SG) by genome-wide transcriptional profiling. A significant proportion of genes preferentially expressed at the stem base are stress-related and classified as being touch-inducible, like *TOUCH2*, and *EXPANSIN L1* (Braam and Davis 1990; Sampedro and Cosgrove 2005; Sehr, Agustí et al. 2010). This led to the speculation, that the expression of such genes at the stem base reflects the mechanical stress due to tissue expansion and/or increasing shoot weight during growth processes.

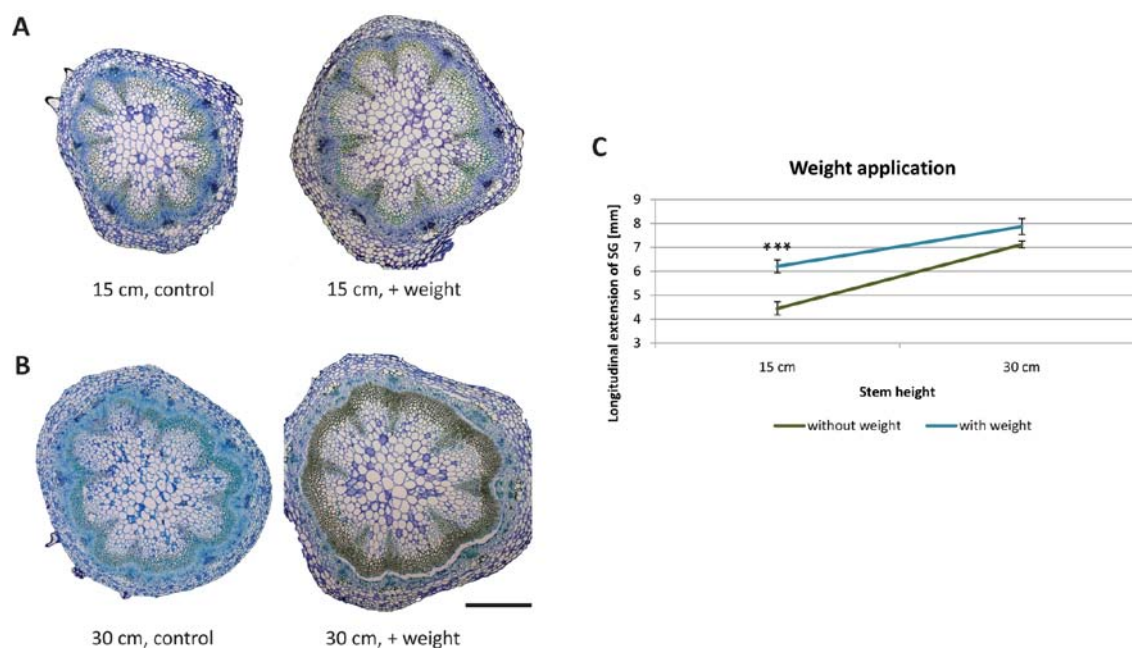


Figure 31: Weight positively influences cambial activity. The continuous application of paperclips as weight onto the inflorescence stem resulted in an increase of cell wall thickness of the IFs (A, B) and lateral extension the ICD in 15 cm as well as in 30 cm high plants (C). The added average weight of 0.95 g led in 15 cm high plants to an increase up to 6.2 ± 0.34 mm in comparison to the control showing 4.4 ± 0.27 mm, whereas in the 1.8 g carrying 30

cm high plants the longitudinal extension of SG was detected until a height of 7.87 ± 0.47 mm in comparison to 7.12 ± 0.16 mm of the control. Scale bar: 500 μ m. Error bars: standard error of means (SE).

A positive effect of shoot weight on the formation of secondary vascular tissue in *Arabidopsis* has been reported previously (Ko et al. 2004). To prove that also IC initiation is influenced by mechanical forces, weight was applied onto the stem in form of paperclips. Indeed, the longitudinal extension of SG in weight-treated plants was increased in comparison to untreated plants (Fig. 31C), demonstrating that weight positively influences IC initiation along the stem.

In our profiling dataset, jasmonic acid (JA) signalling components are among the genes which show the greatest difference in expression between the two samples (Sehr, Agustí et al. 2010). This is true especially for two members of *JAZ* (*JASMONATE-ZIM-DOMAIN*) genes, *JAZ10* and *JAZ7*. JA is a plant hormone known for being involved in stress signalling. To elucidate the role of components of the JA signalling pathway in IC initiation, mutants were analysed, gene expression studies and hormone treatments were performed.

Histological analysis of mutants involved in JA signalling

Analysis of *JAZ* mutants

JAZ proteins are repressors of jasmonate-regulated gene expression by repressing the transcriptional activator MYC2. In the presence of the active form of JA, jasmonic acid-isoleucine (JA-Ile), *JAZ* proteins are targeted by SCF^{COI1} for degradation via the 26S proteasome, thus releasing the transcription factor MYC2 for promoting JA-dependent gene expression (Chini, Fonseca et al. 2007; Browse 2009; Yan et al. 2009; Pauwels et al. 2010). Among the 12 *JAZ* genes, *JAZ10*, *JAZ7* and *JAZ8* were found in our transcriptional profiling to be preferentially expressed at the stem base. T-DNA insertion lines for *JAZ10* and *JAZ7* were analysed in this study, for *JAZ8* no insertion line was available (Sehr, Agustí et al. 2010).

jaz10

It has already been shown that *JAZ10* regulates growth responses related to JA signalling (Yan et al. 2007). To clarify, whether *JAZ10* is involved in IC regulation, two T-DNA insertion lines of *JAZ10* were subjected to further analyses. The SAIL T-DNA is inserted in the 2nd exon, whereas the GABI T-DNA is inserted at the beginning of the 5th intron (Fig. 32A). PCR was performed to test for the presence of the *JAZ10* mRNA (Fig. 32B). The *jaz10-1* allele (lane 3) showed 3 transcripts with the primer combination A+B and no transcript with the combinations A+C and A+D. The *jaz10-2* allele (lane 4) showed transcripts for A+B and A+C. No transcript was detected

with the primer combination A+D. Out of these results *jaz10-2* is considered to be a hypomorphic allele.

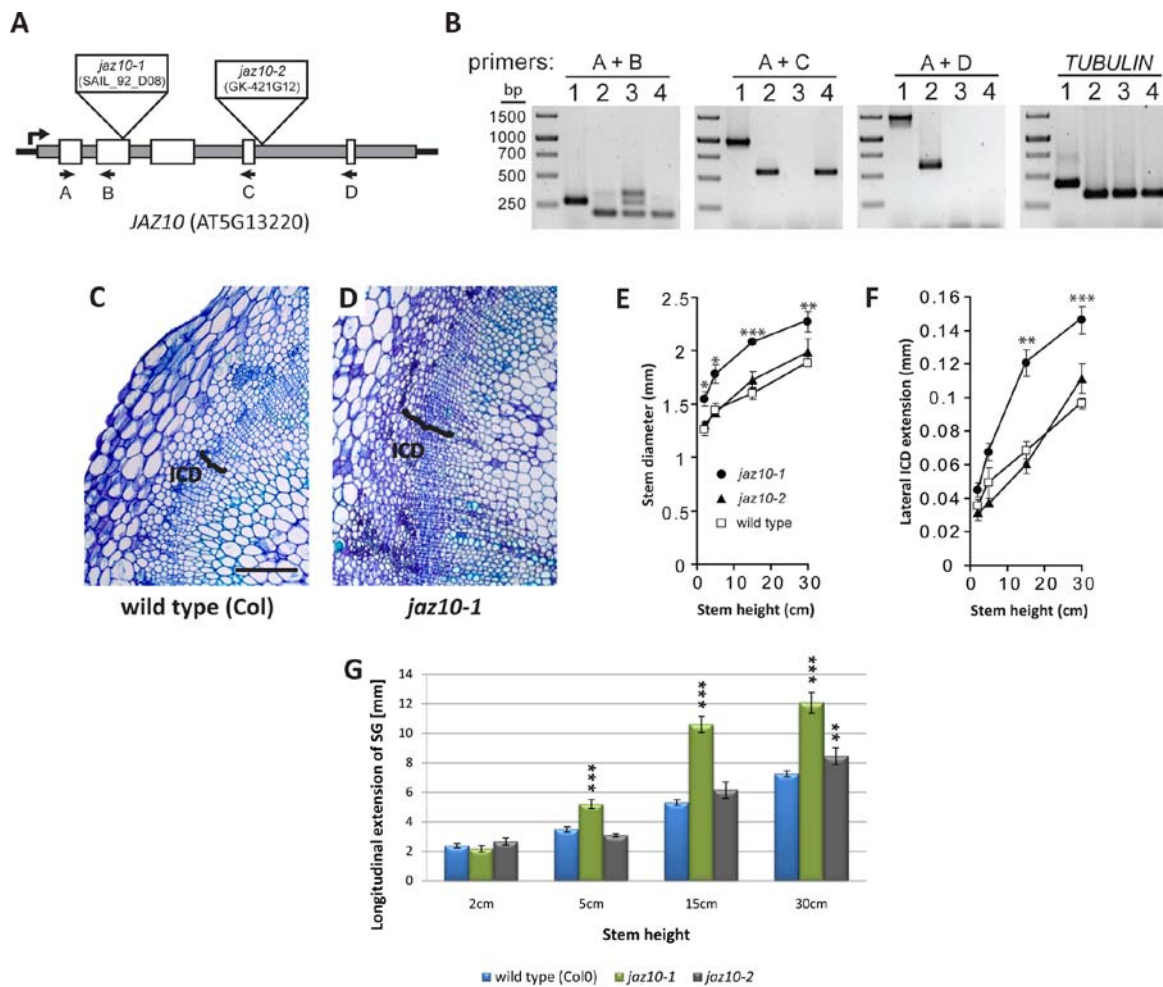


Figure 32: Analysis of *JAZ10*. Intron-exon structure of *JAZ10* (A). The sites of T-DNA insertions are labelled by triangles. The positions of the primers are indicated by arrows. Primer A = *JAZ10_for_ATG*, primer B = *JAZ10_rev_Exon2*, primer C = *JAZ10_rev_Exon4*, primer D = *JAZ10_rev*. The Tubulin primers are *TUBfor3* and *TUBrev2*. Detection of the *JAZ10* transcript is done via RT-PCR (B) using the same primers described in (A). Lane 1 shows the results using wild type genomic DNA as PCR template, lane 2 wild type cDNA, lane 3 *jaz10-1* cDNA and lane 4 *jaz10-2* cDNA. All DNA/RNA samples were extracted from seedlings. The histological analysis of the *JAZ10* mutants revealed that *jaz10-1* had a significantly increased longitudinal extension of SG in comparison to the wild type in almost all stages of development except for the 2 cm high stems and that *jaz10-2* differed from wild type only in 30 cm high plants (G). Furthermore, the lateral extension of the ICD was in *jaz10-1* significantly increased in comparison to the wild type and *jaz10-2* (C, D, and F). The stem diameter at the base was in *jaz10-1* significantly higher than in *jaz10-2* and wild type (E). Scale bar: 100 μ m. Error bars: standard error of means (SE).

The ability to initiate the IC along the stem was significantly increased in the *jaz10-1* mutant in comparison to the wild type, namely 2.17 ± 0.22 mm in 2 cm high stems, 5.2 ± 0.3 mm in 5 cm, 10.6 ± 0.55 mm in 15 cm and 12.06 ± 0.71 mm in 30 cm high plants (Fig. 32E). *jaz10-2* showed a significantly increased longitudinal extension of SG only in 30 cm high stems. At other stages, no difference to the wild type was observed. The same tendency was observed when the activity of the IC, meaning the extension of the ICD at the base of the stem was analysed. While the *jaz10-2*

mutant showed a wild type-like extension, the *jaz10-1* mutant showed an increased extension of the ICD (Fig. 32C, D, G). Furthermore, the diameter of the stem at the base was significantly increased in *jaz10-1* but not in *jaz10-2* (Fig. 32F).

jaz7

A T-DNA insertion line was identified for *JAZ7* in which the open reading frame was disrupted (Fig. 33A), suggesting that the gene function is severely impaired. Indeed, no transcript of *JAZ7* was detected by RT-PCR in the *jaz7-1* sample (lane 3, Fig. 33B). Regarding the longitudinal extension of SG, no differences to the wild type were observed, with the exception of 30 cm tall *jaz7-1* plants which showed a significantly increased longitudinal extension than wild type plants, namely 1.8 ± 0.23 mm in 2 cm, 3.28 ± 0.16 mm in 5 cm, 5.8 ± 0.39 mm in 15 cm, and 8.02 ± 0.15 mm in 30 cm high plants (Fig. 33C).

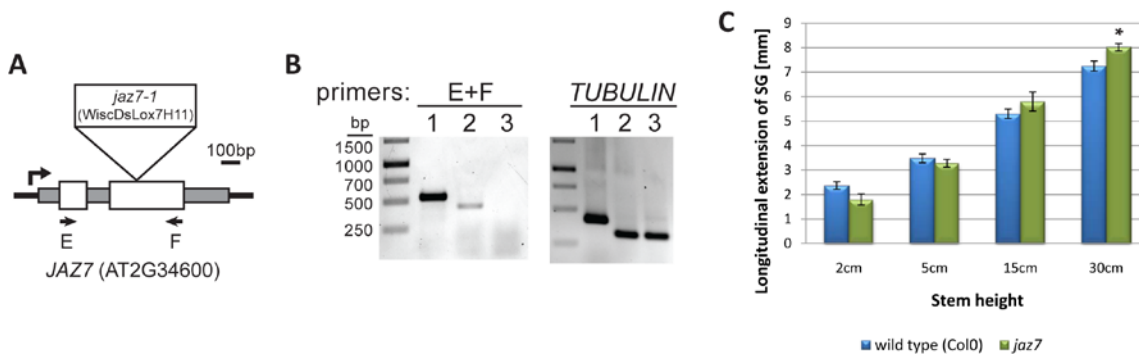


Figure 33: Histological analysis of *jaz7-1*. Exon-intron structure of *JAZ7* (A). The sites of T-DNA insertions are labelled by triangles. The position of the primers is indicated by arrows. Primer E = *JAZ7_for2* and primer F = *JAZ7_rev4*. The Tubulin primers are *TUBfor3* and *TUBrev2*. No transcript in the *jaz7-1* cDNA sample (lane 3) was detected (B). Lane 1 shows the result using wild type genomic DNA as PCR template, lane 2 wild type cDNA, and lane 3 the mutant cDNA. All samples were taken from seedlings. The comparison of the longitudinal extension of SG revealed that only in 30 cm tall stems *jaz7-1* showed a significant increase (C). Error bars: standard error of means (SE).

Taken together, these results suggest, that elements of JA signalling, in particular *JAZ10*, serve as repressors of IC initiation and activity. *JAZ7* seems to be less involved in this process.

Analysis of *MYC* and *COI* mutants

To confirm the influence of the JA signalling pathway on IC initiation and activity, mutants affected in *MYC2* and *COI1* activity were described and analysed.

myc2

MYC2 (also known as *JIN1*) is a bHLH transcriptional activator that binds the G-box (CACGTG) or the T/G-box (AACGTG) in the promoters of JA-regulated genes and serves thus as a key regulator of JA-dependent responses (Lorenzo et al. 2004; Dombrecht et al. 2007). Here, a T-DNA insertion

line for *MYC2* was identified in which the open reading frame was disrupted (Fig. 34A), suggesting that the gene function is severely impaired. Indeed, no transcript of *MYC2* was detected with RT-PCR in the *myc2-3* sample (lane 3, Fig. 34B). No differences between the wild type and *myc2-3* were observed in the anatomical structure by analysing cross sections of the stem base (data not shown). Regarding longitudinal extension of SG, in *myc2* a significantly decreased progression was observed, namely 1.14 ± 0.11 mm in 2 cm, 2.1 ± 0.12 mm in 5 cm, 4.25 ± 0.19 mm in 15 cm, and 5.92 ± 0.38 mm in 30 cm high plants (Fig. 34C).

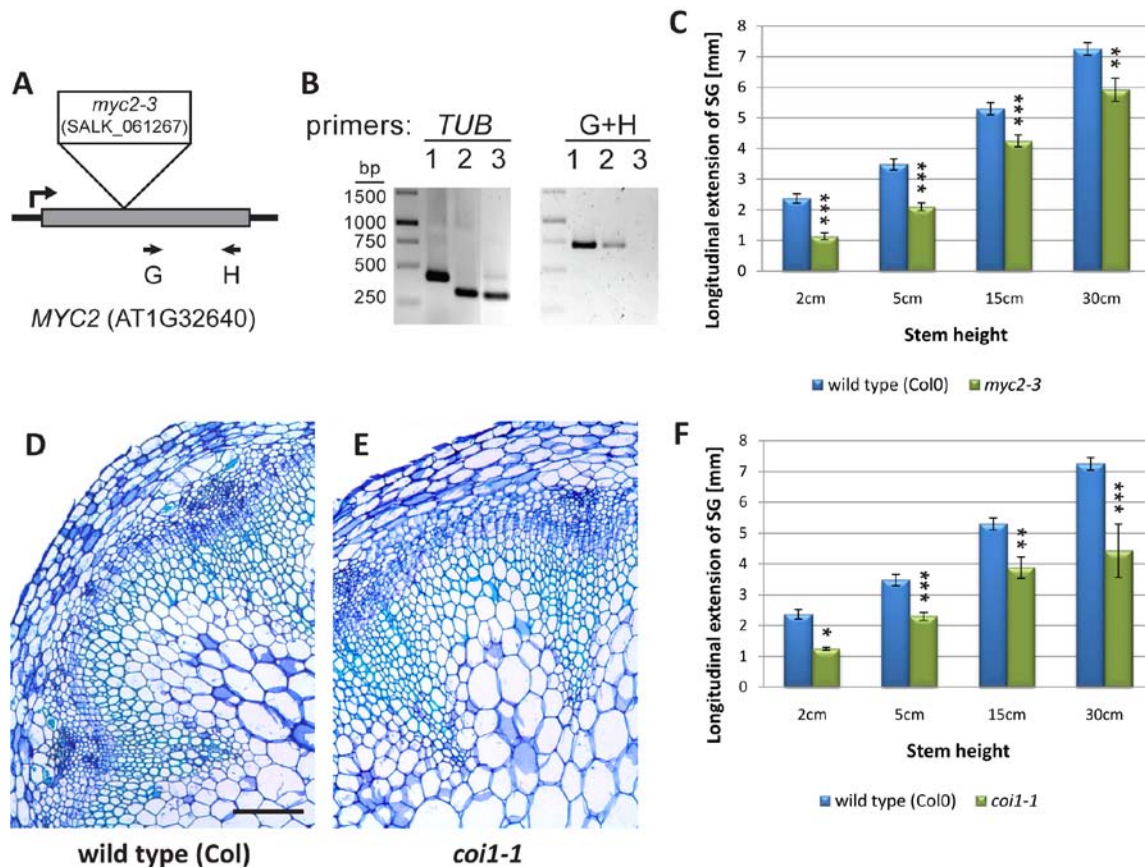


Figure 34: Histological analysis of *coi1-1* and *myc2-3*. Exon-intron structure of *MYC2* (A). The site of the T-DNA insertion is labelled by a triangle. The position of the primers is indicated by arrows. Primer G = MYC2_for and primer H = MYC2_rev2. The Tubulin primers are TUBfor3 and TUBrev2. No transcript in the *myc2-3* cDNA sample (lane 3) was detected (B). Lane 1 shows the result using wild type genomic DNA and lane 2 using wild type cDNA as PCR template. All samples were taken from seedlings. The comparison of the longitudinal extension of SG revealed that all *myc2-3* stems showed a significant decrease (C). No differences in the anatomical structure of cross sections of 30 cm high plants were detected by comparing wild type and the *coi1-1* mutant line (D, E). However, *coi1-1* showed a significant decrease in the longitudinal extension of SG (F). Scale bar: 100 μ m. Error bars: standard error of means (SE).

coi1-1

As already described above, COI1, an F-box protein, is part of the SCF E3 ubiquitin ligase complex and directly binds to JA-Ile and thus recruits JAZ proteins for degradation by the 26S proteasome (Yan, Zhang et al. 2009). In this study the already described mutant line *coi1-1* was used for

further analysis (Xie et al. 1998). By analysing cross sections of the stem base, no anatomical differences between wild type and *coi1-1* were detected (Fig. 34D, E). Regarding the longitudinal extension of SG, *coi1-1* showed a significant decrease, namely 1.25 ± 0.05 mm in 2 cm, 2.3 ± 0.13 mm in 5 cm, 3.88 ± 0.35 mm in 15 cm, and 4.43 ± 0.87 mm in 30 cm high plants (Fig. 34F).

In conclusion, these results identified *MYC2* and *COI1*, which are positive mediators of JA signalling, as factors being positively involved in IC regulation.

Touch-inducibility of *JAZ10*

To confirm an inducibility of *JAZ10* by mechanical forces (Tretner, Huth et al. 2008), a *JAZ10:GUS* marker line was analysed and qRT-PCR was performed to visualise *JAZ10* transcript accumulation.

JAZ10:GUS expression pattern

When analysing the adult plant, the reporter showed a broad expression pattern in flowers. In fruits, expression is reduced to the stigma and the receptacle (Fig. 35A, insert). The expression pattern in leaves was patchy and not specific for a particular tissue. This unspecified and patchy expression pattern was observed in the hypocotyl of seedlings as well (Fig. 35F). Expression in the root was restricted to the stele and the root tip (Fig. 35F, insert). Stems did not show any signal with the exception of the stem base (Fig. 35A).

By analysing the *JAZ10:GUS* expression pattern at the stem base during plant development GUS signal was observed in the first few mm above the rosette of 2 cm, 5 cm and a bit weaker in 15 cm high plants (Fig. 35B, C, arrows). The signal decreased the older the plants were and in 30 cm high plants no signal at all was detected at the base (Fig. 35D, E). Cross sections through the base of agarose-embedded 2 cm and 5 cm tall stems showed that *JAZ10:GUS* expression is restricted to the vascular bundles (predominantly xylem) and to the region of the IFs (Fig. 35I-K, arrows).

To confirm the property of *JAZ10* being touch-inducible, the *JAZ10:GUS* marker line was touched with tweezers at the internode. Already after a slight touch GUS signal was detected (Fig. 35G, arrow) and after touching the stem with damaging the cuticle a stronger GUS signal was visible (Fig. 35H, arrow).

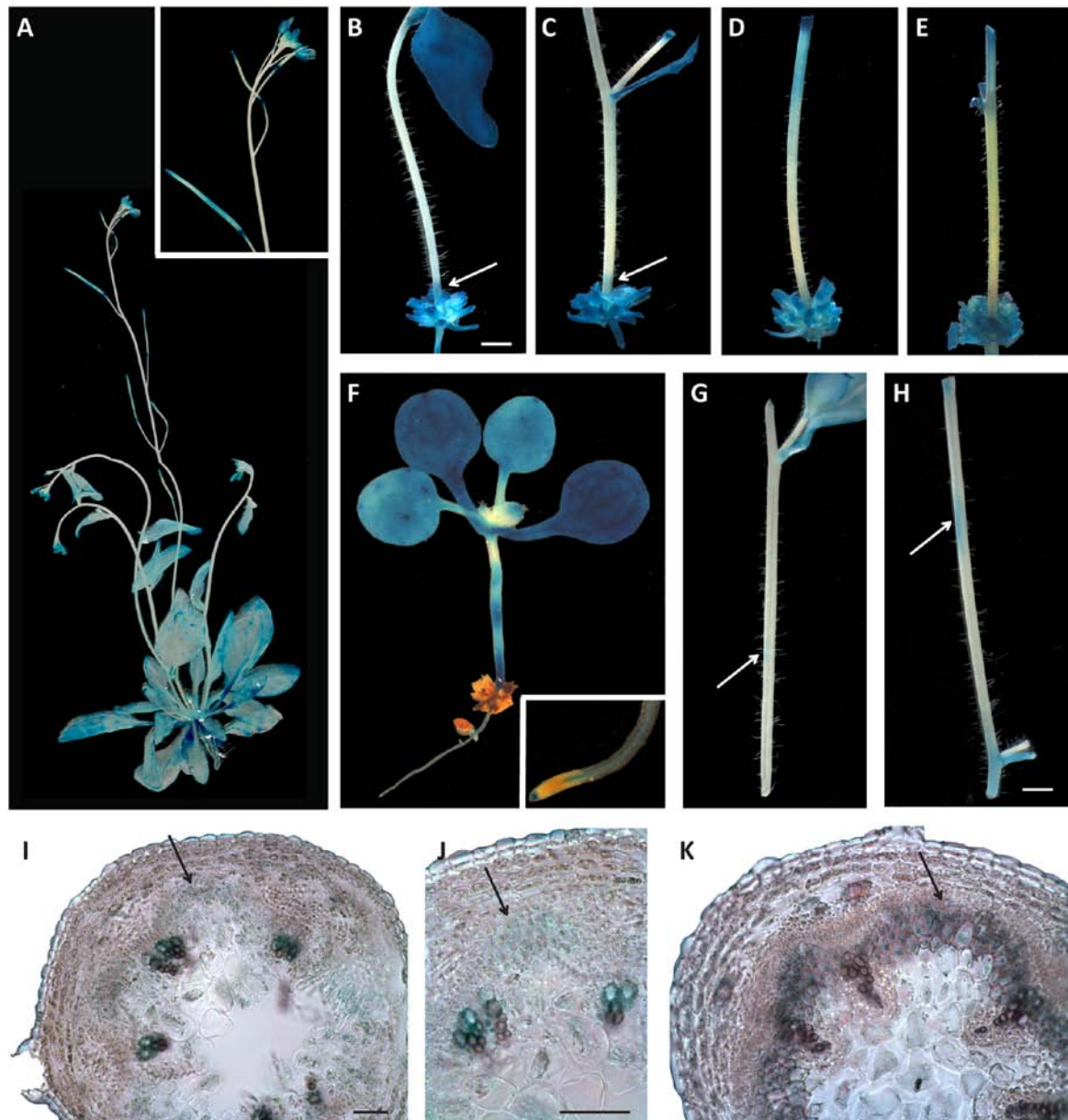


Figure 35: *JAZ10* expression analysis. 15 cm tall *JAZ10:GUS* plants were GUS stained and analysed regarding the overall *JAZ10* expression pattern (A). The insert shows the magnified top of the inflorescence. For more details see text. Expression was analysed at the base of the stem during plant development in 2 cm (B), 5 cm (C), 15 cm (D) and 30 cm (E) tall plants. Whereas GUS signal was detected in 2 cm and 5 cm high plants (B, C, arrows), no signal at the stem base was observed in older plants (D, E). A patchy and unspecific expression pattern was observed in seedlings (F). Expression in the root is restricted to the vasculature and the root tip (F, insert). The *JAZ10:GUS* marker line was touched with tweezers at the internode for about 1 min without damaging the tissue to check whether *JAZ10* is touch-inducible (G, H). After 4 h the plants were harvested and GUS stained. To specify the expression pattern within the stem, cross sections of 2 cm (I, magnified in J) and 5 cm high plants (K) were performed. Scale bars: 2 mm (B, H, true for C-E, G) and 100 μ m (I-K).

qRT-PCR of *JAZ10* upon mechanical and hormonal treatment

To confirm the results concerning the *JAZ10*-dependence on different stimuli, quantitative RT-PCR was performed on seedlings to check the *JAZ10* expression after mechanical and hormonal treatments. *JAZ10* expression increased by a factor of 5 2 h after the mechanical stimulus and

dropped again 4 h after the touching (Fig. 36A). Spraying with meJA clearly resulted in an enhanced expression of *JAZ10* after both, 2 h and 4 h after the treatment, whereas treatment with IAA did not result in any changes of *JAZ10* expression (Fig. 36A). As a positive control, the expression of *IAA5* was tested. This gene is known to be induced upon auxin treatment. Indeed, expression of *IAA5* peaked 2 h and 4 h solely after the treatment with auxin (Fig. 36B).

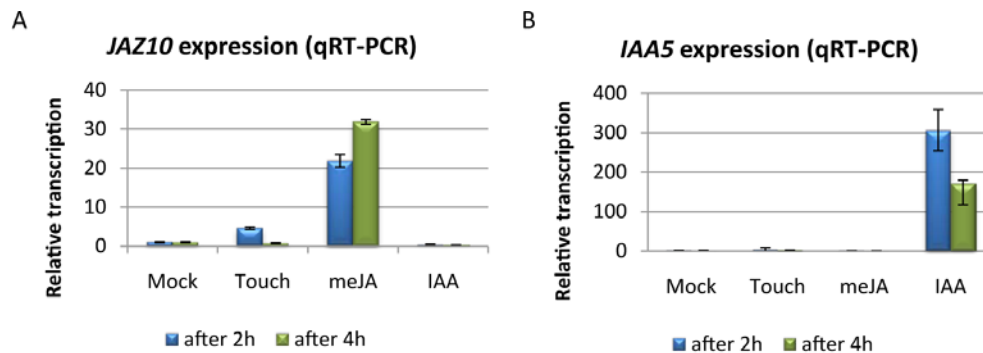


Figure 36: qRT-PCR of *JAZ10* and *IAA5*. Seedlings were subject to mechanical (touched for 30') and hormonal (sprayed with meJA [50 μ M] or IAA [20 μ M]) treatments and harvested 2h and 4h after the treatments. Expression analysis of *JAZ10* (A) was performed utilising qRT-PCR. *IAA5* (B) was used as positive control for the IAA treatment. Error bars: standard error of means (SE).

In conclusion, *JAZ10* expression was detected at the base of the stem in very young plants being consistent with a role of *JAZ10* in IC regulation and SG. However, *JAZ10* is neither expressed in the fascicular cambium nor in the interfascicular cambium. Furthermore, *JAZ10* was expressed after mechanical stimulation (GUS and qRT-PCR analyses) and this result confirms on the one hand the ability of *JAZ10* of being touch-inducible (Lee et al. 2005).

JA watering

To show whether JA is directly involved in IC regulation, plants were continuously watered with JA [0.5 mM] after they have been singularised.

Comparing the phenotype of adult mock- and JA-treated plants, flattened leaves and anthocyanin accumulation in the midvein and stems were observed in the JA-treated plants (Fig. 37A). Measurements of the longitudinal extension of SG showed no difference between JA- and mock-treated plants (Fig. 37B). However, the lateral extension of the ICD zone was significantly increased in the JA-treated compared to the mock-treated plants at all stages of development analysed (Fig. 37C-E). Furthermore, the JA-treated plants showed much thicker secondary cell walls in IF, xylem and phloem cap cells. In the interfascicular region, phloem cells with an enhanced secondary cell wall were observed.

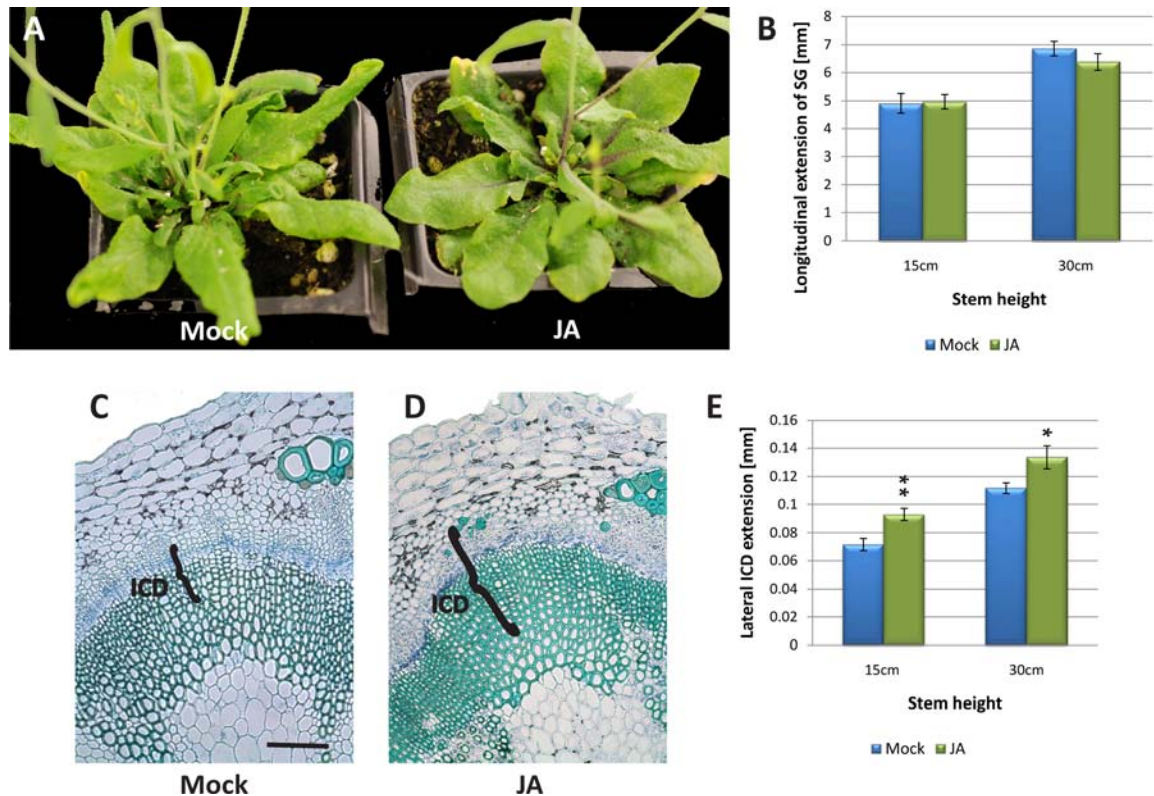


Figure 37: JA watering. JA treatment resulted in an accumulation of anthocyanin and flattened leaves in comparison to mock-treated plants (A). JA-treated plants showed no difference to mock-treated plants regarding the longitudinal extension of SG (B). The lateral extension of the ICD (interfascicular cambium derived cells) zone is significant increased in the JA-treated plants (C-E). In addition, secondary cell walls of the IFs, xylem and phloem cap are much thicker in the JA-treated plants (D). Scale bar: 100 μ m. Error bars: standard error of means (SE).

Together, these observations suggest that general stem stability is positively influenced by JA application (Fig. 37D). Furthermore, these results indicate a positive role of JA signalling in cambium initiation and activity as well as in stem stability.

Histological analysis of mutants involved in ethylene signalling

In the plant kingdom, ethylene is an important hormone playing various roles in development and stress responses (Stepanova and Alonso 2009). It is well known, that on the one hand ethylene biosynthesis is stimulated by mechanical interferences (Takahashi and Jaffe 1984) and on the other hand that ethylene stimulates cambial growth (Biro et al. 1980). Thus, it is suggested that ethylene plays a primary role in mechanical-induced cambial growth responses, e.g. tension wood (Love et al. 2009). In the transcriptional profiling mentioned above a member of the *ERFs* (*ETHYLENE RESPONSE FACTORS*) appeared to be highly expressed at the stem base and was thus further analysed regarding IC dynamics (Sehr, Agustí et al. 2010).

erf104

ERF104 is described as a transcriptional activator which can be induced by mechanical stimulation (touch-inducible) (Nakano et al. 2006). In this study, a T-DNA insertion line of *ERF104* was analysed (Bethke et al. 2009). No differences between the wild type and *erf104* were observed in the anatomical structure by analysing cross sections of the stem base (Fig. 38A, B). Regarding the ability to initiate the IC along the stem, the longitudinal extension of SG was significantly decreased in *erf104* 15 cm and 30 cm high plants in comparison to wild type plants (Fig. 38C).

These results suggest that, next to JA signalling components, ethylene signalling components also play a positive role in IC regulation.

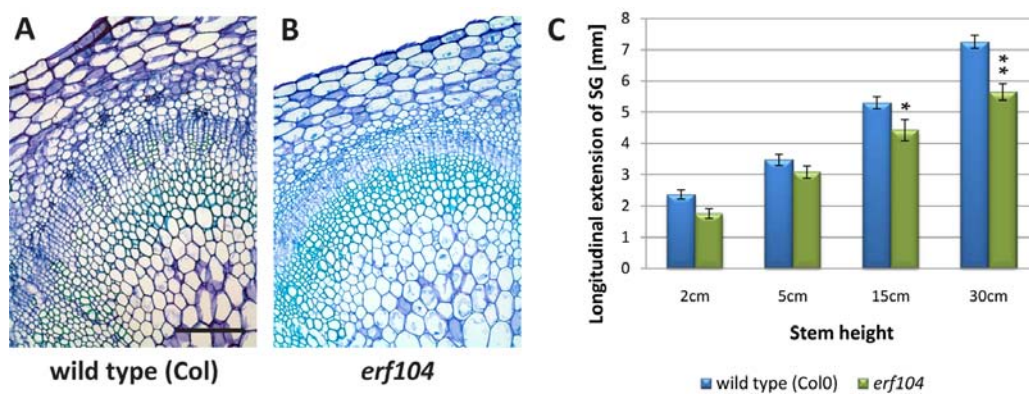


Figure 38: Histological analysis of *erf104*. Cross sections of the base of 30 cm high plant stems are shown for wild type (A) and *erf104* (B). The mutant is comparable to the wild type in its vascular patterning and secondary growth. Regarding its longitudinal extension of SG, *erf104* showed significant less than the wild type at the stage of 15 cm and 30 cm high stems (C). Scale bar: 100 μ m. Error bars: standard error of means (SE).

Discussion

IC initiation and SG

SG in *Arabidopsis* was already a subject in many studies, especially in the root and hypocotyl (Dolan and Roberts 1995). Recently SG in the *Arabidopsis* shoot was taken into consideration (Altamura, Possenti et al. 2001; Chaffey, Cholewa et al. 2002), but so far no detailed description of this process has been performed.

IC initiation progresses acropetally

In this study, the dynamics of IC formation and activity at the base of the *Arabidopsis* inflorescence stem during plant development was shown.

My analysis revealed, that IC formation starts very early in shoot development, since already in 2 cm high stems a zone of cambial activity ranging from the last rosette leaf up to about 2.4 mm is present (Fig. 12A-C, 13A). During plant growth, IC initiation progresses acropetally in the elongating stem, but does not exceed a region of about 1 cm until plants grow 30 cm tall (Fig. 12J-L, 13A). In very young stems (2 cm tall) the IC is initiated in parenchyma cells of the pith adaxially to the starch sheath between the primary bundles (Fig. 12A). During development and acropetal progression, the IC is established more and more in peripheral positions until, in later stages (15-30 cm high plants) the starch sheath serves as precursor for the IC (Fig. 12I, L). This gradual establishment of the IC at the base of the stem from the pith towards the starch sheath confirms the results obtained by Altamura et al. (2001). In that study, the IC at the base of the stem is divided into a nodal and internodal IC. The first is abundant in the lower half of the zone of IC formation and does not include the starch sheath, whereas the latter is abundant in the upper half of the zone of IC establishment and originates partly from the starch sheath (Altamura, Possenti et al. 2001).

The longitudinal progression of IC initiation does not appear in a linear fashion during plant development (Fig. 13A) but ceases at later stages of development, when seed production is well advanced. This cessation of cambial activity has been described in other herbaceous dicots as well, in which at the end of the life cycle the cells of the fascicular cambium differentiate as vascular elements (Larson 1994). For the *Arabidopsis* inflorescence stem, such a stop of cambial activity with further differentiation into vascular elements has been described already to happen in the fascicular cambium (Little, MacDonald et al. 2002). In this study, this terminal differentiation step has been observed in the interfascicular region at the apical margin of the SG inducing segment of 30 cm tall plants too. The cambial cells seem to undergo differentiation

which is suggested by the presence of thickened cell walls (Fig. 12L). These observations could be generally explained by the short life cycle of *Arabidopsis*, in which at the end more energy is put into fruit ripening than in the maintenance of IC initiation.

IC is initiated independently of cell identities in the interfascicular region

Whether the presence of one the two main tissue types in the interfascicular region, namely cortex including starch sheath and interfascicular fibres (IFs), is a prerequisite for IC initiation has been addressed in this work. Although the starch sheath is proposed to be essential for the formation of the IC (Altamura, Possenti et al. 2001), starch sheath-less mutants (*shr-2* and *scr-3*) initiated the IC similarly to wild type proving that starch sheath identity is not crucial for the formation of the IC (Fig. 19A-C). Furthermore, in the IF-less mutant *ifl1/rev-5* IC initiation was likewise not affected. However, its position was shifted towards the centre of the stem (Fig. 19D-G). This suggests that the differentiation of the IFs in the interfascicular region represses the initiation of the IC and “pushes” the IC towards the periphery of the stem into a cell layer which kept the ability to de-differentiate and become meristematic, namely the starch sheath or any other cell layer located distally to the IFs. This proposes an important role of the IFs regarding the position of the IC initiation. The IFs are characterised by extensive secondary cell wall formation which gives this tissue its rigidity. The formation of the secondary cell wall is very complex and involves many genes involved in the biosynthesis of cellulose and lignin. The down-regulation of the lignification of cell walls in the *cad-c cad-d* double mutant (Sibout, Eudes et al. 2005) did not lead to the same shift of the IC position as in the *ifl1/rev-5* mutant. This argues for a minor role of cell wall lignification in the determination of the IC position (Fig. 20) (Sibout, Eudes et al. 2005). The developing IFs (although less lignified) still occupy the space in the interfascicular region in this double mutant and, thus, determine the position of the IC formation. Also the absence of both, lignin and cellulose, in the IFs and partly the secondary xylem in the *nst1 nst3* double mutant (Mitsuda, Iwase et al. 2007) did not lead to a change of the IC position (Fig. 17). Thus, I conclude, that other features of the IFs are responsible for determining the position of the IC in wild type.

The fact that the IC can be established out of various tissue types (starch sheath/cortex and pith) supports the idea of *de novo* establishment of cambium identity without the requirement for a pre-determination of cells of the interfascicular region, similarly to the *de novo* organisation of the vasculature in leaf primordia (Scarpella, Marcos et al. 2006).

Day length affects IC activity

That short day (SD) periods are not necessary to induce SG is demonstrated in this study by growing plants in different photoperiods. Plants grown solely under long day (LD) conditions (16h light/day) can establish the IC similarly as plants grown for 3 and 7 weeks in SD (8h light/day) before shifting to LD (Fig. 14). However, the ability to initiate the IC along the stem (measured by the longitudinal extension of SG, Fig. 14D) and the activity of the IC and stem diameter (Fig. 14A-C) increased the longer the SD period lasted. *Arabidopsis* is a facultative long-day plant requiring an increase of day length as a stimulus for the onset of the reproductive phase, i.e. flowering (Amasino 2010). The longer the SD condition lasts the more time for the vegetative phase is given. SG has been already linked with photoperiodic control of flowering time (Melzer et al. 2008; Sibout et al. 2008). SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1) and FUL (FRUITFULL), two MADS box proteins acting downstream of FT (FLOWERING LOCUS T) in apical meristems as positive regulators of flowering, function redundantly in repressing cambial activity. The double mutant *soc1 ful* shows extensive SG and a shrub-like phenotype with a markedly increased life span similar to perennial plants (Melzer, Lens et al. 2008). Furthermore, the expression of *SOC1* and *FUL* appears to be naturally reduced in Sy-0, an *Arabidopsis* accession from the Isle of Skye, which is characterised as well by a perennial growth characteristic (Poduska et al. 2003; Wang et al. 2007; Melzer, Lens et al. 2008). That plants growing under SD conditions show increased IC initiation in comparison to plants grown solely under LD (see Fig. 14) might be due to delayed onset of the flowering signalling cascade including SOC1 and FUL. During the SD period flowering is not initiated, SOC1 and FUL are just expressed in leaves independently of the flowering signalling cascade and might thus not influence cambial activity (Melzer, Lens et al. 2008). These observations support the idea that herbaceous plants have derived from perennial woody ancestors and that *Arabidopsis* still carries all the molecular equipment to develop woody stems (Kim et al. 2004; Spicer and Groover 2010).

Arabidopsis produces secondary vascular tissues in interfascicular regions

That the *Arabidopsis* IC is able to produce secondary vascular tissues in interfascicular regions resembling to that of trees was confirmed by analysing the marker lines J1721 (GFP enhancer trap line) and *APL:GUS*. Analysing these marker lines, secondary phloem (*APL:GUS*, Fig. 16A, B) and secondary xylem (J1721, Fig. 16C, D) were detected in the interfascicular region at the stem base. Furthermore, the histological analysis of the double mutant *nst1 nst3* (Mitsuda, Iwase et al. 2007) revealed the formation of vessel elements belonging to the secondary xylem in the

interfascicular region (Fig. 17A, B). However, no continuous zone of secondary xylem and secondary phloem was established as it is for example the case in trees. This might be due to the different setup of the primary plant body. In trees, a closed cambial ring is already established in the primary stage (see *Tilia*-type, Fig. 3A, B). Whereas in herbaceous dicots (like *Arabidopsis*) only a few primary bundles are present in the primary stage and a closed cambial ring has to be formed secondarily at the onset of SG (*Ricinus*-type, Fig. 3E, F). This variability in the initiation of SG is supported by phylogenetic analyses showing that the vascular cambium of seed plants in fact share a common evolutionary origin which predates the divergence of angiosperms and gymnosperms. However, out of this ancestral state of the vascular cambium a high variability of the functionality up to a total loss of SG (e.g. monocots) has evolved (Kim, Soltis et al. 2004; Spicer and Groover 2010).

Does IC initiation occur in nodal parts of the stem?

Nodes are the parts of the stem where leaves and side shoots are attached. In *Arabidopsis*, such a node of the main stem consists of a side shoot which is supported by a bract. At each node, part of the vasculature in form of single vascular bundles, so called leaf traces, leaves the main shoot and enters the leaf (Kaussmann and Schiewer 1989). The outgoing leaf trace leaves a so called leaf gap in the main stem behind (Fig. 15H). Whether an IC is established between diverging/merging bundles was addressed here. When nodes along the main inflorescence stem were analysed cambial cell divisions were observed only in the emerging bract/side shoot (Fig. 15A-H). No IC formation was observed in the main inflorescence stem within the node. This clearly demonstrates that IC initiation and SG are well defined processes restricted to the basal part of the main stem and side shoots.

Auxin positively regulates IC initiation

IAA, the most potent native auxin, is a regulator of plant development and also plays a role in SG, most importantly in the establishment of the IC (Little, MacDonald et al. 2002). Especially for woody species it has been demonstrated that decapitation of stems and the resulting decrease of IAA supply inhibits cambial growth in the remaining stump and that restoring the IAA supply by applying IAA to the stump apex promotes cambial growth (Snow 1935). Furthermore, it has been described that the movement of IAA is basipetally, occurs in the cambial region, and can be blocked by ringing with transport inhibitors such as NPA (Sundberg et al. 1994; Davies 1995). Recently, Little et al. (2002) provided evidence, that all those classical IAA/NPA treatments show the same effects in the *Arabidopsis* stem as in the stem of woody species. It has been observed

in several herbaceous dicots, including *Arabidopsis*, that cambial activity commonly ceases in the inflorescence stem, and that the cells of the cambial zone differentiate into vascular elements (Larson 1994; Little, MacDonald et al. 2002), which is supported by the results obtained here (Fig. 12L). This might be explained by a reduced supply of IAA to the vascular cambium. Whether this IAA reduction is due to progressive maturation of the inflorescence, and a resulting decrease of IAA biosynthesis at the apex and, thus, less IAA transport within the vascular cambium is still questionable (Little, MacDonald et al. 2002).

Auxin accumulation/signalling correlates with IC initiation

Here, a positive correlation between the process of IC initiation and auxin accumulation *in vivo* at the base of the inflorescence stem is described. For this purpose, the *DR5rev:GFP* marker line was used which is known to visualise auxin signalling/response as well as auxin concentration (Sabatini, Beis et al. 1999; Benkova, Michniewicz et al. 2003; Bai and Demason 2008; Chandler 2009). Confocal microscopy revealed that auxin accumulation/signalling increases in cells of the interfascicular region before the first cambial cell divisions occur (Fig. 21). That auxin is an activator of vascular formation has been described already in leaves, where the *DR5rev:GFP* signal is preceding provascular cell differentiation (Scarpella, Marcos et al. 2006). This correlation of *DR5rev:GFP* appearance (Fig. 21H, K) and subsequent cambial cell division is supported by the results of the *in vitro* split-plate experiment where upon NAA treatment both IC formation can be initiated (Lichtenberger 2010) and auxin accumulation (visible by *DR5rev:GFP*) in the interfascicular region is enhanced (Fig. 22D, arrow). This leads to the suggestion that auxin is an important signal triggering IC initiation locally by inducing auxin-inducible gene expression at the stem base in cells establishing IC identity.

PAT plays a role in SG

That the site of auxin biosynthesis differs from the site of auxin-induced responses and, furthermore, that auxin is transported in a polar fashion via the vascular cambium basipetally through the plant stem is well documented (Benjamins and Scheres 2008; Tromas and Perrot-Rechenmann 2010). With classical decapitation experiments Little et al. (2002) proved for *Arabidopsis* that upon apical IAA treatment the IC could be initiated in the stem. This initiation typically proceeded laterally from the edges of fascicular cambia towards the centre of the interfascicular region. The IAA-inducibility of the process suggests that IAA is a key factor stimulating IC initiation by diffusing or transported laterally from the main stream being transported polarly in the FC (Little, MacDonald et al. 2002). Polar auxin transport (PAT) is mainly mediated by members of the AUX1/LAX and PIN protein family, which are polarly

localised at the plasma membrane, making those transporters to key regulators in multiple developmental events (Parry, Marchant et al. 2001; Tanaka, Dhonukshe et al. 2006; Krecek, Skupa et al. 2009). In this study, a connection between auxin transporters and IC initiation has been drawn on the basis of histological analyses and quantification of mutant lines. Regarding the *PIN* gene family, the loss-of-function mutants *pin1-613/+*, *pin3-5*, and *pin6-1* showed a significant decrease in the longitudinal extension of SG in comparison to the wild type (Fig. 24K). The same was observed in the *aux1/lax* triple mutant (Fig. 25C). These results suggest that the auxin transport capacity sustained by PIN and AUX1/LAX proteins has a quantitative impact on the IC initiation. The weak effect when removing single factors involved in auxin transport might be due to the extensive functional redundancy among the *PIN* gene family and furthermore on the complex transport mechanism of auxin through the tissues. A stronger effect on IC initiation and SG is expected in *pin* multiple mutants. Speaking of the high complexity of the auxin transport mechanisms, PIN polarity and auxin efflux are at least partly controlled by PID- and D6PK-dependent PIN phosphorylation (Michniewicz, Zago et al. 2007; Zourelidou, Muller et al. 2009; Zhang, Nodzynski et al. 2010). Due to a reduced transport capacity of the PAT machinery in mutants of PID and D6PKs (Bennett, Alvarez et al. 1995; Zourelidou, Muller et al. 2009) a reduced evacuation of auxin from stem tissues into the root is expected leading potentially to auxin accumulation at the stem base. The resulting high auxin concentration at the stem base of these mutants is expected to cause a higher cambial activity and longitudinal extension of the IC than shown in the wild type. This hypothesis is supported only by the mutant phenotype of one of the analysed kinases, namely *pid-3* (Fig. 25G-I), which showed a significant increase of the IC initiation rate. In contrast, the longitudinal extension of SG of the *d6pk d6pk1 d6pk2* triple mutant is significantly reduced in comparison to the wild type (Fig. 25D-F). Thus, those kinases seem to play a role in SG, which depends on a tightly regulated auxin distribution. However, the opposed phenotypes of *pid-3* and the *d6pk* triple mutant support the idea that PID and the D6PK proteins do not have a redundant biochemical function and thus play different roles in SG regulation (Zourelidou, Muller et al. 2009).

How important are the PINs during IC initiation?

The reduced longitudinal initiation of the IC observed in *pin* mutant lines described above (Fig. 24) suggests a role for PIN proteins in SG regulation, especially within IC initiation. Many PIN proteins have specific developmental roles that are largely determined by their highly specific expression pattern (see Fig. 5B) (Krecek, Skupa et al. 2009). Expression analyses revealed that in all analysed GUS marker lines the *PIN* promoter is active mainly in the vascular bundles along the stem (Fig. 26A-L). This is supported by the rather homogenous levels of transcript accumulation

along the stem as revealed by RT-PCR (Fig. 26M). Additionally, especially *PIN1* and *PIN6* promoters are active at the stem base and furthermore at the base of side shoots at nodes. Based on this pattern both, *PIN1* and *PIN6*, are candidates for being regulators of IC initiation and the process of SG.

Since only the PIN protein localisation reveals the function of the PINs in distinct tissues, available PIN-GFP protein marker lines were analysed regarding IC initiation at the stem base (Fig. 27). If *PIN1* serves as one of the main regulators of IC initiation, it is expected to appear very early during this process in the interfascicular region. *PIN1* is already known to localise within the fascicular cambium and young xylem cells in the primary vascular bundles (Gälweiler, Guan et al. 1998), but there are so far no indications about a localisation in the interfascicular region. Data obtained in this study support a localisation of *PIN1* in the fascicular cambium along the stem (Fig. 27A-C) and, additionally, show that *PIN1*-GFP is detected in the IC at the base of the stem, but only in late stages (Fig. 27J-M). Thus, I suggest, that *PIN1*-mediated auxin transport may be important probably only in late stages of IC development. Based on these observations, I hypothesise that *PIN1* might be necessary for draining off auxin which is potentially produced in the interfascicular region after the IC has been established.

The other candidate which might function as a regulator of SG is *PIN6*. Regrettably, no *PIN6*-GFP marker line was available to analyse. Thus, the questions of how and where *PIN6* functions during IC initiation and SG are still open.

Although *PIN3* and *PIN7* transcripts seem to accumulate in the vasculature all along the stem, *PIN3*-GFP and *PIN7*-GFP analysis revealed that the protein levels decrease gradually from top towards the base (Fig. 27D-I). The predominant localisation of *PIN3* in the starch sheath in the stem is already described (Friml, Wisniewska et al. 2002) and could be confirmed in this study, with the addition that *PIN3*-GFP is absent at the base of the stem (Fig. 27F). *PIN3* has been associated mainly to gravitropism since the protein is localised predominantly in gravity-sensing tissues, which are the starch sheath and the columella. Furthermore, a shoot agravitropic phenotype has been observed in the *pin3* mutant line (Fukaki, Wysocka-Diller et al. 1998; Friml, Wisniewska et al. 2002; Harrison and Masson 2008; Morita 2010). In addition to being expressed in gravity-sensing tissues, *PIN3* is active in the pericycle, which is located next to the endodermis and gives rise to lateral roots (Peret et al. 2009). Here, the functional parallelism of pericycle and starch sheath has to be pointed out: both tissues are differentiated but both are able to regain meristematic activity and are, thus, able to produce new tissues (secondary vasculature) and even new organs (lateral roots). With this knowledge I hypothesise that the absence of *PIN3* localisation within the starch sheath at the base of the stem might be important for IC initiation.

In detail, next to its presence and function in gravity-sensing tissues at the top half of the stem, the absence of PIN3 at the base might result in a decreased auxin transport through the starch sheath and thus an accumulation of auxin in the same cell layer. This would then establish a high auxin concentration which is needed for the de-differentiation and onset of meristematic divisions of starch sheath cells or any other cell layer distal to the IFs. Since the PIN7 expression pattern overlaps with the one from PIN3, PIN7 might be also an important factor in IC initiation. In conclusion, the special gradual localisation pattern of PIN3 and PIN7 leads to the hypothesis that the absence of those proteins at the base of mature stems caused by post-transcriptional or post-translational regulation is important for IC initiation.

Does post-translational regulation of PIN3 influence SG?

As many proteins, also PIN proteins are post-translationally regulated by a diverse set of mechanisms, namely protein phosphorylation and ubiquitination, internalisation, endocytic cycling and recycling, thus enabling rapid changes in PIN polarity at the plasma membrane (Titapiwatanakun and Murphy 2009). Like PIN1, PIN3 is able to cycle rapidly and thus show a dynamic actin-dependent subcellular movement which was proven by using a range of diverse inhibitor substances, e.g. BFA and the cytoskeleton disrupting latrunculin B (Geldner et al. 2001; Friml, Wisniewska et al. 2002). Whether this “behaviour” of PIN3 cycling/recycling applies also for the stem base was addressed in this work. Upon treatment with BFA and MG132, accumulation of the PIN3-GFP protein was achieved at a stem region where, under normal conditions, no PIN3-GFP signal is detectable (cf. Fig. 27F, Fig. 29). Since *PIN3* is transcribed all along the stem, this indicates that the plant performs regular protein biosynthesis, but then a proteolysis of the protein occurs mediated by the ubiquitin 26S proteasome. However, so far the question whether the absence of the PIN3 protein at the base of the stem has an influence on IC initiation and SG has not been answered yet. To do so, the stabilisation of the PIN3-GFP protein all along the stem was attempted by two different methodological approaches. The first was the indirect (via the Alc-inducible system, see Deveaux et al., 2003) induction of PIN3-GFP under the SCR promoter which is persistently active in the starch sheath. After ethanol induction the PIN3-GFP signal was detected similarly as in the *PIN3:PIN3-GFP* reporter line in a gradient along the stem (Fig. 28E) indicating that the gradient of PIN3-GFP protein abundance is independently of the promoter used and that the protein is indeed post-translationally regulated. With this approach it was, thus, not possible to stabilise the PIN3-GFP and study its impact on IC initiation and the process of SG. The second approach was to mutate the internalisation motif of PIN3 (see Fig. 11, Fig. 30) and to stabilise the protein at the plasma membrane. This approach was at the

time of writing still in progress. Therefore, the question whether the absence of PIN3 at the base of the stem influences IC initiation and SG still needs further investigation.

JA positively regulates SG

To reveal factors involved in IC initiation in an unbiased manner, transcriptional profiling was performed by Javier Agustí comparing stem segments devoid of SG with stem segments undergoing SG to find novel factors involved in IC initiation (Sehr, Agustí et al. 2010). Genes involved in JA signalling and, to a lesser extent, in ethylene signalling pathways were found to be up-regulated in the stem segment initiating the IC (Sehr, Agustí et al. 2010). Both, JA and ethylene are well known plant stress hormones (Davies 1995). One of the best-studied signal transduction pathways of jasmonates is that of wound response following herbivore attack and mechanical perturbations (Wasternack 2007). Additionally, jasmonates have been implicated in plant thigmomorphogenetic responses (Jaffe 1973; Braam 2005; Tretner, Huth et al. 2008; Chehab, Eich et al. 2009). Regarding SG, no interconnections with jasmonates have been described so far, although jasmonates have been reported to regulate developmental processes like reproductive development, trichome patterning and senescence (Chung, Niu et al. 2009). In this study it could be shown, that upon JA application cambial activity was enhanced (Fig. 37C-E), suggesting a positive role of JA in the process of SG (Sehr, Agustí et al. 2010). Ethylene is associated with a large number of stress responses and also with many developmental processes including the stimulation of cambial cell divisions in *Populus* (Guo and Ecker 2004; Love, Bjorklund et al. 2009).

JA signalling components: repressors and activators of IC activity

The transcriptional profiling revealed an increased transcript accumulation of members of the JAZ (*JASMONATE ZIM DOMAIN*) gene family at the stem base where SG takes place, especially JAZ10 and JAZ7, which are components of the JA signalling pathway (Sehr, Agustí et al. 2010). Histological analysis of the mutant JAZ10 alleles described in this study revealed for the *jaz10-1* mutant a clear enhancement of cambial activity (Fig. 32C-F) and IC initiation (Fig. 32G). In *jaz10-2* and *jaz7-1* a weaker enhancement regarding cambial activity and the longitudinal extension of SG was observed (Fig. 32E-G, 33C). These results suggest a role of JAZ proteins in cambial regulation, in which predominantly JAZ10 might function as a repressor of IC initiation and IC activity. The opposite alteration was observed in *coi1-1* and *myc2-3*, the first serves as a jasmonate receptor (Yan, Zhang et al. 2009) and the second functions as transcriptional activator repressed by JAZ proteins (Boter et al. 2004; Lorenzo, Chico et al. 2004). Both show a significant

decrease in IC initiation along the stem (Fig. 34C-F) suggesting a positive role for COI1 and MYC2 in IC initiation.

In conclusion, I suggest that the JA signalling pathway contributes to the promotion of IC initiation and activity.

Are mechanical forces mediated by stress hormones a trigger for IC initiation?

The positive effect of mechanical forces (especially shoot weight) on cambial activity and SG-related gene expression has been documented already (Brown and Sax 1962; Ko, Han et al. 2004; Lee, Polisensky et al. 2005). A large proportion of genes preferentially expressed in stem fragments in the secondary stage is related to stress signalling pathways and part of those genes is annotated to be touch-inducible (Oh et al. 2003; Ko and Han 2004; Sehr, Agustí et al. 2010). This suggests an involvement of mechanical stimuli on the regulation of gene expression in stems undergoing SG. The weight of the shoot is suggested to be a mechanical trigger of IC initiation (Ko, Han et al. 2004). However, IC initiation along the stem decelerates during plant development (Fig. 31C). This suggests, that shoot weight alone is insufficient to induce IC initiation, but it effects IC activity and IF differentiation positively (Fig. 31A, B). An already well described response to mechanical stress is the formation of tension wood upon leaning/bending of stems (Wilson and Archer 1977). It is possible that mechanical forces generated by cell expansion and/or cell division might be part of the primal stimuli of IC formation. Since SG starts with the activity of the fascicular cambium (FC) in the primary vascular bundles (Beck 2005; Sehr, Agustí et al. 2010), it is tempting to speculate that if cells of the FC expand they generate mechanical stress in peripheral tissues, which then have to react by inducing cell expansion and eventually cell divisions. This response may be important for avoiding tissue disruption. An effect of mechanostimulation on meristematic activity has already been described in the work of Ditengou et al. (2008), in which bending of the main root leads to reprogramming of pericycle cells into founder cells of lateral root primordia. There is strong evidence that mechanostimulation induces JA production and the expression of JA biosynthesis genes and, that this pathway is involved in thigmomorphogenesis, which stands for mechanically induced alterations of morphology (Jaffe 1973; Chung et al. 2008; Tretner, Huth et al. 2008; Chehab, Eich et al. 2009; Glauser et al. 2009; Koo et al. 2009). A JA induction upon touch is potentially triggered by an increase of lipoxygenase (LOX) transcripts which are responsible for the production of JA precursors from free fatty acids. Free fatty acids are released from membrane phospholipids by the phospholipase D (PLD) which localises to the plasma membrane upon elevated cytosolic Ca^{2+} levels. Elevated Ca^{2+} levels can be a direct response to mechanostimulation (Chehab, Eich et al. 2009). That JAZ10, one of the main regulators of IC

activity and initiation along the stem identified in this study, is touch-inducible and, furthermore, is expressed at the base of the inflorescence stem where SG takes place, has been shown in this work using *JAZ10:GUS* and qRT-PCR (Fig. 35B-E, 36A). In detail, *JAZ10* is found to be expressed in very young stems in the interfascicular region next to the starch sheath at the site where both, the IFs differentiate and IC is established (Fig. 35I-K). If *JAZ10* expression reflects tissue tension, these results suggest that such tensions are stronger in early stages of development between the primary bundles where IC initiates and IFs differentiate, suggesting the presence of JA-dependent thigmomorphogenesis in both developmental processes. However, in later stages, *JAZ10* expression in the interfascicular region appears to be solely at the site of the IFs (Fig. 35K). IFs differentiate out of parenchymatous cells into sclerenchymatous cells which have thick secondary cell walls and undergo senescence at the end (Kausmann and Schiewer 1989). JA is described to be involved in controlling senescence as well which would support the expression of *JAZ10* in the domain of the IFs (Watanabe et al. 2000).

My results suggest that, in addition to its essential role as a systemic signal in plant defence and wound response (Chung, Koo et al. 2008), JA signalling could play an important role in developmental processes in which tissue tensions must be released by initiating meristematic activity. Although positive effects on SG and stem stability seem to exist (see Fig. 37), negative effects of JA on cell division, in particular on longitudinal root growth, have been reported (Jaffe 1973; Pauwels et al. 2008; Zhang and Turner 2008). Biologically, it makes sense that, upon mechanical stress, longitudinal growth is inhibited and lateral growth is promoted in order to develop a more robust plant body.

Additionally, ethylene (ET) and its role in mechanical stress growth regulation are well reported. Inhibition of stem elongation, stem swelling, loss of gravitropic sensitivity (Goeschl et al. 1966), and leaf epinasty (Jaffe 1973) are reported actions of ethylene-mediated mechanical stress. Transcriptional profiling revealed a high up-regulation of a member of the ERF transcription factor family, namely ERF104, in stem segments with SG (Sehr, Agustí et al. 2010). ERF104 is a transcriptional activator and additionally touch-inducible (Nakano, Suzuki et al. 2006) and seems to be involved in IC initiation along the stem (Fig. 38C). These results propose a role of ethylene signalling in regulating developmental processes including IC initiation during mechanical stress (Okamoto et al. 2008).

Conclusion – a proposed model of hormonal crosstalk during IC initiation

It is obvious that single hormonal pathways do not act alone during the process of IC initiation and SG but operate in concert. Hormonal crosstalk is often local, may be even tissue-specific, and is spatially and temporally regulated to provide adaptive value to environmental conditions and fine-tuning of responses (Chandler 2009). In the case of IC initiation and activity an additive crosstalk of IAA, JA and ET following mechanical stimuli is proposed and the suggested model of hormonal crosstalk during IC initiation constructed from the results of the current and previous studies is as follows (Fig. 39):

The process of SG starts with the activation of the FC possibly by auxin flow in this tissue (Little, MacDonald et al. 2002; Sehr, Agustí et al. 2010). The proliferating FC and the newly produced cells lead to an increase of the primary vascular bundle which, potentially, generates mechanical forces in the neighbouring cells. These mechanically stressed cells might react by synthesising stress hormones like JA and ethylene. The increase in JA levels leads to the degradation of the JAZ repressors, release of MYC2 and further JA-dependent gene expression. This includes the *JAZ* genes (Chini, Fonseca et al. 2007), in the case of SG especially *JAZ10*. Additionally, auxin biosynthesis could be turned on by the expression of *ASA1*, an auxin biosynthesis gene which has been shown to be JA inducible (Sun, Xu et al. 2009). In addition to JA, also ET might be produced as response to mechanical stress. The ET-signalling cascade leads inter alia to the expression of the ERF transcription factors (Stepanova and Alonso 2009), including *ERF104* (Bethke, Unthan et al. 2009), which turned out to be an additional component in the regulation of SG. ERFs are known to be also JA-inducible (Lorenzo, Piqueras et al. 2003). The expression of ERFs leads to further ET-related gene expression, which might include as well *ASA1* (Stepanova et al. 2005). Upon the ET/JA-inducible expression of *ASA1*, endogenous auxin biosynthesis might be then triggered in the interfascicular region. Additional auxin may be provided by the general main auxin flow within the FC, which would increase IAA concentration in cells that are located in close proximity to FC cells due to auxin moving out of these cells (Little, MacDonald et al. 2002). Thus, the proximity of interfascicular cells to the FC might be the positional trigger for initiation of IC identity (Wilson 1978). Furthermore, IAA is suggested to be transported actively by PAT to the adaxial neighbourhood of the starch sheath in the interfascicular region. This specific IAA localisation domain might be restricted by the differentiating IFs. Whether PIN3 and PIN7, which are present in and around the starch sheath and are degraded long before IC is initiated, are important for establishing the auxin accumulation adaxially to the starch sheath, needs still to be clarified. Surprisingly, both proteins are potentially down-regulated by JA

(Nemhauser et al. 2006), which might explain their spatial distribution which does not overlap with *JAZ10* expression. At the end, the IAA present in the interfascicular region may trigger cell divisions partly by effecting the expression of core cell-cycle regulators (e.g. cyclins and cyclin-dependent kinases) and additionally the turnover of proteins that are necessary for cell proliferation (Snow 1935; Little, MacDonald et al. 2002; Perrot-Rechenmann 2010). Last, but not least, PIN1 seems to be needed in later stages of IC development eventually for draining of the IAA, which has been synthesised in the interfascicular region. Based on this model IAA signalling might play a role downstream of JA signalling during IC initiation.

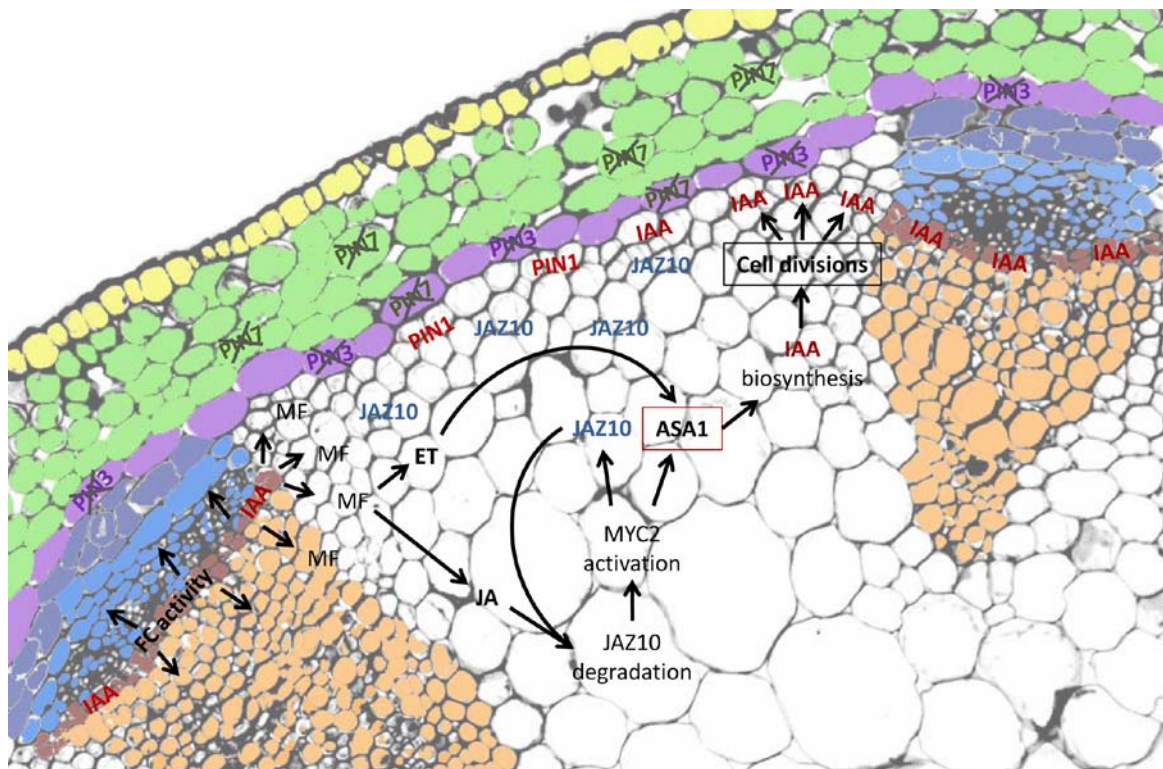


Figure 39: A model of regulatory processes during IC formation. At the stage of the primary stem the fascicular cambium (FC, red) starts to proliferate and might generate mechanical forces (MF) in the surrounding cells which experience thus stress and produce the stress hormones ethylene (ET) and jasmonic acid (JA). The accumulation of JA leads to the degradation of the JAZs repressors and thus to MYC2 activation and further expression of JA-inducible genes, e.g. *JAZ10* which represents a regulatory feedback loop of JA signalling. Presumably, JA- and ET-signalling pathways induce auxin biosynthesis genes like *ASA1* which turns on local auxin (IAA) biosynthesis. Subsequently, IAA might trigger cell divisions observed during IC initiation. Additionally, basipetal polar auxin transport (PAT) mediated by PINs effect IC initiation and activity. PIN3 and PIN7 are predominantly localised in the starch sheath but not within the stem part where secondary growth (SG) takes place. Thus, this absence of both proteins from the plasma membrane at the stem base is supposed to be a prerequisite for IC initiation. However, this clearly needs further investigation. Since PIN1 appears to be in the IC in late stages of development, a role in IAA drainage is proposed.

Taken together, these findings indicate that the IC is initiated *de novo* in interfascicular regions by signals that presumably originate from the FC of primary vascular bundles, and therefore represents a secondary lateral meristem. Moreover, I have shown a positive role for JA signalling in cambium regulation, and hypothesise that it mediates mechanical forces present in the stem,

a structure that is particularly exposed to environmentally induce physical stresses. This finding provides new insights into the mechanisms underlying SG, a source of terrestrial biomass par excellence.

Acknowledgments

This work has been carried out at the Gregor Mendel Institute of Molecular Plant Biology GmbH (GMI) in Vienna, Austria. I would like to thank especially Thomas Greb for giving me the opportunity to work at the GMI and to perform my Ph.D. in his group. Many thanks go to my Ph.D. committee, Marie-Theres Hauser and Klaus Theres, for their support and helpful discussions. I am grateful to the collaborators of the project, Edward E. Farmer, who provided seeds of the *JAZ10:GUS* marker line and participated in writing the publication, Jiří Friml, from whom the *PIN3:PIN3-GFP* marker line and a lot of support regarding PINs was obtained, and Christian Luschnig, for the idea to mutagenise the internalisation motif of PIN3 and the constant scientific support.

Special thanks go to former and current members of the Greb Group Team. It was a pleasure to work with you all! I would like to acknowledge all my colleagues and friends at the institute. You all made the GMI to the best place to work and socialize!

Ganz, ganz herzlich möchte ich mich auch bei all meinen Freunden und Bekannten bedanken, die mit mir nach der Laborarbeit das Leben und die Freizeit genossen haben (und noch immer geniessen). Geht schon, gemma, Vollgas!!

Grosser Dank geht auch an A+H, den zwei Grossspatzen, für ihre ansteckende Begeisterung und Neugierde für Flora und Fauna.

Der allergrösste Dank gilt meiner Familie, allen Edlingers und allen Sehrs, meinen Omas und Opas, Tanten und Onkel, Cousinsen und Cousins. Besonders aber meinen Eltern, Adi und Gerda, und meiner Sista Sabrina, die mich bei allen Entscheidungen und in allen Lebenslagen immer unsterstützt haben und hinter mir gestanden sind. DANKE!

Für den besten Support, den Gedankenaustausch und die vielen Diskussionen und Tratsch, für die geteilten Interessen und für die schöne gemeinsame Zeit möchte ich mich bei Max ganz herzlich bedanken. :*

VIELEN DANK AN EUCH ALLE – MANY THANKS TO ALL OF YOU

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Curriculum vitae

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1995 - 1999	College (Bundesoberstufenrealgymnasium), Treibach-Althofen

Career

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