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Auxin coordinates cell division and cell fate specification during lateral root initiation

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CHAPTER 1 Auxin regulation of cell cycle and its role during lateral root initiation

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Abstract

The plant hormone auxin plays a crucial role in the upstream regulation of many processes, making the study of its action particularly interesting to understand plant development. In this review we will focus on the effects auxin exerts on cell cycle progression, more specifically, during the initiation of lateral roots. Auxin fulfils a dominant role in the initiation of a new lateral root primordium. How this occurs remains largely unknown. Here we try to integrate the classical auxin signalling mechanisms into recent findings on cell cycle regulation. How both signalling cascades are integrated appears to be complex and is far from understood. As a means to solve this problem we suggest the use of a lateral root-inducible system that allows investigation of the early signalling cascades initiated by auxin and leading to cell cycle activation.

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Introduction

For an ideal exploitation of the growth potential it is essential for plants to have an optimally branched root system. Branching of roots occurs, in contrast to shoot branching, by the endogenous formation of new primordial that grow out of the main root to become secondary or lateral roots. Although not as obvious as the phyllotactic positioning of lateral organs in the shoot, there is a certain level of regularity in the spacing of lateral roots along the parent root. In most species lateral roots are exclusively initiated at protoxylem poles resulting in longitudinal rows of lateral roots (Zhang *et al.*, 1999). The mechanisms guiding when and where lateral roots are initiated have a high degree of plasticity, integrating both intrinsic and extrinsic levels of control. Environmental factors, such as water, nutrients, and temperature have significant influences on the root architecture (Dubrovsky and Rost, 2003).

The most important signals for lateral root initiation come from plant hormones and their interplay. Among the plant hormones, auxin is the rooting hormone par excellence. It has become common knowledge that auxins consistently induce lateral and adventitious root formation. Several studies have illustrated this reliably over the years in different species (Blakely and Evans, 1979; Thimann, 1936; Torrey, 1950). Exogenous application of auxin to seedlings, root and stem explants results in most cases in the proliferation of new root primordia. From recent work with *Arabidopsis* it became clear that the endogenous auxin is also essential for the correct positioning of the lateral root primordia. Several mutants that have defects in early auxin responses show no or a reduced number of lateral roots (Casimiro et al., 2003) and when the active cell-to-cell transport (polar auxin transport, PAT) is hindered lateral root initiation is blocked or at least reduced (Casimiro et al., 2001). During lateral root initiation, auxin or an auxin-derived signal is perceived by some of the pericycle cells. The following, clearly visible event of this induction process is an asymmetric, anticlinal division in pericycle cells located adjacent to (a) xylem pole (s) (Casimiro *et al.*, 2001).

However many questions remain unanswered. Nothing is known about the nature of these auxin-derived signals and how only in a few pericycle cells the cell cycle engine can be activated by these signals. On its own this pericycle-specific activation is intriguing, since it is happening outside the root apical meristem implying that the first divisions leading to lateral root initiation would involve a de-differentiation of pericycle cells. However, the common idea of a fully differentiated pericycle has been questioned many times. Shorter cells as a result of enhanced cell division rates have been reported in *Allium cepa*, *Pisum sativum* and *Daucus carota* for those cell files where lateral root initiation occurs (Lloret and Casero,

2002). On the same line, in *Arabidopsis* pericycle cells opposite the protoxylem poles ongoing cell division activity was found (Dubrovsky *et al.*, 2000). Most of these divisions are purely proliferative and result in the shorter cells at the xylem poles in comparison with the size of cells at the phloem poles. Only a few of these extra cell divisions will result in the initiation of a new lateral root and can be specified as formative divisions. Therefore the auxin-induced lateral root initiation may not initially be a matter of starting up the cell cycle engine but rather the promotion of formative divisions in a limited number of cells.

Despite the tremendous increase of our understanding in the molecular regulation of the plant cell cycle over the last 10 years (Dewitte and Murray, 2003), insight into the orchestration of cell division in the context of plant growth is nearly completely lacking. Furthermore, the signalling cascade connecting the auxin signal with the cell division activity is still unidentified. In this respect the initiation of lateral roots could be an ideal system to analyse the connection between auxin signalling and there-initiation of cell division. In this review we want to highlight some of the recent developments in auxin and cell cycle research that may form the basis for an understanding of the intimate link between auxin and the cell cycle during lateral root initiation.

Auxin and cell cycle gene expression

The current understanding of cell cycle regulation was mainly achieved by studying synchronized cell suspensions and revealed its complicated nature (Dewitte and Murray, 2003). Cell cycle regulation in plant cells is an intricate homologue of cell cycle regulation in animal and yeast cells. Basically, it is driven via a complex modulation of the activity of serine/threonine kinases. The catalytic subunit is a cyclin-dependent kinase (CDK) that requires dimerization with a regulatory subunit (cyclin) for activation. Further controls on the kinase-activity occur through phosphorylation/dephosphorylation events of certain conserved amino acids (Mironov et al., 1999) and binding with CDK inhibitory proteins, the so-called Kip-Related Proteins or KRPs (De Veylder et al., 2001). In yeast, animals and plants this principle is highly conserved, but the number of orthologous players differs very significantly. Plants seem to have a core cell cycle machinery that is as complex as that found in mammals as was indicated by the identification of 61 core cell cycle genes in *Arabidopsis* (Vandepoele et al., 2002).

There is not much known on how plant hormones interfere with the cell cycle machinery at the posttranscriptional level, but there are some data available on direct

transcriptional up- or down-regulation of core cell cycle genes. Auxin has been shown to induce the expression of various mitotic cyclins: CYCA2;1, CYCA2;2, CYCB1;1, CYCB2;1, CYCB2;2 (Ferreira et al., 1994a; Ferreira et al., 1994b; Richard et al., 2001; Roudier et al., 2003) and CDKA;1 (Hemerly *et al.*, 1995; John *et al.*, 1993; Martinez *et al.*, 1992; Richard *et al.*, 2001). Furthermore, D-type cyclin levels were shown to be highly responsive to the addition of auxin, but induction could also be provoked by or in combination with other mitogens such as cytokinins, brassinosteroids and/or sucrose (Fuerst *et al.*, 1996; Hu *et al.*, 2000; Richard *et al.*, 2001; Soni *et al.*, 1995). Likewise the plant-specific CDKB1;1 is only weakly induced by auxin alone but a synergistic induction occurs when applied together with cytokinins (Richard *et al.*, 2001). The situation is less clear for the CDK inhibitors or KRPs. In Arabidopsis seven KRPs have been identified (De Veylder *et al.*, 2001) and different transcript accumulation patterns upon mitogen treatments were recorded. At least in cell suspension cultures, auxin seemed to induce KRP1 expression while it was repressing the expression of KRP2 (Richard *et al.*, 2001). This fundamentally different reaction upon auxin suggests that each of the KRPs may play a unique role in the regulation of the cell cycle.

Auxin perception and cell division

Auxin signalling has been the subject of many studies; nevertheless it remains elusive to resolve the many questions and speculations. However, very recently several pieces of the puzzle have been identified. As a central dogma, one can state that signalling starts off with the binding of a ligand to a receptor, activating a cascade of phosphorylations that result in transcriptional regulation. In the case of auxin, a putative receptor (ABP1) has been identified for more than a decade without unambiguous confirmation of its receptor function (Lobler and Klambt, 1985). Even with the biochemical role of ABP1 still uncertain, it is more than likely that this protein can mediate cell division in a direct or indirect manner since homozygous null mutants in ABP1 show aberrant cell division patterns during early embryogenesis. Furthermore, antisense-ABP1 tobacco cell suspensions show defects in auxin-mediated cell elongation and division (Chen et al., 2001). ABP1 transcripts were shown to accumulate at sites of lateral root initiation in sunflower, suggesting that auxin sensitivity of certain cell types might be enhanced at well-defined stages during plant development (Thomas et al., 2003). Whatever the real auxin receptor(s) might be, new data on the potential involvement of GTP binding proteins in controlling the auxin sensitivity towards cell division

were recently provided. GTP-binding proteins are known to couple signals through membrane receptors to downstream effectors. In the classical model, the binding of a ligand to a G protein-coupled receptor results in the conversion of inactive G-proteins (GDP-bound) to the active (GTP-bound) conformation. The binding of GTP results in the release of an alpha-subunit and its activation. Hydrolysis of the bound GTP brings the complex back to its original non-active state (Stryer, 1995). In *Arabidopsis*, the alpha-subunit of a putative heterotrimeric G-protein (GPA) was identified as a positive regulator of cell division (Ullah et al., 2001). More recently, the beta-subunit of the same complex (AGB) was shown to counteract this activity and to play a role in the attenuation of the auxin signal towards cell division. This beta-subunit-dependent attenuation appears to happen upstream of the control of auxin on mRNA steady-state levels (Ullah et al., 2003). Furthermore, it was shown that auxin application was able to repress transcription of the beta-subunit and at the same time to induce the alpha-subunit mRNAs. This reveals the existence of an auto-regulation of this repressive pathway. Moreover the activity of the alpha-subunit and its effect on cell proliferation seems to be controlled by a seven-transmembrane Regulator of G protein Signalling (RGS) protein (Ullah *et al.*, 2003). RGS proteins are known to accelerate the de-activation of the alpha subunits, thereby reducing the G protein-coupled receptor signalling. Whether this part of the receptor signalling is also influenced by auxin has not yet been demonstrated.

Auxin response, protein degradation and cell cycle

Auxin activates 26S-proteasome-mediated degradation of specific proteins. The mechanisms controlling this phenomenon have become clearer recently. The SCF^{TIR1} E3-ligase plays a dominant role in targeting short-lived, nuclear proteins (AUX/IAAs) via oligo-ubiquitination for degradation by the 26S-proteasome (Leyser, 2002). This E3-ligase is a complex composed of three proteins (SKP, Cullin, TIR1). Furthermore, the activity of the SCF^{TIR1}-complex is regulated by cycles of rubiquitination and de-rubiquitination. Up to now, the only targets of the SCF^{TIR1}-complex are members of the AUX/IAA-gene-family (Ramos *et al.*, 2001; Tian *et al.*, 2003). This gene-family consists of 29 members that have conservation in four domains (Hagen and Guilfoyle, 2002). They are generally regarded as repressors of the activity of Auxin Response-regulating transcription Factors (ARFs). The ARFs are active transcription factors when forming dimers within the ARF gene-family, consisting of 23 members (Hagen and Guilfoyle, 2002). The inhibitory action of the

AUX/IAAs finds its origin in disrupting the ARF dimers, resulting in inactive heterodimers. The number of possible combinations between these two gene-families is very high, adding to the complexity of this signalling pathway (Leyser, 2002).

Another piece in this auxin-signalling puzzle is the transcription factor NAC1 (Xie et al., 2000). This transcription factor is considered to be epistatic to TIR1, a component of the SCF^{TIR1}-complex. Modification of the level of transcription of NAC1 strongly correlates with an altered lateral root density. It was hypothesized that NAC1 is a positive regulator of lateral root initiation. A differential display analysis revealed that NAC1 positively regulates the transcription of AIR3 (Xie *et al.*, 2000). This confirms the association of NAC1 with lateral root formation, since AIR3 was earlier identified during another differential display screening for lateral root formation (Neuteboom et al., 1999). AIR3 is expressed at sites of lateral root emergence, suggesting that AIR3 is involved in weakening cell-to-cell connections and thus facilitating lateral root emergence. Furthermore NAC1 is targeted for degradation after ubiquitination through interaction with the E3-ligase SINAT5 (Xie et al., 2002). More recently, it was shown that the microRNA, miR164, targets several NAC-domain transcription factors, among which NAC1 (Mallory *et al.*, 2005). All these levels of regulation on NAC1 suggest that the auxin response mediated through this transcription factor plays a key role in development.

It is not known whether plant cell cycle regulators themselves could be the targets for an auxin-dependent proteolysis nor if their expression is controlled by the AUX/IAA-ARF system. Although without having proof of their functionality, in the promoter region of a considerable number of cell cycle genes auxin response elements (AuxREs) were found (Richard *et al.*, 2001), suggesting that the transcriptional activation of some of them is directly controlled by ARF transcription factors. On the other hand the abundance of the cell cycle inhibitor AtE2Fc is controlled by a SCF-complex (del Pozo et al., 2002). E2Fc acts to repress the entry of cells into S-phase by competitive inhibition for the DPa subunit of the E2Fa/DPa transcription factor complex. The authors could demonstrate that E2Fc is phosphorylated in vitro by both CDKA;1/CYCA2;2 and CDKA;1/CYCD2;1 complexes and that this phosphorylation is essential for the recruitment of the protein by the F-box protein (AtSKP2) and for its ubiquitin-mediated degradation. By inducing the expression of CDKA;1 and cyclins, auxin could promote the degradation of this inhibitor and stimulate the passage through the G1-to-S boundary.

Cell cycle phase-dependent auxin response and sensitivity

In the above paragraphs we referred to some of the possible signalling pathways that could link auxin to the molecular regulation of cell cycle. However, on top of this complexity, it could be that some auxin responses are confined to certain stages of the cell cycle or in other words that cells might acquire the capacity to respond to auxin only at specific phases of the cell cycle. One way to approach this question is to verify if the expression of some of the earlier described genes involved in auxin signalling is cell cycle regulated. A given cell cycle-related temporal expression profile might suggest that the protein is important to translate the auxin response into progression through the cell cycle. In synchronised cell suspensions of *Arabidopsis* cells, IAA17/AXR3 was found to be upregulated and IAA18 showed a S phase peak (Menges and Murray, 2002). Using synchronized tobacco Bright Yellow-2 cells and cDNA-AFLP based expression analysis, early M-phase specific expression of ARF1 and different members of the AUX/IAA gene family could be demonstrated (Breyne and Zabeau, 2001). Although not numerous, these examples indicate that the expression of some of the known auxin signalling genes is cell cycle regulated.

Another gene with a distinct cell cycle-related expression pattern and to some extent essential for a normal auxin response is the HOBBIT gene. It encodes a CDC27 subunit of the anaphase promoting complex (APC) and its expression is restricted to cells undergoing G2-to-M transition. Mutation of the HOBBIT gene leads to severe defects in the root meristem. Interestingly, callus can readily be derived from the mutant, whereas roots derived from mutant callus show the same defects of the mutant root meristem. This strongly suggests that HOBBIT is essential for developmental context-dependent cell cycle regulation (Willemsen et al., 1998). Furthermore, the *hobbit* mutant accumulates high levels of the auxin response inhibitor AXR3/IAA17. This indicates that HOBBIT activity would be involved in targeting such AUX/IAA proteins for degradation. Ideally, this could provide the basis for the restriction of certain auxin-mediated responses to dividing cells (Blilou et al., 2002).

Yet another process that is regulated in a cell cycle dependent manner and is influenced by auxin, is telomerase activity. Telomerases synthesise and maintain specialized nucleoprotein complexes at the ends of linear eukaryotic chromosomes, the so-called telomeres. In mammals, telomerase activity is associated with the differentiation level of the cells. In proliferative cells, telomerase activity is high and dramatically drops when differentiation starts (Sharma et al., 1995). In tobacco BY-2 cells, it was demonstrated that the S-phase-specific telomerase activity was strongly enhanced at early S-phase by addition of

auxin (Tian *et al.*, 2004). Mutants in the *Arabidopsis* telomerase (AtTERT) are viable for a limited number of generations, before showing severe developmental defects (Kurihara and Watanabe, 2004). The capacity to survive without telomerase is in contrast with the immediate catastrophic damage to the genome and the much earlier cell cycle arrest in animals. It is tempting to speculate that the S-phase-specific induction of telomerase activity by auxin is necessary to supply plant cells with sufficient telomeric DNA and thereby guaranteeing prolonged cell proliferation capacity. If auxin-induced cell division goes together with enhanced telomerase activity, the initial pericycle cells in the case of lateral root formation, would be armed with sufficient telomeric DNA, allowing multiple rounds of successive divisions required for the formation of a totally new organ.

Lateral root initiation and cell cycle regulation

Most of the data on cell cycle gene expression were obtained from studies using cell suspensions. The complexity increases tremendously when cell cycle regulation is studied at the whole plant level. Such a complexity can only be examined by reducing the number of variables and by analysing co-expression of genes in the same tissue and process. Despite this reasoning, very few initiatives to assess such problems have been taken. Here we will summarize our data on cell cycle regulation during one particular developmental process, namely the initiation of lateral roots. Tissue-specific expression of known core cell cycle regulators was investigated in the view of lateral root initiation in *Arabidopsis* (Beeckman et al., 2001). This work resulted in the formulation of a model for cell cycle regulation during lateral root initiation. Pericycle cells leaving the root apical meristem remain in G1-phase. The pericycle cells near the protoxylem poles remain susceptible to cell cycle re-entry even after the exit out of the meristem. In radish roots this readiness to divide was interpreted as the pericycle cells having completed DNA synthesis and being in the G2-phase (Blakely and Evans, 1979). Already, within 2 h after exposure, mitotic figures could be noticed in the pericycle cells indicating cell cycle re-entry is positively regulated by the plant hormone auxin. Analysing cell cycle markers in *Arabidopsis* also showed that the G2-to-M marker gene *CYCB1;1* is expressed in dividing cells of the root apical meristem as well as in pericycle cells undergoing the first division of lateral root formation (Beeckman *et al.*, 2001).

Since auxin is regarded as a primary regulator of lateral root initiation, Casimiro *et al.* (2001) assessed the effect of a disturbed auxin distribution on lateral root initiation. Lateral

root initiation was completely abolished in seedlings that were germinated on sufficiently high concentrations of the auxin transport inhibitor N-naphtyl phtalamic acid (NPA). Transfer of such seedlings to media containing the synthetic auxin 1-Naphtalene Acetic Acid (NAA) at appropriate concentrations resulted in asynchronous induction of lateral root initiation. Based on this principle a lateral root inducible system was developed (Himanen et al., 2002). Analysing the expression levels of several core cell cycle genes over time, in the lateral root inducible system, revealed a clear phasing of the cell cycle. The expression levels of cell cycle genes and experiments with G1-to-S inhibitor, hydroxyurea, suggested that all the cells of the pericycle remained in G1-phase until auxin induced re-entry into the cell cycle. This is the first clear demonstration that auxin works on the G1-to-S checkpoint in the case of lateral root initiation. Therefore the cell cycle machinery controlling this transition is most likely the primary target for the auxin signalling cascade. Interestingly, the same cell cycle checkpoint has to be traversed when mesophyl protoplasts of *Petunia hybrida* re-enter the cell cycle after incubation on auxin and cytokinin-containing media (Bergounioux et al., 1988). Later on it was demonstrated that, using the same protoplast cultures, despite the fact that both hormones are necessary for entry into S phase, an auxin pre-treatment allowed cells to enter S phase more rapidly (Tréhin et al., 1998). It could be deduced from these experiments that although auxin alone was not sufficient to push the cells through the G1-to-S transition, it clearly helped in the preparation of the cells to enter S phase.

Moreover, this peculiar auxin-mediated cell cycle progression could explain the failure to induce extra lateral root primordia by ectopic expression of *CYCB1;1* under the control of the *CDKA;1* promoter (Doerner et al., 1996). This ectopic expression enhanced root growth from established meristems but neoplasia could not be induced. Consequently, speeding up the cell cycle on its own is not sufficient to induce lateral root initiation indicating that auxin in the context of lateral root initiation is doing more than purely stimulating cell division. Whether this could account for the lack of reports on lateral root phenotypes in several of the transgenic *Arabidopsis* plants overexpressing or inactivating core cell cycle genes (Cockcroft et al., 2000; Dewitte et al., 2003; Yoshizumi et al., 1999) remains an open question.

KRP genes and lateral root initiation

Corresponding with the auxin-modulated expression of KRP genes in protoplast cultures (Richard *et al.*, 2001), KRPs also did not show a uniform expression regulation in

the lateral root inducible system. *KRP1* and *KRP2* expression were high in the inactive pericycle of NPA-treated roots, and a strong down-regulation could be observed upon transfer to NAA. *KRP4* reacted weakly upon auxin application, whereas *KRP3* showed a completely opposite expression profile being low under lateral root restrictive conditions and induced upon transfer to auxin. The high levels of expression on NPA and auxin induced down-regulation of at least some of the KRPs, suggested a potential role in controlling lateral root initiation by inhibiting CDK activity in pericycle cells that did not receive an auxin signal. Furthermore, overexpression of KRP2 resulted in a strong reduction of the lateral root density (Himanen *et al.*, 2002). Analysis of the tissue-specificity of the expression of this gene supported its role as inhibitor of lateral root initiation. *KRP2* was strongly expressed in pericycle cells near phloem poles, which is consistent with the proposed non-dividing nature of phloem pericycle cells. Furthermore, *KRP2* was also expressed in xylem pericycle cells opposite sites of lateral root formation. This was also in concordance with the observation that these xylem pericycle cells rarely initiate a lateral root. Based on these extra findings a modified model for cell cycle regulation during lateral root initiation has been proposed (Casimiro *et al.*, 2003).

Transcript profiling and lateral root initiation

It is apparent that auxin signalling is a very complex, poorly understood process that is made even more obscure by the tissue specificity of its action. Auxin is undoubtedly involved in lateral root initiation and transcript profiling of this process could contribute largely to our insight into auxin signalling. Analyses on mature root systems before and after auxin treatment have proved to be poorly reproducible, resulting in a very low number of significantly regulated genes (Tian *et al.*, 2002; Ullah *et al.*, 2003). This is mainly due to the heterogeneity of the starting material. Recently we developed a lateral root inducible system allowing the synchronous induction of lateral roots (Himanen *et al.*, 2002). In this system the xylem pole pericycle cells are synchronously activated by auxin transport inhibition followed by auxin application. The details of the inducible system and the major developmental events taking place prior to the first pericycle divisions (represented by the stages 0, I, II and III) are summarized in Fig. 1.1.

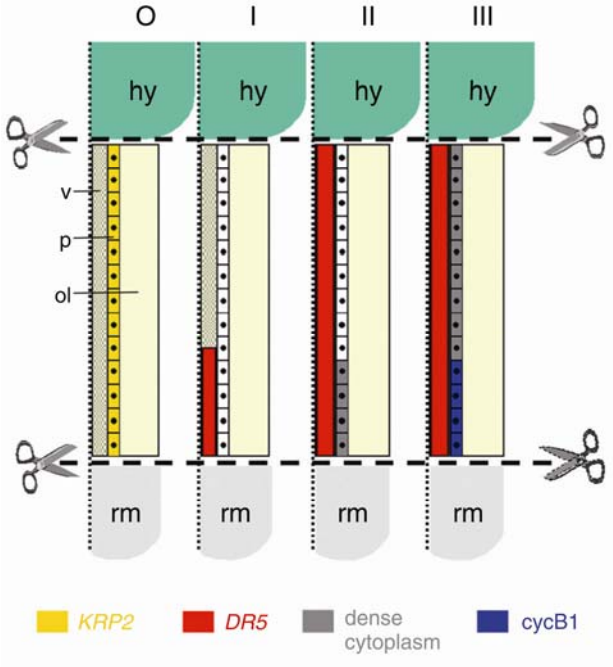

Figure 1.1 Model describing the developmental stages preceding the fist formative cell dividions in the xylem pole pericycle. Four stages (0, I, II, III) could be defined based on the study of gene expression and a detailed cytological study using a lateral root inducible system (Himanen *et al.*, 2002; Himanen *et al.*, 2004). In this system, seedlings were grown in conditions with impeded auxin transport and then transferred to media containing NAA as external auxin source. The scissors and the interrupted lines indicate that part of the root that was used in these studies. Stage 0, G1 cell cycle block is indicated by uniform *KRP2* expression along the pericycle. Stage I, *KRP2* is absent, auxin signal perception and transduction are indicated by the induction of *DR5::GUS*-expression (indicative for cellular auxin responses (Ulmasov *et al.*, 1999)) in the central cylinder. Stage II, *DR5::GUS* is expressed along the whole central cylinder, G1-to-S marker genes are induced, and the cytoplasmic density of the pericycle cells increases (indicative for meristematic cells). Stage III, G2-to-M-specific genes and *CYCB1;1::GUS* are induced, and the entire xylem-pole pericycle is filled with dense cytoplasm. Abrreviations: hy, hypocotyls; ol, outer tissue layers (epidermis, cortex, endodermis); p, xylem pole pericycle; v, central vascular tissue; rem, root meristem.

The auxin transport inhibition induces a G1-cell cycle block accompanied by the expression of the CDK inhibitory KRP2 gene at stage 0, auxin perception and signal transduction at stage I (visualized by the start of DR5::uidA promoter activity), followed by progression over G1-to-S transition at stage II, and G2-to-M transition at stage III, going together with the development of meristematic appearance (dense cytoplasm) of xylem pole pericycle cells. The identification of these ‘pre-cell division’ stages in the process of lateral root initiation is a new perception and was used to determine the sampling time points for a broad transcript profiling study (Himanen et al., 2004). About 20% of all genes under analysis were significantly ($P < 0.005$) differentially expressed. Two of the previously mentioned auxin signalling systems, namely the AUX/IAA and the heterotrimeric G-protein-mediated responses, were activated early on, whereas the ABP1-mediated signalling did not react upon the auxin treatment. Again, the previously mentioned auxin-induced G1-to-S transition

became apparent as the first cell cycle event on the way to lateral root initiation.G1-to-S-specific genes such as E2Fa and Histone H4 were early induced together with DNA replication genes.

The high percentage of significant genes indicates a high reproducibility of the experiment. This transcript profiling validates itself by the presence of previously described regulators of auxin response and cell cycle genes. This dataset is a great source of potential key-regulators of auxin-mediated lateral root induction.

Conclusions and perspectives

Although the molecular control of cell cycle regulation and auxin signalling is the subject of many studies, the pathways linking them together have not yet been uncovered. One thing that is unambiguously clear is the complex nature of the auxin-cell cycle cross-talk. In this review we highlighted some of the pathways that could play a role in this cross-talk. In Fig. 1.2, we summarise the mentioned pathways and hypothesise on the potential links with cell cycle regulation. By focusing on one particular developmental programme such as lateral root initiation and starting from well-designed transcriptome analyses, we hope to get a better insight into the black box between auxin and the cell cycle. Furthermore, studying lateral root initiation as such could contribute to our understanding of developmental biology and in particular root development

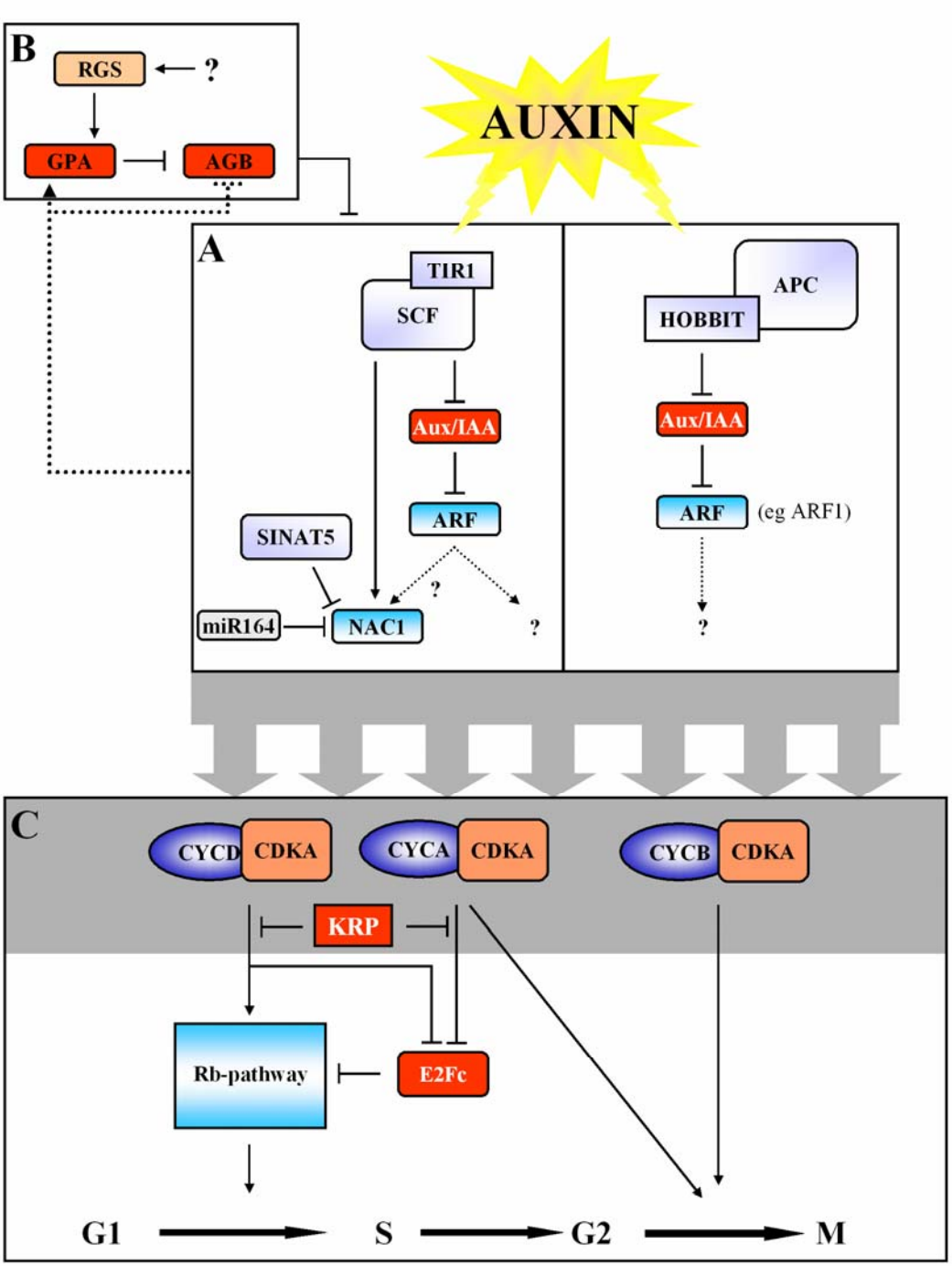

Figure 2.2 Flowchart summarising the discussed auxin signalling pathways and the potential links with cell cycle regulation. (A), Via the SCF^{TIR1}- complex, auxin destabilizes AUX/IAA proteins. Such AUX/IAA proteins act to repress the activity of auxin response factors (ARF). When released, ARFs regulated transcription of auxin responsive genes. One of these genes could be *NAC1*, as it was shown to be epistatic to TIR1. NAC1 is a positive regulator of lateral root initiation. The levels of NAC1 are negatively regulated through proteolysis via SINAT5 and mRNA destabilization through miR164. In this model lateral root initiation is stimulated by positive regulation of G1-to-S and/or G2-to-M transition. This role could be fulfilled by NAC1 alone or in concert with other unknown regulatory factors. Next to this general pathway of auxin signalling there is evidence for auxin signalling pathways that are specific to certain cell cycle phases. The HOBBIT protein was shown to be involved in the destabilisation of certain AUX/IAA proteins during the G2-to-M transition. The targets of these ARFs will potentially be specific to

this cell cycle phase such as ARF1. (B), The auxin signal for cell proliferation is subject to an attenuation mechanism directed via a G-coupled receptor complex. In this system the α-subunit of the heterotrimeric G-protein (GPA) represses the activity of the β-subunit (AGB). The activity of the latter is suggested to attenuate the auxin signal leading to cell proliferation. Upstream of this hetrotrimeric G-protein, a seven-membrane regulator (RGS) stimulates the AGB repression through GPA. Furthermore, it was shown that the transcripts of both subunits are subject to auxin-mediated regulation, resulting in a reduction of this G-protein mediated attenuation of the auxin signal. (C), Auxin is a potent mitogen as is indicated by the auxin inducibility of several types of cyclins and modulation of cell cycle repressors. The light grey one indicates potential auxin modulation of a part of the cell cycle machinery. During G1-to-S transition, D-type cyclins form complexes with CDKA;1 activating the RBR pathway and stimulating entry into S-phase (Gutierrez *et al.*, 2002). Cell cycle inhibitors (KRPs) are potent repressors of this mechanism. A-type and B-type cyclins are also transcriptionally induced by auxin. Together with CDKA;1, A-type cyclins stimulate the degradation of E2Fc which is a negative regulator of the E2F/DP pathway. The B-type cyclins are specific to G2-to-M transition and are thought to fulfil important roles during this transition. Dotted lines indicate transcriptional regulation, full lines postr-transcriptional regulation.

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CHAPTER 2 Auxin fuels the cell cycle engine during lateral root initiation

Vanneste S., Inzé D. & Beeckman T.

Book chapter in: Cell cycle and plant development (Inzé, D., ed.), Blackwell Publishing, London, (in press).

Introduction

Meristems represent sites of mitotic activity in plants. In roots, meristems can be found at the apex and at the tips of lateral roots (primary, secondary, tertiary). The lateral roots are major contributors to the overall root system. The more meristems a root system generates, the more efficiently the soil can be exploited. For a single 16-week old winter rye plant up to an astonishing 13 million branches have been reported, resulting in a total root length of more than 500 km packed in less than 0.05 cubic meters of soil (Dittmer, 1937).

Lateral root development can optimally be studied in a species with a simple diarch root system (having only two protoxylem poles) such as the model plant *Arabidopsis thaliana*.

In *Arabidopsis*, lateral roots originate from 3 files of xylem pole associated pericycle cells at each protoxylem pole. The onset of forming a novel lateral root coincides with restarting cell cycle activity in these formerly non-dividing pericycle cells. Lineage analysis has shown that the majority of cells in a lateral root primordium are derived from the central file of xylem pole pericycle cells (Kurup *et al.*, 2005). Initially, these pericycle cells acquire founder cell identity. After commitment, the founder cells divide asymmetrically resulting in a stage I primordium (Malamy and Benfey, 1997). Subsequently, strictly organized cell divisions result in periclinal growth of the primordium. Between the 3- and 5-layered stage a lateral root primordium is capable of autonomous growth (Laskowski *et al.*, 1995) and evolves into a fully functional lateral root meristem after emergence (Malamy and Benfey, 1997) (Fig 2.1).

Physiological studies have identified a plethora of interactions between most plant hormones during lateral root formation. Among the different plant hormones, auxin stands out for its key role in many, if not all, developmental steps of lateral root development. Indeed, overwhelming evidence shows the involvement of auxin in founder cell specification, cell division and meristem organisation, lateral root emergence and meristem activation. Interestingly, many of the other plant hormone response pathways converge, at least partially, to the modulation of auxin activity.

Here we will summarize the most recent insights into auxin induced lateral root initiation as a model for cell cycle (re)-activation.

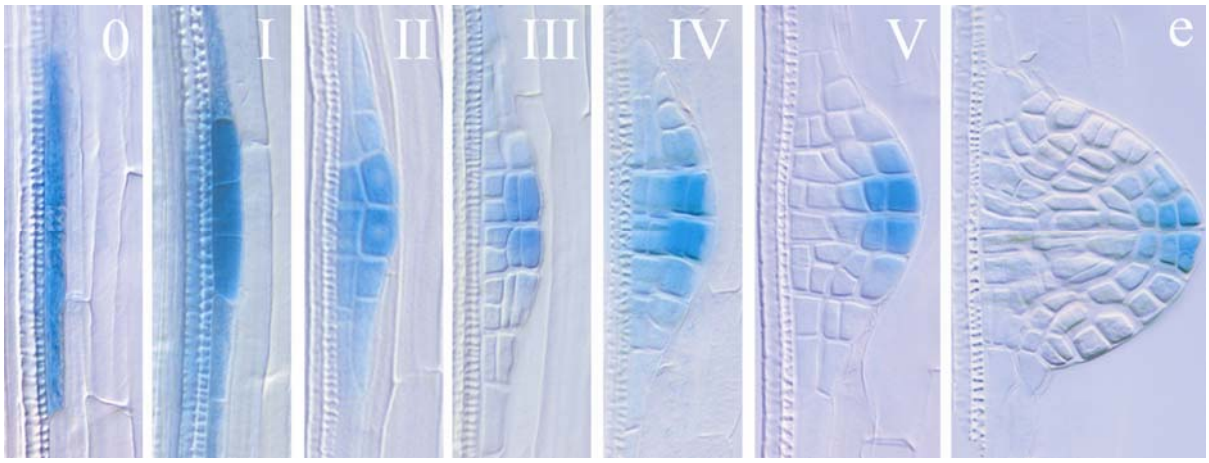

Figure 2.1 Stages of lateral root development and the organisation of an auxin gradient. Blue staining show auxin accumulation as indicated by DR5::GUS activity. Just prior to the asymmetric divisions hallmarking lateral root initiation, stage 0, auxin accumulates in adjacent xylem pole pericycle cells. At stage I, the first anticlinal asymmetric divisions have occurred and the DR5::GUS activity is restricted to the middle cells. At stage II, these cells have undergone their first round of periclinal division and the DR5::GUS maximum is even more restricted. In the following stages (III to V) more rounds of anticlinal and pericyclinal division occur, while the DR5::GUS maximum becomes more and more restricted to define the future lateral root meristem stem cell niche. At emergence (e) a functional lateral root meristem is established. Adapted from Benková *et al.* (2003) with permission from Elsevier ©.

Cell cycle regulation during lateral root development

Like most of the other cell types, xylem pole pericycle cells leave the meristem in G1-phase. Upon triggering into lateral root initiation, G1-to-S transition takes place. The E2F-RB pathway restricts this transition both in mammals and in plants (Shen, 2002). The key players in this pathway are conserved and fulfil similar functions. The RETINOBLASTOMA (RBR) protein inhibits cell cycle progression, at least in part, through the obstruction of E2F/DP transcription factor complexes. D-type CYCLIN/CDK complexes phosphorylate RBR resulting in derepression of E2F/DP complexes followed by G1-to-S transition. In turn, cell cycle inhibitory proteins, called Inhibitors of CDK/Kip-Related Proteins (ICKs/KRPs), can inhibit CYCLIN/CDK activity and thus affect cell cycle progression (reviewed in previous chapters). In roots, *CYCD3;1*, *CYCD3;2*, *RBR*, *E2Fa*, *E2Fc* and *DPa* were up-regulated by auxin treatment, whereas *ICK1*/*KRP1* and *ICK2/KRP2* were down-regulated (Himanen *et al.*, 2002; Vanneste *et al.*, 2005). In somatic tissues and cell suspensions, excessive cell proliferation can be induced by ectopic expression of *CYCD3;1* (Dewitte *et al.*, 2003), *E2Fa/DPa* (De Veylder *et al.*, 2002) and *E2Fb/DPa* (Magyar *et al.*, 2005). In contrast, ectopic overexpression of *ICK1/KRP1* or *ICK2/KRP2* strongly reduced cell cycle progression (De Veylder *et al.*, 2001; Lui *et al.*, 2000). *In situ* hybridisations showed that *ICK2/KRP2* is

expressed in a specific pattern in the root: it is highly expressed throughout the pericycle, except for sites of lateral root initiation. Furthermore, overexpression drastically reduced the number of lateral roots. Therefore, it is believed that the expression of *ICK2/KRP2* is involved in restricting sites of lateral root development (Himanen *et al.*, 2002).

Further progression through S-phase can be inhibited by E2Fc which is a repressor of E2F/DP regulated transcription (del Pozo *et al.*, 2002a). Moreover, CYCA2-CDKA;1 protein complexes have been implicated in phosphorylation of E2Fc, targeting it for proteolysis (del Pozo *et al.*, 2002a). In roots, A-type cyclins *CYCA1;1*, *CYCA2;1*, *CYCA2;4* can be induced by auxin (Himanen *et al.*, 2002; Vanneste *et al.*, 2005) among which, *CYCA2;4* expression may even be primary auxin responsive (Vanneste *et al.*, 2005). Therefore, it is plausible to assume that auxin induced A2-type cyclins stimulates S-phase progression by modulating E2Fc stability in roots. Furthermore, E2Fa/DPa transcription factor complexes directly activate *CDKB1;1* expression (Boudolf *et al.*, 2004b), which is typically expressed from S phase to G2-to-M transition (Segers *et al.*, 1996). In addition, *CDKB1;1* is expressed in xylem pole pericycle cells during lateral root initiation (Beeckman *et al.*, 2001; Vanneste *et al.*, 2005). Overexpression of a dominant negative allele of CDKB1;1 resulted in a reduced G2-to-M transition and enhanced endoreduplication in all tissues tested, including roots (Boudolf *et al.*, 2004a). Consistently, a significant reduction in lateral root density was found in plants overexpressing a dominant negative allele of CDKB1;1 (I. De Smet & T. Beeckman, pers. comm.). Furthermore, CDKB1;1-kinase was shown to regulate ICK2/KRP2 protein abundance (Verkest *et al.*, 2005), strongly suggesting that its kinase activity is correlated with lateral root initiation. *CDKB2;1*, another member of the same family and specific to the G2-to-M transition was also identified as a potential primary auxin responsive cell cycle gene (Vanneste *et al.*, 2005), supporting the idea of a specific auxin signal transduction pathway operating at this cell cycle transition. Still at the same cell cycle phase the APC complex becomes active, regulating proteolysis of cell cycle regulators (see previous chapters). A specific APC-complex subunit, HOBBIT/CDC25b is involved in cell cycle progression and differentiation in the root meristem. Interestingly, the auxin response repressor protein, AXR3/IAA17, accumulates in *hobbit* mutants (Blilou *et al.*, 2002) suggesting HOBBIT/CDC25b might mediate primary auxin response at the G2-to-M transition. It is tempting to speculate that HOBBIT/CDC25b would be involved in regulating AUX/IAA-dependent *CDKB2;1* expression at the onset of lateral root formation.

Stemness of the xylem pole associated pericycle

The terms “stem cell” and “stemness” are becoming increasingly popular in recent plant literature. Whilst no unambiguous definition for a “stem cell” exists (Parker *et al.*, 2005), they are commonly defined as “pluripotent” and “able to reconstitute entire tissues”. Usually, one discerns between “steady-state” and “emergent” stem cells (Shostak, 2006). The steady-state stem cells are self-renewing and give rise to complete tissues and organs through an iteration of asymmetric divisions. Emergent stem cells are transient and are most commonly found in developing tissues, such as developing embryos.

Within the root meristem, one can easily detect steady-state stem cells in a special microenvironment (“stem cell niche”), contacting the quiescent centre (Benfey and Scheres, 2000). These cells undergo asymmetric divisions, in which one daughter cell retains the parental cell fate, whilst the other gives rise to a “proliferating precursor cell” of a particular tissue lineage. When such a stem cell is ablated, a neighbouring cell dedifferentiates and changes its cell fate to replace the ablated stem cell (Xu *et al.*, 2006), suggesting “stemness” diffuses within the meristem.

Beyond the root meristem, a population of “quiescent” emergent stem cells can be found within the pericycle at the xylem poles. Upon stimulation, these cells, previously called “progenitor cells” or “founder cells”, become “true” emergent stem cells as they become part of the pool of cells participating in *de novo* development of a lateral root meristem.

Recently it was found that modulation of the E2F-RB pathway in the root stem cell niche near the quiescence centre of *Arabidopsis* affects rate of differentiation rather than speed of cell division (Wildwater *et al.*, 2005). This is in contrast to the previously reported ectopic proliferation, in non-stem cells, induced by overexpression of stimulatory E2F-RB components (CYCD3;1 and E2Fa/DPa) (De Veylder *et al.*, 2002; Dewitte *et al.*, 2003). These data suggest that the output of the E2F/RB pathway depends on intrinsic levels of stemness of the target cells. Furthermore, the E2F/RB pathway is limiting to lateral root initiation as shown by overexpression of the E2F/RB inhibitory protein, ICK2/KRP2 (Himanen *et al.*, 2002; Vanneste *et al.*, 2005). As xylem pole pericycle cells have high levels of stemness, one might expect a specific output for the E2F/RB pathway in these cells. Overexpression of CYCD3;1 or E2Fa/DPa did not boost lateral root initiation (I. De Smet & T. Beeckman, pers. comm.), suggesting another signal needs to coincide with the activation of the E2F-RB pathway for lateral root initiation to occur. Concordantly, activation of the E2F-RB pathway

in the lateral root-less auxin response mutant, *solitary root-1* simply stimulated proliferative divisions in the xylem pole pericycle, rather than lateral root initiation (Fig 2.2)

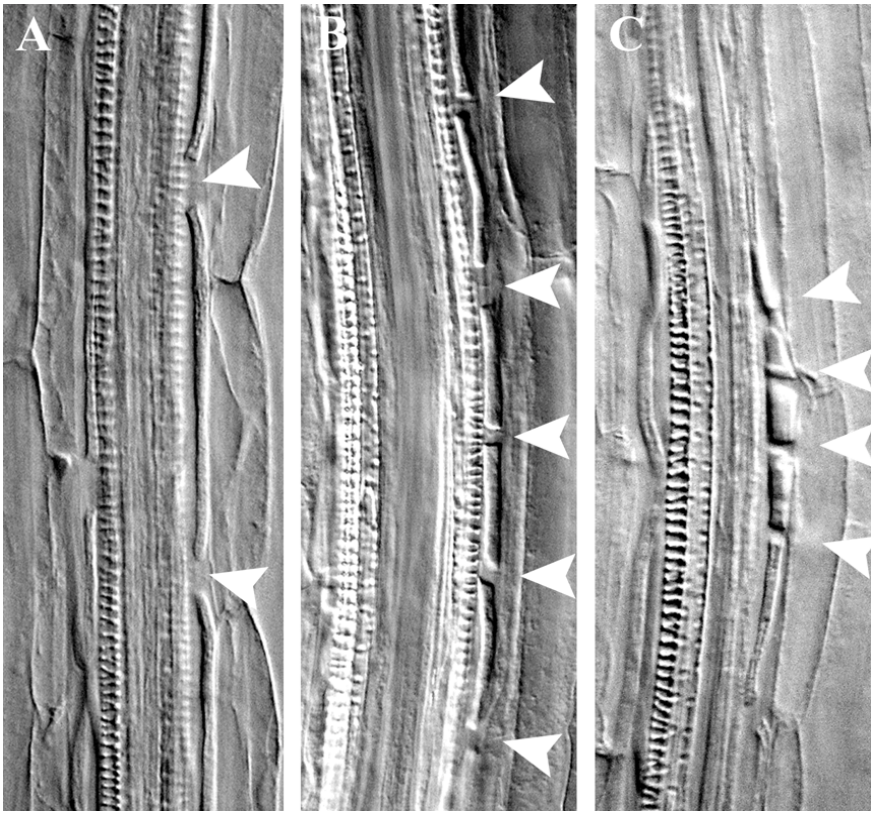

Figure 2.2 Proliferation versus asymmetric division in the xylem pole pericycle. (A) Non-dividing mature pericycle cell. (B) Proliferation of xylem pole pericycle cells upon stimulation of the E2F-RBR pathway in the auxin signalling mutant *solitary root-1*. (C) Formation of a stage I primordium after asymmetric division in xylem pole pericycle cells. Adapted from Vanneste et al. (2005) with permission from ASPB ©.

This implies that an auxin derived signal shifts the output of the E2F-RB pathway from proliferation towards lateral root initiation. The nature of this auxin derived signal remains elusive. However, the identification of novel downstream components of the auxin signalling cascade will be essential to unmask this (these) mysterious component(s).

Auxin signalling during lateral root initiation

Decades of research have been dedicated to the elucidation of the modus operandi of early auxin signalling (Fig 2.3). Only recently, auxin receptors have been identified unambiguously (Dharmasiri *et al.*, 2005a; Kepinski and Leyser, 2005) and comprise a small family AUXIN SIGNALING F-BOX proteins (AFB) (Dharmasiri *et al.*, 2005b), of which TIR1 is the best characterised. These AFBs occur in complexes forming functional

$SCF^{TIR1/AFB1/2/3}$ E3-ligases (Dharmasiri *et al.*, 2005b; Gray *et al.*, 1999). Most of the tested components and regulators of the $SCF^{TIR1/AFB1/2/3}$ complex are found to be highly expressed at sites of lateral root formation, and mutations result in a reduction in lateral root density (del Pozo *et al.*, 2002b; Dharmasiri *et al.*, 2005b; Dharmasiri *et al.*, 2003; Gray *et al.*, 2003) implicating that $SCF^{TIR1/AFB1/2/3}$ mediated ubiquitination is an essential regulatory component for lateral root initiation.

The most notorious targets for $SCF^{TIR1/AFB1/2/3}$ mediated ubiquitination are proteins of the AUX/IAA family (Dharmasiri *et al.*, 2005b; Gray *et al.*, 2001). Direct binding of auxin to $SCF^{TIR1/AFB1/2/3}$ enhances interaction with AUX/IAAs (Dharmasiri *et al.*, 2005a; Kepinski and Leyser, 2005), triggering their oligo-ubiquitination (Gray *et al.*, 2001). After ubiquitination these proteins are rapidly targeted for proteolysis (Ramos *et al.*, 2001; Thrower *et al.*, 2000). This may well be one of the most crucial steps in translating auxin signal into transcriptional information, as AUX/IAAs are known inhibitors of a specific family of transcription factors, called Auxin Response Factors (ARFs).

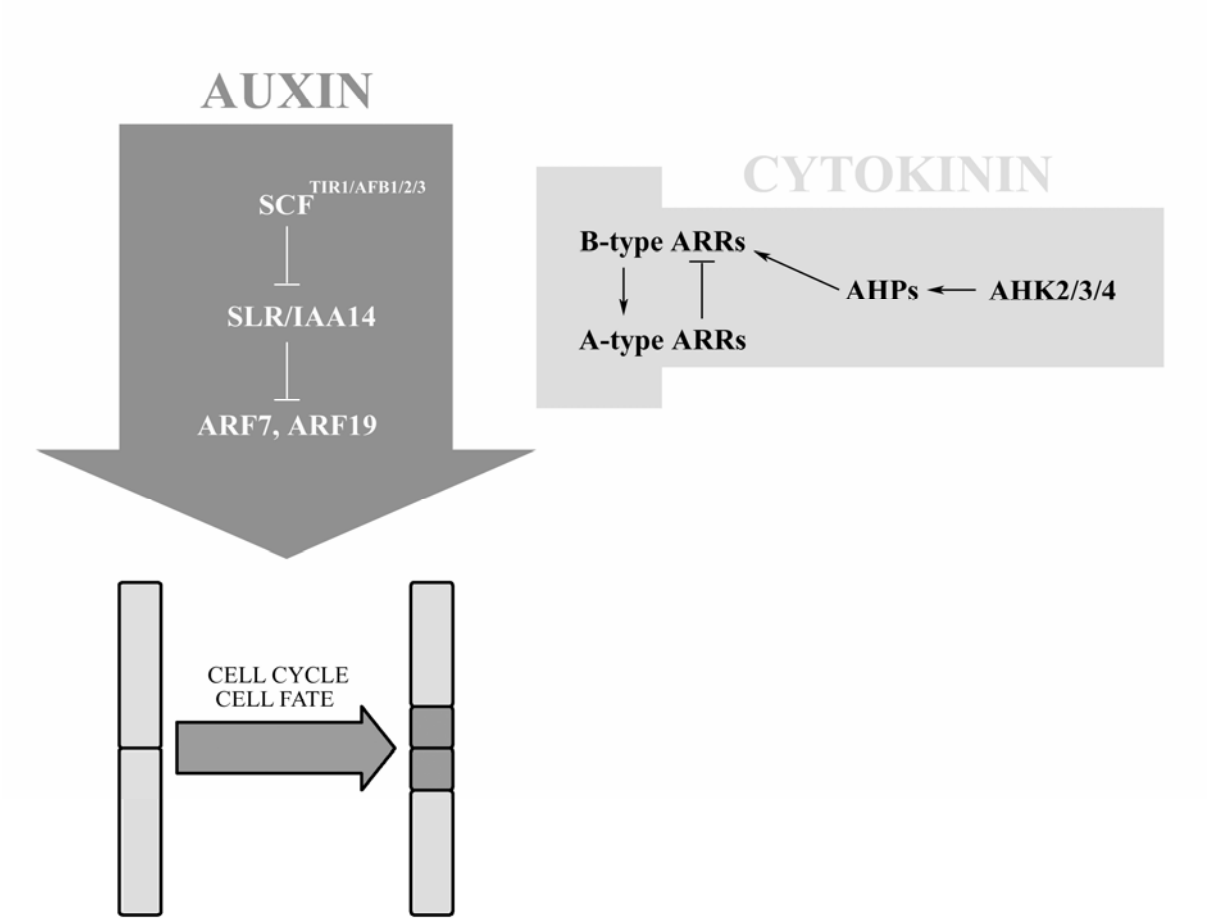

Figure 2.3 Scheme of auxin-cytokinin antagonism during lateral root initiation. When auxin concentrations are high, auxin binds directly to $SCF^{TIR1/AFB1/2/3}$ E3-ligases and dramatically increases their affinity for AUX/IAA proteins such as SLR/IAA14. Upon interaction SLR/IAA14 is ubiquitinated and targeted for proteolysis. SLR/IAA14 degradation derepresses the auxin responsive transcription factors ARF7 and ARF19, which stimulate cell cycle activation and cell fate respecification during lateral root initiation. Cytokinins activate their signalling cascade through binding to membrane-bound AHK2/3/4, which results in the phosphorylation of AHPs that transmit the signal to the nucleus. In the nucleus AHPs phosphorylate B-type ARRs that subsequently activate transcription, among which A-type ARRs that repress B-type ARR activity. B-type ARR activity represses ARF7, ARF19 induced lateral root initiation at an as yet unknown level.

Despite their importance in signal transduction, to date no phenotypes have been observed in loss-of-function *aux/iaa* mutants, suggesting a high functional redundancy within the gene-family (Overvoorde *et al.*, 2005). In the AUX/IAA protein structure, four domains can be discerned (Liscum and Reed, 2002). Conserved residues within domain II are essential for interaction with $SCF^{TIR1/AFB1/2/3}$ complexes (Dharmasiri *et al.*, 2005a; Kepinski and Leyser, 2005; Ramos *et al.*, 2001). Single amino acid changes within domain II impede such interactions, increasing the stability of the AUX/IAA protein. Several of such gain-of-function mutants have been identified, displaying altered root architectures: *axr2-1*, *axr3-1*,

bdl, shy2-2, iaa28-1, slr-1, msg2-1, axr5-1 (Fukaki *et al.*, 2002; Hamann *et al.*, 1999; Rogg *et al.*, 2001; Rouse *et al.*, 1998; Tatematsu *et al.*, 2004; Tian and Reed, 1999; Timpte *et al.*, 1994; Yang *et al.*, 2004). For *msg2-1*, *iaa28-1* and *axr5-1* reductions in lateral root density can be observed while in *axr3-1* and *slr-1* very little or no lateral roots are formed. Inhibition of AUX/IAA-mediated auxin signalling in xylem pole pericycle cells through tissue-specific misexpression of mIAA17 (axr3), mIAA12 (bdl) or mIAA14 (slr) abolishes lateral root initiation (De Smet *et al.*, 2007; Fukaki *et al.*, 2005), suggesting that auxin perception in xylem pole pericycle cells is required for lateral root initiation. In contrast to the high degree of functional redundancy, phenotypes ranging from opposing to similar can be obtained by overexpressing different stabilised (distant or closely related) AUX/IAAs in the same tissue (Knox *et al.*, 2003; Weijers *et al.*, 2005), suggesting some level of functional divergence, even between closely related AUX/IAAs.

The Auxin Response Factor (ARF) family counts 23 members, which are able to stimulate and/or inhibit auxin mediated transcriptional changes. They contain a DNA binding domain and can heterodimerize with AUX/IAA proteins. Due to the presence of a potent inhibitory domain in AUX/IAAs, AUX/IAA-ARF dimers are transcriptional inactive. The phenotypes observed in plants expressing stabilised aux/iaa can be mimicked by knocking out the target ARF such as in *bdl/iaa12* and *mp/arf5* mutants, which both lack the embryonic root (Hardtke *et al.*, 2004). Similarly, *nph4 arf19* double mutants phenocopy the lack of lateral roots in *slr-1* (Okushima *et al.*, 2005; Wilmoth *et al.*, 2005). Furthermore, IAA14 interacts with NPH4/ARF7 and ARF19 in yeast-two hybrid assays (Fukaki *et al.*, 2005), suggesting that IAA14 is a repressor for NPH4/ARF7 and ARF19 both being essential for lateral root initiation.

Not all ARFs are believed to be activators of expression. Stabilising ARF17 (Mallory *et al.*, 2005) or ARF16 (Wang *et al.*, 2005) transcripts strongly repressed lateral root development through modulation of auxin responses.

The main task lying ahead is to identify the down-stream targets of this complex web of stimulatory and inhibitory factors.

Post-transcriptional feed-back mechanisms on auxin signalling

Next to the rigorous transcriptional feed-back mechanisms, an additional layer of post-transcriptional regulation through miRNA-targeted mRNA degradation is emerging. DICER

plays an essential role in processing of miRNA precursors into mature miRNAs (Kurihara and Watanabe, 2004) that guide RNA-Induced RNA Silencing Complexes (RISC) to complementary target mRNAs (Vaucheret *et al.*, 2004). Knocking out DICER-LIKE in *Arabidopsis* resulted in a strong increase in lateral root density (Guo *et al.*, 2005). In contrast, impairing RISC function through *ago1* mutation decreases adventitous rooting in *Arabidopsis* (Sorin *et al.*, 2005). These findings suggest that transcripts of auxin signalling components may be targets of miRNA-mediated mRNA degradation. Indeed, several miRNA targets are clearly involved in lateral root development. The mRNAs of Auxin Binding F-box proteins, identified as a family of auxin receptors (see higher) are targeted for degradation through the pathogen-inducible miR393 (Navarro *et al.*, 2006). ARF8 and ARF6 mRNAs are targeted for degradation through miR167. Moreover, ARF10, ARF16 and ARF17 mRNAs are targets for miR160 (Mallory *et al.*, 2005; Wang *et al.*, 2005). Overexpression of miR160 enhances lateral root development, whereas plants expressing miR160-resistent ARF16 (Wang *et al.*, 2005) or ARF17 (Mallory *et al.*, 2005) display strongly impaired lateral root densities. In addition, NAC1, known as a positive regulator of lateral root formation (Xie *et al.*, 2000; Xie *et al.*, 2002), is targeted by miR164 (Guo *et al.*, 2005).

As miRNAs are just beginning to be understood, it is apparent that miRNA mediated mRNA stability is an important regulatory mechanism to control auxin action.

Polar auxin transport defines lateral root boundaries

Detailed analyses have shown that auxin is present throughout all stages of developing lateral roots. Particularly, in developing lateral root primordia an auxin gradient can be visualised around the stem cell niche, similar to that found in primary root meristems (Fig 2.1; Benková *et al.*, 2003). Chemically interfering with polar auxin transport results in the misspecification of the stem cell niche in the primary root meristem, demonstrated by ectopic quiescent centre identity (Sabatini *et al.*, 1999). Similarly, cell fates are misspecified in lateral root primordia when polar auxin transport is disrupted (Benková *et al.*, 2003; Geldner *et al.*, 2004). Polar auxin transport is dependent on the activity of PIN proteins (Petrášek *et al.*, 2006). Indeed, in higher order *pin* mutants lateral root boundaries are not correctly specified as demonstrated by an continuous sheet of proliferating pericycle cells (Benková *et al.*, 2003). These data suggest a simple organogenesis model, in which orchestrated polar auxin transport accumulates auxin at the site of lateral root initiation and as a consequence deprives the

surrounding tissues of auxin. Subsequently, an auxin gradient is set up within the developing lateral root meristem with an optimum at the tip and auxin deprivation at the lateral root base. Such a model of auxin accumulation-deprivation is becoming more and more an accepted model for auxin-driven organogenesis. In the shoot apex, polar auxin transport has already been extensively implicated in lateral organ formation and positioning (Reinhardt, 2005). Computer models based on real PIN1 dynamics in the shoot epidermis have shown that polar auxin transport is responsible for phyllotactic spacing of leaves. In this model auxin is pumped to the cell with the highest auxin concentration and ensures a local auxin accumulation and a peripheral auxin deprivation, defining the site of leaf initiation (Jonsson *et al.*, 2006; Smith *et al.*, 2006). Indeed when PAT function is abolished, ring-shaped organs could be induced by local auxin application to the shoot apex (Reinhardt *et al.*, 2003). In the root, an equivalent situation is found in the formation of a continuous sheet of proliferating pericycle cells (Benková *et al.*, 2003; Geldner *et al.*, 2004). Given the strong positive regulatory effect of auxin on cell cycle activity, it is apparent that a local auxin gradient results in differential cell cycle activity, shaping the organ.

Despite the high importance of polar auxin transport in the different stages of lateral root development it seems unlikely to be sufficient to fulfil the requirements for organogenesis. Due to similarities between the primary root meristem and lateral root meristems, we will briefly discuss cell fate determinants that act in primary root organogenesis as well as in lateral root development. One of the earliest induced genes determining root identity in embryogenesis is *PLETHORA* (*PLT1*) as it is expressed at a position correlating with the embryonic root stem cell niche. Furthermore, strong overexpression of *PLT* can induce homeotic transformation of shoot to root identity. Interestingly, *PLT1* expression is downstream of the AUX/IAA-ARF auxin signalling cascade, and thus correlates with sites of auxin accumulation, such as lateral root initiation. Yet, *plt1plt2* mutants produce many lateral roots, which rapidly terminally differentiate (Aida *et al.*, 2004). As discussed above, auxin accumulation in developing lateral root primordia is imposed by polar auxin transport. Furthermore, PLT activity is also required for *PIN* gene expression to stabilize auxin accumulation, sustaining its own expression (Blilou *et al.*, 2005). Several of the PIN genes have been shown to be rapidly auxin inducible (Vieten *et al.*, 2005), whereas *PLT* expression was much slower (Aida *et al.*, 2004). Nevertheless, it is not the level of *PIN* expression *per se* that drives the auxin flow, but rather their subcellular localisation. During QC regeneration, cell fate changes brought about by PLT precede PIN polarity changes (Xu *et al.*, 2006). Similarly, for different stages of lateral root development, PIN

polar localisations rapidly changes (Benková *et al.*, 2003), suggestive of ever-changing cell fates within the developing lateral root primordium.

Cytokinins inhibit lateral root development

The balance of auxins and cytokinins is believed to be a key determinant in many developmental processes. In tissue cultures, high auxin-to-cytokinin ratios are used to induce rooting, while low auxin-to-cytokinin ratios favour shoot development. In roots, cytokinins seem to have a negative effect on lateral root formation (Fig 2.3). This is supported by the observed reduction of lateral root formation in tobacco plants overproducing cytokinin (Li *et al.*, 1992). On the other hand decreasing cytokinin content by overexpression of cytokinin oxidases dramatically promoted root expansion by more extensive branching and higher rates of root growth (Werner *et al.*, 2003; Werner *et al.*, 2001). These experiments suggest that next to auxin, cytokinin content is a major determinant of lateral root formation.

Cytokinin is perceived through a small family of sensor histidine kinases, AHK2, AHK3 and CRE1/AHK4. Mutant analysis showed that AHK2 and AHK3 are the main contributors to the cytokinin-mediated lateral root repression (Riefler *et al.*, 2006). The cytokinin receptors are believed to transmit the signal through phosphotransfer proteins (AHPs) leading ultimately in an altered phosphorylation state of the Arabidopsis Response Regulators (ARRs). Furthermore, it was inferred that higher order AHP mutants show decreased cytokinin sensitivity and consistent developmental defects (Hutchison *et al.*, 2006). Type-B ARRs have a receptor and a DNA binding domain, and may serve as transcription factors that translate the cytokinin signal into a primary transcriptional cytokinin response (Hosoda *et al.*, 2002; Lohrmann *et al.*, 2001; Sakai *et al.*, 2001). Overexpression of *ARR1* or *ARR2* increases sensitivity to cytokinin (Hwang and Sheen, 2001; Sakai *et al.*, 2001). Conversely, at cytokinin concentrations limiting for lateral root formation in wild type, still several lateral roots were present in higher order type-B ARR mutants (Mason *et al.*, 2005), consistent with a decreased cytokinin sensitivity. Type-A *ARR*s are primary cytokinin responsive genes (D'Agostino *et al.*, 2000; Taniguchi *et al.*, 1998) that are believed to be directly activated through type-B ARRs (Rashotte *et al.*, 2003). In contrast to type-B ARRs, type-A ARRs are negative regulators of cytokinin response, and thus repress their own expression (Hwang and Sheen, 2001). In accordance, hexuple mutants in type-A ARRs display an increased cytokinin sensitivity coupled to a reduced lateral root density (To *et al.*,

2004). All these data point unanimously toward an inhibitory role for cytokinins in root development. However, abrogating cytokinin response in the root by knocking out all cytokinin receptors results in a complete loss of meristem function (Higuchi *et al.*, 2004), suggesting that a minimal cytokinin response is required for root development. Recently it was shown that cytokinins inhibit lateral root initiation specifically at the level of G2-to-M transition. Expression of G2-to-M regulatory genes, such as A- and B-type cyclins, was shown to be repressed by cytokinin treatment, whereas expression of G1-to-S regulatory genes was not affected (Li *et al.*, 2006).

As reduction in cytokinin content or signalling results in higher lateral root densities, it is beyond doubt that cytokinins antagonise auxin activity. On the other hand auxin readily stimulates oxidative breakdown of cytokinins (Zhang *et al.*, 1995) and represses cytokinin biosynthesis, whereas cytokinin only mildly affects auxin content (Nordström *et al.*, 2004). In accordance to these data, the primary cytokinin responsive type-A ARR5 is found to be down-regulated at sites of lateral root development (Lohar *et al.*, 2004). These data are consistent with the established idea that a balanced response to auxin and cytokinin is required for lateral root initiation.

Brassinosteroids regulate auxin transport

Brassinosteroids have recently been shown to promote lateral root development synergistically with auxin (Bao *et al.*, 2004). Consistently, the signalling pathways for auxin and brassinosteroids have been shown to converge at the level of transcription during hypocotyl elongation (Mockaitis and Estelle, 2004; Nemhauser *et al.*, 2004). However, as auxin responsive genes respond much slower to brassinosteroids than to auxins (Goda *et al.*, 2004), it seems more likely that brassinosteroids affect auxin responsive gene expression indirectly. Recently, Li *et al.* (2005) showed that brassinosteroids stimulate basipetal polar auxin transport in roots which was correlated with an increased *PIN1* and *PIN2* expression. Furthermore, PIN2 appears to be regulated by brassinosteroids also at the post-transcriptional level. In plants overexpressing PIN2-GPF only a small domain of expression can be observed. However, by applying brassinosteroids, the PIN2-GFP expression domain could be enlarged considerably (Li *et al.*, 2005). Furthermore in *pin2* mutants the promotive effect of brassinosteroids on lateral root formation is nearly completely abolished (Li *et al.*, 2005), suggesting a link between brassinosteroids, PIN2 abundance and lateral root formation. In

contrast to this, auxin stimulates PIN2 degradation (Abas *et al.*, 2006) probably with the involvement of the SCF regulatory protein AXR1 (Sieberer *et al.*, 2000). It will be of interest to identify the F-box protein that mediates PIN2 ubiquitination and at which level brassinosteroids interfere with PIN2-degradation.

Light alters auxin sensitivity

Light is one of the fundamental elements required for sustaining autotrophic plant growth. The most apparent example of the role of light in plant growth and development is photomorphogenesis. Although roots remain predominantly in the dark, light also appears to play a role in regulating root development, at least in part by regulating auxin sensitivity. Unexpectedly, light might reach roots, even when submerged in the soil as it can be conducted via the vascular system (mainly xylem) of both woody (Sun *et al.*, 2003) and herbaceous plants (Sun *et al.*, 2005).

The molecular mechanisms of light signalling are well studied and characterised. COP1 is an E3 ubiquitin ligase that represses light signalling by targeting signal transduction machinery for degradation (Yi and Deng, 2005). One of the best characterised targets of COP1 is the bZIP transcription factor HY5 (Osterlund *et al.*, 2000). Mutants in *cop1* and *hy5* impose opposite effects on lateral root development, in which *cop1* exhibits a defect in lateral root density whereas the *hy5* mutation enhances lateral root initiation and lateral root elongation (Ang *et al.*, 1998; Oyama *et al.*, 1997). These phenotypes are most likely to be achieved through alterations in auxin sensitivity. Indeed, HY5 has recently been implicated in direct binding to the promoters of the auxin-signalling inhibitors AXR2/IAA7 and SLR/IAA14 (Cluis *et al.*, 2004). In light conditions, COP1 is inactive and HY5 is stabile to induce expression of AXR2/IAA7 and SLR/IAA14, thereby repressing auxin signalling and lateral root formation. Therefore, the COP1/HY5 dependent control on root development might represent the naturally occurring mechanism to avoid rooting in the above-ground parts of the plant.

Conclusions and perspectives

Little is known on the molecular mechanisms of auxin-driven cell cycle progression. During lateral root initiation, auxin signalling converges onto cell cycle activation.

Nevertheless, cell cycle activation in xylem pole pericycle cells does not necessarily result in the formation of a lateral root. This implies that lateral root initiation requires additional processes, such as the specification of a differential cell fate between the daughter cells within a lateral root initiation site. It will be of interest to identify components involved in the interplay between cell cycle progression and the respecification of cell fates during lateral root initiation. Due to the recent development of a lateral root inducible system (Himanen *et al.*, 2002) it is possible to use high-throughput molecular tools, such as microarray analysis, to study auxin-regulated lateral root initiation. Carefully dissecting lateral root initiation will yield many novel and exciting insights into cell cycle regulation, auxin signalling cascades as well as the complex phenomenon of hormonal crosstalk.

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CHAPTER 3 Cell cycle progression in the pericycle is not sufficient for SOLITARY-ROOT/IAA14-mediated lateral root initiation in *Arabidopsis*

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Abstract

To study the mechanisms behind auxin-induced cell division, lateral root initiation was used as a model system. By means of microarray analysis, genome-wide transcriptional changes were monitored during the early steps of lateral root initiation. Inclusion of the dominant auxin signalling mutant *solitary root1* (*slr1*) identified genes involved in lateral root initiation that act downstream of the auxin/indole-3-acetic acid (Aux/IAA) signalling pathway. Interestingly, key components of the cell cycle machinery were strongly defective in *slr1*, suggesting a direct link between Aux/IAA signalling and core cell cycle regulation. However, induction of cell cycle in the mutant background by overexpression of the D-type cyclin (*CYCD3;1*) was able to trigger complete rounds of cell division in the pericycle that did not result in lateral root formation. Therefore, lateral root initiation can only take place when cell cycle activation is accompanied by cell fate respecification in pericycle cells. The microarray data also yielded evidence for the existence of negative and positive feedback mechanisms that regulate auxin homeostasis and signal transduction in the pericycle, thereby fine-tuning the process of lateral root initiation.

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INTRODUCTION

Auxins have long been put forward as potent stimulators of cell division (Gautheret, 1939), but although considerable progress has been made in our understanding of both auxin signalling (Weijers and Jürgens, 2004) and cell cycle progression (Inzé, 2005), the molecular mechanisms by which these processes are connected remain poorly understood (Vanneste *et al.*, 2005). The initiation of lateral roots, marked by specific cell divisions in the pericycle (Casimiro *et al.*, 2001), is triggered by auxin (Torrey, 1950) and is, therefore, an ideal model system for studying how auxin signalling activates cell cycle progression.

The onset of lateral root formation coincides with the occurrence of a series of anticlinal, asymmetric divisions in the xylem pole pericycle (Malamy and Benfey, 1997); hence, cell cycle activation is inherently connected with lateral root initiation. Activation and progression through the major phases of the cell cycle (G1, S, G2 and M) are governed by the control of cyclin-dependent kinases (CDKs). The activity of these CDKs can be modulated through interacting regulatory components called cyclins (Dewitte *et al.*, 2003; Lee *et al.*, 2003; Roudier *et al.*, 2003) and CDK subunits (De Veylder *et al.*, 1997), inhibitory components (inhibitors of CDKs and Kip-related proteins) (De Veylder *et al.*, 2001; Wang *et al.*, 1997) or through stimulatory (Shimotohno *et al.*, 2004), and inhibitory phosphorylation (Sun *et al.*, 1999). Beeckman *et al.* (2001) showed that in *Arabidopsis thaliana*, pericycle cells leaving the root apical meristem remain in G1 phase. Under normal conditions, only the pericycle cells at the xylem pole retain the capability to respond to an inductive signal, such as auxin, to initiate lateral roots. Interestingly, the expression of the cell cycle inhibitor KRP2 is strongly down-regulated in xylem pole pericycle cells, when roots are subjected to auxin treatment (Casimiro *et al.*, 2003; Himanen *et al.*, 2002). However, it can be anticipated that numerous regulatory proteins will be involved in lateral root initiation.

For global studies of developmental processes at the molecular level, relatively large quantities of material are required, but are almost impossible to obtain for lateral root initiation because of the small subsets of pericycle cells involved (Kurup *et al.*, 2005; Laskowski *et al.*, 1995). Based on the observation that inhibition of polar auxin transport prevents lateral root initiation (Casimiro *et al.*, 2001), a lateral root-inducible system (LRIS) was developed and characterized (Himanen *et al.*, 2002; Himanen *et al.*, 2004). Germination on media with sufficiently high levels of 1-*N*-naphthylphthalamic acid (NPA) results in roots without lateral root initiation sites, which can be induced synchronously throughout the pericycle by

subsequent transfer to media with high auxin concentrations (Himanen *et al.*, 2002). This system allows the monitoring of the sequential signalling and cell cycle progression during lateral root initiation (Himanen *et al.*, 2004).

Prior to induced cell division, auxins have to be perceived and transmitted to turn on the cell cycle machinery. The current understanding of early auxin signal transduction is focused on the 26S proteasome-dependent proteolysis of specific small short-lived nuclear proteins, designated AUX/IAA (Dharmasiri and Estelle, 2004). AUX/IAA proteins belong to a family of 29 members (Liscum and Reed, 2002) and act as negative regulators by repressing auxin response factors (ARFs) (Tiwari *et al.*, 2004). When the auxin concentration increases, the interaction between the SCF^{TIR1} E3-ligase and AUX/IAA proteins is stimulated by direct binding of auxin to a family of auxin-binding F-boxes ((Dharmasiri *et al.*, 2005a; Dharmasiri *et al.*, 2005b; Kepinski and Leyser, 2005), resulting in oligo-ubiquitination of AUX/IAA proteins (Gray *et al.*, 2001). Such ubiquitinated proteins are usually targeted for 26S proteasome-mediated proteolysis (Hershko and Ciechanover, 1998). Thus, high auxin concentration derepresses ARF activity allowing the primary auxin response to take place. When AUX/IAA proteins are mutated in domain II, essential for the interaction with the SCF^{TIR1} complex (Ramos *et al.*, 2001), their stability increases dramatically, resulting in auxin-resistant phenotypes (Ouellet *et al.*, 2001; Ramos *et al.*, 2001). Over the years, an increasing number of dominant and semi-dominant *Aux/IAA* mutants have been described that exhibit point mutations in domain II (Liscum and Reed, 2002). When mutated in domain II, *solitary root1* (*slr1*) mutants develop a primary root without any sign of lateral root initiation (Fukaki *et al.*, 2002). Therefore, the SLR/IAA14 protein is probably a central regulator of lateral root initiation.

Here, lateral root initiation was used as an *in planta* model to study the interaction between the auxin signalling pathway and cell cycle activation. For this purpose, the lateral rootless phenotype of the early auxin signalling mutant *slr1* was utilized to compare on a genome-wide level the transcriptional changes that occur in root segments of wild type and *slr1* during auxin-induced lateral root initiation. Complementation by overexpression of *CYCD3;1* in the *slr1* pericycle was able to trigger cell divisions, but no lateral root initiation, suggesting that more is involved than the activation of cell cycle progression in the pericycle. The transcriptional data suggest that during lateral root initiation, counteracting feedback regulation acts on auxin homeostasis and the signal transduction machinery. Furthermore, the analysis of the transcriptional changes in wild type versus *slr1* allowed some cell cycle regulators to be pinpointed as potential targets of the primary auxin signalling pathway

RESULTS

slr1 as a tool to link auxin signalling to lateral root initiation

The dominant mutation in *SLR/IAA14* results in complete absence of detectable lateral root initiation sites, formation of aberrant root hairs, and agravitropic root growth (Fukaki *et al.*, 2002). For a better notion of the tissues and cell types that become the primary targets of the reduced auxin response in the mutant, we analyzed the expression pattern of *SLR/IAA14* at the cellular level by anatomical sections of 3-day-old roots of transgenic plants harbouring a promoter IAA14/β-glucuronidase (P_{IAA14}*::GUS*) construct. Expression of mutant *slr/iaa14* under control of this promoter sequence has been shown to be sufficient to phenocopy the root phenotype of *slr1* (Fukaki *et al.*, 2002). Furthermore, tissue-specific expression of stabilized SLR/IAA14 in the xylem pole pericycle conferred the lateral rootless phenotype of *slr1* (Fukaki *et al.*, 2005). The expression of P_{IAA14}*::GUS* was strongest in the xylem pericycle cells, in the xylem-associated cells of mature root tissues, and almost absent from phloem pole pericycle cells (Fig. 3.1 A,B). This stronger expression at the xylem pole, the site of lateral root initiation in *Arabidopis*, suggests a causal link between the reduced auxin responsiveness and the lack of lateral roots in the mutant. During lateral root development, GUS activity was strongest in the youngest lateral root primordia, whereas it was nearly absent in mature lateral root meristems (Suppl. Fig. 3.1). In epidermal cells of mature root tissues, the expression was also low (Fig. 3.1 A). In the elongation zone, expression was strongest in epidermal cells (Fig. 3.1 C), whereas more distally, it was restricted to the lateral root cap (Fig. 3.1 D). Plants harboring the P_{IAA14}*::GUS* construct were crossed into the *slr1* background, resulting in F1 plants exhibiting a reduced level of expression compared to that of the wild type (Fig. 3.1 E,F). Anatomical sections showed that the tissue specificity of P_{IAA14}*::GUS* expression was maintained in *slr1* (data not shown).

When the primary root length was compared between 2-week-old wild-type and mutant seedlings, no significant differences were observed (data not shown), suggesting that the meristematic activity of the *slr1* primary roots was not affected. To verify whether *slr1* mutants still normally specified the pericycle, the enhancer trap line, J0121, a marker for xylem pericycle cell identity (Casimiro *et al.*, 2001), was crossed into the *slr1* mutant. The green fluorescent protein expression pattern of J0121 did not change significantly when compared to the wild-type situation (Fig 3.1 G,H). Because CDKA;1 is a central regulator of cell cycle

progression and its expression is associated with competence to divide (Hemerly *et al.*, 1995), the $P_{CDKA;1}$*::GUS* fusion was analyzed. Its expression was equally strong in the pericycle of wild type and *slr1* (Fig 3.1 I,J). Also, expression of P_{ALF4}*::GUS*, fused with ALF4, a protein with unknown function that is required for maintaining pericycle cells competent to form lateral roots (DiDonato *et al.*, 2004), was unaltered in *slr1* (data not shown). These observations indicate that the changes in auxin response and cell cycle activation in the mutant are probably not due to an altered pericycle identity or a reduced competence to divide.

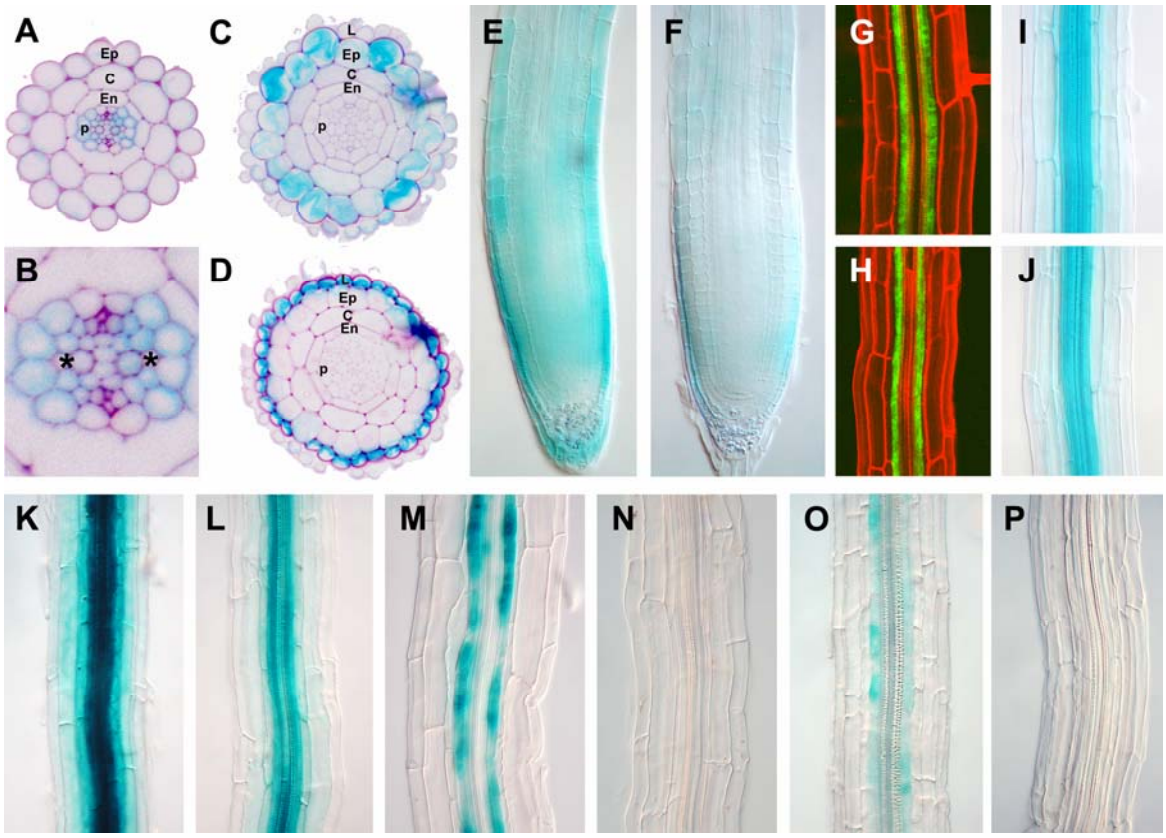

Figure 3.1 Expression analysis in wild type and *slr1* (A-D) Anatomical sections of P_{IAA14}*:GUS* mature root tissue (A), detail of stele (B), elongation zone (C), and root meristem (D) in wild type background. (E) and (F) P_{IAA14}*:GUS* expression in root apical meristem in wild type and in *slr1*, respectively. (G) and (H) Xylem pole pericycle-specific green fluorescent protein expression in mature root segment of J0121 in wild type and in *slr1*, respectively. (I) and (J) $P_{CDKA;1}$*:GUS* expression in wild type and in *slr1*, respectively. (K) to (P) Expression in roots germinated on 10 µM NPA and transferred 72 h after germination to 10 µM NAA for 12 h of P_{DR5}*:GUS* expression in wild type (K), P_{DR5}*:GUS* expression in *slr1* (L), $P_{CYCB1;1}$*:GUS* in wild type (M), $P_{CYCB1;1}$*:GUS* in *slr1* (N), $P_{CDKB1;1}$*:GUS* in wild type (O), and $P_{CDKB1;1}$*:GUS* in *slr1* (P). C, cortex; En, endodermis; Ep, epidermis; L, lateral root cap; p, pericycle. * indicates protoxylem cells.

To compare the transcriptional changes associated with auxin induction in the wild-type and the *slr1* situations, we used the recently developed LRIS that allows the synchronization of lateral root initiation in roots devoid of lateral roots (Himanen *et al.*, 2002). The possibility that this LRIS might induce lateral root initiation in the mutant was first addressed before initiating large-scale experiments. Therefore, specific GUS reporter lines were crossed into the mutant

background and their response to a 12-h auxin treatment following a 72-h growth on NPA was characterized. In 72-h NPA-treated seedlings, no expression of the auxin-responsive P_{DR5}*::GUS* construct could be detected in either genotype (data not shown). When seedlings had been transferred to α-Naphthalene Acetic Acid (NAA) for 12 h, P_{DR5}*::GUS* activity was strongly induced throughout the root in wild type and to a lesser extent in *slr1* (Fig. 3.1 K,L), suggesting that the *slr1* mutation also results in reduced auxin responsiveness within the LRIS. To assay cell cycle progression within the LRIS, plants harbouring GUS reporter constructs for the G2-to-M transition-associated genes *CYCB1;1* and *CDKB1;1* were used. The expression of *CYCB1;1* is strong in pericycle cells undergoing the first divisions of lateral root initiation (Beeckman *et al.*, 2001; Himanen *et al.*, 2002), whereas that of *CDKB1;1* starts from the S phase into the G2-to-M transition (Menges and Murray, 2002; Porceddu *et al.*, 2001). With these two GUS reporter lines, no expression was detected in the pericycle in either genotype when germinated on NPA (data not shown). When transferred to auxin for 12 h, wild-type pericycle cells had very strong GUS activity for both markers, hinting that these pericycle cells divide actively (Fig. 3.1 M,O). The destruction box fused to GUS in $P_{CYCB1;1}$*::GUS* results in proteolysis during the late mitosis. Therefore, because of its patchy GUS pattern, the GUS-stained pericycle cells might have undergone at least one cycle of cell division during the 12-h NAA treatment. On the other hand, no GUS activity could be induced in *slr1* pericycle cells (Fig. 3.1 N,P), reflecting the complete absence of mitotic activity, following 12 h of auxin treatment. These data legitimize the use of the *slr1* mutant in a genome-wide transcriptional analysis of lateral root initiation.

Microarray set-up and statistical analysis

Within the LRIS, pericycle cells have been shown to be blocked in the G1 phase when germinated on NPA (Himanen *et al.*, 2002; Himanen *et al.*, 2004). Subsequent transfer to auxin media was sufficient to trigger the primary auxin response within 2 h, resulting in a synchronous induction of lateral root initiation over the entire pericycle (visualized by $P_{CYCB1;1}$*::GUS* expression in Fig. 3.2). To gain insight into the early events of lateral root initiation, samples for microarray analysis were taken at the time points 0 h NAA (= 72 h NPA), 2 h NAA and 6 h NAA for both wild type and *slr1* (highlighted stages in Fig. 3.2). Per time point, approximately 1000 root segments were sampled and two independent biological replicates were performed. To minimize contamination with non-relevant tissues and dividing

cells, root segments were cut above the root apical meristem and below the root-hypocotyl junction. These segments were further subjected to the required steps for microarray analysis with the ATH1 Affymetrix chips harbouring 22,746 probe sets (see Methods). ANOVA analysis on the raw gene expression data assessed the significance of three major sources of variability affecting the expression level: the duration of the auxin treatment (time), the genotype, and the interaction between these two. Correction for multiple comparison was performed by controlling the false discovery rate, and q-values were calculated (Storey and Tibshirani, 2003). At a stringency level of $p<0.001$, 3110 genes had a significantly modulated expression profile during the experiment. Furthermore, none of them could be rejected based on the calculated q-values ($q<0.05$) (Suppl. Table 3.1).

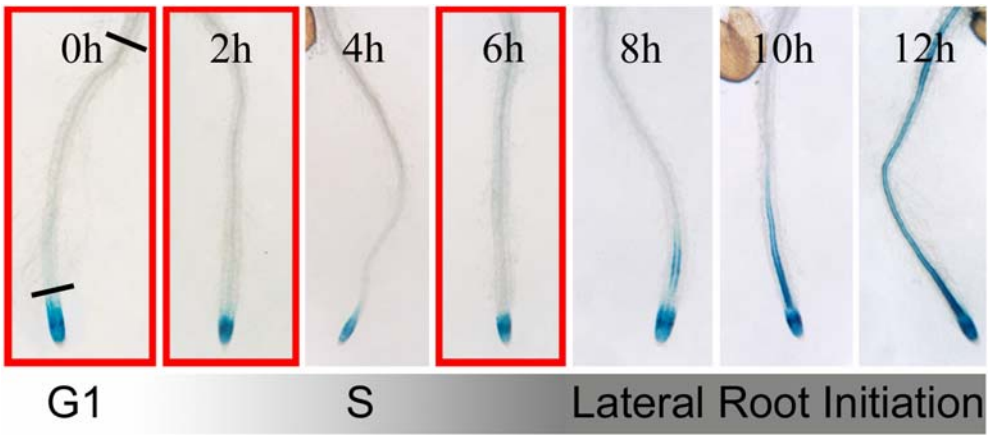

Figure 3.2 Schematic representation of the LRIS. Seeds are germinated on medium supplemented with NPA to inhibit lateral root initiation. The pericycle cells of seedlings germinated on NPA are in G1 phase (0 h). Subsequent transfer to NAA-supplemented medium induces gradual cell cycle progression over S, G2, and M phases, corresponding to synchronized lateral root initiation. $P_{CYCB1;1}$:*GUS* activity marks G2-to-M transition. Red rectangles indicate time-points used for the microarray. The segment between root tip and root-hypocotyl junction (black lines at 0 h) was used for the microarray analysis.

"Cross-table clustering" to identify "lateral root initiation" genes

The generally used approaches of hierarchical (Eisen *et al.*, 1998) and K means (Soukas *et al.*, 2000; Tavazoie *et al.*, 1999) clustering allow the classification of gene expression patterns of single-series microarray time courses. However, for the comparison of multiple-series microarray time courses, no standard algorithms are established. Most reports on such issues restrict themselves to fold-change comparisons (Tian *et al.*, 2002; Ullah *et al.*, 2003) or basic diagrams (Puthoff *et al.*, 2003; Taji *et al.*, 2004). Less frequently, clustering is used to identify

differences between expression profiles in different genotypes (De Paepe *et al.*, 2004). Here, a new approach was developed based on the commonly used single-series time course K means clustering algorithm (Fig. 3.3 A). By combining the data irrespective of the genetic background (0 h-2 h-6 h) with the K means clustering algorithm, the gene expression profiles could be classified into 14 clusters (Fig. 3.3 B). Subsequently, the clusters were plotted at the headings of rows and columns of a cross-table. In this cross-table format, the expression profile of each gene is summarized by a uniquely identifiable cluster combination corresponding to the expression pattern in wild type and in *slr1*. Per cluster combination, the gene frequency was calculated and included into the cross-table; a colour code was implemented to assess easily the differential dynamics of expression between both genotypes (Fig. 3.3 C; Suppl. Table S1). Hereafter, we will refer to this methodology as "cross-table clustering". This approach allows a high-resolution representation of gene-expression patterns across multiple genotypes and provides a tool to compare the transcriptional changes in wild type versus mutant within the framework of a unified set of expression profiles.

In order to gain further insight into the dataset, the functional relevance of all types of cluster combinations was evaluated. The wild-type expression profiles were subdivided into three major patterns: up-regulated (Fig. 3.3 B, clusters 1-6), constitutive (Fig. 3.3 B, clusters 7-11), and down-regulated (Fig. 3.3 B, clusters 12-14). These expression patterns were compared with the corresponding expression profiles in *slr1*. The genes represented by cluster combinations on the diagonal of the cross-table (gray) exhibit expression profiles that are similar in both genotypes, whereas above (red and orange) and below (green and blue) the diagonal, the induction rates in the wild type are higher and lower than those in the mutant, respectively. The Gene Ontology classification for Biological Function (Berardini *et al.*, 2004) was applied on all annotated genes of each subgroup and subsequently compared to all annotated significant genes within our experiment with the EASE 2.1 software (Hosack *et al.*, 2003). The 913 genes that were up-regulated dependent on wild-type SLR/IAA14 stability (selection in Fig. 3.3C; Suppl. Table 3.2) were significantly enriched ($P_{Bonferoni} < 0.01$) in proliferation-related Biological Functions, such as "Cell Cycle", "Nucleic Acid Metabolism", and "Protein Metabolism" (Fig. 3.4), but restricting this selection to the earliest induced genes (Fig. 3.3 B, cluster 1-2-3) resulted in a significant enrichment ($P_{Bonferoni} < 0.01$) in the Biological Functions "Response to Endogenous Stimulus" and "Response to Auxin Stimulus" (Suppl. Table 3.4). These observations strongly correlate to the previous observations of Himanen *et al.* (2004), who found that within the LRIS, genes related to signalling were up-regulated early prior to cell cycle activation.

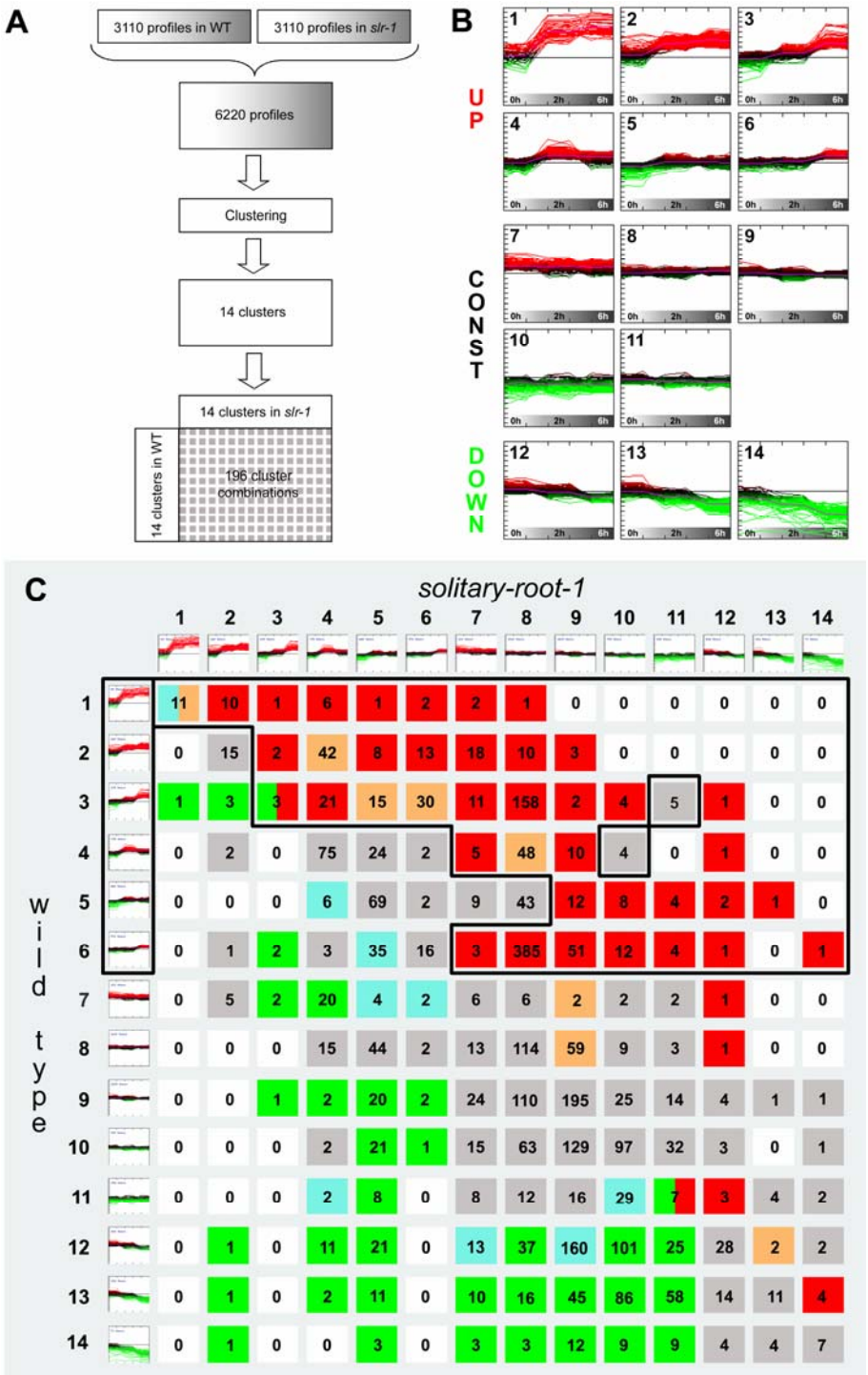

Figure 3.3 Cluster analysis of the expression data. (A) Schematic representation of the "cross-table clustering" methodology. A two-series dataset (wild type and *slr1*) of 3110 profiles is combined into a single-series dataset corresponding to 6220 profiles irrespective of genotypic background. This dataset was clustered into 14 clusters and includes the major patterns within the single-series dataset. Subsequently, the clusters were plotted in a cross-table format: the wild-type and *slr1* expression clusters were plotted in front of the rows and at the head of the columns, respectively. All expression profiles over the different series are summarized by 14^2 (196) cluster combinations. (B) Clusters illustrating the major patterns of the combined dataset. In a white-gray gradient, the time points of the single-series are shown. Each time point is characterized by the individual biological repeated values (0 h = 72 h NPA, 2 h = 0 h + 2 h NAA and 6 h = 0 h + 6 h NAA). Clusters 1-6, 7-11, and 12-14 correspond to up-regulated. constitutive, and down-regulated expression profiles, respectively. (C) Cross-table representation of the expression profiles within both genotypes. The frequencies of each cluster combination within the dataset are indicated in each square. Gray marks no significant difference in induction rates between both genotypes, whereas red and

orange and green and blue indicate that the induction rates are higher and lower in wild type than in *slr1*, respectively. The black line encircles the selected 913 *LRI* genes.

In conclusion, the cross-table clustering methodology on our dataset reliably identified 913 genes that depend on rapid SLR/IAA14 degradation for normal auxin responsiveness. Because *slr1* does not initiate lateral roots, even upon auxin treatment, such genes might encode potential regulatory proteins required for lateral root initiation and will be designated as "lateral root initiation" (*LRI*) genes hereafter.

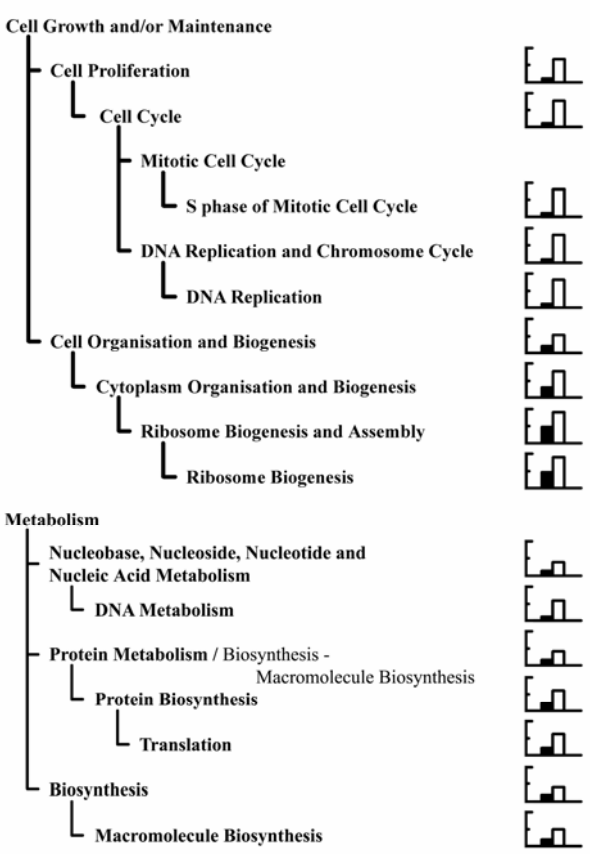

Figure 3.4 Overrepresented functional categories within the SLR/IAA14-mediated up-regulated genes. For each significantly overrepresented functional category, the corresponding percentage of annotated genes in the gene ontology is indicated among the 3110 significant profiles versus the complete array (black bars) and 913 *LRI* genes versus the 3110 significant genes (white bars). Ordinate is 100%.

Validation of *LRI* genes by database comparison

With the same lateral root-inducible system, we had performed previously a microarray study based on cDNA microarrays representing approximately 4,600 genes (Himanen *et al.*, 2004). At a stringency level of 0.005, 906 genes had been found to be significantly expressed. The profiles of 343 genes were clustered into three up-regulated clusters. From the selected 913

LRI genes, defined as auxin inducible in a SLR/IAA14-dependent manner, 122 genes were also represented in the earlier dataset (Table S3.5). This result suggests that, despite the differences in type of array (cDNA array vs Affymetrix gene chip) and the differences in statistical analysis, a high level of reproducibility was obtained by the lateral root-inducible system and implies also that a high level of confidence can be attributed to the profiles associated with the *LRI* genes, as suggested by the q-values.

The *arf7arf19* double mutants have been shown to phenocopy the lateral rootless phenotype of *slr1* to a large extent (Okushima *et al.*, 2005; Wilmoth *et al.*, 2005), indicating that SLR/IAA14 inhibits the activity of these ARFs to block lateral root initiation. Recently, a yeast two-hybrid assay confirmed that SLR/IAA14 interacts with ARF7 and ARF19 (Fukaki *et al.*, 2005). Therefore, both *slr1* and *arf7arf19* mutants can be expected to have similar target genes. The auxin inducibility in *arf7arf19* has recently been assessed by Okushima *et al.* (2005), who assayed the effects of a 2-h treatment with 5 μM IAA on 5-day-old seedlings of wild type, *arf7*, *arf19*, and *arf7arf19* in a microarray that gave a robust overview of early auxin-induced genes. The respective ARF7- and ARF19-dependent auxin inducibility of the 913 *LRI* l genes was extracted from the published data set (Table S3.6). Of the 913 *LRI* genes, 99 were induced more than two-fold in wildtype, whereas the auxin inducibility of these genes was abolished in *arf7arf19* double mutants (Table S3.7). Because in this experimental set-up only a 2-h IAA treatment was used, a large percentage of these genes belong to the early induced *LRI* genes (83 out of 365 genes in wild-type clusters 1-3). Due to large experimental differences, we cannot exclude that more *LRI* genes act downstream of ARF7 and ARF19. These results indicate that the use of the lateral root-inducible system provides a high reproducibility among divergent experiments, giving a high resolution image on differential expression during lateral root initiation.

Cell cycle progression during lateral root initiation

Within these 913 *LRI* genes, cell division-related genes were identified, such as *APC8/CDC23*, *PCNA1*, *RNR1*, *MCM3*, *MCM4*, *MCM7/PROLIFERA*, *RPS18A/PFL1, RPS13A/PFL2*, *FtsZ1-1*, *FtsZ2-1*, *CYCD3;2*, *CYCA2;4*, *CYCB2;5*, *CDKB2;1*, and *CKL3* (Table 1). APC8/CDC23 is part of the anaphase-promoting complex (APC) that is involved in targeting A- and B-type cyclins for degradation during mitosis (Capron *et al.*, 2003). Proliferating cell nuclear antigen (PCNA1) belongs to the DNA replication machinery and its

expression is associated with the S phase (Hübscher *et al.*, 2002). MiniChromosome Maintenance (MCM) proteins are conserved eukaryotic replication factors involved in the initiation of DNA replication (Mariconti *et al.*, 2002) and ribonucleotide reductase large subunit (RNR1) is part of a rate-limiting enzyme in the synthesis of nucleotides (Elledge *et al.*, 1993). The ribosomal proteins encoded by *POINTED FIRST LEAVES1* (*PFL1*) and *PFL2* are produced during lateral root formation (Ito *et al.*, 2000; Peters *et al.*, 2004). FtsZ is involved in plastid divisions (Osteryoung *et al.*, 1998). On the other hand, we recovered very few core cell cycle genes (Vandepoele *et al.*, 2002). The represented genes consisted of one G1-to-S (*CYCD3;2*), an S-phase-related (*CYCA2;4*) and two G2-to-M related genes (*CYCB2;5* and *CDKB2;1*), and a recently identified CDK-like protein-encoding gene (*CKL3*) (Menges *et al.*, 2005). However, in the root part concerned, most core cell cycle genes are expressed in low abundance at this high stringency level ($p<0.001$). When the stringency level was reduced to $p<0.01$, the number of significantly modulating cell cycle genes increased to 28 ($q<0.05$) (Table S3.8).

Three cell cycle genes of the stringent selection, *CYCD3;2*, *CYCA2;4* and *CDKB2;1*, contain at least one auxin-responsive element (ARE; TGTGTC or GAGACA) within a sequence 1000 bp upstream of their 5' untranslated region (Fig. 3.5 A), suggesting these genes may be part of the primary auxin response with their expression controlled by unstable AUX/IAA proteins (Ulmasov *et al.*, 1999). Consistent with this, treating 5-day-old roots with the protein synthesis inhibitor, cycloheximide (CHX) for 2 h and 4 h, resulted in a progressively strong induction of *CYCA2;4* and *CDKB2;1*, respectively (Fig. 3.5 B). However, for *CYCD3;2*, only a weak CHX-mediated induction was observed, suggesting the AREs in its promoter may not be functional.

Consistent with the observation that their induction is strongly dependent on normal SLR/IAA14 degradation, *CYCA2;4* and *CDKB2;1* are probably directly regulated through a labile repressor, such as SLR/IAA14. Taken together, these results argue for a direct link between auxin signalling and cell cycle activation during lateral root initiation both at the S phase as at the G2-to-M transition.

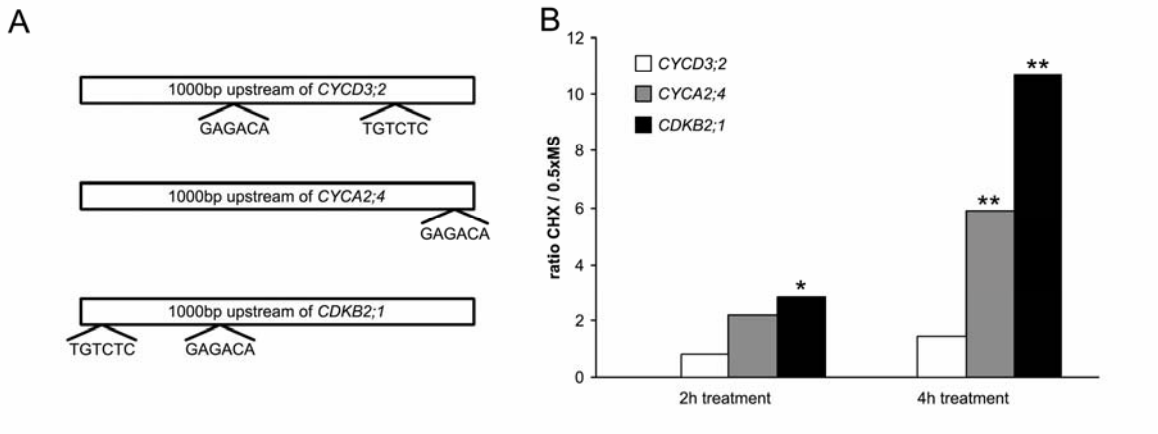

Figure 3.5 Analysis of primary auxin responsiveness of *CYCD3;2*, *CYCA2;4*, and *CDKB2;1*. (A) AREs within the 1000-bp sequence upstream of the 5' untranslated region of *CYCD3;2*, *CYCA2;4*, and *CDKB2;1*. (B) Ratio of expression levels of *CYCD3;2*, *CYCA2;4*, *CDKB2;1* between CHX treatment and 0.5 x Murashige and Skoog mock-treatment for 2 h and 4 h.

Cell cycle progression in the pericycle is not sufficient to complement the lack of lateral roots in *slr1*

Overexpression of G1/S regulators, such as *CYCD3;1* (Dewitte *et al.*, 2003) or the transcription factor complex *E2Fa/DPa* (De Veylder *et al.*, 2002), is sufficient to trigger several rounds of cell division in cell types that normally remain quiescent. Can overexpression of these cell cycle regulators induce pericycle cell proliferation and possibly even lateral roots in the absence of a normal SLR/IAA14-dependent auxin response? To address this question, *slr1* was crossed into the transgenic lines and their respective wild types. The root phenotypes of the F1 seedlings were analyzed in detail. All the wild-type (Columbia [Col-0] and Landsberg *erecta* [L*er*]) and *CYCD3;1*^{OE} (in L*er* background) plants formed normal roots (Fig. 3.6 A,E,F). However, *E2Fa/DPa*^{OE} (in Col-0 background) showed a clear reduction in primary root length and in visible lateral roots (Fig. 3.6 B). The reduced lateral root density of *E2Fa/DPa*^{OE} was also observed in cleared 10-day-old roots and can be rescued by auxin treatment (I. De Smet, unpublished results). When *slr1* was crossed into Col-0, *E2Fa/DPa*^{OE}, L*er*, or *CYCD3;1*^{OE}, no lateral roots could be observed (Fig. 3.6 C,D,G,H). Surprisingly, after closer microscopic inspection of the root, only in *CYCD3;1*^{OE} x *slr1* were regions seen in which the pericycle cells were shorter (Fig. 3.6 L) than those of mature L*er* and L*er* x *slr1* (Fig. 3.6 I,K). These series of short cells were found exclusively in the pericycle cell layer and are most probably the result of one or more extra rounds of cell division. In *E2Fa/DPa*^{OE} x *slr1* and Col-0 x *slr1* roots, no such regions with shortened pericycle cells could be observed (data not shown). Unlike in a stage I primordium, which is normally composed of small radially swollen cells in the midst of larger

elongated cells (Fig. 3.6 J; Malamy and Benfey, 1997), the short pericycle cells in *CYCD3;1*^{OE} x *slr1* roots had a uniform cell size and were not radially swollen (Fig. 3.6 L). Also absent were stage-II lateral root primordia, which are the result of a periclinal division of stage-I primordium cells.

To verify whether these regions of shortened cells were the consequence of extra rounds of cell division after leaving the meristem, the expression levels of the S-phase-associated *CYCA2;4* gene (Fig. 3.6 M) and the G2/M-specific *CYCB1;1* (Fig. 3.6 N) and *CDKB1;1* (Fig. 3.6 O) were analyzed via real-time reverse transcription-polymerase chain reaction (RT-PCR) in the *CYCD3;1*^{OE} x *slr1* background. In *slr1* background, the expression of these cell cycle genes was strongly reduced, whereas that of both genes appeared to be restored by *CYCD3;1* overexpression. To assess whether this cell division activity in the pericycle of the mutant was associated with a recovered capacity to initiate a new organ, the expression of the *PLETHORA1* (*PLT1*) gene was analyzed in *CYCD3;1*^{OE} x *slr1* roots. *PLT1* encodes a transcription factor that has been shown to be associated with quiescent centre specification downstream of the auxin signal (Aida *et al.*, 2004). Because a new quiescent centre has to be specified during lateral root formation, *PLT1* expression can be used as a marker for early stages of lateral root organogenesis (Suppl. Fig. 3.2). Nevertheless, *PLT1* expression remained low in *CYCD3;1*^{OE} x *slr1* roots (Fig. 3.6 P). These results indicate that the requirements for organogenetic processes, such as lateral root initiation, are more complex than simple activation of the cell cycle and that additional SLR/IAA14-dependent signalling is needed to develop a new organ.

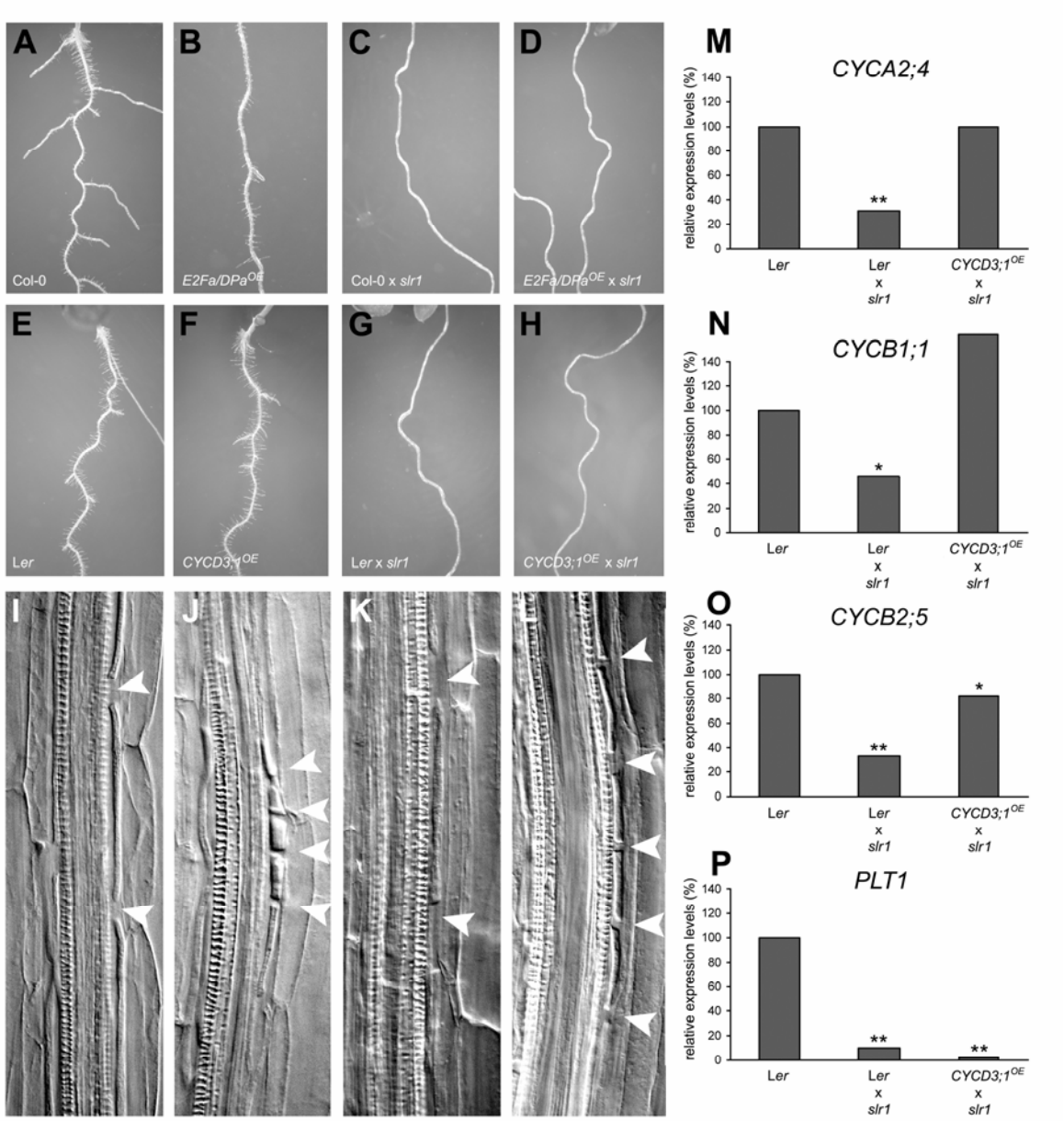

Figure 3.6 Complementation of cell cycle defect in *slr1*. **(A) to (H) Overview of root phenotype of 5-day-old seedlings of Col-0 (A), *E2Fa/DPa*^{OE} (B), Col-0 x *slr1* (C), *E2Fa/DPa*^{OE} x *slr1* (D), L*er* (E), *CYCD3;1*^{OE} (F), L*er* x *slr1* (G), and *CYCD3;1*^{OE} x *slr1* (H). (I) to (L) Microscopic analysis of the pericycle after clearing of mature L*er* pericycle cell (I), L*er* stage I lateral root primordium (J), mature L*er* x *slr1* pericycle cell (K), and zone of shortened pericycle cells in *CYCD3;1*^{OE} x *slr1* (L). Arrowheads mark pericycle cell size. (M) to (P) Real-time PCR analysis on L*er*, L*er* x *slr1* and *CYCD3;1*^{OE} x *slr1* roots of *CYCA2;4* (M), *CYCB1;1* (N), *CDKB1;1* (O), and *PLT1* (P).**

Complex auxin signalling-dependent mechanisms regulate lateral root initiation

As expected, besides cell cycle related genes, several central regulators of auxin signalling were recovered among the *LRI* genes (Table 1). Nine *Aux/IAA* genes were identified within this selection. For two of them, gain-of-function mutants with defects in lateral root formation have been described before (*axr5/iaa1* (Park *et al.*, 2002; Yang *et al.*, 2004); and *msg2-1/iaa19* (Tatematsu *et al.*, 2004)). The remaining seven *Aux/IAA* genes have not been characterized functionally. In addition to this type of negative feedback regulation, several potential *Aux/IAA* targets, such as *ARF4*, *ARF16* and *ARF19*, were also strongly up-regulated in a SLR/IAA14-dependent fashion, especially ARF19, which seems to play an important role in regulating lateral root formation, because *arf7arf19* double mutants exhibit a *slr1*-like phenotype (Okushima *et al.*, 2005; Wilmoth *et al.*, 2005). Furthermore, both ARF7 and ARF19 were shown to interact with SLR/IAA14 in a yeast two-hybrid assay (Fukaki *et al.*, 2005). In conclusion, our microarray data show SOLITARY-ROOT/IAA14-dependent expression of both *Aux/IAA* and *ARF* genes, suggesting that at the level of auxin signalling, lateral root initiation is tightly regulated through both negative and positive feedback.

Table 1 Known auxin-and cell cycle-relatd genes within the *LRI* genes. [a] Genes in bold depend on ARF7 and ARF19 function for auxin inducibility (see Suppl. Table 3.7 for expression profiles). [b] Correspond to the clusters representing the expression profiles of the respective genes in Col-0 and in *slr1*, respectively (see Fig. 3.3).

Description	Gene[a]	AGI Code	Cluster Coordinates
Cell cycle	*APC8/CDC23*	At3g48150	6.8
	PCNA1	At1g07370	3.6
	RNR1	At2g21790	3.8
	MCM3	At5g46280	3.8
	MCM4	At2g16440	3.6
	MCM7/PRL	At4g02060	3.8
	RPS18A/PFL1	At1g34030	6.8
	RPS13A/PFL2	At3g60770	6.8
	FtsZ1-1	At5g55280	6.9
	FtsZ2-1	At2g36250	6.8
	CYCD3;2	At5g67260	3.9
	CYCA2;4	At1g80370	2.9
	CKL3	At1g18670	2.4
	CYCB2;3	At1g20610	6.10
	CDKB2;1	At1g76540	6.10

Auxin signalling	***IAA1/AXR5***	At4g14560	2.4
	IAA4/AUX2-11	At5g43700	2.5
	IAA5/AUX2-27	At1g15580	3.6
	IAA11	At4g28640	1.2
	IAA13	At2g33310	2.4
	IAA18	At1g51950	4.8
	IAA19/MSG2	At3g15540	1.1
	IAA20	At2g46990	3.6
	IAA29	At4g32280	1.2
	ARF4	At5g60450	2.8
	ARF16	At4g30080	6.8
	ARF19	At1g19220	3.4
Auxin transport	*AUX1*	At2g38120	2.4
	LAX3	At1g77690	3.5
	PIN1	At1g73590	2.4
	PIN3	At1g70940	2.4
	PIN7	At1g23080	2.4
	PINOID/PID	At2g34650	1.1
	PINOID-like	At3g20830	4.8
	TCH3	At2g41100	2.4
	PGP1	At2g36910	4.8
Auxin conjugation	***GH3.1***	At2g14960	1.2
	GH3.3	At2g23170	1.1
	GH3.4	At1g59500	1.1
	GH3.5/AtGH3a	At4g27260	1.2
	GH3.6/DFL1	At5g54510	1.4
	UGT84B1	At2g23350	6.8
	IAR1	At1g51760	9.8
Auxin biosynthesis	CYP79B2	At4g39950	13.11
	CYP79B3	At2g22330	13.11
	ATR1	At5g60890	13.10
	YUCCA	At4g04180	4.2

Attenuating and maintaining auxin levels during lateral root initiation

Auxin conjugation, transport, and biosynthesis contribute to local auxin homeostasis. The dataset presented (Table 3.1) indicates that these three processes are a part of lateral root initiation.

The *GH3* gene family consists of the first identified auxin-responsive genes (Hagen *et al.*, 1991) and has been divided into three major groups, according to their substrate specificity and sequence similarities (Staswick *et al.*, 2002). Six group-II GH3 proteins have been shown to act as IAA-amido synthetases, providing a mechanism to reduce the level of active auxin by conjugation to amino acids (Staswick *et al.*, 2005). Interestingly, all five *GH3* genes identified as *LRI* genes belong to the group-II GH3 family and, besides ARF8 (Tian *et al.*, 2004), they also seem to be regulated through ARF7 and ARF19 transcription factors (Okushima *et al.*, 2005). Also *UGT84B1*, coding for an enzyme involved in the conjugation of glucose to IAA (Jackson et al., 2002), was induced in wild type, but not in *slr1*. In contrast, *IAR1*, which codes for a putative ZIP family transporter protein and is necessary for the production of IAA out of IAA-amino acid conjugates (Lasswell *et al.*, 2000), was strongly repressed in wild type and to a lesser extent in *slr1*. A negative feedback mechanism interfering with the level of free auxin is therefore likely to be active during lateral root initiation.

On the other hand, many components of the auxin transport machinery were recovered within the *LRI* genes. Not only did the putative auxin influx carriers *AUX1* and *Like-AUX1 3* (*LAX3*) (Parry *et al.*, 2001) emerge, but so did the auxin efflux facilitators, such as *PIN1*, *PIN3*, and *PIN7* (Paponov *et al.*, 2005), and some proteins involved in their correct localization, such as PINOID/PID (Friml *et al.*, 2004), a PINOID-like, and a PID-interacting protein, TCH3 (Benjamins *et al.*, 2003). Moreover, the multidrug resistance protein involved in polar auxin transport encoded by *PGP1* (Lin and Wang, 2005) was found among the *LRI* genes. The corresponding mutants display defects in lateral root initiation (Benková *et al.*, 2003; Bennett *et al.*, 1996). The auxin inducibility of polar auxin transport genes (influx and efflux) was also observed within the vascular cambium of hybrid aspen (Schrader *et al.*, 2003). Interestingly, *PIN1*, *PIN3*, and *PIN7* were also shown to depend on ARF7 and ARF19 for their auxin inducibility (Okushima *et al.*, 2005) and their Aux/IAA-dependent inducibility has been implicated in their functional redundancy (Vieten *et al.*, 2005).

Recently, auxin has been shown to negatively regulate its own biosynthesis (Ljung *et al.*, 2005). Two genes encoding enzymes involved in the Trp-dependent IAA biosynthesis pathway (*CYP79B2* and *CYP79B3*)(Azumi *et al.*, 2002) and their transcriptional regulator *ATR1/MYB34* (Ljung *et al.*, 2005) were down-regulated upon auxin treatment in wild type, but to a lesser extent in *slr1*. Interestingly, *YUCCA*, a gene encoding another rate-limiting enzyme of the Trp-dependent IAA biosynthesis pathway (Zhao *et al.*, 2001), was more induced in *slr1* than in wild type.

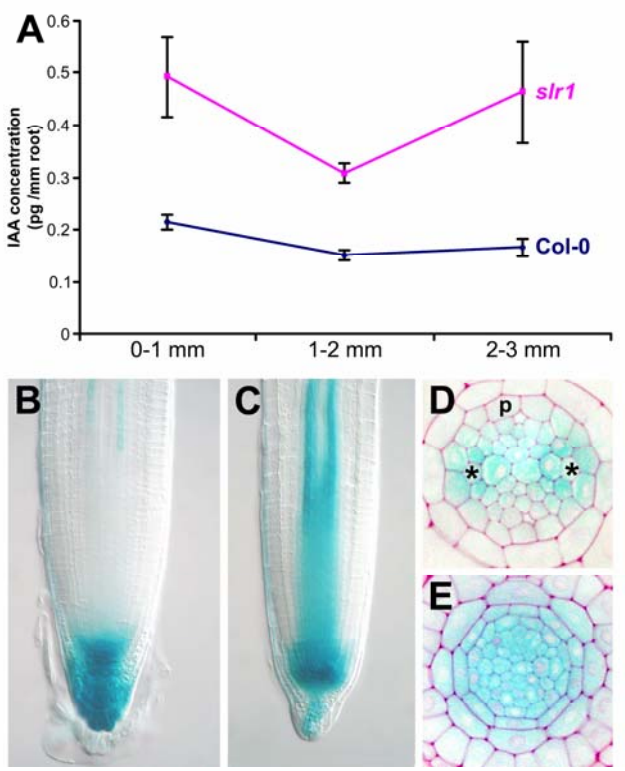

Figure 3.7 Analysis of auxin content in Col-0 and *slr1*. (A) IAA concentration of Col-0 and *slr1* of the 3-mm most apical part of the primary root. (B) and (C) P_{DR5}*:GUS* expression in 5-day-old root apical meristems in Col-0 and *slr1*, respectively. (D) and (E) Anatomical analysis of P_{DR5}*:GUS* in *slr1* of the elongation zone and the root apical meristem, respectively. p, pericycle. * indicates protoxylem cells.

Taken together, these data suggest that auxin homeostasis is disturbed in *slr1*. Therefore, the auxin content of seedling roots of wild type and *slr1* was determined by gas chromatography-selected reaction monitoring-mass spectrometry (Ljung *et al.*, 2005). Indeed, a significantly higher auxin concentration was found in the most apical 3 mm of *slr1* primary roots than that in wild type (Fig. 3.7 A). Correspondingly, the increased auxin concentration in *slr1* could be visualized with P_{DR5}*:GUS* (Fig. 3.7 B-E).

DISCUSSION

The results of our genome-wide transcriptome analysis allow us to present a model describing a regulatory network that integrates some of the most likely occurring processes upon auxin perception during the onset of lateral root initiation (Fig. 3.8). The auxin signalling cascade starts off by the auxin-stimulated targeting of AUX/IAA proteins for degradation, thereby derepressing the transcriptional activity of ARFs (Dharmasiri and Estelle, 2004). Therefore, the auxin-mediated degradation of the SLR/IAA14 protein might be the first crucial event in the preamble toward lateral root initiation (Fig. 3.8 A).

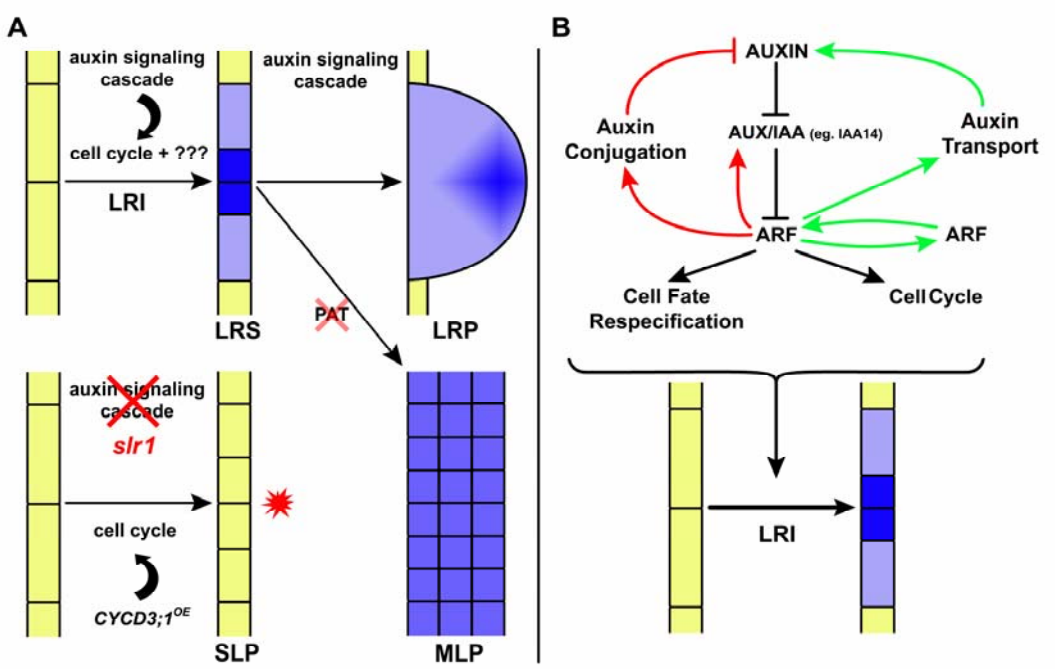

Figure 3.8 Model of SLR/IAA14-dependent lateral root initiation. (A) Induction of lateral root initiation (LRI) in the pericycle resulting in a lateral root initiation site (LRS) when the auxin signalling cascade is intact. Further development toward a lateral root primordium is also dependent on auxin. When the polar auxin transport machinery is intact, auxin gradients are set up to organize the lateral root primordium (LRP). Disturbing the polar auxin transport (PAT) does not inhibit the auxin-induced developmental program of lateral root formation, but provokes rather an unorganized multilayered proliferating zone (MLP) (Benková *et al.*, 2003; Geldner *et al.*, 2004). When the auxin signalling cascade is defective (*slr1*), no lateral roots can be initiated. Complementation of the cell cycle defect in *slr1* by overexpression of *CYCD3;1* is not sufficient to activate the developmental program of LRI; nevertheless, it can induce some proliferative divisions in the pericycle, resulting in a single-layered proliferating zone (SLP). (B) Model for AUX/IAA-mediated LRI. (Black path) In the early auxin signalling cascade, increased auxin levels stimulate the degradation of AUX/IAA proteins, such as SLR/IAA14. AUX/IAA proteins repress the transcriptional activity of one or more ARFs. Downstream of this signalling cascade lays the activation of the developmental program of LRI that includes the coordinated action of cell fate respecification and cell cycle progression. (Red paths) The auxin signalling cascade induces auxin conjugation and may negatively influence auxin content. Also, increased auxin levels induce AUX/IAA proteins that repress ARF transcriptional activity. (Green paths) Increased auxin levels can induce auxin transport, leading to even higher auxin levels. Furthermore, auxin induces ARF production, promoting downstream auxin signal transduction.

The importance of cell cycle progression during lateral root initiation

When the auxin-induced transcriptional changes in root segments of wild type and *slr1* are compared, the inhibition of auxin signal transduction in the mutant is correlated with the failure to induce cell cycle-related genes. Although the role of auxin in cell cycle progression is generally accepted, we found evidence that this stimulation may occur via an AUX/IAA–ARF-dependent pathway. Indeed, *CYCA2;4* and *CDKB2;1* both contain at least one ARE in their promoter sequence and their auxin inducibility has been shown to depend on the normal

degradation of SLR/IAA14. Furthermore, both genes are induced by the inhibition of protein synthesis, suggesting a labile repressor is controlling their transcription. Because AUX/IAA proteins are regarded as labile repressors (Ouellet *et al.*, 2001), it is tempting to consider *CYCA2;4* and *CDKB2;1* to be primary targets of the SLR/IAA14 signalling. These results are indicative for a potential molecular mechanism by which auxin signalling may feed into the cell cycle. Nevertheless, the functionality of the respective AREs remains to be proven.

However, stimulation of cell cycle was not sufficient to trigger the formation of lateral roots in the *slr1* background. Only *CYCD3;1*^{OE} was capable of initiating a round of cell divisions in *slr1* pericycle cells, giving rise to a single layered proliferation zone (Fig. 3.8 A) that did not progress further into lateral root developmental stages, let alone lateral root primordia.

Cell fate respecification of the pericycle

The inability to form lateral roots after cell cycle stimulation of the pericycle suggests that besides cell cycle progression, *slr1* is unable to respecify the identity of pericycle cells into that of lateral root primordia. Analogous to the situation found in early embryogenesis, the formation of lateral roots also seems to depend on cell fate alteration through the occurrence of asymmetric divisions. In case of embryogenesis and primary root development, polar auxin transport installs an auxin maximum, visualized by the auxin reporter construct P_{DR5}*:GUS* (Sabatini *et al.*, 1999), restricting *PLETHORA* (*PLT*) expression which specifies stem cell identity (Aida *et al.*, 2004; Blilou *et al.*, 2005).

Likewise, polar auxin transport is required to form lateral roots because mutants defective in polar auxin transport fail to produce lateral roots (Benková *et al.*, 2003; Geldner *et al.*, 2004). Transcripts of the polar auxin transport machinery appear to be unable to accumulate normally in auxin-treated *slr1* roots, preventing the installation of an auxin maximum. Therefore, it might be argued that fine-tuned polar auxin transport could be the missing factor to specify stem cell identity for lateral root initiation in *slr1*. Other mutants defective in polar auxin transport were unable to produce individual lateral root primordia upon auxin supplementation; instead, multilayered proliferation zones (Fig. 3.8 A) developed (Benková *et al.*, 2003; Geldner *et al.*, 2004). Nevertheless, none were detected in the *slr1* root that has increased auxin levels, even when cell division was stimulated by *CYCD3;1*^{OE}. However, *PLT* expression was not restored by *CYCD3;1*^{OE} in *slr1*, suggesting the induced pericycle cell divisions do not correspond to

lateral initiation sites. Thus, the impaired auxin transport in *slr1* would probably not be the only element responsible for its inability to form lateral roots. Polar auxin transport is necessary for providing auxin to the pericycle and for organizing the auxin gradient in the developing lateral root primordium; induction of other factors are probably also needed to turn into organogenesis.

Such factors, which are indispensable for induction of asymmetric divisions, acquisition of different cell fates, and organogenesis, might be represented by members of the *WUSCHEL-related homeobox* (*WOX*) family. During embryogenesis, *WOX* genes are expressed in restricted areas of the embryo and are believed to be involved in cell fate specification (Haecker *et al.*, 2004). In this respect, it is worth mentioning that one member of the *WOX* family, *WOX13*, was retrieved among the *LRI* genes. The SLR/IAA14-mediated auxin inducibility (cluster combination 6.8) of *WOX13* suggests that it is involved in the respecification of pericycle cells during lateral root initiation. Moreover, the functional analysis of other proteins encoded by the *LRI* genes will probably result in the identification of potential cell fate respecification factors and will provide new insights into this intriguing developmental process.

A binary switch mechanism is involved in lateral root initiation

Our results suggest that lateral root initiation is regulated through positive as well as negative feedback loops (Fig. 3.8 B, green and red arrows). Two types of regulation with negative effects on auxin concentration and/or signal transduction can be distinguished from the dataset (Fig. 3.8 B, red arrows). One type is brought about by the strong up-regulation of genes encoding enzymes involved in auxin conjugation. Conjugation of IAA to amino acids or sugars can reduce the free auxin level, although some conjugates of IAA and amino acids are reversible and can contribute to the pool of free IAA (Lopéz-Bucio *et al.*, 2005). However, the contribution of IAA oxidation is believed to be much more important for auxin homeostasis than that of conjugation (Kowalczyk and Sandberg, 2001). Secondly, in contrast to the auxin-induced instability of AUX/IAA proteins (Gray *et al.*, 2001), their expression was found to be strongly induced within the LRIS. Because these proteins encode potent inhibitors of ARF-mediated transcription (Tiwari *et al.*, 2004), their induction provides a direct negative feedback onto the auxin signal transduction.

An example of a positive feedback loop (Fig. 3.8 B, green arrows) is the active auxin transport that has been shown to be auxin inducible in a SLR/IAA14-dependent manner. The

installation of its own transport machinery matches the self-organizing nature of auxin transport suggested in the canalization hypothesis (Sachs, 1988). This supposition implies that high auxin levels in the pericycle would be actively reinforced by the locally increased auxin transport potential. Furthermore, the down-regulation of IAA biosynthesis genes and the up-regulation of the IAA conjugation potential in early stages of lateral root initiation suggest that young primordia depend on auxin import for their development. Later on, in emerged lateral root primordia, a regained capacity for auxin biosynthesis has been observed (Ljung *et al.*, 2005). Indeed, only excised lateral root primordia at later stages of development can grow in culture without added hormones (Laskowski *et al.*, 1995). Furthermore, expression of several ARFs is also induced during lateral root initiation, reducing the AUX/IAA versus ARF ratio with an increased sensitivity toward auxin as a consequence and amplification of the auxin signal to stimulate the onset of lateral root initiation.

In summary, in the proposed model lateral root initiation is subjected to two counteracting regulatory circuits that result in a binary switch mechanism. When the auxin concentration is low, the auxin response acts to dampen small fluctuations in auxin concentration and no lateral roots are initiated. When this negative regulation is overcome, auxin concentration and responsiveness are actively reinforced by a positive feedback mechanism. This positive spiral activates the developmental program of lateral root initiation. When *SLR/IAA14* is mutated, no lateral roots can be produced. Also auxin homeostasis is mis-regulated in *slr1*, leading to auxin accumulation. Thus our model is consistent with the experimental data and provides a basis to understand the complex self-regulatory mechanisms involved in lateral root initiation.

MATERIALS & METHODS

Plant material and growth conditions

In this study, we analyzed the *Arabidopsis thaliana* (L.) Heynh. ectotypes Col-0, L*er*, the mutant *slr1* (Fukaki *et al.*, 2002), the promotor fusions P_{IAA14}*:GUS* (Fukaki *et al.*, 2002), $P_{CDKA;1(cdc2a)}$*:GUS* (Hemerly *et al.*, 1995), $P_{CDKB1;1}$*:GUS* (de Almeida Engler *et al.*, 1999), $P_{CYCB1;1}$*:GUS* (Colon-Carmona *et al.*, 1999), P_{DR5}*:GUS* (Ulmasov *et al.*, 1997), P_{ALF4}*:GUS* (DiDonato *et al.*, 2004), *E2Fa/DPa*^{OE} (De Veylder *et al.*, 2002), *CYCD3;1*^{OE} (Dewitte *et al.*, 2003), and the xylem pole pericycle-specific GAL4 enhancer trap line J0121

(http://www.plantsci.cam.ac.uk/Haseloff/geneControl/catalogues/Jlines/record/record_0.html) were analyzed. Seeds were always germinated on medium derived from standard Murashige and Skoog medium on vertically oriented square plates (Greiner Labortechnik, Frickenhausen, Germany) in a growth chamber under continuous light (110 $\mu E.m^{-2}.s^{-1}$ photosynthetically active radiation, supplied by cool-white fluorescent tungsten tubes [Osram, München, Germany]) at 22°C. Supplements consisted of 10 μM *N*-1-naphthylphthalamic acid (NPA; Duchefa, Haarlem, The Netherlands), 10 μM α-1-naphthalene acetic acid (NAA; Sigma-Aldrich, St. Louis, MO), or 30 μM cycloheximide (CHX; Sigma-Aldrich).

Histochemical and histological analysis

The β-glucuronidase (GUS) assays were performed as described (Beeckman and Engler, 1994). For microscopic analysis, samples were cleared by mounting in 90% lactic acid (Acros Organics, Brussels, Belgium) (analysis of GUS stainings) or using the clearing method described by Malamy and Benfey (1997) (analysis of pericycle cell length). All samples were analyzed by differential interference contrast microscopy (Leica DMLB, Leica, Vienna, Austria).

For anatomical sections, GUS-stained samples were fixed overnight in 1% glutaraldehyde and 4% paraformaldehyde in 50 mM phosphate buffer (pH 7). Samples were dehydrated and embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's protocol. For proper orientation of the samples, we used a two-step embedding methodology, based on a pre-embedding step to facilitate orientation in 0.5-ml Eppendorf tubes (De Smet *et al.*, 2004). Sections of 5 μm were cut with a microtome (Minot 1212; Leitz, Wetzlar, Germany), dried on Vectabond-coated object glasses, counterstained for cell walls with 0.05% ruthenium red for 8 min (Fluka Chemica, Buchs, Switzerland), and rinsed in tap water for 30 s. After drying, the sections were mounted in DePex medium (British Drug House, Poole, UK) and covered with cover slips.

Photographs were taken with a CAMEDIA C-3040zoom digital camera (Olympus, Tokyo, Japan) and processed with Photoshop 7.0 (Adobe, San José, CA).

Microarray analysis and data processing

Col-0 and *slr1* seeds were germinated on medium containing 10 μM NPA and transferred 3 days after germination under continuous light to 10 μM NAA, according to the points of the time course (0 h-2 h-6 h). All sampling points were performed in duplicate. For each sampling, approximately 1000 root segments between root apical meristem and root-hypocotyl junction were pooled. From the pooled LRIS, RNA of root segments was extracted with the RNeasy Kit (Qiagen, Hilden, Germany). Out of 5.8 μg total RNA, biotinylated copy RNA was produced (Hennig *et al.*, 2003), of which 20 μg was fragmented and hybridized to the ATH1 arrays (Affymetrix, Santa Clara, CA, USA). Washing, detection, and scanning were performed as described by Hennig *et al.* (2003). Raw data were processed with the statistical algorithm of the Affymetrix Microarray Suite 5.0 (Liu *et al.*, 2002). Box plots of $\log_2$-transformed normalized value distributions of all arrays show that most array-to-array effects were taken care of by the normalization procedure (Suppl. Fig. 3.3). The normalized data were subjected to two-factor ANOVA analysis with Microsoft Excel (Seattle, WA, USA). The false positives were controlled by measuring the false discovery rate (q-value) (Storey and Tibshirani, 2003) with the freely available software QVALUE (http://genomine.org/qvalue/). Significant profiles were preprocessed (Fig. 3.2 A) prior to clustering. The optimal number of clusters was estimated with the Figure of Merit calculations (Yeung *et al.*, 2001) and K means clustered (Soukas *et al.*, 2000) in the Multiple Experiment Viewer 2.2 of The Institute for Genome Research (Saeed *et al.*, 2003). The cross-table was constructed with Photoshop 7.0 (Adobe).

Real-time PCR

RNA was extracted with the RNeasy kit (Qiagen). Poly(dT) cDNA was prepared from 1 μg total RNA with the Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and quantified on an iCycler apparatus (Bio-Rad, Hercules, CA) with the qPCR Core Kit for SYBR green I (Eurogentec, Seraing, Belgium). PCR was carried out in 96-well optical reaction plates heated for 10 min to 95°C to activate hot start Taq DNA polymerase, followed by 50 cycles of denaturation for 60 sec at 95°C, and annealing-extension for 60 sec at 58°C. Target quantifications were performed with specific primer pairs designed with the Beacon Designer 4.0 (Premier Biosoft International, Palo Alto, CA). Expression levels were first normalized to *ACTIN2* expression levels that did not show clear systematic changes in Ct-value, and then to

the respective expression levels in wild type (L*er*). The primers used to quantify gene expression levels were At1g42970/*GAPDH*, 5'-TCTTTCCCTGCTCAATGCTCCTC-3' and 5'-TTTCGCCACTGTCTCTCCTCTAAC-3'; At3g18780/*ACTIN2*, 5'-TTGACTACGAGCAGGAGATGG-3' and 5'-ACAAACGAGGGCTGGAACAAG-3'; At1g80370/*CYCA2;4*, 5'-GCTCCAGATCGCCTCCAAG-3' and 5'-CACGCAGGTTGTAGTAGATG-3'; At3g54180/*CDKB1;1*, 5'-GGTGGTGACATGTGGTCTGTTGG-3' and 5'-CGCAGTGTGGAAACACCCGG-3'; At3g20840/*PLT1*, 5'-ACGATATGCCTTCCAGTGATG-3' and 5'-TTCAGACCCATTCCTTGTGC-3'.

Quantification of IAA

Wild-type and mutant *slr-1* seedlings were grown on vertical plates (1 x Murashige and Skoog medium, 1% sucrose, 1% agar, pH 5.7) under long-day conditions (16 h light, 8 h darkness). Samples were collected, extracted, and purified as described by Ljung *et al.* (2005). Of the primary root, the apical 3 mm was collected from 7-day-old seedlings and cut in 1-mm sections. For each sample, sections from 50 seedlings were pooled. Endogenous IAA content was analyzed by gas chromatography-selected reaction monitoring-mass spectrometry (Edlund *et al.*, 1995). Isotopic dilution was calculated based on the addition of 100 pg $^{13}C_6$-IAA per sample. Four replicates were analyzed for each sample.

Accession numbers and data deposition

All microarray data will be made available in the Genevestigator database (Zimmermann et al., 2004; https://www.genevestigator.ethz.ch) and in the public repository Gene Expression Omnibus upon publication (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE3350. The accession numbers of the genes used in this study are At4g14550 (*SLR/IAA14*), At3g54180 (*CDKB1;1*), At3g48750 (*CDKA;1*), At4g37490 (*CYCB1;1*), At4g34160 (*CYCD3;1*), At2g36010 (*E2Fa*), At5g02470 (*DPa*), At5g20730 (*ARF7*), At5g37020 (*ARF8*), At5g1030 (*ALF4*), At3g20840 (*PLT1*) and At4g35550 (*WOX13*). Accession numbers of genes discussed in the text are listed in Table 1.

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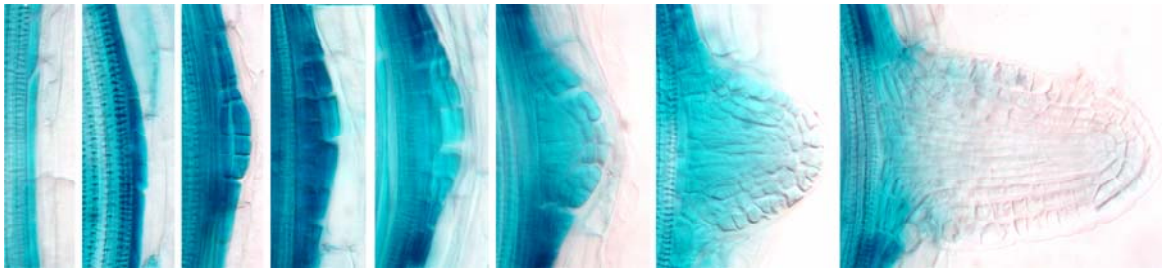
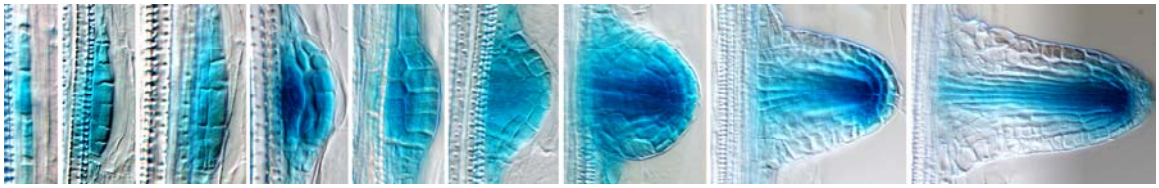
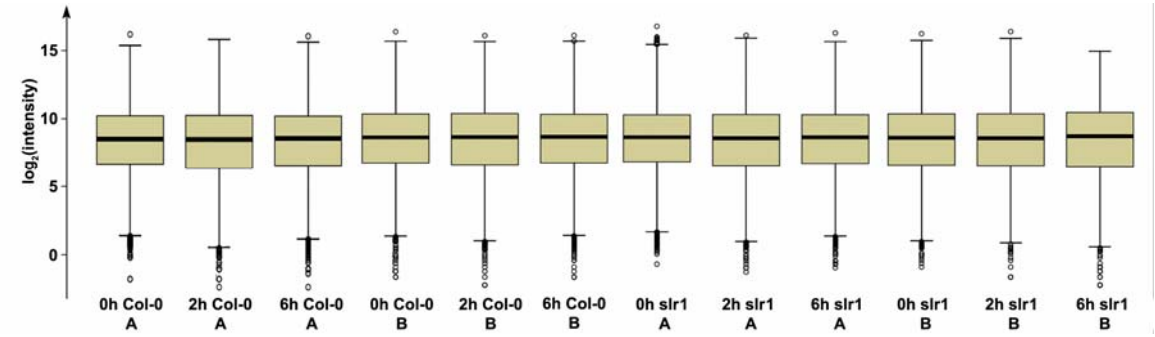


Supplemental Figure 3.1 ***P_{IAA14}::GUS*** **activity throughout different stages of lateral root development**



Supplemental Figure 3.2 Histochemical localisation of *plt1-1::GUS* activity using a promoter-trap line during different stages of lateral root formation



Supplemental Figure 3.3 Box plots of Log_2-transformed normalized value distributions of all arrays.

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CHAPTER 4 Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression

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Abstract

Plant development displays an exceptional plasticity and adaptability that involves the dynamic, asymmetric distribution of the phytohormone auxin. Polar auxin flow, which requires polarly localized transport facilitators of the PIN family, largely contributes to the establishment and maintenance of the auxin gradients. Functionally overlapping action of PIN proteins mediates multiple developmental processes, including embryo formation, organ development and tropisms. Here we show that PIN proteins exhibit synergistic interactions, which involve cross-regulation of PIN gene expression in *pin* mutants or plants with inhibited auxin transport. Auxin itself positively feeds back on PIN gene expression in a tissue-specific manner through an AUX/IAA-dependent signalling pathway. This regulatory switch is indicative of a mechanism by which the loss of a specific PIN protein is compensated for by auxin-dependent ectopic expression of its homologues. The compensatory properties of the PIN-dependent transport network might enable the stabilization of auxin gradients and potentially contribute to the robustness of plant adaptive development.

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INTRODUCTION

Plants cannot respond to the environment by a motile behavioural response but have evolved a highly flexible and adaptive developmental programme instead. In postembryonic development, meristems, which contain stem cell populations, provide new cells for growth at both ends of the main body axis. New structures, such as flowers, leaves and lateral shoot and root branches form *de novo* and connect to existing body structures by newly differentiated vascular strands. Both shoots and roots modify their growth direction in response to external signals such as light and gravity (Jürgens, 2003). Differential distribution of the plant hormone auxin within tissues and organs underlies adaptation processes, including the regulation of root meristem activity (Sabatini *et al.*, 1999), organogenesis (Benková *et al.*, 2003) and vascular tissue differentiation (Mattsson *et al.*, 2003), as well as tropic growth (Friml et al., 2002b). These dynamic auxin gradient result from an active , directional (polar) auxin transport between cells, which requires differentially expressed auxin transport facilitators of the PIN family.

The direction of the auxin flow is believed to be determined by the asymmetric cellular localization of PIN proteins as auxin transporters is still lacking, but numerous circumstantial evidences demonstrate that multiple PIN proteins play a central role in auxin transport (Friml and Palme, 2002). Despite the proposed uniform function of PIN proteins in auxin transport, genetic analysis implicates different PINs in various, seemingly unrelated developmental processes (Friml, 2003). In *Arabidopsis*, PIN1 mediates organogenesis and vascular tissue differentiation (Benková *et al.*, 2003; Galweiler *et al.*, 1998; Reinhardt *et al.*, 2003), PIN2 root gravitropic growth (Müller *et al.*, 1998), PIN3 shoot differential growth (Friml *et al.*, 2002b), PIN4 root meristem activity (Friml *et al.*, 2002a), and PIN7 early embryo development (Friml *et al.*, 2003b). Strong, embryo lethal phenotypes of *pin1,2,4,7* quadruple mutants, suggest a functional redundancy within the PIN gene family (Friml *et al.*, 2003b). Moreover, recent analysis of various combinations of *pin* mutants revealed ectopic expression of PIN proteins in some mutant combinations (Blilou *et al.*, 2005), but the underlying mechanism and biological importance of this effect is unclear. The PIN-dependent auxin distribution system displays an enormous plasticity at the subcellular level. It represents an entry point for both environmental (such as gravity) as well as developmental signals, which can modulate the polarity of PIN localization and hence the direction of auxin flow (Benková *et al.*, 2003; Friml, 2003; Friml *et al.*, 2002b). It remains unclear how the multiple

environmental and developmental signals are integrated and can result in a stabilized modulations of the PIN-dependent auxin distribution network, which are required to initiate and perpetuate a particular adaptation response.

Here we identify and describe synergistic interactions within the auxin transport network, which correlate with specific ectopic expression and proper polar targeting of PIN proteins in certain cells. This phenomenon involves feedback between auxin distribution and PIN gene expression as well as PIN stability. The identified complex regulations provide a mechanistic basis for compensatory properties of a functionally redundant auxin distribution network.

RESULTS

PIN functional redundancy in embryo development involved cross-regulation of PIN gene expression

Previous analysis of embryo (Friml *et al.*, 2003b), lateral root (Benková *et al.*, 2003) and primary root (Blilou *et al.*, 2005) development in multiple *pin* mutants demonstrated functionally redundant of PIN genes. To gain more insights into the mechanism of PIN functional redundancy, we examined root and embryo development in various multiple *pin* mutants in conjunction with PIN localization patterns. In the early embryo, following zygote division, only two PIN members have been detected. PIN1 resides in the apical side of suspensor cells. *PIN4* and *PIN3* expression is detected only at the globular and heart stage, respectively, in the root pole region (Friml *et al.*, 2003b) (Fig. 4.2 A). *pin7* mutants were shown to have defects at early embryo stages; however, they recover to a large extent by redundant activity of the remaining embryonically expressed PINs (Friml *et al.*, 2003b). *pin1,7* early embryo phenotypes did not differ visibly from *pin7* single mutants (Fig. 4.1 B). Remarkably, a more detailed analysis of defects in multiple *pin* embryos revealed that *pin1,3,4,7* embryos are more severely affected than *pin1,7* or *pin7* embryos also at early, preglobular stages. When compared with *pin1,7* embryos, *pin1,3,4,7* display novel phenotypes with compressed embryos consisting of densely packed, non-elongated cells (Fig. 4.1 A-C) and the frequency of early embryo defects increased (Col-0: 5.7%, 10/177; *pin1,7*: 16.1%, 35/218; *pin1,3,4,7*: 28.8% 61/212). These findings on a redundant role for PIN1,3,4,7 in early embryogenesis could not easily be reconciled with the lack of *PIN3* and *PIN4*

expression at this stage and suggested functional cross-regulation between distinct members of the PIN gene family. Indeed, when we examined expression and localization patterns of PIN proteins in *pin7* embryos, *PIN4* was found ectopically expressed in the *PIN7* expression domain as early as the preglobular stage, when *PIN4* is normally not expressed (Fig. 4.1 D-G). Remarkably, ectopically expressed PIN4 protein exhibited the same polar localization as the PIN7 protein that had been replaced. Such as cross-regulation of *PIN4* expression in the *pin7* mutant potentially explains the observed synergistic interactions in early embryo development.

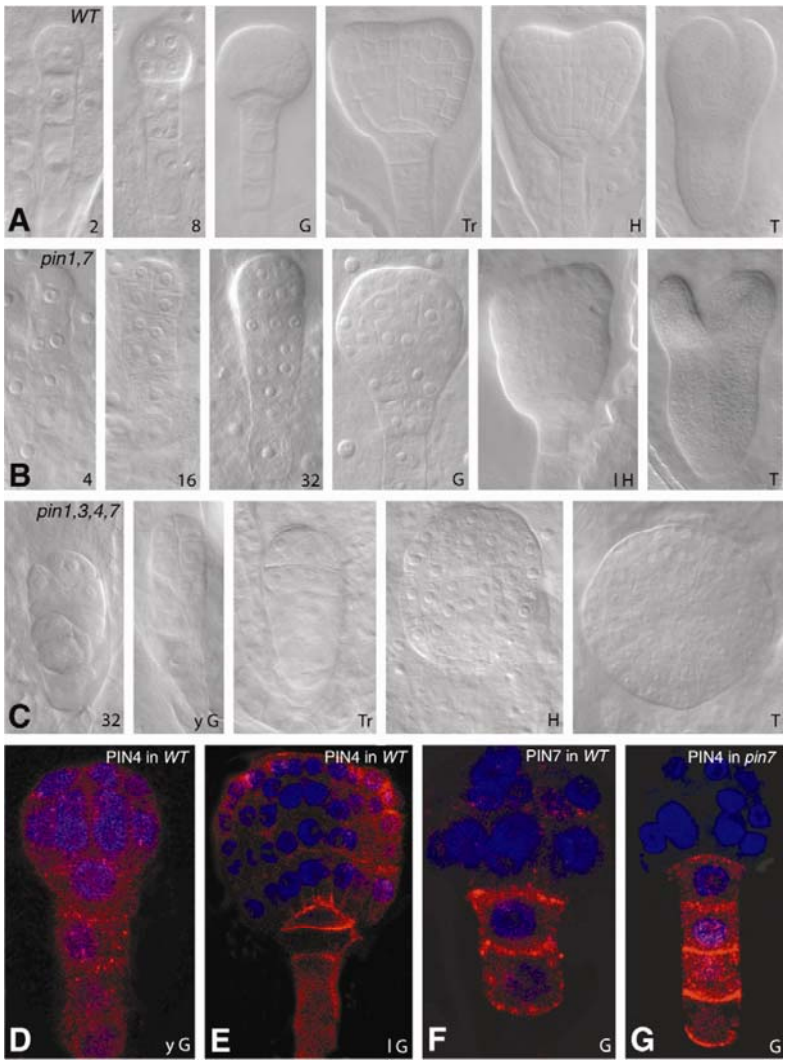

Figure 4.1 Cross-regulation of Pin expression and function in embryo development. (A-C) Novel embryo phenotypes in *pin1,3,4,7* multiple mutants (C) compared with wild-type (A) and *pin1,7* mutant (B) embryos. (D-G) Immunostaining showing ectopic *PIN4* expression in the suspensor of the *pin7* preglobular embryo (G) in a pattern similar to that of *PIN7* expression in wild type (F). No *PIN4* expression in wild type at this stage (D) and expression restricted to root meristem precursors at later stages (E). For the embryo stages the numbers indicate the developmental stage according to the actual number of pro-embryo cells of the corresponding wild-type stage. G, globular; H, heart; l, late; T, Torpeda; Tr, triangular; y, young.

PIN functional redundancy in root development involves cross-regulation of *PIN* gene expression

In the root meristem, five PIN genes are known to be expressed (Fig. 4.2 A). The *PIN1* expression pattern is somewhat variable, but under our experimental conditions PIN1 could be found predominantly at the basal (lower) side of stele and endodermis cells with occasional weak expression in the quiescent centre and up tot the four youngest epidermis and cortex daughter cells (Friml *et al.*, 2002a) (Fig. 4.2 D). *PIN2* is expressed in a non-overlapping pattern in the lateral root cap and older epidermis and cortex cells with apical (upper) polarity in the epidermis and predominantly basal polarity in the cortex (Friml *et al.*, 2003a; Müller *et al.*, 1998) (Fig. 4.2 G). *PIN4* is expressed in the central root meristem with a polar subcellular localization pointing predominantly towards the columella initials (Friml *et al.*, 2002a) (Fig. 4.2 J). By contrast, PIN3 (Friml *et al.*, 2002b) and PIN7 (Blilou *et al.*, 2005) are localized in largely overlapping patterns in columella and stele of the elongation zone. However, with the exception of PIN2, which when mutated causes agravitropic root growth, removal of any of the other PINs causes no, or rather subtle, root phenotypes (Friml *et al.*, 2002a; Friml *et al.*, 2003b; Sabatini *et al.*, 1999). By contrast, *pin1,2* double mutants displayed strong root growth defects reflected in significantly shorter roots and the formation of a smaller root meristem, when compared with either single mutant (Fig. 4.2 B,C) or any other double mutant combination (Blilou *et al.*, 2005). These strong synergistic interactions between PIN1 and PIN2 may indicate a functional cross-regulation similar to that observed with PIN4 and PIN7 in the embryos. Indeed, the analysis of expression and abundance of PIN1 and PIN2 in the respective mutants reveals that *PIN* became ectopically induced in the *PIN2* expression domain in cortex and epidermis cells of *pin2* (Fig. 4.2 E). Reciprocally, in *pin1* mutants, *PIN2* was ectopically expressed in the endodermis and weakly in the stele (Fig. 4.2 H) along with ectopic upregulation of the *PIN4* expression in the stele (Fig. 4.2 K). Remarkably, ectopically expressed PIN proteins exhibited the polar localization of the PIN protein that had been replaced. PIN2 and PIN4 were basally localized, when upregulated in endodermis or stele of *pin1* (Fig. 4.2 I,L), whereas PIN1 showed apical localization in epidermis and basal localization in cortex cells when upregulated in roots of the *pin2* mutant (Fig. 4.2 F). These findings demonstrate that the functional redundancy of PIN proteins involves cross-regulation of PIN gene expression and polar targeting in a cell-specific manner, which potentially explains the observed synergistic interactions. Accordingly, ectopic

upregulation of PINs, as observed in *pin* mutants, could be sufficient to – at least partially – compensate for the function of missing PIN genes.

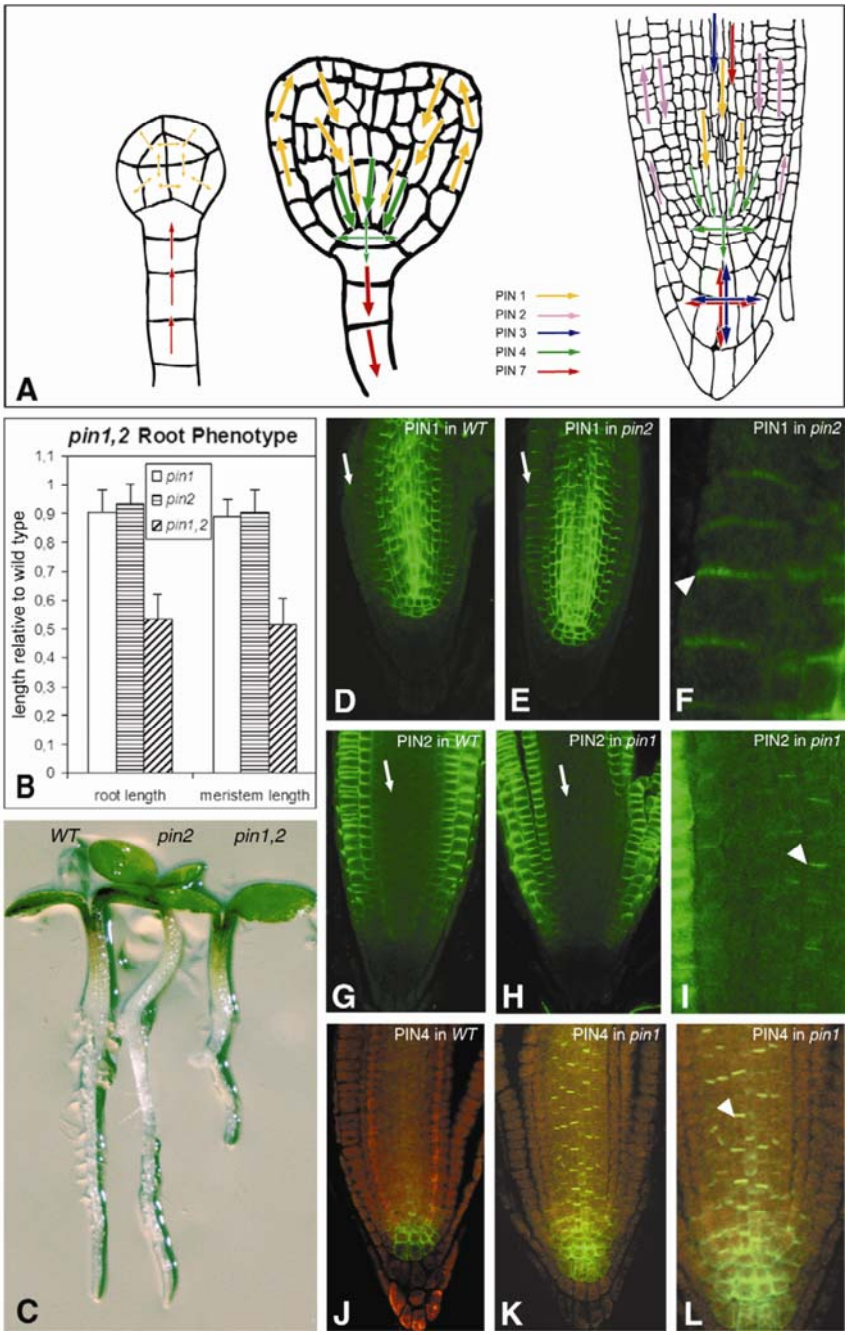

Figure 4.2 Cross-regulation of PIN gene expression in root. (A) Symbolic depiction of PIN gene expression and polar localization in preglobular, early heart embryos and in seedling root. Arrows indicate presumed directions of auxin flow based on subcellular PIN polarity. (B) Comparison of root and meristem length between *pin1* and *pin2* single mutants and *pin1,2* double mutants. (D-L) Immunostaining showing cross-regulation of PIN expression. PIN1 is upregulated in the epidermis of *pin2* root (E) compared with wild type (D). Detail showing polar PIN1 localization in epidermis of *pin2* root (F). PIN2 is ectopically expressed in the stele of *pin1* root (H) compared with wild type (G). Detail showing polar PIN2 localization in *pin1* stele (I). PIN4 is upregulated in the stele of *pin1* root (K,L) compared with wild type (J). Arrows indicate corresponding expression domains; arrowheads polarity of PIN localization.

Cross-regulation of *PIN* gene expression is related to changes in polar auxin transport

In order to investigate how the observed cross-regulation of PIN gene expression might be regulated, we modified auxin distribution by blocking auxin transport with the auxin transport inhibitor NPA. To exclude possible cross-hybridization with related PIN proteins, we performed the experiments on the *PIN1::PIN1:GFP*, *PIN4::PIN4:GFP* and *PIN2::PIN2:HA* transgenic lines and analysed either GFP or HA-tag expression. In *PIN1::PIN1:GFP* (Benková *et al.*, 2003), the GFP fluorescence was detected in the stele and endodermis cells of primary roots with occasional weak expression in the quiescent centre and a few youngest cortex and epidermis cells (Fig. 4.3A), as is normal for anti-PIN1 immunolocalization results. Following NPA treatment, ectopic upregulation of PIN1:GFP fluorescence was observed in the epidermis (Fig. 4.3B). In untreated *PIN4::PIN4-GFP* roots, the expression of PIN4:GFP was restricted to the central root meristem and columella with only a faint signal or no signal in the stele (Fig. 4.3E), whereas PIN2:HA expression in *PIN2::PIN2:HA* plants was detected only in cortex, epidermis and lateral root cap cells (Fig. 4.3 C). Following NPA treatment, PIN4:GFP as well as PIN2:HA expression became strongly upregulated in the stele (Fig. 4.3 F,D). In *PIN1::GUS*, *PIN2::GUS* and *PIN4::GUS* transgenic plants, a similar ectopic upregulation of GUS activity following NPA treatment could be observed (Fig. 4.3 G,H and not shown), demonstrating an effect on the PIN promoter activity rather than post-transcriptional regulations. These data demonstrate that the chemical inhibition of auxin transport can modulated PIN gene expression in a way similar to that observed in *pin* mutants, suggesting a link between NPA-sensitive auxin transport and the regulation of PIN gene expression.

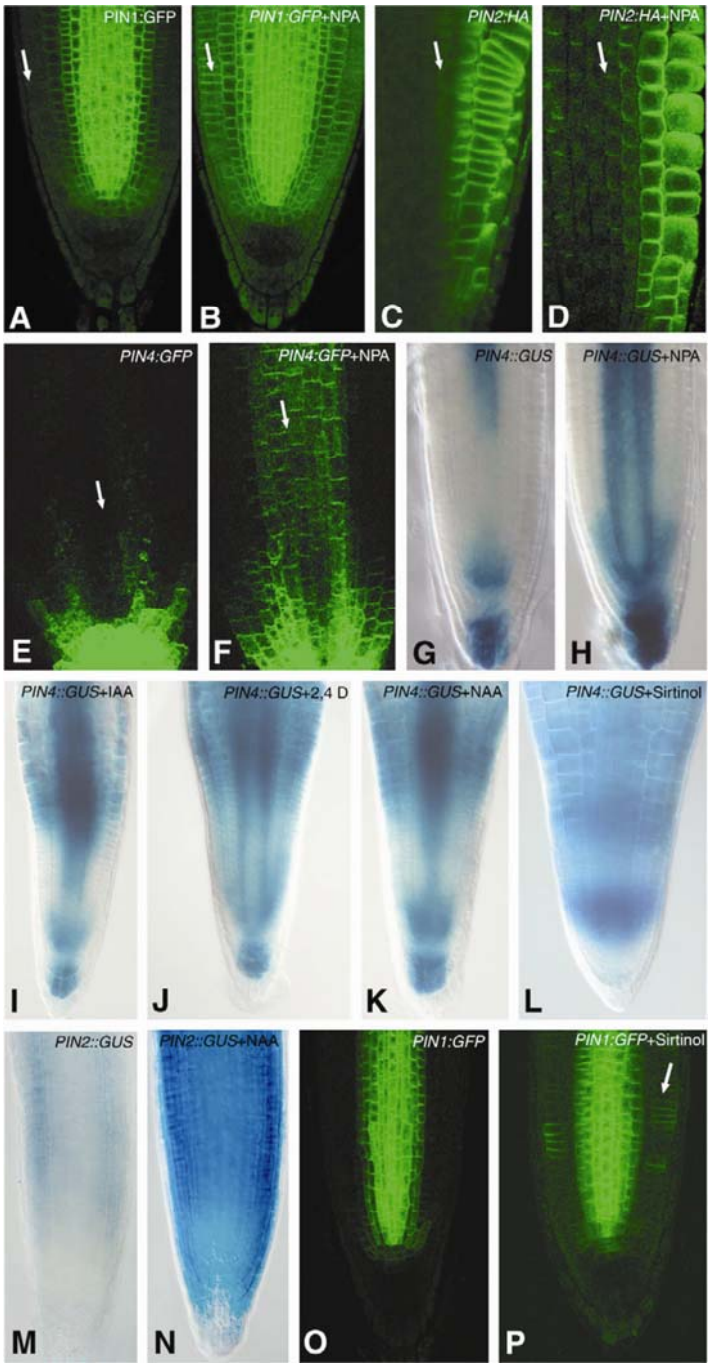

Figure 4.3 Manipulation of auxin homeostasis leads to ectopic PIN gene expression. (A-H) Inhibition of auxin transport by NPA (50 µM for 24 hours) leads to upregulation of *PIN1::PIN1:GFP* in epidermis and cortex (B); *PIN2::PIN2:HA* (D), *PIN4::PIN4-GFP* (F) and *PIN4::GUS* (H) in the stele compared with untreated controls (A,C,E,G). (I-L) Treatment for 24 hours with biologically active auxins such as IAA (50 µM, I), 2,4-D (0.1 µM, J) and NAA 10µM, K) or for 5 days with auxin precursor sirtinol (20µM, L) leads to an upregulation of *PIN4::GUS* expression compared with control (G). (M,N) Upregulation of *PIN2::GUS* expression in root following treatment with 50µM NAA for 24 hours (N) compared with untreated control (M). (O,P) Treatment for 5 days with auxin precursor sirtinol (20µM) leads to upregulation of *PIN1::PIN1:GFP* in epidermis and cortex cells (P) compared with control (O).

Auxin-dependent signalling controls PIN gene expression in a situ-specific manner

Next we addressed potential mechanisms underlying the observed cross-regulation of PIN gene expression. As NPA treatment and various *pin* mutants change the pattern of auxin distribution in roots and embryos (Friml *et al.*, 2002a; Friml *et al.*, 2003b; Luschnig *et al.*, 1998; Sabatini *et al.*, 1999), we tested whether auxin itself can directly influence PIN gene expression. Treatment with different biologically active auxins such as IAA, NAA and 2,4-D led to an increase in GUS activity in *PIN4::GUS* (Fig. 4.3 I-K) and PIN2::GUS (Fig. 4.3 M,N and not shown) roots. Importantly, both NAA and 2,4-D, which differ in their transport properties (Delbarre *et al.*, 1998), induced PIN gene expression in a similar way, indicating that auxin influences PIN gene expression without the need of the active auxin transport. This was further confirmed by analysis of the effects of sirtinol –a compound that is not a substrate of the auxin transport but is converted to a substance with auxin effects (Dai *et al.*, 2005; Zhao *et al.*, 2003). The effect of sirtinol seemed to be somewhat delayed when compared to auxin effects, but prolonged treatments had the same impact on the induction of PIN gene expression as auxins, as shown, for example, by the upregulation of *PIN4::GUS* and PIN1:GFP (Fig. 4.3 L,O,P).

To quantitatively asses the effect of auxin on PIN gene expression, we performed a quantitative real-time RT-PCR (Q-RT-PCR) following a treatment with NQQ. To address possible differences in the effect of auxin on PIN gene expression in different parts of the seedling, we examined PIN gene expression in cotyledons, hypocotyls and roots separately. The expression of all tested PIN genes (*PIN1,2,3,4,6,7*) clearly responded to auxin treatments but showed prominent differences in different parts of seedlings (Fig. 4.4 A). In cotyledons, the response to auxin was more divergent and varied from strong upregulation of *PIN1*, *PIN7*; to a somewhat weaker response of *PIN6* and to no upregulation of *PIN2*, *PIN3* and *PIN4* expression. In hypocotyls, hardly any effect of auxin on PIN gene expression was detected, whereas in roots, all PIN genes showed a clear increase in expression (up to 22-fold in the case of *PIN7*) following auxin treatment. The apparent downregulation of PIN2 and PIN6 is not significant due to the very low expression levels of these genes in this tissue. The auxin effect on PIN gene promoter activity was further confirmed by testing PIN gene promoter activity in GUS transcriptional fusions. Indeed, *PIN1,2,3,4,6,7::GUS* transgenic plants responded to auxin treatment by upregulation of GUS expression in a tissue-specific manner (Fig. 4.4 B-D and data not shown). For example, in cotyledons, the *PIN1::GUS*, but not *PIN3::GUS* or *PIN4::GUS* seedlings showed increased GUS activity following auxin treatment, but neither of the transgenic lines showed any upregulation in hypocotyls (Fig. 4.4

B-D) under the same conditions. However, the GUS activity in *PIN1::GUS*, *PIN3::GUS* and *PIN4::GUS* was clearly increased in roots. Closer examination of the induction pattern (e.g. in *PIN3::GUS* and *PIN4::GUS* roots) confirmed tissue and cell-specific response to auxin treatment, as in some cells more upregulation occurred than in others (Fig. 4.4C-D).

In summary, these results show that the expression of *PIN*s is directly or indirectly controlled by auxin in a tissue-specific manner, which provides a plausible mechanism for the observed cross-regulations in PIN functional redundancy.

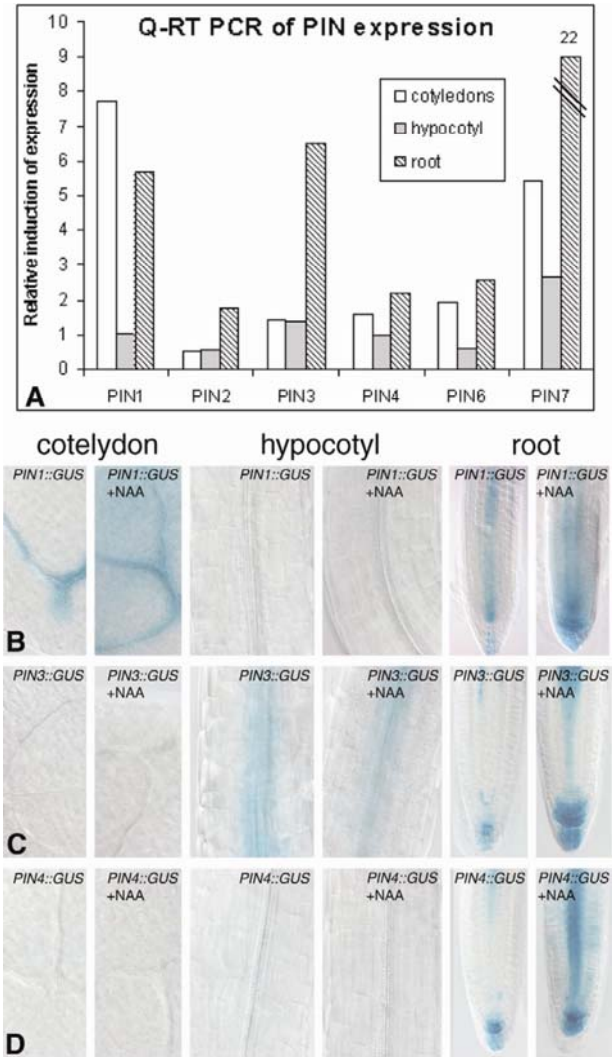

Figure 4.4 Tissue-specific regulation of PIN gene expression by auxin (A) Quantitative RT-PCR showing upregulation of PIN gene expression in cotyledons, hypocotyls and roots following auxin treatment (10µM NAA for 3 hours). Induction of PIN gene expression is depicted relative to the non-induced controls. (B-D) Induction of *PIN1::GUS* (1 µM, B), *PIN3::GUS* (0.5 µM, C) and *PIN4::GUS* (0.5 µM, D) expression in cotyledons, hypocotyls and roots after growing the plants for 4 days on medium containing NAA.

The auxin effect on PIN gene expression is time- and concentration-dependent

Analyses of GUS activity in *PIN1::GUS*, *PIN3::GUS*, *PIN4::GUS* and *PIN7::GUS* rots revealed that the auxin effect on the activity of PIN promoters is time- and concentration-dependent (Fig. 4.5 A-D). Staining conditions here were chosen to maximize the dynamic range of staining intensities in order to better resolve the differences in GUS expression levels after auxin treatment rather than to obtain optimal overall staining patterns. Thus, for example, untreated *PIN7::GUS* seedlings, when optimally stained, also showed GUS signal in the stele (Fig. 4.5 D inset), which is in accordance with earlier observations of PIN7 expression (Blilou *et al.*, 2005). Interestingly, independently of the time and concentration of the auxin treatment, the upregulation remained largely confined to the same tissues, further confirming the cell-type-specific effect on PIN gene expression. The kinetics and concentration-dependence of the auxin effect on PIN gene expression were evaluated by Q-RT-PCR for *PIN1*, *PIN2*, *PIN3*, *PIN4* and *PIN7* in roots. The experiment revealed that expression of different *PIN*s displays different kinetics in their auxin-dependent responses. Following auxin (2,4-D) application, *PIN1* (t-test: $P<0.01$), *PIN3* ($P<0.01$) and *PIN7* ($P<0.005$) showed a significant upregulation after 15 minutes and a steady increase in RNA levels up to 6 hours after induction. By contrast, *PIN2* ($P<0.005$) and *PIN4* ($P<0.005$) showed a delayed reaction with significant upregulation only after 1 hour of 2,4-D incubation showed a maximal response after 2 hours (Fig. 4.6 A). Also, concerning the maximal effective auxin concentrations that lead to the upregulation of expression, differences between PINs were found. *PIN2* ($P<0.01$) expression was induced by hormone concentrations as low as 10nM 2,4-D, whereas for the induction of *PIN1* ($P<0.01$), *PIN3* ($P<0.005$), *PIN4* ($P<0.01$) and *PIN7* ($P<0.005$) expression, concentrations as high as 100nM 2,4-D were needed to obtain significant changes in expression (Fig. 4.6 B). For the induction of PIN1 and PIN7 expression, the optimal concentration was around 1μM 2,4-D, whereas for PIN2, PIN3 and PIN4 it was higher than 10μM 2,4-D. Northern blot analysis and quantitative analysis of GUS activity confirmed the Q-RT-PCR results, as shown, for example, by analysis of *PIN2* expression in response to NAA (Fig. 4.6 E,F). These results show that while expression of PIN proteins is induced by auxin, induction kinetics and effective concentrations exhibit variability within the PIN gene family.

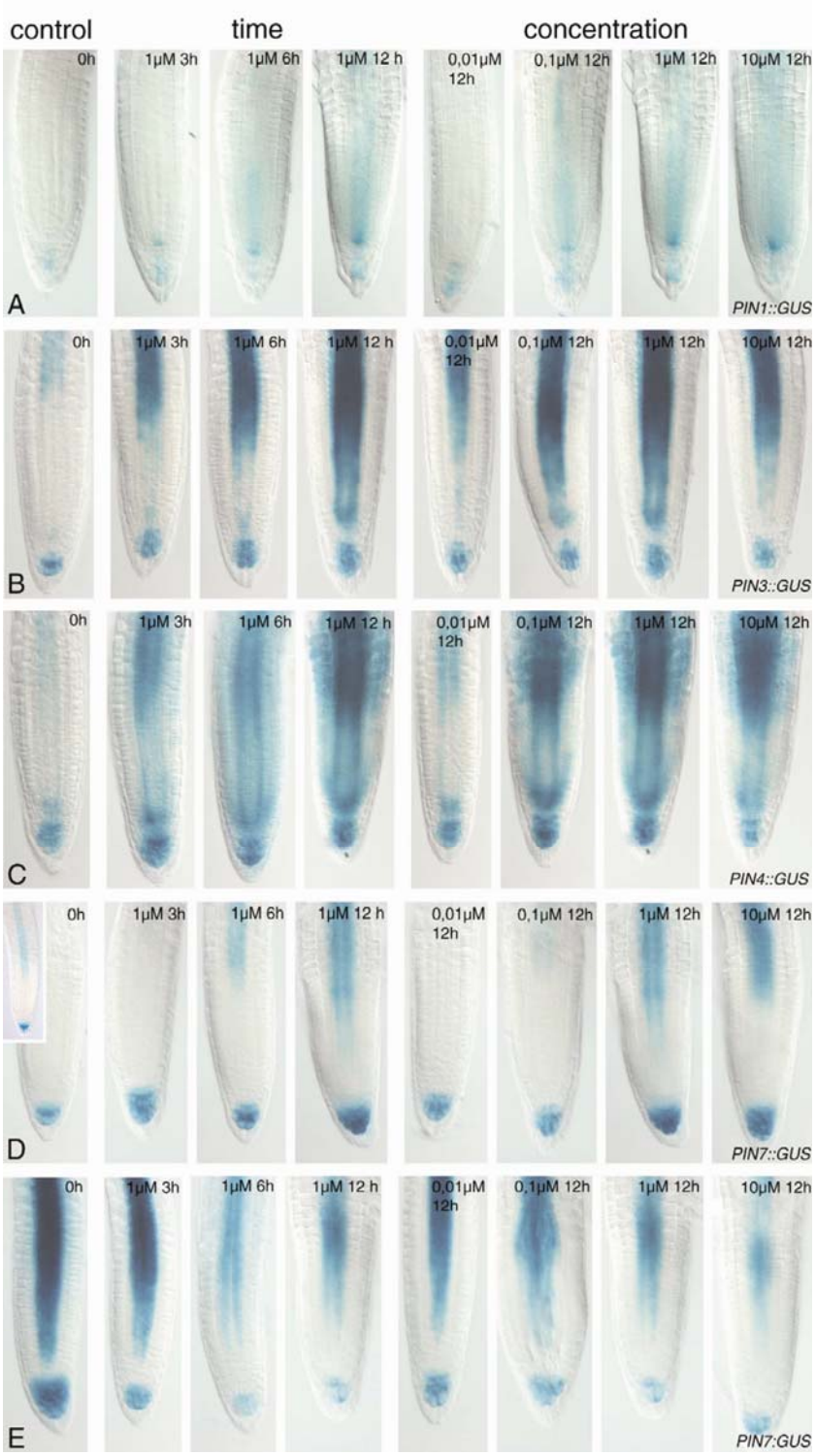

Figure 4.5 Time- and concentration-dependence of auxin-regulated PIN gene expression (A-D) Upregulation of *PIN1::GUS* (A), *PIN3::GUS* (B), *PIN4::GUS* (C) and *PIN7::GUS* (D) expression after different times and different concentrations of 2,4-D incubation. Inset (D) shows untreated *PIN7::GUS* roots after prolonged GUS staining. (E) Downregulation of PIN7:GUS fusion protein abundance in seedlings under the same conditions.

Auxin regulates PIN gene expression through an Aux/IAA-dependent pathway

We then addressed the molecular mechanism by which auxin regulates PIN gene expression. Even when the protein synthesis was inhibited by cycloheximide, auxin induced the expression of PIN proteins (not shown), demonstrating that the auxin-dependent PIN upregulation does not require *de novo* synthesis of any factors and thus *PIN*s are primary response genes. Significantly, a treatment with cycloheximide alone was sufficient to induce expression of *PIN1*, *PIN2*, *PIN3* and *PIN4* to roughly maximum levels (Fig. 4.6 C), implying that PIN gene expression is controlled by an unstable transcriptional repressor. The auxin effect on gene expression is known to involve a rapid, auxin-dependent degradation of the Aux/IAA transcriptional repressors (Gray *et al.*, 2001). Indeed, in the *solitary root1* (*slr1*) mutant, which carries the stabilized version of the AA14 repressor (Fukaki *et al.*, 2002), auxin-dependent upregulation PIN gene expression is severely compromised (Fig. 4.6 C), suggesting that auxin utilizes Aux/IAA-dependent signalling to regulate PIN gene expression. In addition, we used transgenic plants harbouring a stabilized version of IAA17 under the control of a heat-shock promoter (*HS::axr3-1*) (Knox *et al.*, 2003). The induction of axr3 expression by 2 hours of 37°C treatment concomitantly abolished the auxin-dependent upregulation of *PIN1*, *PIN2*, *PIN3*, *PIN4* and *PIN7* expression (Fig. 4.6 D), directly linking the regulation of PIN gene expression to the Aux/IAA signal transduction pathway. These conclusions gained additional support from global expression analysis following auxin-dependent induction of lateral root formation (Vanneste *et al.*, 2005). Microarray-based analysis was performed at different time points after auxin application to seedlings that were grown under inhibited auxin transport (by NPA). Only the differentiated part of the primary root was analysed to minimize the influence of different tissue- and developmental-stage-specific factors (Vanneste *et al.*, 2005). Under these conditions, the expression of *PIN1*, *PIN3* and *PIN7* was rapidly and strongly induced by auxin, along with a number of well-known primary auxin response genes as well as *PINOID* and related genes (Fig. 4.6 G). Expression of other PIN genes was also analyses (e.g. PIN genes were also on the ATH1 Affymetrix chip) but were not induced in this experiment (data not shown). Importantly, the observed auxin-dependent induction of PIN gene expression was completely abolished when the expression profiling experiment was performed in the *slr1* mutant (Fig. 4.6 G). In summary, these experiments demonstrate that tissue-specific PIN gene expression is regulated by auxin through Aux/IAA dependent signalling.

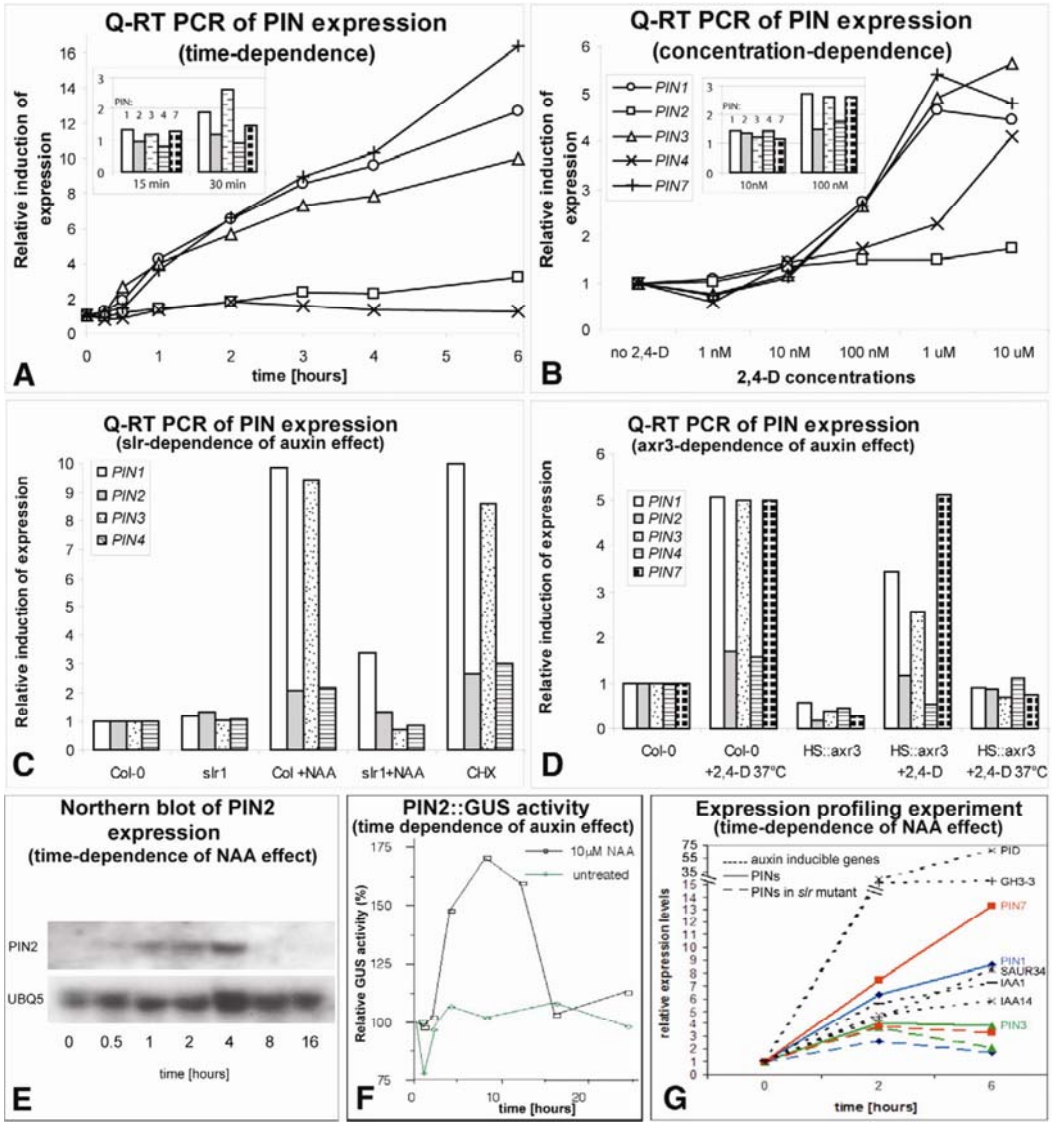

Figure 4.6 Quantitative evaluation of auxin-regulated PIN gene expression. (A,B) Quantitative RT-PCRs showing time-dependence (A) and concentration-dependence (B) of the effect of auxin (1µM 2,4-D in A) on PIN expression. Insets show higher magnifications of early time points (A) and low concentrations (B). The legend in B also applies to A. (C,D) Auxin (1µM 2,4-D) does not induce PIN expression in *slr-1* mutants (C) or after induction of axr3 expression in in *HS::axr3-1* lines (D), as shown by quantitative RT-PCR. Cycloheximide alone induces PIN expression (C). (E,F) Northern blot (E) and quantitative GUS assays (F) show the time-dependence of auxin (10µM NAA) effect on *PIN2* expression. (G) An expression profiling experiment shows auxin-dependent upregulation of PIN genes, *PID* and selected primary auxin response genes in differentiated parts of the root grown on NPA. The auxin-dependent upregulation of PIN gene expression is abolished in the *slr-1* mutant. Induction of PIN gene expression is depicted relative to the non-induced controls.

Auxin-dependent post-transcriptional downregulation of PIN proteins

Our results suggest that auxin is able to modulate PIN levels by regulating PIN gene expression in a highly specific way. Additional levels of regulations might occur due to effects on PIN protein stability, as at least PIN2 degradation was shown to be regulated by auxin levels (Sieberer *et al.*, 2000). To address the post-transcriptional effects of auxin on the

abundance of PIN proteins, we utilized GUS and GFP translational fusions with PIN1, PIN2, PIN4 and PIN7. Comparisons of the auxin effects on *PIN7::GUS* and *PIN7::PIN7:GUS* clearly showed a time- and concentration-dependent transcriptional upregulation of *PIN7* promoter activity (Fig. 4.5 D), but a downregulation of PIN7:GUS levels (Fig. 4.5 E). Similarly, PIN7:GFP (Fig. 4.7 A) and PIN2:GFP (Fig. 4.7 B) abundance decreased at higher auxin concentrations (higher than 100nM 2,4-D). However, at lower concentrations, the PIN2 and PIN7 protein amount increased (best at 10nM 2,4-D), suggesting that both the transcriptional and the post-transcriptional auxin effects on PIN expression overlap. In support of this notion, the transcriptional upregulation of *PIN2* expression in the stele, which occurs in *PIN2::GUS* seedlings following auxin treatment (Fig. 4.3 N), cannot be observed in *PIN2::PIN2:GFP* seedlings (Fig. 4.7 B). In *PIN1::PIN1:GFP* roots, the optimal 2,4-D concentration for the PIN1:GFP upregulation in epidermal cells was 100nM. At higher concentrations, the PIN1:GFP level decreased also in its stele expression domain (Fig. 4.7 C), albeit to a lesser extent than in the case of PIN2 and PIN7 reporter proteins. However, there was no visible decrease in the PIN4:GFP amount following auxin treatment (not shown). These results show that auxin, besides modulating PIN gene expression, reduces the abundance of specific PIN proteins post-transcriptionally at higher concentrations. This provides an additional level of regulation for modulating of different PIN protein amounts in different cells.

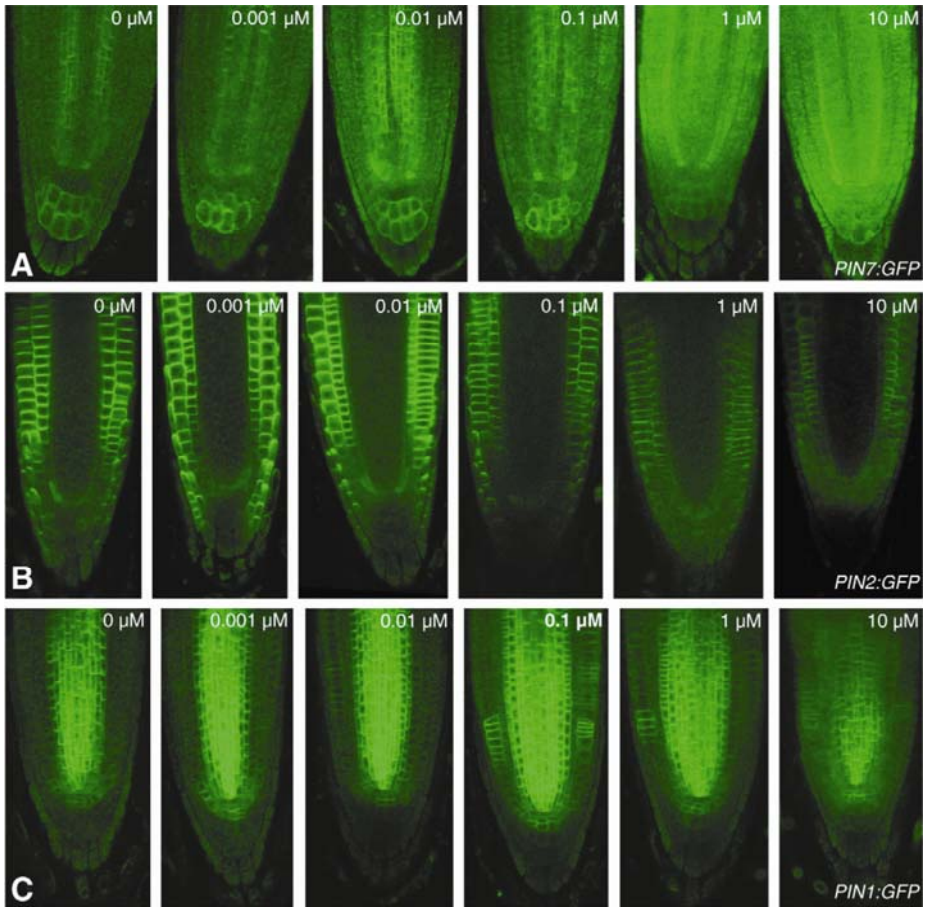

Figure 4.7 Increased auxin levels lead to a decrease in PIN levels in *PIN1::PIN1:GFP* roots. (A-C) The PIN7:GFP (A), PIN2:GFP (B) and PIN1:GFP (C) protein levels decrease at higher auxin concentrations. Four-day-old roots were treated with different concentrations of 2,4-D for 24 hours.

DISCUSSION

Functionally redundant PIN-dependent auxin distribution as a common mechanism in plant development

The local accumulation of the plant signalling molecule auxin in certain cells (auxin gradients) underlies an unexpected variety of developmental processes. Embryo development, postembryonic formation of various organs, such as lateral roots, leaves, flowers, floral organs, and ovules, vascular tissue differentiation, the regulation of root meristem activity and directional growth responses – all these processes are accompanied by, and require, locally elevated auxin activities (Benková *et al.*, 2003; Friml *et al.*, 2003b; Mattsson *et al.*, 2003; Reinhardt *et al.*, 2003; Sabatini *et al.*, 1999). The current model proposes that increased auxin levels in different cells activate a signalling cascade, which via a network of AUX/IAA transcriptional repressors and ARF transcription factors leads to the expression of a specific set of genes and to the activation of specific developmental programmes (Weijers and Jürgens, 2004). The importance of contributions of tissue-specific auxin synthesis and degradation are not entirely clarified yet (Ljung *et al.*, 2002), but it seems that the major mechanism by which auxin accumulates in given cells is an intercellular, directional flow of auxin. The auxin distribution network is molecularly characterized by polarly localized PIN auxin efflux facilitators (Friml and Palme, 2002). Whether or not different PIN proteins have the same molecular function is still unclear, as they mediate different developmental processes. However, the synergistic effects of multiple *pin* mutant combinations on embryo and root development demonstrate functional redundancy (Blilou *et al.*, 2005; Friml *et al.*, 2003b). Also the findings that different *PIN*s get ectopically expressed in *pin* mutants and thus can at least partially compensate for the function of the missing PIN protein suggest that different PIN proteins are to some extent functionally interchangeable (Blilou *et al.*, 2005). Thus it seems that the molecular function of different PIN proteins in auxin transport is similar, although they obviously differ in the regulation of their expression, as shown by the differential expression pattern as well as by their different responsiveness to increased auxin levels. It is likely that PIN proteins will differ also in the regulation of their proteasome-dependent turnover (Sieberer *et al.*, 2000) and regulation of their subcellular polarity in different cell types. Such properties of a functionally redundant PIN-dependent auxin

distribution network would allow for the integration of various signals at different levels, thereby determining local auxin distribution in different parts of plants.

Model for feedback regulations and compensatory properties in auxin distribution

Plant development is characterized by its flexibility and adaptability, which allow the optimal adjustment of plant shape according to the environment. The auxin distribution network is supposed to enable the integration of multiple environmental and developmental signals to allow the flexible changes in auxin accumulation patterns that underlie the adaptive nature of plant development. The regulation of PIN polar targeting, degradation and differential regulation of expression are potential upstream control points for mediating the dynamic auxin gradients. For example, the PIN3 polarity can be rapidly modulated by environmental signals such as gravity, which through asymmetric auxin distribution ultimately leads to gravitropic bending (Friml *et al.*, 2002a). Also developmental signals can mediate dynamic changes in PIN polarity and thus mediate apical-basal axis specification in embryos (Friml *et al.*, 2003b), trigger specific patterns of organ positioning (Reinhardt *et al.*, 2003) or perpetuate organ primordium development (Benková *et al.*, 2003).

Under conditions of an ever-changing environment and constant stimulation, a dynamic system such as the PIN-dependent auxin transport network requires a mechanism(s), which would at some point stabilize and perpetuate its readjustments. For this purpose, biological systems typically accommodate feedback regulatory loops. Long-standing physiological models, such as the canalization hypothesis, proposed that auxin itself can modulate its own transport and its polarity (Sachs, 1988) and thus mediate regenerative properties of plant development, especially the *de novo* formation of vascular strands (Berleth and Sachs, 2001; Sachs, 2000). The canalization hypothesis assumes, besides positive feedback on transport activity, a directional polarization of auxin flow relative to the position of the auxin source. Our data show that auxin itself, together with cell-type-specific factors, can positively control PIN transcription, which involves the activity of Aux/IAA transcriptional repressors. The complementary evidence for the influence of auxin distribution on PIN gene expression came from the expression profiling experiments in poplar (Schrader *et al.*, 2003) and from the analysis of flavonoid mutants, where both the auxin transport and the distribution of PIN proteins are affected (Peer *et al.*, 2004). However, the effect of auxin

on PIN polarity or on polarity of auxin flow has not been demonstrated so far. Our data show that ectopically expressed PIN proteins in various *pin* mutants always adopt the correct polar localization, suggesting a tight cell-type-based control, apparently requiring direct or indirect regulation by auxin. Such a functional link is also provided by the recent analysis of regulators of PIN polarity, such as the Ser/Thr protein kinases of the PINOID type (Friml *et al.*, 2004). It has been reported previously that *PINOID* is a primary auxin response gene. Also our expression profiling data show that *PINOID* and homologous genes are upregulated along with the PIN genes in the same tissues. It is thus conceivable that auxin mediates changes of cellular PIN polarity via control of *PINOID* expression. In such a scenario, both cellular PIN levels and PIN localization can be influenced by auxin itself. Such feedback regulations may contribute to the compensatory properties of the auxin distribution network. In the simplest model, the defect in auxin flow caused, for example by mutation in a specific PIN protein, would lead to auxin accumulation within affected cells. This in turn would lead to the upregulation of expression a polar retargeting of other PIN family member(s), which, in this manner, could functionally compensate. This unique, so far undescribed, type of regulatory redundancy explains observed genetic redundancy and provides a possible mechanism for the stabilization of changes in auxin distribution. The fine interplay between the modulating external signals and the stabilizing internal feedback in the PIN-based auxin transport network might thus contribute to both the flexible and robust nature of plant development.

MATERIALS AND METHODS

Used materials

The PIN1,2,3,4,7::GUS, *pin4-3*, *pin3-2*, *pin7-1*, *pin7-2*, *pin1,7*, *pin1,3,4,7*, PIN1::PIN1-GFP (Benková *et al.*, 2003), *pin1,2*, *pin1,7* (Blilou *et al.*, 2005), PIN7:GUS (Friml *et al.*, 2003b), PIN2::PIN2-GFP (Xu *et al.*, 2006), HS::axr3-1 (Knox *et al.*, 2003), pin1 (Okada *et al.*, 1991), *slr-1* (Fukaki *et al.*, 2002), *agr1* (Chen *et al.*, 1998) and *eir1-1* (Luschnig *et al.*, 1998) have been described previously. PIN4::PIN4-GFP was generated by insertion of mGFP into the PIN4 coding sequence (nucleotides 1032 to 1035 from ATG). The PIN2::PIN2:HA construct was generated by fusion of the PIN2 promoter (1302 bp) and the

PIN2 (AF086906 cDNA with the nine-amino acid HA epitope tag at the C-terminus in the kanamycin version of the pS001 plasmid (Reiss *et al.*, 1996).

Growth conditions and drug treatments

Arabidopsis seedlings were grown in a 16 hours light/8 hours dark cycle at 18-25 °C on 0.5xMS with sucrose. Short-time exogenous drug application was performed by incubation of 4-5 day old seedlings in liquid 0.5xMS with or without 1% sucrose supplemented with indole-3-acid (IAA); 2,4-Dichlorophenoxyacetic acid (2,4-D); N-1-naphthylphthalamic acid (NPA) or 1-naphthalene acetic acid (NAA). The sirtinol treatment was done by growing the seedlings for 5 days on 0.5xMS with 1% sucrose plus 10µM sirtinol

Quantitative RT-PCR and Northern Blot analyses

RNA was extracted using the RNeasy kit (QIAGEN) from root samples. Poly(dT) cDNA was prepared out of 1 µg total RNA using Superscript III Reverse Transcriptase (Invitrogen, Belgium) as recommended by Invitrogen. Quantifications were performed on a Bio-Rad Icycler apparatus with the qPCR Core Kit for SYBR green I (Eurogentec) upon recommendations of the manufacturer. PCR was carried out in 96-well optical reaction plates heated for 10 minutes to 95 °C to activate hot start Taq DNA polymerase, followed by 40 cycles of denaturation for 60 seconds at 95 °C and annealing-extension for 60 seconds at 58 °C. Target quantifications were performed with specific primer pairs designed using Beacon Designer 4.0 (Premier Biosoft International, Palo Alto, CA). Expression levels were normalized to *ACTIN2* expression levels. All RT-PCR experiments were at least performed in triplicates and the presented values represent means. The statistical significance was evaluated by t-test. Northern analysis of *PIN2* expression was performed with Col-0 seedlings (6 DAG) grown in liquid 0.5xMS under continuous illumination. Prior to the experiment, seedlings were transferred into the dark for 16 hours. NAA (10 µM) was added and samples were harvested at indicated time points. Total RNA (10 µg) was loaded per lane. As a loading control, *UBQ2* was used. For the quantification of PIN2::GUS activity, the GUS activity was determined as described (Sieberer *et al.*, 2000). *PIN2::GUS* seedlings (6 DAG) were pre-adapted in the dark for 16 hours, treated with 10 µM NAA and subsequently processed at indicated time points. Protein concentrations were normalized with Bradford reagent (Biorad).

Expression profiling experiments

Growth conditions were as described (Himanen *et al.*, 2004). For the time-course experiments, plants were grown on 10μM NPA for 72 hours before they were transferred to 10μM NAA containing medium. For the RNA preparation only the differentiated segments were used. The root apical meristems were off and the shoots were removed below the root/shoot junction. RNA was isolated using RNeasy kit according to the manufacturer's instructions. A more detailed description of the microarray, including the data evaluation is given elsewhere (Vanneste *et al.*, 2005).

In situ expression and localization analysis

Histochemical staining for GUS activity and immunolocalization were performed as described (Friml *et al.*, 2003a). For PIN2::GUS, stainings were performed with 10-fold lower concentrations of the X-GLUC substrate. The following antibodies and dilutions were used: anti-PIN1 (1:500) (Benková *et al.*, 2003), anti-PIN2 (1:400) (Paciorek *et al.*, 2005) and anti-PIN4 (1:200) (Friml *et al.*, 2002a), anti-HA (mouse) (Babco, 1:1000); and FITC (1:200) and CY3-conjugated anti-rabbit (1:500) or anti-mouse (1:500) secondary antibodies (Dianova). For GFP visualization, samples were fixed for 1 hour with 4% paraformaldehyde, mounted in 5% glycerol and inspected. Microscopy was done on a Zeiss Axiophot equipped with an Axiocam HR CCD camera. For confocal laser scanning microscopy, a Leica TCS SP2 was used. Images were processed in Adobe Photoshop and assembled in Adobe Illustrator.

Phenotype analysis

For embryo phenotype analysis, for each condition and stage, at least 40 embryos were analysed as described (Friml *et al.*, 2003b). Root phenotypes were examined in 4-day-old seedlings. Root length was measured from hypocotyls junction to root apex, and root meristem size from the position in which epidermis cells rapidly elongate to quiescent centre as described (Blilou *et al.*, 2005). Microscopy inspection of roots and embryos was done on a Zess Axiophot equipped with an Axiocam HR CCD camera using differential contrast optics.

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CHAPTER 5 FOUR LIPS/MYB124 and MYB88 restrict divisions during lateral root development

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Abstract

During plant organogenesis, the shape and positioning of the organs is determined by local auxin accumulation. Extensive physiological and genetic studies have revealed a key role for PIN auxin efflux facilitators in the organization of the auxin distribution patterns. However little is known on the mechanisms by which their expression is regulated. Here, we show that a small family of related MYB transcription factors (FOUR LIPS/MYB124 and MYB88) are recruited to the chromatin in the *PIN3* promoter region thereby enhancing PIN3 expression. Furthermore, *flpmyb88* double mutants showed a reduced number of lateral roots and developed a multilayered proliferating pericycle upon auxin treatment, a characteristic reminiscent of mutants defective in polar auxin transport. Our data suggests that FLP and MYB88 act as direct regulators of *PIN3* expression thereby installing a peculiar auxin gradient that is crucial for normal organogenesis.

In preparation

INTRODUCTION

Plants display an amazing potential for post-embryonic growth and are able to adapt their growth and development according to ever-changing environmental conditions. The architecture of the root system can be altered in order to optimize mining of the soil for water and nutrients. This is achieved through the extensive and continuous formation of new branches, called lateral roots. The mechanisms by which roots regulate their branching pattern are poorly understood.

One of the most important regulators of lateral root development is the plant hormone auxin. Auxin is involved during initiation, meristem organisation and outgrowth. Therefore, insight into the molecular mechanism of auxin action is crucial for our understanding of the branching process in roots. Central in the auxin signalling pathway is the regulation of Aux/IAA stability (Gray *et al.*, 2001). Aux/IAAs make up a family of transcriptional repressors. They repress the activity of Auxin Responsive transcription Factors (ARFs) that drive primary auxin responsive gene-expression. Aux/IAA stability is inversely correlated with intercellular auxin levels as auxin directly stimulates interaction with SCF^{TIR1} E3-ligases. This interaction results in the targeting of the Aux/IAAs for proteolysis and subsequently in derepression of ARF activity. Stabilising mutations in Aux/IAA proteins expressed in the pericycle often result in strong defects in lateral root development (Fukaki *et al.*, 2002; Rogg *et al.*, 2001; Tatematsu *et al.*, 2004; Tian and Reed, 1999; Yang *et al.*, 2004). Plants expressing stabilised SOLITARY-ROOT/IAA14 (SLR/IAA14) result in the complete absence of lateral root initiation even upon exogenous auxin application (Fukaki *et al.*, 2002). Similar defects in lateral root development were observed in double mutants of ARF7 and ARF19 (Okushima *et al.*, 2005; Wilmoth *et al.*, 2005). Furthermore, SLR/IAA14 has been shown to interact with ARF7 and ARF19 by yeast-two hybrid assays (Fukaki *et al.*, 2005), suggesting SLR/IAA14, ARF7 and ARF19 form an important regulatory module for auxin-induced lateral root formation. However, little is known on the downstream targets of this module.

Active polar auxin transport plays an important role in lateral root development. It regulates delivery of auxin to the pericycle and formation of an auxin gradient that determines the shape of the developing meristem (Benková *et al.*, 2003; Casimiro *et al.*, 2001; Geldner *et al.*, 2004). In order to correctly establish this auxin gradient, several members of the PIN family are differentially expressed at different stages of lateral root development (Benková *et al.*, 2003). These PIN proteins are rate-limiting factors for auxin efflux (Petrášek *et al.*, 2006) and have specific subcellular localisations that determine the direction of auxin flow

(Wisniewska *et al.*, 2006). Auxin has the amazing ability to stimulate its own transport, in part through Aux/IAA-dependent transcriptional induction of PINs (Vieten *et al.*, 2005). However, nothing is known on the upstream driving mechanism that guarantees the differential PIN expression during lateral root development.

During stomatal development asymmetric and symmetric divisions occur in a highly coordinated fashion. After commitment to the stomatal pathway, a meristemoid mother cell divides asymmetrically giving rise to a meristemoid cell and a larger neighbouring cell. The meristemoid cell can undergo several rounds of asymmetric divisions before differentiating into a guard mother cell. Next, the guard mother cell differentiates producing two guard cells that represent the stoma (Nadeau & Sack, 2003). Recently, several transcription factors have been implicated in regulating various steps of the stomatal lineage (Lai *et al.*, 2005; Macalister *et al.*, 2006; Ohashi-Ito and Bergmann, 2006; Pillitteri *et al.*, 2006). The MYB transcription factors FOUR LIPS (FLP/MYB124) and MYB88 act to repress guard mother cell identity after symmetric division. Loss of FLP function results in ectopic and symmetric divisions, giving rise to stomatal tumors (Lai *et al.*, 2005). Thus, FLP plays an important role in regulating the switch between proliferation and differentiation within the stomatal lineage.

A correct balance between proliferation and differentiation is inherent to every developmental program. In the case of lateral root development the tight correlation between these two processes is reflected in the various clearly defined development stages that can easily be followed microscopically (Malamy and Benfey, 1997). However, it is unclear how this interplay between proliferation and differentiation is regulated. Here we identified and characterized a novel role for the guard cell differentiation factor FLP. We found that FLP is downstream of SLR/IAA14-ARF7,ARF19 auxin signalling and that it is required for auxin-induced *PIN3* expression. Furthermore, deregulation of *PIN3* expression was correlated with a decrease in lateral root emergence in *flpmyb88* mutants. Our findings provide new insights into auxin-induced lateral root organogenesis.

RESULTS

FOUR LIPS is downstream of SLR/IAA14-ARF7,ARF19 auxin signalling

In a previous transcript profiling (Vanneste *et al.*, 2005), *FOUR LIPS/MYB124* (*FLP*), a transcription factor involved in guard cell differentiation (Lai *et al.*, 2005), was found to be rapidly induced by auxin in roots, in a *solitary-root-1* dependent manner (Suppl. Fig. 5.1). In contrast, its closest orthologue *MYB88* did not respond to auxin (Suppl. Fig. 5.1). We evaluated the kinetics of auxin treatment on the expression of *FLP* and *MYB88* using Q-RT-PCR in roots. Following auxin treatment (1μM 2,4-D), *FLP* was strongly induced within 2h and reached a plateau after 3h. On the other hand *MYB88* did not show any response to auxin even after 6h of auxin treatment. Previously, *FLP* expression was recorded in late guard mother cells of the stomatal lineage and in developing guard cells (Lai *et al.*, 2005). Using the same *FLP::GUS* reporter line, in which 8.7 kb of 5' *FLP* region drives GUS-GFP expression (Lai *et al.*, 2005), we could induce GUS activity gradually all over the pericycle by auxin treatment in seedling roots (Suppl. Fig. 5.2). In lateral root forming roots, two weeks after germination, we detected FLP::GUS activity in the columella of primary root meristems (Fig. 5.1 C) and in all stages of lateral root development (Fig. 5.1 D,E). This supports the notion that FLP is auxin inducible as these tissues have high auxin contents. Moreover, in the lateral rootless mutant *slr-1*, no FLP::GUS activity could be detected upon auxin treatment (Suppl. Fig. 5.4). The tissue-specific auxin inducibility suggests an important role for FLP in lateral root development. These data illustrate the rapid auxin inducibility of *FLP* in roots and confirm our previous microarray data.

Next, we addressed the mechanism of *FLP* auxin inducibility. The microarray analysis suggested that *FLP* auxin inducibility was abolished in the *solitary-root-1* (*slr-1*) mutant that expresses a stabilised version of SLR/IAA14 (Suppl. Fig. 5.1). Furthermore, it has been suggested that ARF7 and ARF19 interact with SLR/IAA14, and are thus likely candidates to mediate SLR/IAA14-repressed transcription. Indeed, both in *slr-1* and *arf7arf19 FLP* auxin inducibility was completely abolished compared to mock-treated controls (Fig. 5.1 B). Both *slr-1* and *arf7arf19* display strong defects in lateral root development, even upon auxin treatment (Fukaki *et al.*, 2002; Okushima *et al.*, 2005). The lack of *FLP* inducibility in these mutants might however not be linked to the absence of auxin response itself, but rather be a consequence of their inability to produce lateral roots. To rule this out, we included an

overexpression line of the cell cycle inhibitory protein KRP2 (*35S::KRP2*) in the experiments. This transgenic line also shows strong defects in lateral development (Himanen *et al.*, 2002), but is not expected to interfere with auxin signalling. In contrast to the auxin signalling mutants (*slr-1* and *arf7arf19*), *35S::KRP2* showed, in the absence of a normal level of lateral root production, *FLP* auxin inducibility similar to that of WT (Fig. 5.1 B). Taken together these data suggest that *FLP* expression might be directly regulated through SLR/IAA14-ARF7,ARF19 auxin signalling. Indeed, in *arf7arf19* mutants complemented with ARF7-GR under control of its endogenous promoter, FLP auxin inducibility could be restored by dexamethasone (DEX) treatment (Suppl. Fig. 5.3) (Okushima *et al.*, 2007). Interestingly, in these lines, *FLP* expression could be induced in absence of DEX by cycloheximide (CHX), but not by auxin. This suggests that an unknown unstable repressor regulates *FLP* expression, independently of ARF7 and ARF19.

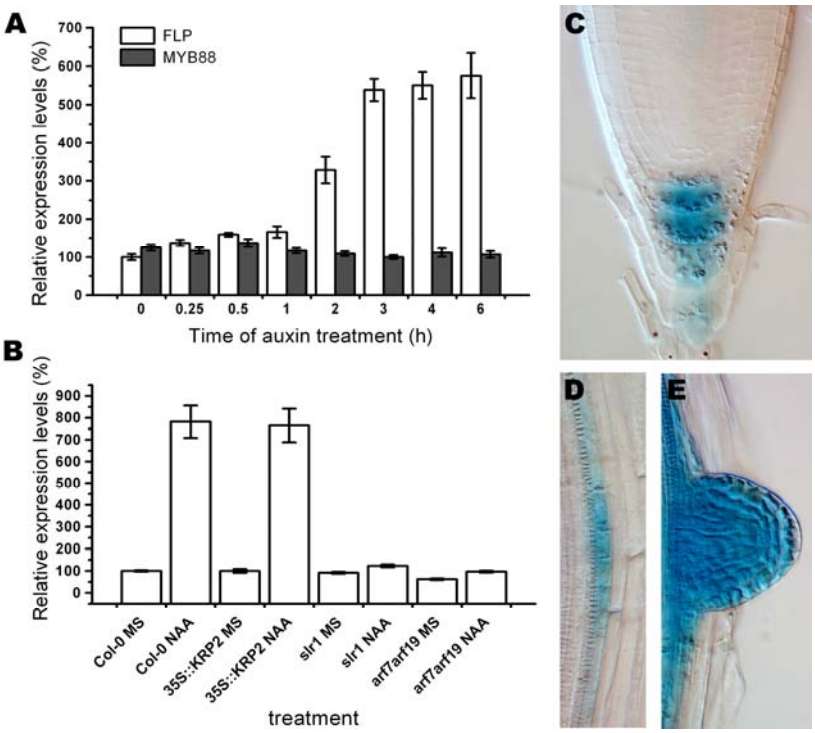


Figure 5.1 FLP is downstream of SLR/IAA14-ARF7,ARF19 auxin signalling. (A) Time-course analysis of *FLP* and *MYB88* transcripts upon auxin treatment (1µM 2,4-D). (B) Analysis of *FLP* transcripts after mock and auxin (10µM NAA) treatment for 6h in WT (Col-0), *35S::KRP2*, *slr-1* and *arf7arf19* mutants. (C-E) FLP::GUS activity in (C) columella of primary root tip, (D) stage I lateral root primordium and (E) emerged lateral root meristem. Error bars indicate standard deviation.

FOUR LIPS acts redundantly with *MYB88* in lateral root development

The specific expression pattern in the root, i.e. in columella cells and cells involved in lateral root formation and its prominent auxin-inducibility is indicative for a potential role during root development. In the shoot, FLP and its closest orthologue, MYB88, are important regulators of stomatal development (Lai *et al.*, 2005). In order to avoid the potential of redundant functions of the two genes, we analyzed *flpmyb88* double mutants to get insight into their roles in root development.

Given the specific expression in all stages of lateral root development we assessed lateral root density. We measured root lengths of 5 day old plants that were subsequently subjected to microscopic analysis to quantify the densities of emerged and non-emerged lateral roots by making use of cleared root samples. Both in *flp7myb88* and *flp1myb88* a significant reduction in emerged lateral root density ($P<0.001$) was observed (Fig. 5.2 A). The microscopic analysis of emerged and non-emerged lateral roots showed that *flpmyb88* mutants had a bigger fraction of non-emerged lateral roots compared to wild type (Fig. 5.2 B). This suggests that FLP function is involved in lateral root emergence. Next, both wild type and mutant roots were exposed to exogenous auxin for 5 days. In the auxin-treated wild-type roots we observed distinct lateral roots emerging across the primary root (Fig. 5.2 C,E), while in *flpmyb88* mutants, developing lateral root primordia were less well defined and seemed retarded during development (Fig. 5.2 D,F). In order to get insight into the aberrant response of the mutants upon auxin treatment, we analyzed the expression pattern of the lateral root boundary marker *CUC3::GUS* (Vroemen *et al.*, 2003) in *flp7myb88* and wild type roots treated with NAA for 3 days (Fig. 5.2 I,J). In WT, *CUC3::GUS* was strongly expressed in sites flanking developing lateral roots, whereas in *flp7myb88*, no *CUC3::GUS* activity could be observed. This suggests that the specification of lateral root boundaries is compromised in *flpmyb88* mutants.

Table 5.1 Frequency of cells in the outer layer of stage II primordia in WT and *flpmyb88* double mutants

	Number of cells in the outer layer of stage II primordia			
Genotype	3	4	5	6
Col-0	3.4%	41.4%	44.8%	10.3%
L*er*	11.8%	35.3%	44.1%	8.8%
flp1myb88	11.8%	29.4%	41.2%	17.6%
flp7myb88	18.2%	21.2%	30.3%	30.3%

During the early developmental stages of lateral root development a fixed sequence of cell divisions occur (Malamy and Benfey, 1997) and deviations from the normal cell division pattern can be recorded easily. Lateral root initiation is hallmarked by coordinated asymmetric divisions in xylem pole pericycle cells. After a series of anticlinal divisions, the most central cells undergo a single periclinal division giving rise to a stage II primordium. We determined the number of cells in the outer layer of stage II primordia in WT and *flpmyb88* (Table 5.1), as a relative measure of the number of cells in lateral root initiation sites. In WT, about 44% of stage II primordia contained 5 cells in their outer layer and only 10% contained 6 cells. In contrast, in *flpmyb88* mutants we found a strong increase in frequency of stage II primordia with 6 cells in their outer layers, 18% in *flp1myb88* and 30% in *flp7myb88*. These data suggest that FLP acts to restrict cell division, also during the earliest stages of lateral root development.

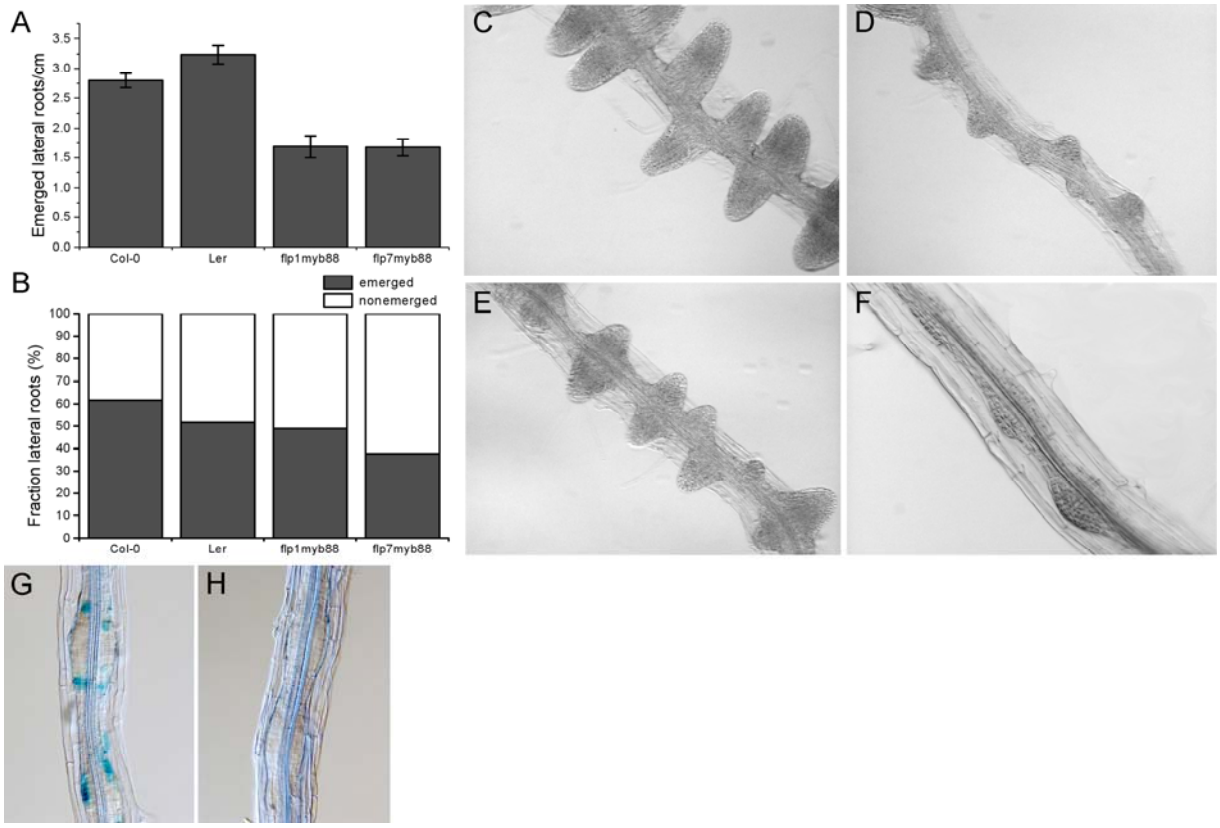


Figure 5.2 Lateral root phenotype of *flpmyb88* mutants. (A) Lateral root density in 5 day old roots. (B) Fraction of emerged vs. non-emerged lateral root primordial in 5 day old roots. (C-F) Roots treated for 5 days with 10µM NAA of (C) Col-0, (D) *flp1myb88*, (E) L*er*, (F) *flp7myb88*. (G-H) CUC3::GUS activity in auxin treated (G) WT and (H) *flp7myb88*.

FLP is a positive regulator of auxin induced *PIN3* expression

Similar defects in lateral root emergence and boundary definition have been reported earlier for mutants known to be defective in auxin distribution (Benková *et al.*, 2003; Geldner *et al.*, 2004). Furthermore, during lateral root development, several members of the PIN family show specific and overlapping expression patterns that can be used as markers for the directionality of the auxin distribution (Friml *et al.*, 2003; Wisniewska *et al.*, 2006). This has led to the insight of an early PIN-mediated installation of an auxin gradient during lateral root initiation (Benková *et al.*, 2003). Interestingly, auxin itself strongly induces *PIN* gene expression (Vieten *et al.*, 2005), suggesting that *PIN* auxin-inducibility is an important aspect for installing an auxin gradient in a developing lateral root.

The observed lateral root defects in *flpmyb88* mutants suggest that polar auxin transport might be affected in these mutants. Therefore, we examined auxin-inducible *PIN* expression levels in *flpmyb88* mutants (Fig. 5.3 A-F). Roots of 7 day old WT and *flp1myb88* plants were treated with auxin (10 μM NAA) for 6h. Real-time PCR analysis of *PIN* transcripts showed that *PIN1*, *PIN3* and *PIN7* were strongly up-regulated in WT. *PIN1* and *PIN7* auxin inducibility was similar in WT as in *flp1myb88*, while *PIN3* auxin inducibility was clearly reduced in *flp1myb88* compared to WT (Fig. 5.3 A). Similarily, PIN3::GUS activity was strongly reduced in *flp7myb88* (Fig. 5.3 B,C). These data suggest that FLP and MYB88 might be required for normal *PIN3* expression levels.

Next we tested if the FLP protein could be directly involved in of *PIN3* expression by ChIP analysis (Fig. 5.3 F). Within a region 2kb upstream of the *PIN3* start codon, 4 primer pairs were chosen for semi-quantitative PCR after ChIP. Both in Col-0 and *flp1myb88* we compared relative quantities of each fragment in the pre-ChIP sample (input), an IgG negative control (IgG) sample and a sample precipitated with specific anti-FLP-MYB88 antibodies (FLP). For all fragments, a strong band could be detected in the pre-ChIP sample. Fragment A showed a weak background band after IgG ChIP in Col-0. In contrast FLP-ChIP showed a clear band for fragment C for Col-0 but not for the *flp1myb88* double mutant. For fragment D we could detect after FLP-ChIP a weak band both in Col-0 and *flp1myb88* suggesting the detection is probably the result of aspecific binding (Fig. 5.3 F). These data suggests that FLP might be recruited to the chromatin the *PIN3* promoter at fragment D. However, EMSA analysis could not show a direct interaction of FLP with this fragment (data not shown),

which is suggestive for the recruitment of FLP and MYB88 to the *PIN3* promoter chromatin via an adaptor protein. Taken together, these data illustrate the involvement of FLP and MYB88 in the control on *PIN3* transcriptional activity.

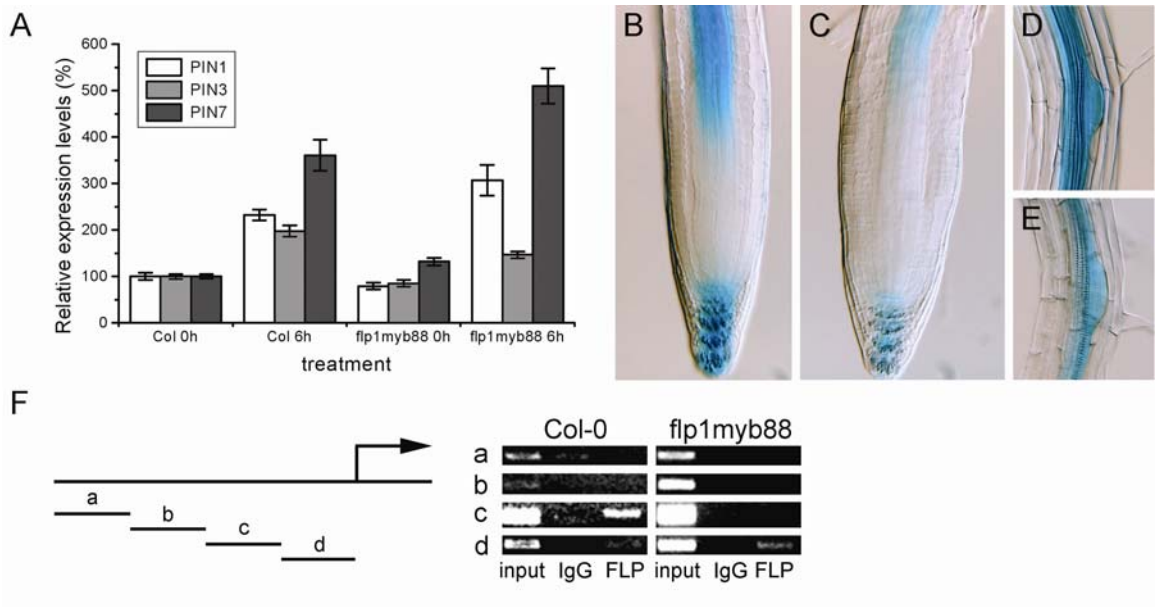


Figure 5.3 FLP and MYB88 regulate *PIN3* expression. (A) Real-time PCR analysis of PIN1, PIN3 and PIN7 transcripts upon auxin treatment (10 µM NAA) in WT and *flp1myb88*. (B-E) PIN3::GUS activity in (B, E) WT and (C, E) *flp7myb88*. (F) Left: Schematic representation of PIN3 promoter with indication of regions targeted for semi-quantitative PCR after ChIP. Right: ChIP on *PIN3* promotor using WT and *flp1myb88* chromatin.

DISCUSSION

The auxin inducible FLP is involved in patterned cell division in lateral roots

In an attempt to identify downstream targets of auxin signalling during lateral root initiation, a previous transcript profiling experiment has pinpointed the FLP/MYB124 transcription factor as one of the most promising candidates due to its early and auxin-inducible expression during lateral root initiation and supported by the notion that it has an important role in restricting cell divisions in stomatal development (Lai *et al.*, 2005; Vanneste *et al.*, 2005).

In the present study, we could detect *FLP* expression in all stages of lateral root development. Moreover, we found that its expression depends on the SLR/IAA14-ARF7,ARF19 auxin signalling module. Loss of FLP functions resulted in a reduction in density of emerged lateral roots. Interestingly, we found defects in the patterned cell division of developing lateral roots in *flpmyb88* mutants. As a consequence, cell fates are not correctly established as suggested by the absence of CUC3::GUS activity in the flanks of lateral roots and a retarded lateral root emergence.

Taken together, we were able to show that FLP acts to restrict cell divisions also in developing lateral roots from the earliest stages onwards, similar to its role in the stomatal lineage.

FLP enhances auxin induced *PIN3* transcription

Central to many organogenetic processes is the formation of an auxin gradient (Benková *et al.*, 2003; Friml *et al.*, 2003; Sabatini *et al.*, 1999). Auxin gradients are set in place and maintained through activity of members of the PIN family of auxin efflux mediators (Benková *et al.*, 2003). PIN proteins have peculiar subcellular polar localisations. It has been shown that their polar localisations correlate with the direction of auxin flow (Wisniewska *et al.*, 2006). During different steps of lateral root development several PINs are expressed in specific spatio-temporal patterns (Benková *et al.*, 2003). However, besides their AUX/IAA-mediated auxin inducibility (Vieten *et al.*, 2005), little is known on the mechanism underlying transcriptional regulation of PINs. We have identified FLP and MYB88 as potential

enhancers of *PIN3* auxin inducibility as observed by lateral root defects, expression analysis and ChIP. However, direct binding of FLP to the PIN3 promoter could not be shown, suggesting that an adapter protein is required for FLP recruitment. Interestingly, FLP recruitment to the *PIN3* promoter could no longer be observed in a preliminary ChIP experiment in *arf7arf19* (Z. Xie & E. Grotewold, pers. comm.), suggesting that the presumed adapter protein depends on ARF7 and/or ARF19 function. Yet, more experiments are required to support this notion.

In summary, we propose the following model for FLP-regulated *PIN3* expression during lateral root development (Fig. 5.4). Auxin triggers proteolysis of SLR/IAA14 that results in derepression of ARFs, among which ARF7 and ARF19. The activity of these ARFs induces expression of both *FLP* and *PIN3*. The newly formed FLP enhances ARF mediated *PIN3* expression locally after recruitment to *PIN3* promoter chromatin. FLP recruitment occurs either directly through ARF7 and/or ARF19 or through an ARF7 and/or ARF19 regulated protein. The *FLP* auxin inducibility allows local fine-tuning of *PIN3* levels needed to set in place an auxin gradient essential to lateral root meristem formation.

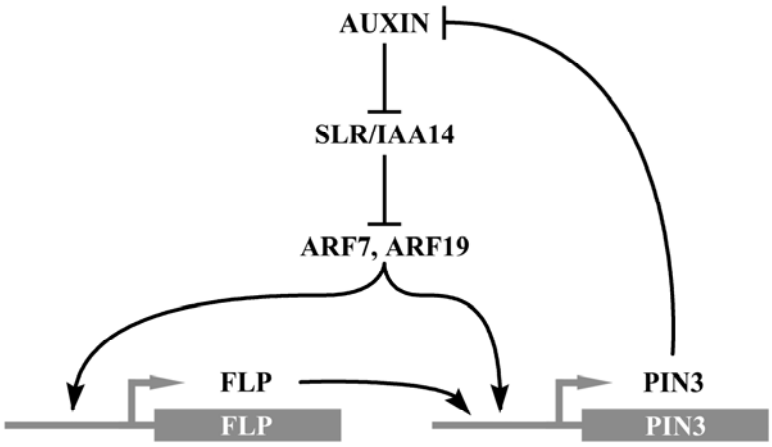


Figure 5.4 Scheme of FLP mediated PIN3 auxin inducibility.

MATERIALS & METHODS

Plant material and growth conditions

In this study, we analyzed the *Arabidopsis thaliana* (L.) Heynh. ectotypes Col-0, L*er*, the mutants *slr1* (Fukaki *et al.*, 2002), *arf7arf19* (Okushima *et al.*, 2005), *flp-1*, *flp-7*, *myb88*, *flp-1 myb88* and *flp-7myb88* (Lai *et al.*, 2005), the promoter fusions *FLP::GUS* (Lai *et al.*, 2005), *PIN3::GUS* (Friml *et al.*, 2002) and overexpression line *35S::KRP2* (De Veylder *et al.*, 2001). *Arabidopsis* seedlings were grown under continuous light conditions at 18-25 °C on 0.5xMS with sucrose. Exogenous drug application was performed by incubation of 4-5 day old seedlings in 0.5xMS with 1% sucrose supplemented with 2,4-Dichlorophenoxyacetic acid (2,4-D) (1 μM) or 1-naphthalene acetic acid (NAA) (10 μM).

Quantitative RT-PCR

RNA was extracted using the RNeasy kit (QIAGEN) from root samples. Poly(dT) cDNA was prepared out of 1 μg total RNA using Superscript III Reverse Transcriptase (Invitrogen, Belgium) as recommended by Invitrogen. Quantifications were performed on a Bio-Rad Icycler apparatus with Platinum SYBR Green qPCR Supermix-UDG kit (Invitrogen, Belgium) upon recommendations of the manufacturer. PCR was carried out in 96-well optical reaction plates heated for 10 minutes to 95 °C to activate hot start Taq DNA polymerase, followed by 40 cycles of denaturation for 60 seconds at 95 °C and annealing-extension for 60 seconds at 58 °C. Target quantifications were performed with specific primer pairs designed using Beacon Designer 4.0 (Premier Biosoft International, Palo Alto, CA). The primers used to quantify gene expression levels were FLP_FW (CGAAATGCCACTGGTATTGATAGC), FLP_RW (CACCATCACTCTCATTCACATTGC), MYB88_FW (GAGGAGATTCACT TTCGGCTTTTAG), MYB88_RW (AGGATTGCTTGTTGTGTTAACTCAG), PIN1_FW (TACTCCGAGACCTTCCAACTACG), PIN1_RW (TCCACCGCCACCACTTCC), PIN3_FW (GAGGGAGAAGGAAGAAAGGGAAAC), PIN3_RW (CTTGGCTTGTAA TGTTGGCATCAG), PIN7_FW (GTCCGTTAGGCACTTCCTTTACCC), PIN7_RW (TCAAGGCGGTGCAAAAGAGATTCG), EF-1-alfa_FW (CTGGAGGTTTTGAGGCTG GTAT), EF1-alfa_RW (CCAAGGGTGAAAGCAAGAAGA) Expression levels were normalized to EF-1-alfa (At1g07940) expression levels. All RT-PCR experiments were

performed in triplicates and the data was processed using qBase v1.3.4 (Hellemans *et al.*, 2007)

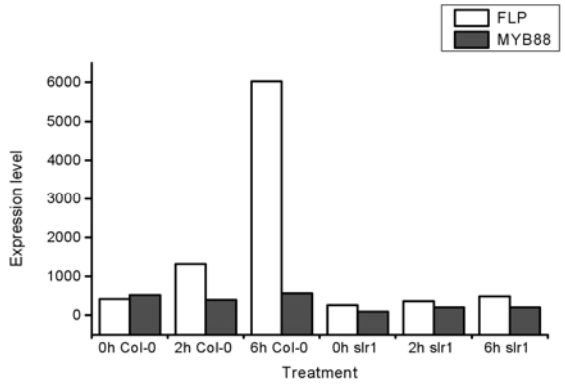
Histochemical analysis

The β-glucuronidase (GUS) assays were performed as described (Beeckman and Engler, 1994). For microscopic analysis, samples were cleared by mounting in 90% lactic acid (Acros Organics, Brussels, Belgium) (analysis of GUS stainings) or using the clearing method described by Malamy and Benfey (1997). All samples were analyzed by differential interference contrast microscopy (Leica DMLB, Leica, Vienna, Austria). Photographs were taken with a CAMEDIA C-3040 zoom digital camera (Olympus, Tokyo, Japan) and processed with Photoshop 7.0 (Adobe, San José, CA).

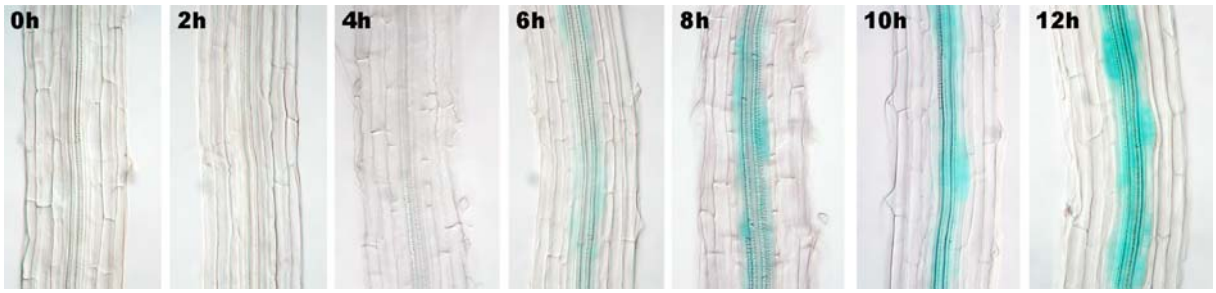
Chromatin Immunoprecipitation

Roots of 1 week old plants were treated with 10µM NAA for 4h. After crosslinking under vacuum, the tissue was grinded and DNA was sheared by sonication. After pre-clearing with salmon sperm / protein A, the sample was used for IP with the specific anti-FLP-MYB88 antibody and IgG as a control. After lysis, DNA was extracted by using PCR purification kit (QIAGEN). 1 µl was used for semi-quantitative PCR.

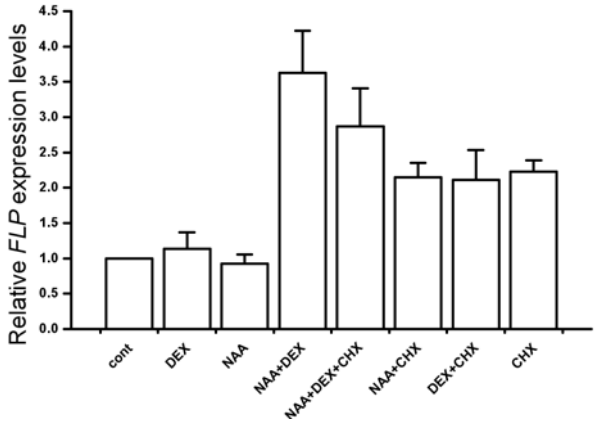
Supplemental data



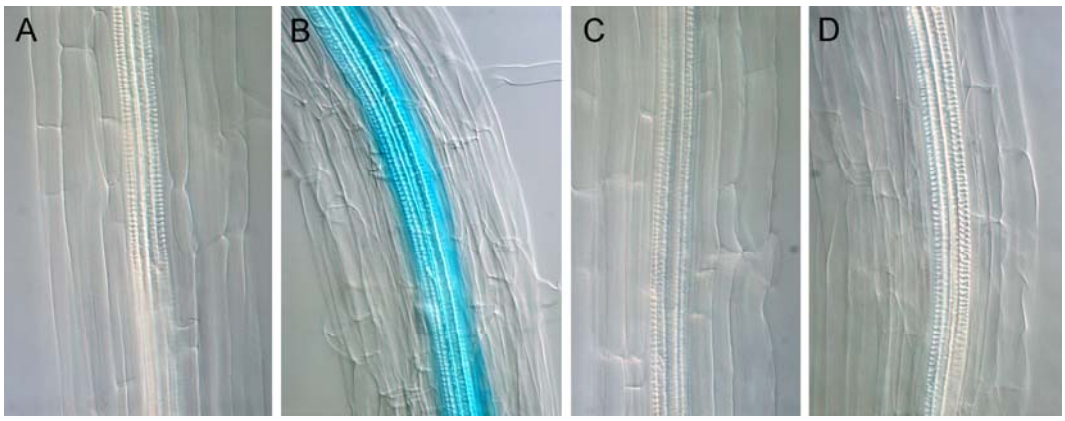
Supplemental Figure 5.1 Microarray data of *FLP* and *MYB88* transcripts in response to the lateral root inducible system, comparing WT (Col-0) to the auxin insensitive mutant *slr-1*. 0h corresponds to 72h-old seedlings germinated in presence of the auxin transport inhibitor (10µM NPA). Such seedlings were transferred to auxin (10µM NAA) for 2h and 6h respectively.



Supplemental Figure 5.2 Auxin responsiveness of FLP::GUS. 0h corresponds to 72h old FLP::GUS seedlings germinated in presence of NPA. Such seedlings were transferred to auxin (10µM NAA). After 6h of auxin treatment, GUS activity could be detected in the roots.



Supplemental Figure 5.3 Real-time PCR analysis of ARF7-dependent *FLP* auxin inducibility. Seedlings of *arf7arf19* complemented with ARF7-GR under control of its endogenous promoter, was able to restore *FLP* auxin inducibility only in presence of DEX. CHX was able to induce *FLP* expression in absence of DEX in these lines.



Supplemental Figure 5.4 *solitary-root-1* dependent auxin-inducibility of FLP::GUS activity. (A-D) FLP::GUS activity in WT (A-B) and *slr-1* (C-D) before (A,C) and after (B,D) auxin treatment.

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CHAPTER 6 A2-type cyclins at the end of cell division

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Abstract

Plants regulate their growth and development through a tight coordination between cell division and differentiation. The equilibrium between mitosis and differentiation is rigorously controlled by mitotic CDK activity. As A2-type cyclins are at the centre of mitotic regulation through CDK activity, we used double and triple CYCA2 mutant combinations as tools to untangle the interplay between differentiation and proliferation in plants. In general, all mutants were characterized by a deceleration of the cell cycle with a delay in G2-to-M transition. However, differentiation events occurred as if cell division was normal, resulting in stomatal precursor cells with guard cell fate, aberrant cell division patterns in early lateral root primordia and aberrant vascular patterning in developing leaves. We conclude that, in accordance to animals, plant developmental programmes do not show a cell cycle dependent checkpoint but that rather a regulatory hierarchy between cell fate establishment and cell division.

In preparation

INTRODUCTION

Cell division is a fundamental aspect of the development of all multicellular organisms. After fertilisation the zygote undergoes multiple rounds of cell division, laying down, maintaining and expanding the body plan. Correct morphogenesis requires a strict orchestration of cell fate establishment and cell division. Both in animals as in plants, little is known on how differentiation processes are intertwined with the core cell cycle machinery.

As cell division is of such critical importance for any organism, it is subject to rigorous regulation. A typical cell division cycle can be subdivided in 4 distinct phases: G(ap)1, S(ynthesis), G(ap)2 and M(itosis) phase. Transitions between G1 and S or G2 and M are regulated by specific Ser/Thr kinases and might represent gateways through which developmental programmes feed into the cell cycle and/or vice versa. These Ser/Thr kinases are dimers composed of a catalytic subunit (Cyclin Dependent Kinase, CDK) and a regulatory subunit (Cyclin). Most of the basic cell cycle regulatory machinery is considerably conserved among eukaryotes. However, plants have a higher complexity of cell cycle regulation compared to animals demonstrated by the presence of multiple copies of most cell cycle regulators (Vandepoele *et al.*, 2002).

Differentiated cells are by definition non-dividing, nevertheless, they often undergo consecutive cycles of DNA synthesis without mitosis (endoreduplication). This infers that differentiation is not necessarily uncoupled of CDK activity but clearly associated with the absence of mitotic CDK activity. Indeed, mitotic cyclins (CYCAs) are specifically down regulated in differentiating tissues (Roudier *et al.*, 2003). The mechanisms underlying the coordination between cell division and differentiation are however poorly understood.

During mitosis, kinase activities of both A- and B-type CDK are high (Magyar *et al.*, 1997; Porceddu *et al.*, 2001). Reduction of the respective kinase activities through dominant negative approaches resulted in premature onset of endoreduplication (Boudolf *et al.*, 2004b; Verkest *et al.*, 2005). Yet, CDK kinase activities depend on binding with specific cyclin partners. A- and B-type cyclins are highly expressed during mitosis (Menges *et al.*, 2005), and are thus potential interaction partners to these CDKs. For instance CYCA2;2 and CYCA2;3 have been shown to interact with CDKA;1 *in vitro* (del Pozo *et al.*, 2002; Imai *et al.*, 2006). Furthermore, *cyca2;1* and *cyca2;3* mutants showed enhanced endoreduplication levels, whereas overexpression of CYCA2;3 or Nicta;CYCA3;2 dramatically reduced endoreduplication levels (Imai *et al.*, 2006; Yoshizumi *et al.*, 2006; Yu *et al.*, 2003). Taken

together, A-type cyclins in plants are required for G2-to-M transition, and are most likely negatively regulated during the switch between proliferation and endoreduplication.

Here we used A2-type cyclin mutants as tools to untangle the interplay between differentiation and proliferation in plants. Indeed, our data support a role for A2-type cyclins at G2-to-M transition. Despite a slowed down G2-to-M progression in triple mutants, differentiation events occurred as if cell division occurred normally, suggesting an uncoupling of cell fate establishment and cell division.

RESULTS

CYCA2 expression is associated with tissues competent to divide

In order to gain insight into A2-type cyclin function, we analysed the promoter activities of all 4 A2-type cyclins by histochemical GUS staining (Fig. 6.1). All promoter::GUS lines showed activity in shoot apical meristems (Fig. 6.1 A-D), developing lateral roots (Fig. 6.1 E-L) and primary root meristems (Fig. 6.1 M-P). In root apical meristems, *CYCA2;2* and *CYCA2;4* showed a remarkably similar expression pattern (Fig 6.1 N-P), with highest expression in the vascular cylinder, while *CYCA2;1* and *CYCA2;3* were more homogeneously expressed (Fig 6.1 M,O). Interestingly, besides expression in vascular tissues, no GUS activity could be detected in other differentiated tissues.

These expression patterns are largely consistent to those reported by Imai *et al.* (2006), with the exception that our reporter line for *CYCA2;4*, seemed to have lower expression levels.

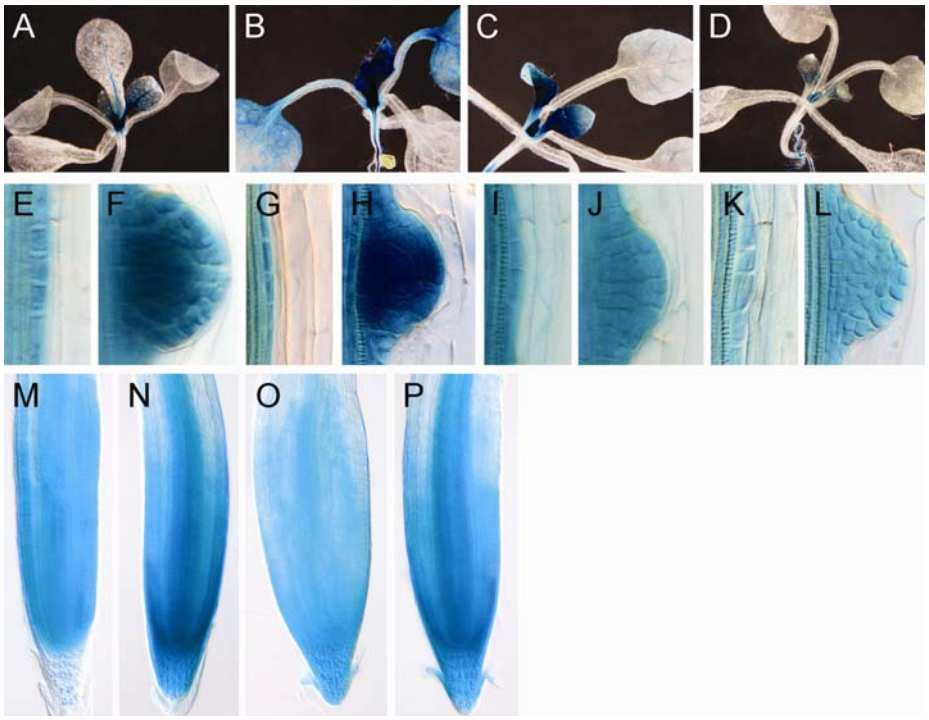


Figure 6.1 CYCA2::GUS expression analysis. *CYCA2;1::GUS* activity (A,E,F,M) in shoot (A), stage I LRP (E), stage V LRP (F) and root apical meristem (M). *CYCA2;2::GUS* activity (B,G,H,N) in shoot (B), stage I LRP (G), stage V LRP (H) and root apical meristem (N). *CYCA2;3::GUS* activity (C,I,J,O) in shoot (C), stage I LRP (I), stage V LRP (J) and root apical meristem (O). *CYCA2;4::GUS* activity (D,K,L,P) in shoot (D), stage I LRP (K), stage V LRP (L) and root apical meristem (P).

Triple mutants in CYCA2s have defects in cell cycle progression

As is the case for the majority of cell cycle regulators in plants, redundancy hinders the assessment of the biological function for cyclins in *Arabidopsis* through mutants. Yet, for each A2-type cyclin at least one full knock-out allele was found in publicly available mutant collections of SALK, GABI-KAT and EXOTIC (Fig. 6.2 A). Using representative knock-outs we constructed three triple mutant combinations (*cyca2;124*, *cyca2;134* and *cyca2;234*) and used these to score several cell cycle parameters in roots and shoots (Fig. 6.2 B-M). After 10 days of growth on vertically oriented plates under continuous illumination, root length and lateral root densities were determined (Fig. 6.2 B,C). In *cyca2;124* root length was not significantly altered compared to WT, in contrast, both *cyca2;134* and *cyca2;234* showed a reduction in root length of about 20% ($P < 0.0001$). Interestingly, there was no clear difference between *cyca2;134* and *cyca2;234* primary root lengths. The lateral root densities showed a gradual decrease over the analysed triple mutant combinations (Fig. 6.2 C). In *cyca2;124* lateral root density was only slightly reduced (10 %; $P < 0.05$), in *cyca2;134* by 34 % ($P < 0.0001$) and in *cyca2;234* by more than 50 % ($P < 0.0001$). Similarly to the defects in root architecture, we could find increasing reductions in leaf size in different triple mutants (Fig. 6.2 D-G). In contrast, size of epidermal cells of 3-week old 1st leaves was increased in the mutants (Fig. 6.2 H-K). Furthermore, we found that both in cotyledons and leaves, endoreduplication levels were enhanced again showing a gradient of severity over the different triple mutant combinations (Fig. 6.2 L,M).

In conclusion, we detected remarkable graduations in strength of phenotypes over the triple mutants (*cyca2;124* < *cyca2;134* < *cyca2;234*). Generally, the strongest cell cycle defects were found when CYCA2;3 and CYCA2;4 functions were lacking. In all cases the observed mutant phenotypes highlight the importance of A2-type cyclins as positive regulators of proliferation.

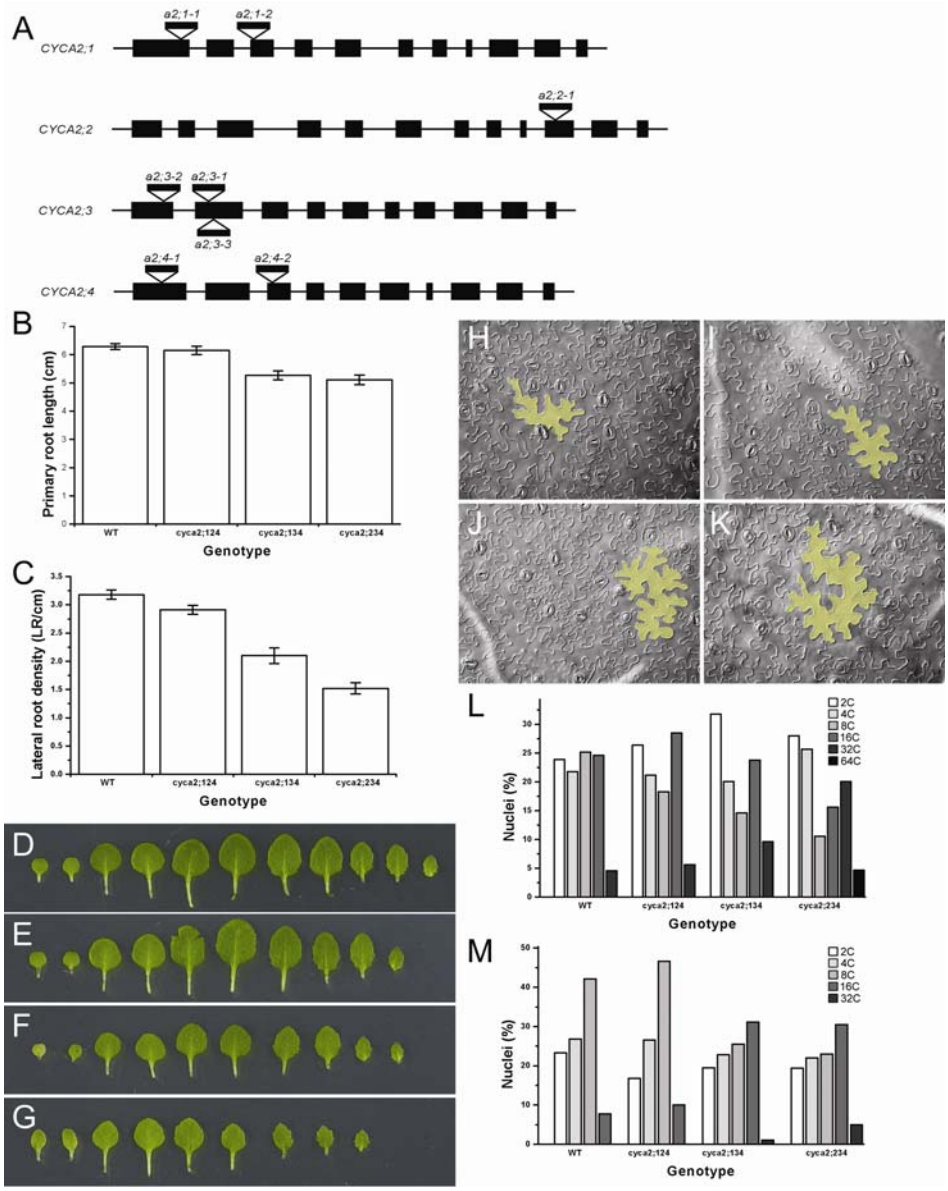


Figure 6.2 Analysis of A2-type cyclin mutants. (A) Graphic reproduction showing the positions of the T-DNA insertions in the different mutant alleles. (B) Primary root length in 10 day old triple mutant. (B) Lateral root densities of 10 day old triple mutant roots. (D-G) Developmental leaf series of (D) WT, (E) *cyca2;124*, (F) *cyca2;134* and *cyca2;234*. (H-K) Epidermal cell size of (H) WT, (I) *cyca2;124*, (J) *cyca2;134* and (K) *cyca2;234*. (L) Endoreduplication levels in cotyledons of triple mutants. (M) Endoreduplication levels in leaves of triple mutants.

A2-type cyclins are involved in stomatal development

Detailed analysis of the promoter::GUS fusions showed, next to higher described general expression patterns, detectable GUS activity in the stomatal lineage for *CYCA2;2* and *CYCA2;3* (Fig. 6.3 A,B). This suggests that A2-type cyclins might have an important role in regulating cell divisions during stomatal development. Indeed, microscopic inspection of the epidermis of triple mutants revealed a frequent occurrence of circular and kidney-shaped cells

that showed morphological traits of mature guard cells: characteristic cell wall thickening and chloroplast accumulations (Fig. 6.3 C, D). Furthermore, we found that their position in the leaf epidermis was correlated with underlying intercellular air cavities in the leaf mesophyll normally found beneath stomata, reminiscent of hypostomatal cavities (Suppl. Fig. 6.1). These characteristics suggest that these cells descend from stomatal lineages and will therefore be referred to as "aberrant stomata".

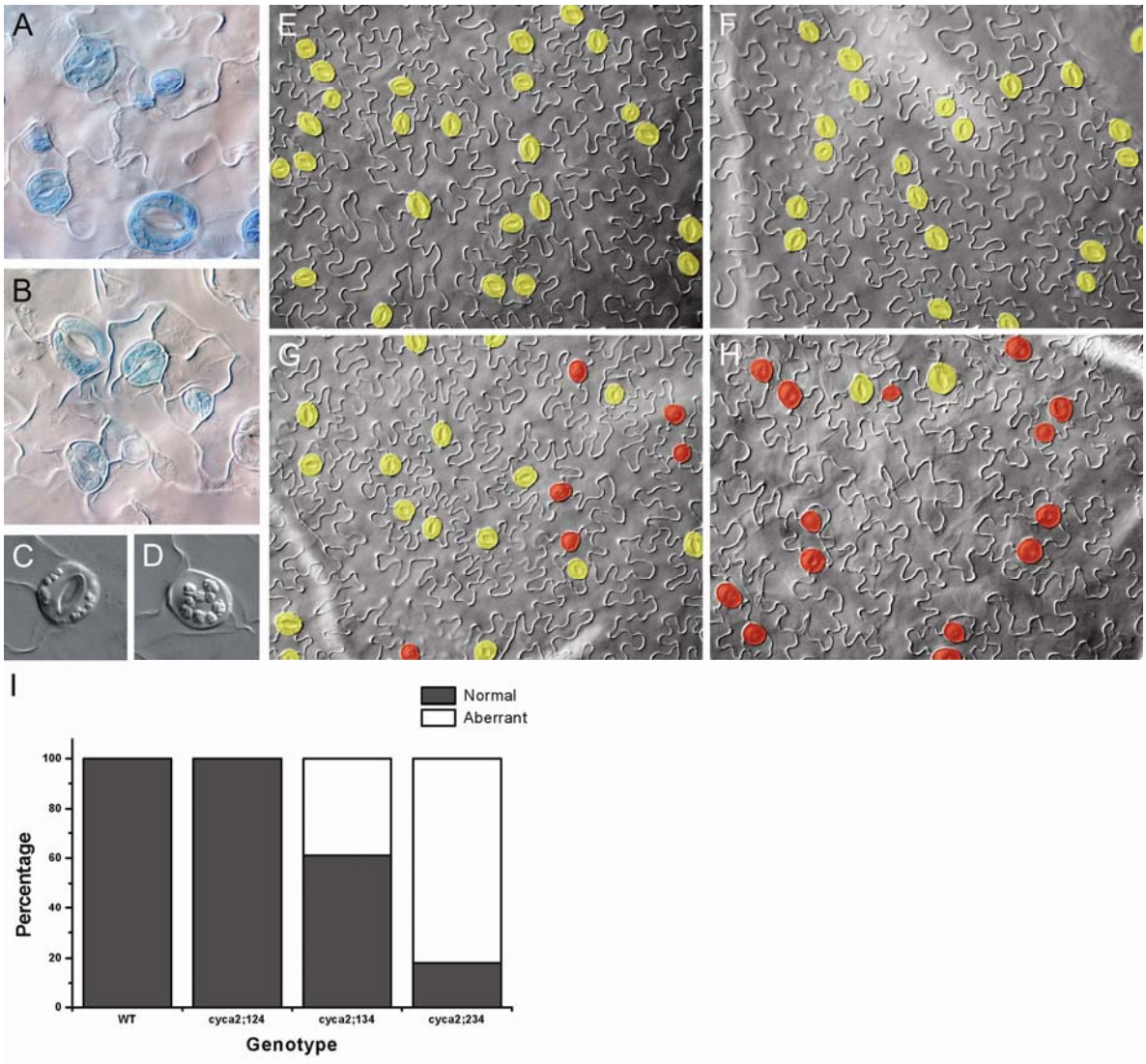


Figure 6.3 A2-type cyclins are involved in stomatal development. (A, B) Stomatal expression of (A) *CYCA2;2::GUS* and (B) *CYCA2;3::GUS*. (C,D) Detail of (C) a normal stoma and (D) an aberrant stoma. (E-H) Lower epidermis of 3 week old first leaves of (E) WT, (F) *cyca2;124*, (G) *cyca2;134* and (H) *cyca2;234* (yellow indicates normal, red aberrant stomata). (I) Frequency analysis of normal vs. aberrant stomata.

We could detect aberrant stomata in *cyca2;134* and *cyca2;234*, but not in wild type and *cyca2;124* (Fig. 6.3 E-I). Furthermore, such cells were only found when CYCA2;3 was mutated (*cyca2;3*, *cyca2;23*, *cyca2;34*). In the lower epidermis of 2-week old cotyledons of

the various mutant combinations, we determined the frequencies of normal and aberrant stomata. In all alleles of *cyca2;3* we could only find 1 to 2 percent of aberrant stomata, whereas double mutants with *cyca2;2* and *cyca2;4* alleles showed a dramatic increase in phenotype penetrance (up to 30%). Moreover, *cyca2;234* mutants nearly did not form any normal stomata.

Taken together, these data suggest that A2-type cyclins are required for stomatal precursor cell division.

Table 6.1 Analysis of stomatal phenotype in various genotypic backgrounds

Genotype	Counts	Normal	Aberrant	Normal (%)	Aberrant (%)
Col-8	501	501	0	100	0
cyca2;1-1	501	501	0	100	0
cyca2;1-2	478	478	0	100	0
cyca2;2-2	965	965	0	100	0
cyca2;3-1	845	830	15	98	2
cyca2;3-2	960	939	21	98	2
cyca2;3-3	988	975	13	99	1
cyca2;4-1	805	805	0	100	0
cyca2;4-2	824	824	0	100	0
cyca2;12	546	546	0	100	0
cyca2;14	464	464	0	100	0
cyca2;24	474	474	0	100	0
cyca2;23	831	699	132	84	16
cyca2;34	1149	913	236	79	21
cyca2;3-2cyca2;4-2	1286	899	387	70	30
cyca2;3-3cyca2;4-2	964	681	283	71	29
cyca2;124	529	529	0	100	0
cyca2;134	744	457	287	61	39
cyca2;234	734	42	692	6	94

Anatomical analysis of *cyca2;234*

As *cyca2;234* displayed the most dramatic phenotypes, we performed an anatomical analysis of various meristematic tissues in this triple mutant (Fig. 6.5). The wild-type shoot apical meristem is composed of small isodiametric cells with a high frequency of newly formed thin cell walls as a sign of intensive cell division activity. (Fig. 6.5 A). In *cyca2;234*,

such cell walls could not be found or were at least less numerous, suggesting that the meristem function is reduced in the mutant (Fig. 6.5 A,B). Meristems are typically structured in 2 important periclinal units. The tunica is composed of the 2 outermost periclinal cell layers, whereas the mass of cells beneath the tunica is designated the corpus. Although being smaller, both important structures were maintained in the mutant, suggesting that meristem structure remained intact, albeit with a reduced meristematic activity.

In the root apical meristem, we found that the columella was composed of only two to three columns of cells whereas a typical wild-type columella contains four columns (Fig. 6.3 C-F). Interestingly, starch accumulation could not be detected in the columella stem cell layer by lugol-staining, indicating that stem cells did not prematurely differentiate to columella cells when CYCA2 function was impaired (Fig. 6.5 E,F).

Also during lateral root formation, we found dramatic defects in morphology as a result of a defect in A2-type cyclin activity (Fig 6.5 E-H; Suppl. Fig. 6.2). The centre of a stage II lateral root primordium is typically composed of 2 layers, each containing 4 to 6 cells in the central region. (Fig. 6.5 G). In *cyca2;234*, stage II primordia could be detected composed of fewer, enlarged cells (Fig. 6.5 I). In later stages of lateral root development, similar defects could be observed, finally resulting in a more or less normal looking lateral root meristem, harbouring fewer, but enlarged cells (Fig 6.5 H,J; Suppl. Fig. 6.2). Despite these serious cell division defects during early lateral root initiation in the mutant, the lateral root developmental program was not obstructed and could successfully proceed as if all cells were present to form a functional lateral root meristem.

Next to the described cell division defects, we found a dramatic reduction in fertility in this mutant combination. After fertilisation, sepals and petals senesced and the pistil rapidly elongated to form a silique in WT (Fig. 6.5 I). In *cyca2;234* pistils did not elongate after sepals and petals have senesced, suggesting that fertilisation did not occur (Fig. 6. J). To address whether a this is due to a defect in *cyca2;234* pollen development, we performed an anatomical analysis of anthers of flowers shortly before opening (Fig. 6.5 K,L). In WT, several pollen grains could be found in each pollen sac, whereas in *cyca2;234* fewer pollen grains could be retrieved per pollen sac. Often several pollen grains seemed to be aborted. Furthermore, the mutant pollen sacs were made up of bigger cells presenting a higher degree of sclerification of the cell walls. These data suggest that fertility in *cyca2;234* could be affected both at the level of microgametogenesis itself, as well as at the level of a mechanical impediment, preventing release of those pollen grains that did develop normally.

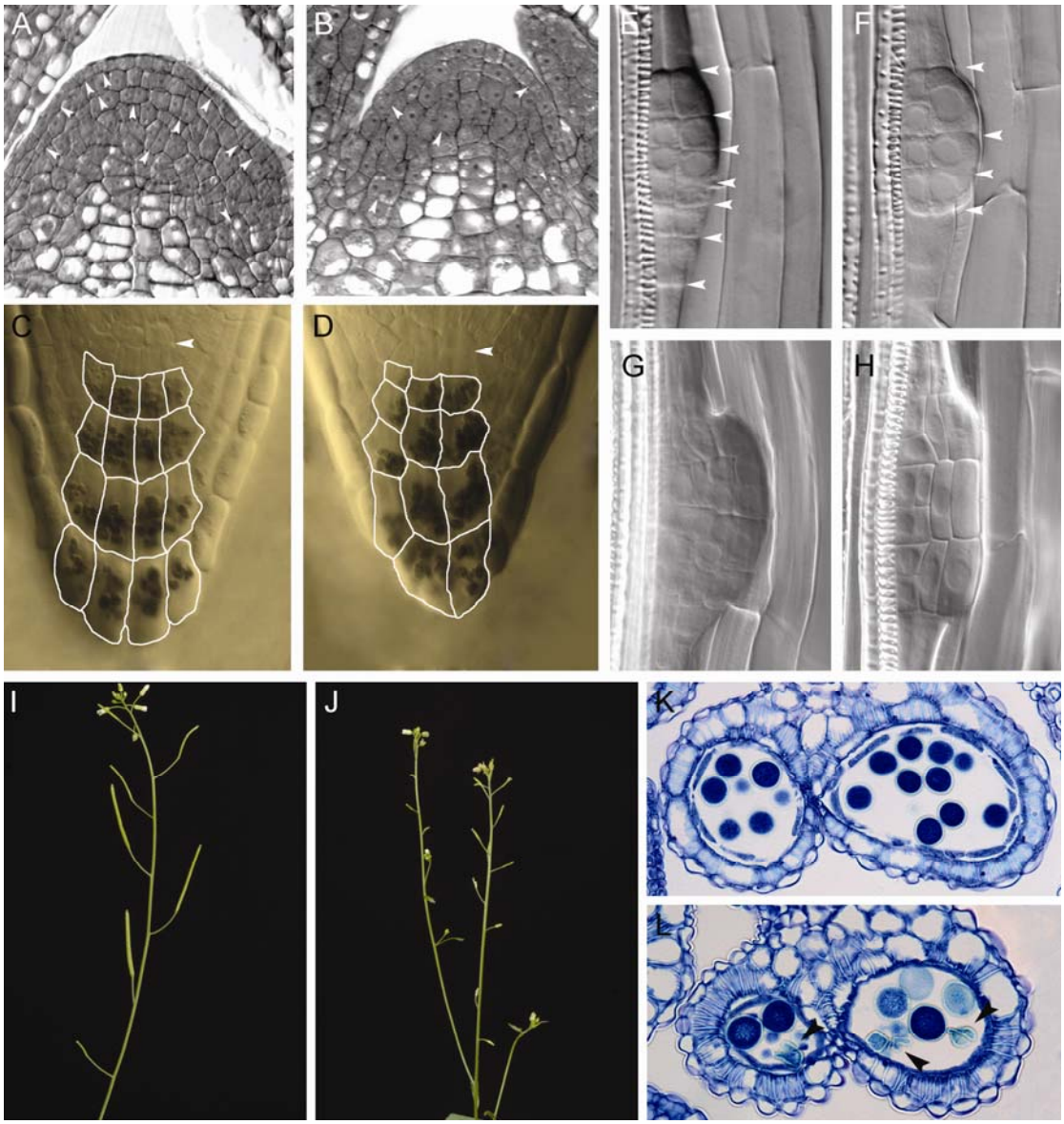


Figure 6.4 Anatomical and morphological defects in *cyca2;234*. (A,B) Section through shoot apical meristems of 10-day old (A) WT and (B) *cyca2;234* (Arrowheads indicate newly formed cell walls). (C,D) Lugol staining of root tips of (C) WT and (D) *cyca2;234* (Arrowheads indicate cell wall where quiescence centre contacts columella stem cells; white marking highlights columella columns). (E-F) DIC images of stage II lateral root primordium of (E) WT and (F) *cyca2;234* (Arrowheads indicate periclinal cell walls). (G,H) Stage V lateral root primordium of (G) WT and (H) *cyca2;234*. (I,J) Developing inflorescence of (I) WT and (J) *cyca2;234*. (K,L) Sections though pollen sacs of (K) WT and (L) *cyca2;234*, arrowheads indicate aborted pollen in the mutant.

A2-type cyclins repress differentiation in vascular tissue

Besides cambial activity during secondary growth, primary vascular tissues most often do not display any cell cycle activity. Nevertheless, several cell cycle genes are constitutively

expressed in these non-dividing tissues. Also for A2-type cyclins we could observe expression in vascular tissues of leaves (Fig. 6.6 A-D). Strongest expression was observed in young developing leaves. *CYCA2;1* and *CYCA2;4* showed highest expression in young vascular cells (Fig. 6.6 A,D). Furthermore, both *CYCA2;2* and *CYCA2;3* expression could be observed in cells associated to differentiated vascular cells (Fig. 6.6 B,C).

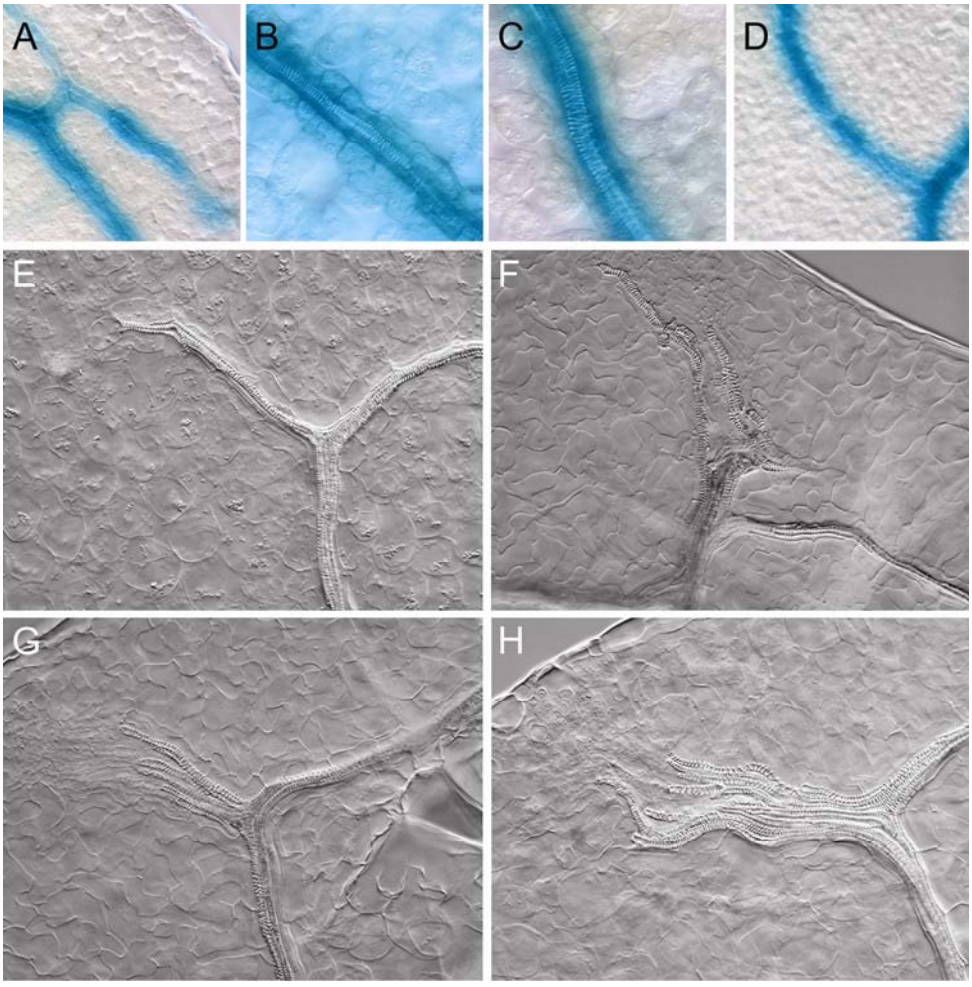


Figure 6.5 A2-type cyclin function in vascular differentiation. (A-D) GUS activity in vascular tissues for (A) *CYCA2;1::GUS*, (B) *CYCA2;2::GUS*, (C) *CYCA2;3::GUS* and (D) *CYCA2;4::GUS*. (E-H) Detail of vascular tissues near hydatodes in (E) WT, (F) *cyca2;124*, (G) *cyca2;134* and (H) *cyca2;234*.

Next, we analysed vascular development in the first leaf pair of 3 week old 1st leaves of WT, *cyca2;134*, *cyca2;124* and *cyca2;234* (Fig. 6.6 E-H). In wild type, the vasculature near hydathodes shows an open ending, consisting of a few xylem vessels, phloem and vascular parenchyma (Fig. 6.6 E). In all triple mutants analysed we could observe an excessive amount of xylem vessels in the proximity of hydathodes (Fig. 6.6 F-H). These data suggest that A2-type cyclins are involved in vascular differentiation.

Cotyledons show the most basic vascular pattern in the shoot. The pattern consists of a mid vein, two marginal primary veins each having one branch connecting back to the mid vein (Fig. 6.7 A). We found deviating vascular patterns in all triple mutants, with a tendency to become less complex in different mutant combinations (Fig. 6.7 C-D). The most rudimental vascular pattern was found in *cyca2;234* cotyledons, with only one mid vein and two marginal primary veins (Fig. 6.7 D).

Taken together, these data suggest that A2-type cyclins fulfil important roles in vascular differentiation and patterning.

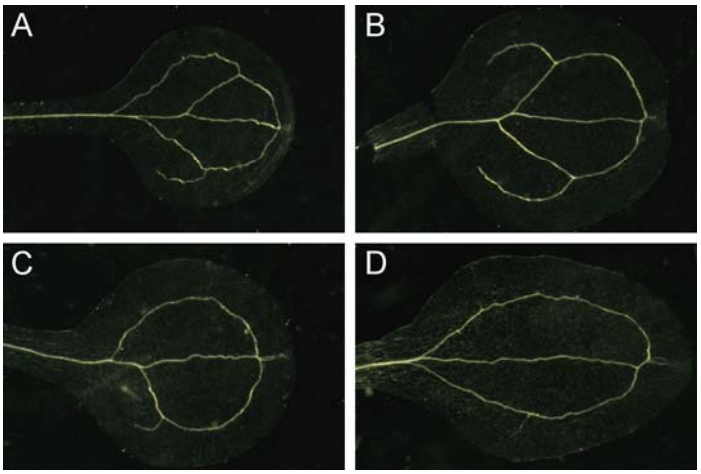


Figure 6.6 Vascular patterning defects in A2-type cyclin mutants. (A-D) Cotyledons of (A) WT, (B) *cyca2;124,* (C) *cyca2;134* and (D) *cyca2;234*.

CYCA2s are interaction partners for CDKA;1 and CDKBs

Given the proposed role for A2-type cyclins in cell cycle regulation we tested their potential for interaction with several CDKs through a Bimolecular Fluorescence Complementation assay (Fig. 6.4).

Fused to GFP all A2-type cyclins (Fig. 6.4 A-D), CDKA;1, CDKB1;1, CDKB1;2, CDKB2;1 (Fig. 6.4 E-H) and CDKB2;2 (Suppl. Fig. 6.3) localised in the nucleus of tobacco epidermal cells after transfection. Next to their nuclear localisation, CYCA2;2, CDKA;1, CDKB1;1, CDKB1;2 and CDKB2;2 also showed cytoplasmic localisation. In order to assay CYCA2-CDK interactions we fused each CYCA2 to one half of GFP and CDKs to the other half of GFP and performed co-transfection of tobacco leaves. Upon interaction of expressed proteins, fluorescent properties of GFP are restored and can be detected upon excitation. Using this technique (BiFC) (Walter *et al.*, 2004), we could demonstrate interaction between all A2-type cyclins and CDKA;1, CDKB1;1, CDKB1;2 and CDKB2;1 (Fig 6.4 I-X), but not

with CDKB2;2 (data not shown). All interactions were restricted to the nucleus. Interestingly, CYCA2-CDK interactions did not always occur uniformly throughout the nucleus, but showed remarkable speckles of high intensity within the nucleus. Taken together, our data suggest that A2-type cyclins can interact with several CDKs. Kinase assays will be required to quantify the contributions of A2-type cyclins to CDK kinase activities.

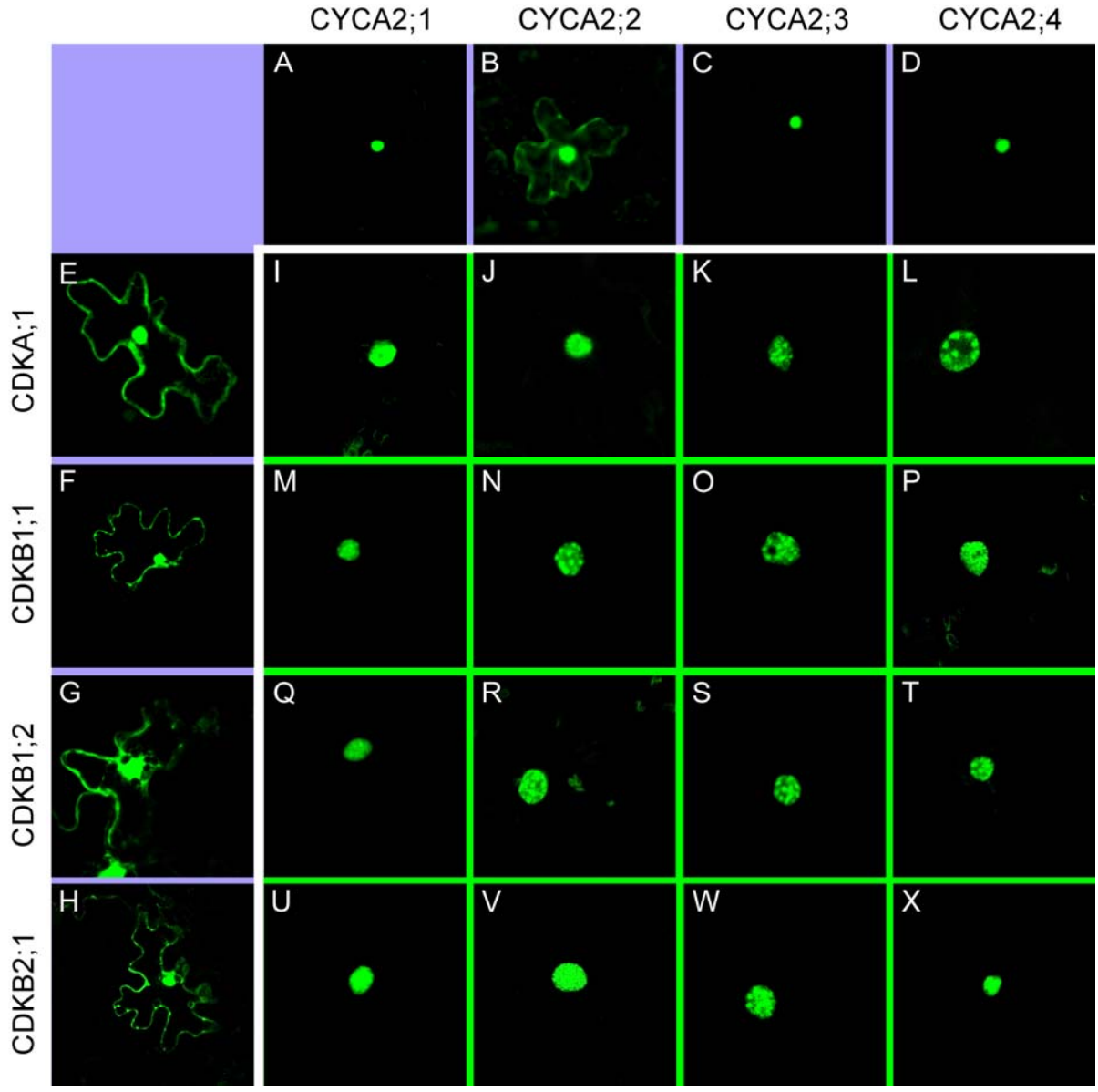


Figure 6.7 Localisation of A2-type cyclins and interactions with CDKs. (A-H) Subcellular localisation of (A) CYCA2;1-GFP, (B) CYCA2;2-GFP, (C) CYCA2;3-GFP, (D) CYCA2;4-GFP, (E) CDKA;1-GFP, (F) CDKB1;1-GFP, (G) CDKB1;2-GFP and CDKB2;1-GFP. (I-X) BiFC analysis for interactions between (I-L) CDKA;1, (M-P) CDKB1;1, (Q-T) CDKB1;2 and (U-X) CDKB2;1 with (I,M,Q,U) CYCA2;1, (J,N,R,V) CYCA2;2, (K,O,S,W) CYCA2;3 and (L,P,T,X) CYCA2;4.

DISCUSSION

Are CYCA2s cofactors to a Mitosis Inducing Factor?

In plants, ectopic overexpression of E2Fa-DPa transcription factors strongly stimulated S-phase progression, resulting in either excessive cell division cycles or in endoreduplication, depending on the cell type (De Veylder *et al.*, 2002). The tissues undergoing endoreduplication correlated with highly differentiated tissues, whereas those undergoing cell division corresponded to cell types with a high competence to divide. A model was proposed that postulated the existence of a Mitosis Inducing Factor (MIF) to be essential for the choice between cell division and endoreduplication. Interestingly, overexpression of a dominant negative allele of CDKB1;1 could suppress partially E2F-DP induced extra proliferation. Together with its peak of expression at G2-to-M boundary, it was proposed that CDKB1;1 could be part of such a MIF. Yet, overexpression of CDKB1;1 did not stimulate proliferation, suggesting that CDKB1;1 is not rate-limiting to mitosis. This is consistent with the notion that CDKs require binding to cyclins for full activity.

Triple mutants in CYCA2s showed defects in cell division and displayed enhanced levels of endoreduplication. All A2-type cyclins were expressed in tissues with high competence to divide, such as meristems and vascular tissue, and were down-regulated in differentiated tissues. Furthermore, overexpression of A-type cyclins strongly repressed endoreduplication, while stimulating cell division (Imai *et al.*, 2006; Yu *et al.*, 2003). (pollen) These data suggest that A2-type cyclins are rate-limiting to G2-to-M transition, thus fulfil, at least partial, MIF-functions.

A- and B-type CDK kinase activities peak during G2-to-M transition. Interestingly, their kinase activity strongly depends on binding to timely expressed cyclin cofactors. Indeed, A- and B-type cyclins are expressed during mitosis. Furthermore, CYCA2;2 and CYCA2;3 can interact with CDKA;1 (del Pozo *et al.*, 2002; Imai *et al.*, 2006). Moreover, we confirmed interaction between A2-type cyclins with CDKA;1 and could also show their interaction with several B-type CDKs through BiFC, including CDKB1;1. Together with the role for CDKB1;1 in cell cycle regulation it is likely that CYCA2-CDKB1;1 complexes form functional MIFs. However, our data does not exclude that other CYCA2-CDK complexes also act as MIFs.

A2-type cyclins and vascular development

Our *cyca2* mutants show strong defects in cell division at the level of G2-to-M transition as indicated by enhanced endoreduplication and meristem defects. Unexpectedly, we found aberrations both in vascular patterning and differentiation. As the vascular pattern is laid down through polar auxin flow, A2-type cyclins might act downstream of auxin signalling. Indeed, *CYCA2;4* has previously been reported to be highly auxin-inducible (Vanneste *et al.*, 2005).

During vascular differentiation auxin is known to play a dual role. Depending on tissue context, it stimulates either proliferation or differentiation. The peculiar phenotypic aspects observed in the triple mutants, allows us to postulate the following model for the role of A2-type cyclins in vascular development: Auxin is synthesized throughout young leaf primordia where it stimulates expression of cell cycle regulatory genes, such as A2-type cyclins (Vanneste *et al.*, 2005), and PIN auxin transport regulators (Scarpella *et al.*, 2006). Due to the self-regulatory nature of PIN expression, discrete paths of auxin transport are laid down (Sauer *et al.*, 2006; Scarpella *et al.*, 2006). This causes auxin to be drained towards these auxin transport routes, depleting the surrounding source cells and resulting in a downregulation of A2-type cyclins. The downregulation of the cyclins allows the differentiation of these cells into mesophyll tissue. Cells in the centre of the auxin paths are exposed to high auxin concentrations, which sets up a chain of events leading to differentiation as xylem and phloem. Cells at the edge of the auxin paths are exposed to medium high auxin concentrations and will differentiate into bundle sheath cells and maintain CYCA2 expression.

The strength of this model is illustrated by the fact that it can elegantly explain the observed vascular defects in the triple mutants. Firstly, in *cyca2* mutants, normal vascular patterning is disturbed, probably due to differentiation defects. Moreover, near the hydathodes, we observed ectopic xylem differentiation. Hydathodes are sites of auxin biosynthesis in the leaf. From these points auxin is taken up in the vascular system through bundle sheath cells, which maintain *CYCA2* expression. We suggest that this *CYCA2* expression represses the auxin-induced xylem differentiation. Due to loss of CYCA2 function, bundle sheath cells near the hydathodes differentiate into xylem vessels. As a consequence, auxin accumulates and stimulates the formation of new bundle sheath cells, that subsequently

differentiate into xylem. Iteration of this process results in the observed arrays of ectopic xylem in *cyca2* mutants.

CYCA2 function is required for terminal division in the stomatal lineage

During stomatal development, asymmetric and symmetric divisions occur in a highly coordinated fashion. After commitment to the stomatal pathway, a meristemoid mother cell divides asymmetrically giving rise to a meristemoid cell and a larger neighbouring cell. The meristemoid cell can undergo several rounds of asymmetric divisions before forming a guard mother cell. Next the guard mother cell divides symmetrically and differentiates, resulting in two guard cells that comprise the stoma (Nadeau & Sack, 2003).

Recently it was shown that overexpression of a dominant negative allele of CDKB1;1 showed a defective stomatal development, resulting in circular- and kidney-shaped cells with guard cell identity in the epidermis (Boudolf *et al.*, 2004a). The occurence of these aberrant cells in the epidermis appeared to be a consequence of a G2 cell-cycle arrest preceding guard cell formation of meristemoid cells and guard mother cells. It was concluded that CDKB-kinase activity is essential for meristemoid and guard mother cell division.

Our data suggest that A2-type cyclins can form complexes with CDKB1;1. Interestingly, *CDKB1;1*, *CYCA2;2* and *CYCA2;3* show overlapping promoter activity in the stomatal lineage, suggesting that CYCA2;2-CDKB1;1 and CYCA2;3-CDKB1;1 complexes can be formed during stomatal development. Consistently, we found in various *cyca2* mutant combinations stomatal defects similar to those described when CDKB kinase activity was impaired. Taken together our data strongly suggest that A2-type cyclins control terminal divisions in the stomatal lineage through regulation of CDKB1;1-kinase activity.

A role in terminal divisions for Cyclin A has previously been proposed in *Drosophila* embryogenesis (Reber et al., 2006). During embryogenesis most epidermal cells are programmed to stop dividing after 16 rounds of cell division. In these cells, Cyclin E expression is down-regulated to allow timely cell cycle exit. During G2-to-M transition, both Cyclin E and Cyclin A can repress activation of APC-mediated proteolysis of mitotic cyclins. During mitosis 16 the stability of mitotic cyclins solely depends on Cyclin A function. Loss of Cyclin A activity results in premature degradation of essential mitotic regulators (Cyclin B and cdc25), preventing terminal mitosis. Analogous to the Drosophila epidermal cell determination, a plant guard mother cell undergoes terminal division to form two mitotically

quiescent guard cells. We could show that loss of CYCA2 function prevents this terminal division suggesting that plant A2-type cyclins might fulfil evolutionary conserved functions in regulating terminal mitosis. However, it is not known whether plant A-type cyclins affect APC activity.

Regulatory hierarchy between cell fate establishment and cell division

As important as the maintenance of cell division activity, is the orchestrated regulation of cell cycle exit and the exact site and timing of cellular differentiation. It is obvious that during plant morphogenesis an intensive cross-talk is at play that guarantees optimal plant architecture. How the differentiation processes are intertwined with the core cell cycle machinery is currently not known.

When cell cycle progression is impaired in specific tissues of Drosophila and Xenopus, precursor cells acquire either daughter cell fates, or mixed cell fates (Harris and Hartenstein, 1991; Hartenstein and Posakony, 1990). In plants lacking SCARECROW transcription factor function, asymmetric division of cortex-endodermis precursors does not occur. Even in the absence of the division, characteristics of both endodermis and cortex are found in the resulting cell lineage (Di Laurenzio *et al.*, 1996).

The stomatal lineage shows a high coordination between asymmetric and symmetric divisions in which cell fates are readily established. We found that by slowing down cell division by mutating CYCA2s, stomatal precursors acquire guard cell identity without undergoing the normal terminal division. In this case terminal differentiation occurs independently of cell division. Also the lateral root developmental program proceeds, more or less normally even when specific divisions are missing. Nevertheless, all cell types are eventually specified to form a functional lateral root meristem, suggesting that other factors such as positional cues are at play during lateral root development. This suggests that also in plants, cell differentiation can be uncoupled from cell fate specification.

Interestingly, the FOUR LIPS (FLP) and FAMA transcription factors has been implicated in coordinating cell cycle exit and guard cell fate establishment (Lai *et al.*, 2005; Ohashi-Ito and Bergmann, 2006). The corresponding mutants shows extra cycles of symmetric division in guard mother cells and do not fully acquire guard cell identity. Moreover, FAMA overexpression leads to aberrant stomata (Ohashi-Ito and Bergmann,

2006), similar to those observed when CYCA2 function was lost. Therefore, it will be of interest to analyse if CYCA2s are targets of FLP and/or FAMA.

MATERIALS & METHODS

Plant material and growth conditions

In this study we used *Arabidopsis* seedling of the ecotype Col-0 and L*er* and mutants for the various A2-type cyclins from publicly available collections. *Arabidopsis* seedlings were grown under continuous light conditions at 18-25 °C on 0.5xMS with sucrose.

Mutant description and genotyping

We acquired insertions in the first and third exon of *CYCA2;1* (SALK_121077 = *cyca2;1-1* and SALK_123348 = *cyca2;1-2*), an insertion in 10th exon of *CYCA2;2* (GABI_120D03 = *cyca2;2-1*), insertions in first and second exon of *CYCA2;3* (SALK_092515 = *cyca2;3-1*, SALK_086463 = *cyca2;3-1* and SALK_043246 = *cyca2;3-3*) and insertions in first and third exon of *CYCA2;4* (SALK_070301 = *cyca2;4-1* and GAT_5.10009 = *cyca2;4-2*) (Fig. 6.2 A).

For detection of T-DNA inserts we used primers specific to the left border of the T-DNAs used for mutagenesis (LBC1, LB_GABI and LB_EXOTIC) in combination with gene-specific primers (Table 1) The alleles cyca2;1-1, *cyca2;2-1*, *cyca2;3-1* and *cyca2;4-1* are representative knock-out alleles and have been used for analysis unless stated otherwise.

RNA extraction and Real-Time PCR

RNA was extracted with the RNeasy kit (Qiagen). Poly(dT) cDNA was prepared from 1 µg total RNA with the Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and quantified on an iCycler apparatus (Bio-Rad, Hercules, CA) with the Platinum SYBR Green qPCR Supermix-UDG kit (Invitrogen, Belgium). PCR was carried out in 96-well optical reaction plates heated for 10 minutes to 50°C to allow UNG activity, followed by 10 minutes of 95°C to activate hot start Taq DNA polymerase, and 40 cycles of denaturation for 60 seconds at 95°C and annealing-extension for 60 seconds at 58°C. Target quantifications

were performed with specific primer pairs designed using Beacon Designer 4.0 (Premier Biosoft International, Palo Alto, CA). Expression levels were normalized to EF-1-alfa (At1g07940) expression levels. All RT-PCR experiments were performed in triplicates and the data was processed using qBase v1.3.4 (Hellemans *et al.*, 2007).

Histochemical staining and anatomical analysis

The β-glucuronidase (GUS) assays were performed as described (Beeckman and Engler, 1994). For microscopic analysis, samples were cleared by mounting in 90% lactic acid (Acros Organics, Brussels, Belgium) (analysis of GUS stainings) or using the clearing method described by Malamy and Benfey (1997). Lugol staining was performed as described (Sabatini *et al.*, 1999). All samples were analyzed by differential interference contrast microscopy (Leica DMLB, Leica, Vienna, Austria).

For anatomical sections, GUS-stained samples were fixed overnight in 1% glutaraldehyde and 4% paraformaldehyde in 50 mM phosphate buffer (pH 7). Samples were dehydrated and embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's protocol. For proper orientation of the samples, we used a two-step embedding methodology, based on a pre-embedding step to facilitate orientation in 0.5-ml Eppendorf tubes (De Smet *et al.*, 2004). Sections of 5 μm were cut with a microtome (Minot 1212; Leitz, Wetzlar, Germany), dried on Vectabond-coated object glasses, counterstained for cell walls with 0.05% ruthenium red for 8 minutes (Fluka Chemica, Buchs, Switzerland), and rinsed in tap water for 30 s. After drying, the sections were mounted in DePex medium (British Drug House, Poole, UK) and covered with cover slips.

Photographs were taken with a CAMEDIA C-3040zoom digital camera (Olympus, Tokyo, Japan) and processed with Photoshop 7.0 (Adobe, San José, CA).

Flow cytometry

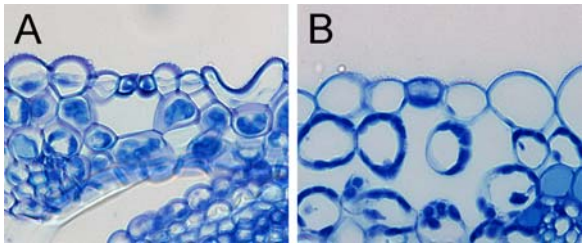
Flow cytometry was done on 1st leaves or cotyledons of 3-week old seedlings using a CyFlow® ML (Partec) flow cytometer as described (Galbraith *et al.*, 1983).

Bimolecular Fluorescence Complementation (BiFC)

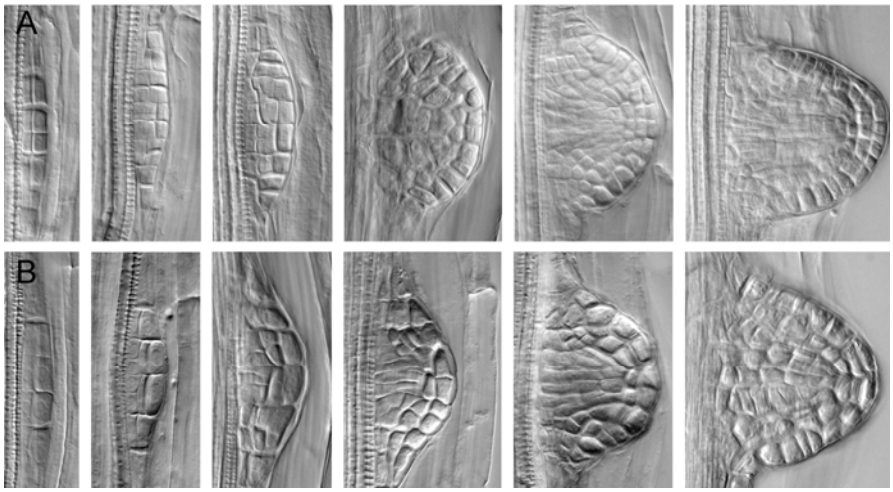
We use the bimolecular fluorescence complementation (BiFC) approach developed by Hu et al. (2002) for simple and direct visualization of protein interactions in living cells. The BiFC approach is based on the formation of a fluorescent complex when two fragments of a fluorescent protein are brought together by an interaction between proteins fused to the fragments. For the BiFC analysis, GFP was truncated at residue 465 and split in two parts: N-terminal part consisting of 465 bp and C-terminal part consisting of 252 bp and cloned into pDONRp2Rp3 entry clones, making them multisite Gateway compatible. Furthermore, ORFs for CYCA2;1, CYCA2;2, CYCA2;3, CYCA2;4, CDKA;1, CDKB1;1, CDKB1;2, CDKB2;1 and CDKB2;2 were cloned without stop-codon in pDONR221 for rapid subcloning with the various parts of/and full length GFP

The abaxial epidermis of young leaves of 2 to 4-week old *Nicotiana benthamiana* plants were infiltrated with a dilution series of LBA4404 *Agrobacterium tumefaciens* harbouring the various constructs. Fluorescence was assessed 3 to 5 days after infiltration with a LSM510 confocal laser scanning microscope. As controls, the ORFs fused to full eGFPs were assayed in this system.

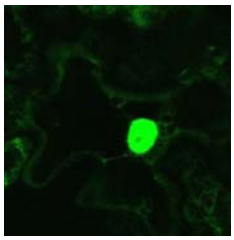
Supplemental data



Supplemental Figure 6.1 Sections through stomatal pore of (A) WT and (B) *cyca2;234*



Supplemental Figure 6.2 Stages of lateral root development of (A) WT and (B) *cyca2;234*



Suplemental Figure 6.1 Localisation of CDKB2;2-GFP in tobacco epidermal cells

Acknowledgements

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CHAPTER 7 Conclusions and perspectives

Lateral root initiation is the event in which two adjacent xylem pole pericycle cells undergo coordinated asymmetric divisions. The plant signalling molecule auxin is a major regulator of lateral root development and is involved in all steps of this developmental process. However, little is known on how auxin exerts its regulatory effect on lateral root development. Furthermore, lateral root initiation provides an excellent model to answer fundamental developmental questions such as positional patterning, growth axis establishment as well as respecification of cell fate and cell polarity. Therefore it is of high importance to study the molecular mechanisms of root branching.

Transcript profiling as a tool to study lateral root initiation

Auxin is one of the main regulators of lateral root development. Central to the major, identified auxin signalling mechanisms is the de-repression of specific transcription factors, suggesting that auxin exerts its effect through transcriptional regulation. Therefore we aimed to identify auxin signalling components through a time-course based transcript profiling on specific root fragments. Microarray technology allows analysing transcriptional changes at a near genome-wide scale (more or less 22,000 probe sets) in a single experiment, resulting in an enormous amount of data to process before coming to candidate genes. The use of time-courses allowed us to follow timely differences, and served at the same time as internal controls to reveal inductive or repressive trends in transcriptional activity.

Using very stringent selection criteria ($P < 0.001$), we retained over 3000 significantly modulated genes. This suggested that our samples showed a high reproducibility, and thus provides data of very high quality. Indeed, we were able to identify several known genes that were previously not characterised as auxin inducible i.e. genes encoding auxin transporters (PINs, AUX1, LAX3). Furthermore, several other interesting genes were differentially expressed in this dataset. For example, the guard cell differentiation factor FOUR LIPS (Lai *et al.*, 2005), several AP2 genes that are involved in cytokinin signalling (Rashotte *et al.*, 2006), a WRKY transcription factor involved in primary root patterning (W. Grunewald, G. Gheysen & T. Beeckman, unpublished results) and several cell cycle regulators. Phenotypic analysis of mutants of these genes suggests that most of them are important regulators of lateral root initiation.

Taken together, this suggests that our transcript profiling dataset might contain several other key regulators of auxin signalling and lateral root organogenesis. However, one should be aware that post-transcriptional signalling events will also be at play and can not be detected through transcript profiling.

A role for auxin in stomatal development?

One of the most intriguing findings of our transcript profiling was the observation that the guard cell differentiation factor FOUR LIPS (FLP) was strongly induced by auxin in roots (Chapter 5). We found evidence that FLP indeed plays a role in lateral root development through regulation of expression of polar auxin transporters. It is recruited to the chromatin of

the promoter of the *PIN3* auxin efflux facilitator, where it might be involved in stimulating *PIN3* expression. The observations we made in the root, raise the question whether auxin also is involved in stomatal development. So far, little or nothing has been reported on a function for auxin in stomatal development. Yet, several auxin inducible genes are expressed in developing stomata (pers. comm. F. Sack).

We postulate here a presumptive model for the role of auxin in FLP mediated stomatal development. Also in the stomatal lineage, auxin acts as a mitogenic signal and triggers cell division. At the time that the guard mother cell divides to give rise to two guard cells, auxin also induces *FLP* expression. FLP promotes auxin efflux through upregulation of polar auxin transport components (such as PIN3), which results in removal of the mitogenic auxin and saveguards the terminal differentiation into guard cells.

FLP coordinates differentiation and cell division through negative regulation of CYCA2s?

We found that mutants in CYCA2s lack specific cell divisions in lateral root and stomatal development (Chapter 6). Moreover, cell differentiation proceeded as if cell division had occurred normally. This is in contrast to the phenotypes of *flpmyb88* mutants, which have ectopic guard mother cell divisions (Lai *et al.*, 2005) and ectopic divisions during lateral root development (Chapter 5). This suggests that FLP acts to restrict cell division through negatively regulating CYCA2 expression while allowing progression of cell differentiation. It is not yet clear whether FLP mediates this effect directly or indirectly. For this purpose, we will test *CYCA2* expression levels in *flpmyb88* mutants and analyse lateral root and stomatal phenotypes of *flpmyb88 cyca2* mutants.

These experiments will greatly enhance our insights into the coordination of cell division and differentiation.

Future perspectives

This work has greatly contributed to our insights in lateral root development. Moreover, we found several parallels to other developmental programs, such as stomatal development. Therefore, the characterisation of other genes identified through this transcript profiling is very likely to further broaden our understanding of lateral root initiation and

stomatal development. Recently, we have found *in vivo* evidence for coordinated nuclear movement in xylem pole pericycle cells prior to lateral root initiation. Upon tissue-specific inhibition of the auxin response in the xylem pole pericycle by transactivation of mIAA17, lateral root initiation was efficiently inhibited. However, a strong enrichment of dislocated nuclei were observed (De Smet *et al.*, 2007), suggesting that this process is regulated independently of the classical auxin signalling cascade. Therefore, genetic screens will be required to complement the transcriptional characterisation of lateral root initiation.

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SUMMARY

Most plants rely on roots for uptake of water and nutrients and anchorage. The root architecture can be elaborated through formation of branches, called lateral roots. The more a root system is branched, the more efficient it can fulfil its functions. Furthermore, root branching provides an excellent model to answer fundamental developmental questions such as positional information during patterning, growth axis establishment as well as respecification of cell fate and cell polarity. Therefore it is of high importance to study the molecular mechanisms of root branching.

Endogenous and environmental cues are integrated to control the formation of a new lateral root. In *Arabidopsis thaliana*, lateral roots originate from a specific tissue within the root, the pericycle. The first events physically hallmarking the formation of a new lateral root (lateral root initiation) are coordinated asymmetric divisions of two neighbouring xylem pole pericycle cells (Beeckman *et al.*, 2001). The plant signalling molecule auxin is a major regulator of lateral root development and is involved in all steps of this developmental process (Blakely *et al.*, 1988). However, little is known on how auxin exerts its regulatory effect on lateral root development (**Chapter 1 & 2**).

My PhD work was aimed at identifying and characterizing new molecular regulators of lateral root development. One of the major challenges was to perform transcript profiling on the early steps of auxin-mediated lateral root initiation. For this purpose we needed to control lateral root initiation. Therefore, we developed a lateral root inducible system (LRIS), in which we were able to synchronize lateral root initiation. Inhibition of lateral root initiation events was achieved through chemical interference with polar auxin transport, followed by exogenous auxin application that triggered lateral root initiation events across the length of the root (Himanen *et al.*, 2002). An exploratory transcript profiling, covering about 4600 genes, demonstrated the power of this approach as it allowed a timely separation of signalling events and cell cycle activation (Himanen *et al.*, 2004).

Next, we used the LRIS in a near genome-wide transcript profiling to compare auxin responses between roots of the wild type and an auxin-insensitive mutant, *solitary-root-1* (*slr-1*), which is impaired in lateral root initiation (Vanneste *et al.*, 2005). Our data showed that auxin-dependent activation of cell cycle was strongly defective in *slr-1*. In order to test the hypothesis that cell cycle stimulation in the pericycle would be sufficient for lateral root development, we overexpressed G1-to-S stimulators CYCD3;1 and E2Fa/DPa in *slr-1*.

Instead of inducing lateral roots, we merely induced proliferative cell divisions in the pericycle, suggesting that cell cycle activation is not sufficient for auxin-induced lateral root initiation (Vanneste *et al.*, 2005).

In order to further investigate the role of cell cycle regulators in lateral root development we addressed the role of the A2-type cyclin family by mutant analysis. In specific triple mutant combinations we could find a strong reduction in lateral root density. Furthermore, we observed that in some triple mutants lateral root primordia were composed of fewer cells. Despite the defect in cell number, all cell types defining a functional root meristem were present, suggesting that cell fate specification occurs independently of cell cycle progression. Furthermore, in the epidermis of CYCA2 mutants we detected unicellular structures that displayed guard cell characteristics. In accordance with the lateral root primordia in the mutants, these aberrant stomata might also be the result of cell fate establishment uncoupled from cell cycle progression (**Chapter 6**).

Work of other laboratories complemented our findings and identified mutants or conditions, in which cell fate specification in developing lateral roots was impaired (Benková *et al.*, 2003; Geldner *et al.*, 2004). Both reports indicated that transport-dependent local auxin accumulation is essential for cell fate specification during lateral root development. Within developing lateral roots, polar auxin transport regulators of the PIN family show distinct and overlapping expression patterns (Benková *et al.*, 2003). Our near-genome-wide microarray analysis provides further important insights into this mechanism. We found that *PIN* genes were amongst the earliest auxin inducible genes, which are upregulated during lateral root initiation. Moreover, we could show that regulation of PIN transcription is downstream of the classical auxin signalling cascade in which auxin regulates transcription through destabilisation of labile repressors (Vieten *et al.*, 2005). This finding has been shown to be an essential component in modelling auxin transport-dependent development (Jonsson *et al.*, 2006). Moreover, our microarray data revealed that the AUX1- auxin influx carrier homolog, LAX3, was auxin inducible. Recently, Swarup et al. (submitted) showed that LAX3 is involved in regulating lateral root emergence. LAX3 allows auxin to be taken up into the cortex and endodermis, where it triggers transcriptional activation of cell wall modifying enzymes.

In addition to the cell cycle regulators and auxin transport components we pinpointed several transcription factors, which act downstream of auxin and which play a potential important role in auxin signalling. Among these, we found the guard-cell-differentiation transcription factor, FOUR LIPS/MYB124 (Lai *et al.*, 2005). Asides being regulated by auxin,

we also showed that it is expressed in all stages of lateral root development. Moreover, FLP function was found to be involved in restricting cell divisions in developing lateral roots. The mutant phenotype was reminiscent of defects in polar auxin transport. Indeed, we could show that FLP is recruited to chromatin of PIN3 promoter, where it can act as an enhancer of PIN3 transcription providing new insights into the mechanisms of auxin-driven lateral root patterning (**Chapter 5**).

In conclusion, our detailed analysis of early auxin-induced transcripts in roots has led to several important new insights in lateral root initiation. Therefore, it will be of interest to further explore this dataset for other important regulators of lateral root development.

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SAMENVATTING

Met behulp van hun wortels zijn planten in staat zich te verankeren in de bodem, waar ze ook dienen voor de opname van water en nutriënten en verankering in de bodem. Het volume bodem dat door de wortels efficiënt gebruikt wordt, hangt in sterke mate af van de complexiteit van het wortelsysteem. De complexiteit van wortelarchitectuur kan verhoogd worden door de vorming van vertakkingen, zijwortels. Naast het fundamentele biologisch belang van zijwortelvorming voor planten, kan dit ontwikkelingsproces ook dienen als een uitstekend model om cruciale vragen te beantwoorden zoals, welke positionele signalen liggen aan de basis van patroonvorming, groei-as-bepaling en de herspecificatie van celidentiteit en –polariteit. Daarom is het belangrijk om de moleculaire mechanismen van zijwortelvorming te bestuderen.

Endogene en omgevingsfactoren controleren samen de vorming van een nieuwe zijwortel. In *Arabidopsis thaliana*, ontstaan zijwortels in een specifiek weefsel binnenin de wortel, de pericyclus. De eerste gebeurtenissen die geobserveerd kunnen worden tijdens zijwortelvorming zijn de gecoördineerde asymmetrische celdelingen in twee aangrenzende xyleempool-geassocieerde pericycluscellen (Beeckman *et al.*, 2001). De signaal molecule in planten, auxine, is één van de belangrijkste regulatoren van zijwortelontwikkeling en is betrokken in alle stappen van dit ontwikkelingsproces (Blakely *et al.*, 1988). Desalniettemin is er weinig geweten over de moleculaire mechanismen waarmee auxine zijwortelvorming beïnvloedt (**Chapter 1 & 2**).

Mijn doctoraatsonderzoek was gericht op de identificatie en karakterisering van nieuwe moleculaire regulatoren van zijwortelontwikkeling. Eén van de grootste uitdagingen was de analyse van transcriptionele veranderingen tijdens de vroege stappen van auxine-geïnduceerde zijwortelvorming. Om dit gericht te kunnen doen hebben we een systeem ontwikkeld om dit proces te manipuleren. Met behulp van het zijwortelinduceerbaar systeem (ZWIS) zijn we in staat zijwortelinitiatie gesynchroniseerd te induceren over gans de lengte van de wortel (Himanen *et al.*, 2002). Via transcriptionele analyse op ongeveer 4600 genen konden we aantonen dat binnen het ZWIS, signaaltransductie en celcyclusactivatie van elkaar konden gescheiden worden in de tijd (Himanen *et al.*, 2004).

Vervolgens gebruikten we het ZWIS in een bijna genoom-wijde analyse van transcriptionele veranderingen die optreden in wortels van wild type en een auxine-

ongevoelige mutant, *solitary-root-1* (*slr-1*), die geen zijwortels kan maken (**Hoofdstuk 3;** Vanneste *et al.*, 2005). Onze data toonde aan dat auxine-afhankelijke celcyclusactivatie sterk verstoord was in *slr-1*. Om te testen of celdelingsstimulatie in de pericyclus voldoende zou zijn om het zijworteldefect in *slr-1* te complementeren, hebben we de G1-S regulatoren, CYCD3;1 en E2Fa/DPa, tot overexpressie gebracht in deze mutant. In plaats van het induceren van zijwortels, kon enkel proliferatieve celdeling waargenomen worden in de pericyclus. Dit suggereerde dat celdeling niet voldoende is voor auxine-geïnduceerde zijwortelvorming (Vanneste *et al.*, 2005).

Om de rol van celcyclusregulatoren in zijwortelontwikkeling verder te onderzoeken analyseerden we de functie van de CYCA2 genfamilie via mutanten. In specifieke drievoudige mutanten vonden we een sterke reductie in zijworteldensiteit. Daarenboven vonden we dat zijwortels van de mutanten opgebouwd waren uit minder cellen. Desondanks het cellulaire defect, waren alle celtypes van een functioneel zijwortelmeristeem aanwezig. Dit suggereert dat celidentiteit bepaald wordt, onafhankelijk van celdelingsvoortgang. In de epidermis van CYCA2 mutanten vonden we unicellulaire structuren die sluitcelkarakteristieken vertoonden. Dit is in overeenstemming met de zijworteldefecten in de mutanten aangezien ook hier celidentiteitsbepaling en celdelingsvoortgang onafhankelijk gebeurden (**Hoofdstuk 6**).

In de literatuur werden mutanten en behandelingen beschreven waarin celidentiteitsbepaling in ontwikkelende zijwortels verstoord was (Benková *et al.*, 2003; Geldner *et al.*, 2004). Beide rapporten toonden aan dat transport- afhankelijke lokale auxine-accumulatie essentieel is voor celidenteiteitsbepaling tijdens zijwortelvorming. Regulatoren van polair auxine-transport van de PIN-familie hebben specifieke en overlappende expressie patronen tijdens zijwortelvorming (Benková *et al.*, 2003). Onze bijna-genoom-wijde analyse van transcriptionele veranderingen tijdens zijwortelinitiatie leverde ook in belangrijke mate bij tot nieuwe inzichten in dit mechanisme. We vonden dat PIN-genen bij de vroegst auxine-geïnduceerde genen horen. Daarenboven vonden we dat hun transcriptionele opregulatie afhankelijk was van de klassieke auxinesignaalcascade. In deze signaaltransductieweg beïnvloedt auxine transcriptie door de destabilisering van labiele repressoreiwitten (**Hoofdstuk 4**; Vieten *et al.*, 2005). Deze bevinding bleek uiteindelijk een essentiële component te zijn voor computermodellen van auxinetransport-afhankelijke ontwikkelingsprocessen (Jonsson *et al.*, 2006). Daarenboven vonden we dat ook het AUX1 homoloog LAX3 auxine-induceerbaar was. Onlangs werd door Swarup *et al.* (submitted) aangetoond dat LAX3 betrokken is in zijworteluitgroei. LAX3 is essentieel voor de opname

van auxine in de endodermis- en cortexcellen in de nabijheid van zijwortelprimordia, waar het vervolgens transcriptie van celwandmodificerende enzymen stimuleert, zodat de zijwortel kan uitgroeien.

Bovenop de celcyclusregulatoren en auxinetransport-componenten hebben we verschillende transcriptiefactoren geïdentificeerd die potentieel een belangrijke rol spelen in auxinesignalisatie. Zo vonden we dat de stomatale differentiatie transcriptiefactor, FOUR LIPS/MYB124 (Lai *et al.*, 2005), naast de eigenschap van auxine-induceerbaar te zijn, eveneens tot expressie komt in alle stadia van zijwortelvorming. De mutant vertoonde zijworteldefecten die deden denken aan defecten in polair auxinetransport. We konden inderdaad aantonen dat FLP gerecruteerd wordt naar het chromatine in de PIN3-promotor, waar het PIN3-transcriptie stimuleert. Deze bevinding verhoogt ons inzicht in de mechanismen van auxine-gestuurde zijwortelvorming (**Hoofdstuk 5**).

Tot besluit kunnen we stellen dat onze gedetaileerde analyse van vroege auxine-geïnduceerde transcripten in wortels geleid heeft tot verscheidene belangrijke nieuwe inzichten in zijwortelinitiatie. Het zal dus interessant zijn om in de verkregen dataset te zoeken naar belangrijke regulatoren van zijwortelontwikkeling.

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ADDITIONAL PUBLICATIONS

Auxin-mediated cell cycle activation during early lateral root initiation.

Himanen, K., Boucheron, E., Vanneste, S., de Almeida Engler, J., Inzé, D., and Beeckman, T.

Abstract

Lateral root formation can be divided into two major phases: pericycle activation and meristem establishment. In Arabidopsis, the first lateral root initiation event is spatially and temporally asynchronous and involves a limited number of cells in the xylem pericycle. To study the molecular regulation during pericycle activation, we developed a lateral root-inducible system. Successive treatments with an auxin transport inhibitor and exogenous auxin were used to prevent the first formative divisions and then to activate the entire pericycle. Our morphological and molecular data show that, in this inducible system, xylem pericycle activation was synchronized and enhanced to cover the entire length of the root. The results also indicate that the inducible system can be considered a novel in planta system for the study of synchronized cell cycle reactivation. In addition, the expression patterns of Kip-Related Protein2 (KRP2) in the pericycle and its ectopic expression data revealed that the cyclin-dependent kinase inhibitor plays a significant role in the regulation of lateral root initiation. KRP2 appears to regulate early lateral root initiation by blocking the G1-to-S transition and to be regulated transcriptionally by auxin.

Article in: Plant Cell, 14, 2339-2351 (2002)

An easy and versatile embedding method for transverse sections.

De Smet, I., Chaerle, P., Vanneste, S., De Rycke, R., Inzé, D., and Beeckman, T.

Abstract

In several research areas, transverse sections are indispensable for studying structural aspects of specimens. However, the oriented embedding of small cylindrical samples can become problematic, especially when transverse sections at right angles to the main axis of the object are desired. Here, we describe an easy and low-cost technique for oriented embedding of small (< 500 mum) as well as of larger specimens (> 500 mum). The usefulness of the technique is demonstrated for roots and stamens of Arabidopsis thaliana and for adventitious roots of Asplenium demerkense, as examples of small and larger cylindrical samples, respectively. Furthermore, several types of resin (glycol methacrylate, epoxy and acrylic resins) were successfully tested, showing the applicability of the technique for light and electron microscopy and for immunolocalizations. In conclusion, the principle of the technique can be extended to several resins and a wide variety of specimen types, such as stems, leaves and textile fibres. The originality of the technique lies in its simplicity combined with its high efficiency to produce well-oriented transverse sections.

Article in: Journal of Microscopy-Oxford 21, 76-80 (2004)

Transcript profiling of early lateral root initiation.

Himanen, K., Vuylsteke, M., Vanneste, S., Vercruyse, S., Boucheron, E., Alard, P., Chriqui, D., Van Montagu, M., Inzé, D., and Beeckman, T.

Abstract

At the onset of lateral root initiation in Arabidopsis thaliana, the phytohormone auxin activates xylem pole pericycle cells for asymmetric cell division. However, the molecular events leading from auxin to lateral root initiation are poorly understood, in part because the few responsive cells in the process are embedded in the root and are thus difficult to access. A lateral root induction system, in which most xylem pole pericycle cells were synchronously activated by auxin transport inhibition followed by auxin application, was used for microarray transcript profiling. Of 4,600 genes analyzed, 906 significantly differentially regulated genes were identified that could be grouped into six major clusters. Basically, three major patterns were discerned representing induced, repressed, and transiently expressed genes. Analysis of the coregulated genes, which were specific for each time point, provided new insight into the molecular regulation and signal transduction preceding lateral root initiation in Arabidopsis. The reproducible expression profiles during a time course allowed us to define four stages that precede the cell division in the pericycle. These early stages were characterized by G, cell cycle block, auxin perception, and signal transduction, followed by progression over G(1)/S transition and G(2)/M transition. All these processes took place within 6 h after transfer from N-1-naphthylphthalamic acid to 1-naphthalene acetic acid. These results indicate that this lateral root induction system represents a unique synchronized system that allows the systematic study of the developmental program upstream of the cell cycle activation during lateral root initiation.

Article in: Proceedings of the National Acadamy of Sciences USA 101, 5146-5151 (2004)

Lateral root initiation or the birth of a new meristem.

De Smet, I., Vanneste, S., Inzé, D. & Beeckman, T.

Abstract

Root branching happens through the formation of new meristems out of a limited number of pericycle cells inside the parent root. As opposed to shoot branching, the study of lateral root formation has been complicated due to its internal nature, and a lot of questions remain unanswered. However, due to the availability of new molecular tools and more complete genomic data in the model species Arabidopsis, the probability to find new and crucial elements in the lateral root formation pathway has increased. Increasingly more data are supporting the idea that lateral root founder cells become specified in young root parts before differentiation is accomplished. Next, pericycle founder cells undergo anticlinal asymmetric, divisions followed by an organized cell division pattern resulting in the formation of a new organ. The whole process of cell cycle progression and stimulation of the molecular pathway towards lateral root initiation is triggered by the plant hormone auxin. In this review, we aim to give an overview on the developmental events taking place from the very early specification of founder cells in the pericycle until the first anticlinal divisions by combining the knowledge originating from classical physiology studies with new insights from genetic-molecular analyses. Based on the current knowledge derived from recent genetic and developmental studies, we propose here a hypothetical model for LRI.

Review in: Plant Molecular Biology 60: 871-887 (2006)

Auxin-dependent regulation of lateral root positioning in the basal meristem of Arabidopsis.

De Smet, I., Tetsemura, T., De Rybel, B., Frei dit Frei, N., Laplaze, L., Casimiro, I., Swarup, R., Naudts, M., Vanneste, S., Audenaert, D., Inzé, D., Bennett, M.J. & Beeckman, T..

Abstract

In plants, the developmental mechanisms that regulate the positioning of lateral organs along the primary root are currently unknown. We present evidence on how lateral root initiation is controlled in a spatiotemporal manner in the model plant Arabidopsis thaliana. First, lateral roots are spaced along the main axis in a regular left-right alternating pattern that correlates with gravity-induced waving and depends on AUX1, an auxin influx carrier essential for gravitropic response. Second, we found evidence that the priming of pericycle cells for lateral root initiation might take place in the basal meristem, correlating with elevated auxin sensitivity in this part of the root. This local auxin responsiveness oscillates with peaks of expression at regular intervals of 15 hours. Each peak in the auxin-reporter maximum correlates with the formation of a consecutive lateral root. Third, auxin signaling in the basal meristem triggers pericycle cells for lateral root initiation prior to the action of INDOLE-3-ACETIC ACID14 (SOLITARY ROOT).

Article in: Development 134: 681-690 (2007)