Regulation of stem cell maintenance and cell differentiation states in *Arabidopsis* root development

Het reguleren van stamcellen en celdifferentiatie

tijdens de

wortelontwikkeling van *Arabidopsis thaliana*

(met een samenvatting in het Nederlands)

Proefschrift

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Voor mijn ouders

“Forty-six and two are just ahead of me”
MJK, 1996

Cover: Expression of PLETHORA2 in the shoot apex induces the production of root (like) tissue instead of new leaves. Designed by Fred Zurel, F&N Eigen Beheer, Amsterdam.

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Contents

Preface

During the development, growth and maintenance of an organ, a multitude of internal and external signals need to be collectively integrated and processed into the appropriate genetic response. Transcription factors often play essential roles in directing the genetic response program by controlling the rate of downstream gene transcription. In plants, transcription factors have been identified in almost every thinkable aspect of growth and development; often because identification is simplified by their pronounced loss-of-function effect. For example, loss of WUSCHEL (WUS) function, a homeobox transcription factor expressed in the organizing centre of the shoot stem cell niche, results in plants with prematurely terminated shoot organs. In roots, knockout of the GRAS transcription factors SHORT ROOT (SHR) and SCARECROW (SCR) or AP2-domain transcription factors PLETHORA1 (PLT) and PLT2 results in misspecification of the stem cell organizer and cessation of root growth. To further dissect these complex cellular systems it is essential to identify the transcription factors involved and reveal the genetic downstream programs regulated by these developmental control genes.

The experiments presented in this thesis topic the role of transcription factor family members in regulating growth, development, and maintenance of the *Arabidopsis* root. We demonstrate a conserved homeobox transcription factor regulates distal stem cell maintenance and expand the notion that the PLT family of transcription factors specifically regulates stem cell properties to a significantly broader role in root development. In addition, we show that members of the *PLT* gene family can activate transcriptional targets in a dose-dependent fashion to establish a feed-forward network that regulates root growth and development.

Chapter **one** reviews the molecular genetical approaches that have been adopted to address *Arabidopsis* root development. These efforts have resulted in the identification of numerous genes, involved in as many regulatory processes of root growth and development. Incidentally, conserved mechanisms and genetic factors that act in root and shoot growth have been uncovered, revealing general principles of plant development. Transport-mediated graded distribution of the phytohormone auxin, for example, acts as a global organizer that is locally translated into distinct cellular responses by specific pairs of signaling mediators. In this chapter, we focus on recent advances in our understanding of *Arabidopsis* root development. Taking embryogenesis as a starting point, we describe the genes and mechanisms involved in root meristem and stem cell patterning and maintenance.

In chapter **two** we demonstrate a role for the homeobox gene *WOX5* (*WUSCHEL-RELATED 5*), a homologue of WUS, in regulating distal stem cell maintenance in a non-cell autonomous fashion downstream from the SHR/SCR signaling pathway. While loss-of-function leads to stem cell differentiation, overexpression of WOX5 results in the accumulation of distal stem cells that operate independent from quiescent centre (QC) signaling. By reciprocal expression experiments we show that

WOX5 and WUS are functionally equivalent in stem cell control, indicating that the organizers of both stem cell systems employ related regulators to provide stem cell maintenance signaling.

Chapter **three** describes the functional analysis of four PLT family members that are redundantly required for embryonic root development and post-embryonic root growth in a dose-dependent fashion. Transcriptional and translational studies of the PLT proteins reveal their graded expression spanning the root meristem with highest expression in the stem cell area, intermediate levels in the transit amplifying zone, and low levels in the differentiation zone. By changing the shape of the PLT expression patterns we show that cell fate within the root meristem depends on the amount of PLT cells are subjected to; high PLT levels maintain stem cell fate, intermediate levels control cell division, and low levels allow cell elongation.

Chapter **four** elaborates on the PLT dosage-dependent control of root cell fate by demonstrating that direct targets of PLT2 are expressed in non-overlapping expression domains within the root meristem. By manipulating PLT2 protein levels in the root meristem, we demonstrate a corresponding shift in the target expression domain; indicating PLT2 can regulate its transcriptional targets in a concentration-dependent manner. In addition, the varied identity of downstream targets suggests that PLT2 controls diversified developmental pathways associated with cell proliferation and growth. Interestingly, the identification of auxin transport facilitators, biosynthetic regulators and signaling components as direct PLT2 targets indicate a multi-level regulatory feedback into the auxin gradient that specifies root patterning.

In chapter **five** the results presented in the previous chapters will be discussed in the light of recent insights in stem cell maintenance and specification, morphogen theory and transcriptional networks to give directions to future research.

Chapter 1

A general introduction to *Arabidopsis* root development

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1. **Introduction**

A unique property of plants is their life-long ability to grow and to continuously develop, elaborating on the basic body plan laid down in the embryo. Therefore, plants depend on the incessant activity of confined populations of stem cells located at opposite ends of the apical-basal body axis. With each asymmetric stem cell division, one daughter cell is maintained as a stem cell, while the other will differentiate to form specific tissues. In this way, a limited number of stem cells can generate organs the size of trees.

1.1 Arabidopsis root morphology

In the root of the dicotyledonous model plant *Arabidopsis thaliana*, a small amount of stem cells generate all the different tissues that can be distinguished along the symmetrical radial axis (Fig. 1A). Because their rigid cell walls make it impossible for plant cells to move, the stereotyped division pattern of the root stem cells organizes the separate tissues in concentric columns or cell files. From outside to inside, these layers are designated as lateral root cap, epidermis, cortex, endodermis, and pericycle as the cell files that surround the central vascular tissue. Clonal analysis and ablation studies indicate that cell lineage does not necessarily determinate cell fate and pattern formation, but that plant cells are flexible and rather rely on positional information for adapting their final fate (Dolan *et al*., 1993; Scheres *et al*., 1994; van den Berg *et al*., 1995). At the basal end, a set of stem cells give rise to the central portion of the root cap, known as the columella. Internal to and contacting all the stem cells is a small number of mitotically less active cells; the quiescent center (QC).

Along the apical-basal axis of the root, stem cell daughters continuously travel through time, crossing the zone of cell division (meristematic zone), the zone of cell expansion and elongation (elongation zone) and, ultimately, meet their destiny in the differentiation zone. In other words, at any time, all developmental stages are present within the root tip. Together with the predictable fate of all individual cells that compose the root, these features make the *Arabidopsis* root an excellent system to study the genetic control of organ development.

First, we will briefly discuss the fundamental principles of auxin transport and action because these processes have become particularly important for our understanding of *Arabidopsis* root development.

1.2 Principles of auxin transport and signaling

During the past century, the phytohormone auxin has been identified and extensively studied for its effects on development in many plant species (reviewed in Srivastava,

2002). Auxin is transported through the plant in a directional cell-to-cell fashion, called polar auxin transport that is mediated by the auxin influx carrier AUX1 (Bennett *et al*., 1996), the PINFORMED (PIN) family of auxin efflux facilitators (Paponov *et al*., 2005), and members of the P-glycoprotein subfamily of ATP-binding cassette transporters (Geisler and Murphy, 2006). PIN membrane proteins are frequently localized polarly to the cell membrane and have been shown to act as the main direction-determining factor of polar auxin transport (Wiśniewska *et al*., 2006).

Blocking the auxin efflux machinery with 1-*N*-naphtylphtalamic acid (NPA) or application of the auxin analog 2,4-dichlorophenoxyacetic acid (2,4-D), disrupted auxin transport and distribution in *in vitro* cultured embryos and resulted in embryo defects, such as cup-shaped embryos with no functional root pole or ball-shaped embryos lacking any distinct apical-basal axis (Liu *et al*. 1993; Hadfi *et al*., 1998; Friml *et al*., 2003). These studies suggest an important link between auxin distribution and early embryo development. We will discuss further evidence in support of this hypothesis.

The specificity of the response to auxin is thought to be generated by optimized pairs of interacting transcriptional regulators, designated Auxin/Indole-3-Acetic Acid (Aux/IAA) proteins and Auxin Response Factor (ARF) transcription factors present in the auxin-responsive cell (Hamann *et al*., 2002; Tiwari *et al*., 2004; Weijers *et al*., 2005). Binding of auxin to the Transport Inhibitor Response 1 (TIR1), an auxin receptor and F-box subunit of the SCF^{TIR1} E3 ubiquitin ligase (Dharmasiri *et al*., 2005; Kepinski and Leyser, 2005), promotes degradation of the Aux/IAA proteins via the ubiquitin proteasome pathway and releases the repressive hold on the interacting ARF transcription factor proteins (reviewed in Quint and Gray, 2006).

Activity of the synthetic auxin-responsive promoter DIRECT REPEAT5 (DR5) is often used as an indirect marker to visualize auxin distribution (Sabatini *et al*., 1999; Friml *et al*., 2003). Although the DR5 reporter activity is merely an output of the cellular auxin response, its activity has been shown to correspond well with auxin levels by using immunolocalization with an anti-IAA antibody (Friml *et al*., 2003).

1.3 Chapter outline

In this chapter, we will discuss the critical stages particularly relevant for setting up the root pattern during the *Arabidopsis* embryogenesis, followed by the developmental processes important in post-embryonic root growth. The main topics discussed are (1) the general importance of auxin transport and signaling in all of the illustrated developmental processes, (2) the combinatorial code utilizing *PLETHORA* and *SHORT-ROOT*/*SCARECROW* genes that positions and specifies the stem cell niche

to the auxin machinery with feedback loops, (3) the role of *WUSCHEL-RELATED HOMEODOMAIN (WOX)* genes extending toward elucidating common factors in stem cell maintenance from root and shoot organizers, (4) radial patterning, (5) regulation of root zonation, and (6) briefly, the integration and crosstalk of hormones regulating root growth and development.

2. Specification of the apical and basal cell lineage

Embryogenesis starts with the elongation of the zygote followed by an asymmetric division producing two daughter cells with different fate and characteristics. In *Arabidopsis*, the small apical cell develops into an eight-cell embryo proper after three rounds of stereotypic divisions forming two layers with four cells each (Jürgens, 2001; Fig. 1B). While the apical layer of cells gives rise to the shoot meristem and the larger part of the cotyledons, descendents of the basal pro-embryo layer form the remaining part of the cotyledons, the hypocotyl, the embryonic root, and the proximal root stem cells. The larger basal cell of the divided zygote produces a file of seven to nine cells by repetitive horizontal divisions that embody the extra-embryonic suspensor that serves as a conduit for nutrients and growth regulators to support the embryo proper development (Yeung and Meinke, 1993). From this basal cell lineage, the uppermost cell (the presumptive hypophysis) is sequestered by the embryo to adopt an embryonic fate and participate in development (Fig 1B). During heart stage of embryogenesis, the hypophyseal cell divides asymmetrically to generate an upper lens-shaped cell that will form the QC and a basal columella root cap progenitor. When the QC is specified, it induces the surrounding cells to become the root meristem stem cells (Dolan *et al*., 1993; Scheres *et al*., 1994; Fig. 1B). Post-embryonic development initiates from these stem cells that are laid down in the heart of the root meristem.

2.1 Zygote and early embryo development

The first step toward root formation is the establishment of the apical-basal axis of the developing zygote. Over the last two decades, several factors involved in this early patterning event have been identified and linked at the genetic level.

The early expression dynamics of several members of the *WOX* transcription factor family coincides with specific cell fate changes during early embryo development (Haecker *et al*., 2004). Before fertilization, *WOX2* and *WOX8* are expressed in the egg cell and the central cell of the embryo sac and, thereafter, in the elongating zygote. After division of the zygote, *WOX2* mRNA is restricted to the apical cell, whereas *WOX8* mRNA is found exclusively in the basal cell accompanied by *WOX9* expression. At the octant (8-cell) stage, *WOX2* and *WOX8* mRNAs remain

confined to the corresponding embryo domains of the apical and basal cell derivatives, respectively. *WOX9* expression expands into the central domain of the embryo, crossing the clonal boundary established at the first zygotic division, and weakens in the uppermost suspensor cell (Haecker *et al*., 2004). Although this dynamic *WOX9* expression pattern is not always observed, the early expressed *WOX* genes have been shown to share redundant functions during embryo development (Wu *et al*., 2007). Strong *wox9* loss-of-function mutants arrest development two to three divisions after the zygote stage. Eradicating *WOX8* function in the *wox9* background enhances this defect, arresting embryos after the first division. Likewise, *wox8* increases apical embryo defects caused by an insertion mutation in *WOX2*, resulting in abnormal cotyledon separation (Wu *et al*., 2007).

Development of the basal cell lineage has been shown to require a mitogen-activated kinase kinase (MAPKK) kinase, named YODA *(*Lukowitz *et al*., 2004). In *yoda* loss-of-function mutants, the elongation of the zygote is suppressed and the cells of the basal lineage divide in an unpredictable manner. As a result, these cells fail to form the suspensor and are ultimately incorporated into the developing embryo. The apical cell initially develops normally into a wild-type octant embryo, indicating a specific role for the MAP kinase signaling pathway in the correct specification of the basal cell lineage (Lukowitz *et al*., 2004), perhaps through the partitioning of fate determinants, such as the *WOX* genes.

After division of the zygote, a profound auxin response maximum in the apical daughter cell is quickly established as visualized by fluorescent *DR5* reporter expression (Friml *et al*., 2003). Expression of *PIN1* is restricted to the apical cell and the PIN1 protein is distributed within the cell membrane in a non-polar fashion. In contrast, PIN7 is polarly localized to the apical membrane of the basal cell facing the apical embryo pole. The asymmetric localization of PIN1 and PIN7 proteins present at the first steps of embryogenesis suggests the auxin transport routes from the maternal tissues to establish the auxin gradient and to initiate polarization (Friml *et al*., 2003; Fig. 1B). In a small, but reproducible, number of early *pin7* mutant embryos, the apical cell divides horizontally rather than vertically, accompanied by misexpression of the *DR5* marker in the extra-embryonic suspensor. At later stages, these *pin7* embryos display irregular division patterns in the lower embryo regions and, occasionally, fail to establish a pro-embryo (Friml *et al*., 2003). Interestingly, around the globular (32-cell) stage, *pin7* embryos start recovering from these defects, eventually resulting in fertile plants with no apparent phenotype. Double, triple, and quadruple *pin* mutant embryos show more severe patterning defects and do not recover, indicating functional redundancy among the *PIN* genes (Friml *et al*., 2003; Blilou *et al*., 2005; Vieten *et al*., 2005). PIN proteins in overlapping expression domains compensate for the loss of one another, even to the extent of ectopic *PIN2* expression in *pin3pin4pin7* embryos, whereas *PIN2* is normally not expressed at

these embryonic stages (Blilou *et al*., 2005), implying the presence of a flexible compensatory mechanism for the loss of PIN proteins in embryos.

Defects in cultured NPA-treated embryos and early *pin7* embryos resemble the phenotypes of the *gnom/emb30* (*gn*) mutant (Mayer *et al*., 1993). *GN* encodes a Brefeldin A (BFA)-sensitive membrane-associated guanine nucleotide exchange factor for ARF GTPase involved in the regulation of intracellular endosomal trafficking (Steinmann *et al*., 1999; Geldner *et al*., 2003). Strong loss-of-function *gn* mutants display a nearly symmetric division of the zygote, followed by oblique divisions of the apical cell. Unable to compensate these defects, *gn* mutants fail to specify the hypophysis and, ultimately, develop into seedlings without a functional root meristem. In *gn* embryos, the *DR5* reporter activity was detected ectopically in the suspensor, mimicking the *DR5* expression upon auxin efflux inhibition (Friml *et al*., 2003). Coordinated localization of PIN1 is perturbed in *gn* embryos (Steinmann *et al*., 1999), but with a BFA-resistant form of GN, the PIN1 localization is no longer sensitive to BFA, whereas other trafficking processes remain affected (Geldner *et al*., 2003). Thus, GN is responsible for mediating the intracellular trafficking of PIN1-containing endosomes. Mutants of the vacuolar protein sorting 29 (VPS29), a member of the retromer complex, display similar embryonic defects as those reported for *gn* (Jaillais *et al*., 2007). Moreover, the PIN1 localization is also affected in these mutants. Genetic analysis indicates that the VPS29 function is required downstream of GN for proper PIN1 protein cycling. Although more PIN protein family members have been shown to rapidly cycle between the plasma membrane and endosomal compartments and to internalize upon BFA treatment, GN action does not seem to mediate all PIN protein trafficking to the same extent (Friml *et al*., 2003; Grebe *et al*., 2003, Geldner *et al*., 2003). Recently, a PIN2-specific endosomal cycling route has been described that depends on the SORTING NEXIN 1 (SNX1) protein, hinting at the existence of at least two different endosomal factors for the trafficking of auxin transport facilitators (Jaillais *et al*., 2006). As in weak *gn* mutants, growth is impaired in *snx1* roots and the normal auxin distribution is perturbed. Double homozygous mutants for *vsp29* and *snx1* could not be obtained, suggesting that loss the loss of function of these two genes is either gametophytic or embryonically lethal (Jallais *et al*., 2007). As SNX1 and VPS29 are possible components of the retromer complex in plants, VPS29 might contribute to the SNX1-PIN2 pathway as well.

Molecular analysis of the auxin-insensitive mutants *monopteros* (*mp*) and *bodenlos* (*bdl*) highlighted a role for auxin in embryonic root formation (Berleth and Jurgens, 1993; Hamann *et al*., 1999). Initial abnormalities in *mp* and *bdl* are manifest in the apical region as early as the two-cell stage. In contrast to wild type, the apical cell in *mp* and *bdl* embryos divides horizontally, with twice the number of tiers at the octant stage as a consequence. At later stages, mutant embryos fail to specify the hypophysis correctly (see below) resulting in the complete absence of a seedling

root. The abnormal divisions in the pro-embryo are apparent before any defect can be discerned in the basal cell lineage, suggesting instructive communication across the clonal boundary between hypophysis and pro-embryo. Interestingly in this respect, the *WOX9* expression does not shift into the central embryo domain in *mp* and *bdl* mutants, nor is it downregulated in the hypophysis, implying that *WOX9* acts as a downstream component of auxin signaling (Haecker *et al*., 2004). The *mpbdl* double mutant embryos closely resemble the single-mutant phenotype, indicative for a role of both genes in the same developmental pathway (Hamann *et al*., 1999). Indeed, the auxin response inhibitor BDL/IAA12 has been identified as an *in planta* interacting partner of the *MP/ARF5* transcription factor (Hardtke and Berleth, 1998; Hamann *et al*., 2002; Weijers *et al*., 2006). The *bdl* mutant phenotype is caused by a dominant mutation that enhances protein stability, thereby constitutively inhibiting MP-dependent responses (Hamann *et al*., 2002). During early embryo development, *MP* and *BDL* transcripts are co-expressed in the apical embryo domain as early as the first division of the zygote, and, from the globular stage onward, both mRNA species become gradually restricted to the provascular cells (Hamann *et al*., 2002, Weijers and Jürgens, 2005). Recently, a stabilized version of the BDL paralog IAA13 has been demonstrated to cause similar embryonic defects as those seen in *bdl* (Weijers *et al*., 2005). Since *IAA13* is expressed in the same domain as *BDL*, both BDL and IAA13 are assumed to need to be degraded in early embryogenesis for MP to promote root specification. Recently, binding of the TOPLESS (TPL) protein to BDL was found to be required for BDL repression of MP activity. In accordance, *tpl* can suppress the patterning defects of the *bdl* mutant (Szemenyei *et al*., 2008). Together, these data suggest that correct auxin homeostasis and signaling are required for axis formation and specification of basal cell fates.

The embryonic patterning activity of *MP* is largely dispensable when the presumptive glutamate carboxypeptidase *ALTERED MERISTEM PROGRAM 1* (*AMP1)* is not functional (Vidaurre *et al*., 2007). *amp1* suppresses the phenotype of *mp* during embryo development and *amp1mp* mutants frequently form hypocotyls and roots post-embryonically. In *amp1* embryos, a failure of basal cell descendents to attain suspensor cell fate leads to abnormal divisions, generating additional cell tiers in the embryo proper. Together with the overlapping expression domains of both genes, *MP* might interfere locally with *AMP1*-promoted cell differentiation to maintain the basal meristematic region (Vidaurre *et al*., 2007).

In contrast to *mp* and *bdl* mutants, early patterning defects in *hobbit* (*hbt*) and *auxin resistant 6* (*axr6*) develop first in the basal cell lineage (Willemsen *et al*., 1998; Hobbie *et al*., 2000). Instead of the typical horizontal partitioning, the uppermost derivative of the basal cell displays vertical divisions at the four-cell stage. At later developmental stages, *hbt* and *axr6* fail to develop a functional root meristem and, similarly to *mp* and *bdl*, result in seedlings without root. The

HBT gene encodes a subunit of the anaphase-promoting complex (APC), a class of ubiquitin protein ligase (Blilou *et al*., 2002). Unlike CDC27A and core APC/C subunits that are constitutively expressed (Blilou *et al*., 2002; Capron *et al*., 2003; Kwee and Sundaresan, 2003), HBT/CDC27B is restricted to mitotically active and elongating cells and is mostly excluded from differentiated tissues. Analysis of weak *hbt* alleles and loss-of-function clones indicates that the primary function of HBT is to mediate cell division and endoreduplication, contributing to meristem activity and cell expansion (Serralbo et al., 2006; Pérez-Pérez *et al*., 2008). Consistent with a role for the APC in protein degradation, auxin response regulators were found to be stabilized in *hbt* plants (Blilou *et al*., 2002). The induced loss-of-function *hbt* clones, however, indicate that cell division and expansion defects caused by HBT removal are not primarily caused by altered perception or altered auxin distribution in the root meristem (Serralbo et al., 2006). Moreover, it remains to be seen whether Aux/IAA proteins are direct substrates of the APC complex. Mutations in HBT suggest that alterations in cell cycle can interfere with specific signaling events as a consequence of modified cell division patterns. The connection between cell cycle and embryonic patterning is evident from studies of the *tilted1* mutant (Jenik *et al*., 2005). This mutant carries a viable mutation in the catalytic subunit of DNA polymerase ϵ that lengthens the cell cycle throughout embryo development, with aberrant hypophyseal cell divisions and ultimately displacement of the root pole from its normal position on top of the suspensor as a consequence.

Loss-of-function mutations in *AXR6*, which encodes the CULLIN subunit of the SCF^{TIR1} E3 ubiquitin ligase complex, result in accumulation of the AXR2/IAA7 protein and, most probably, other Aux/IAA proteins. Potential Aux/IAA candidates to accumulate in *axr6* are the BDL protein and/or its functional paralog IAA13 (Hellman *et al*., 2003).

2.2 Specification of the hypophysis

The pro-embryo induces the uppermost extra-embryonic suspensor cell to become the hypophysis that is destined to generate the QC and columella root cap. Based on morphological observations, this hypophysis specification has been speculated to take place between the octant and dermatogen (16-cell) stage of embryogenesis (Jürgens and Mayer, 1994; Jürgens, 2001). However, besides the transient expression of *WOX9* in the uppermost suspensor cell at the four-cell stage, stable molecular markers have not been identified at these stages to characterize the specification of the hypophysis. The first genetic markers, indicative of hypophysis identity, appear some time between the transition from dermatogen to globular stage with the onset of *WOX5* and *PIN4* gene expression (Haecker *et al*., 2004; Friml *et al*., 2002a). Moreover, between dermatogen and globular stage, the suspensor has reached its

final cell number (7-9 cells) and will no longer divide (Mansfield and Briarty, 1991). Based on these morphological and gene expression criteria, specification of the hypophysis might well occur between dermatogen and globular stage rather than before these stages (Fig. 1B).

Coinciding with hypophysis specification, a dramatic shift in the apical-basal auxin response gradient can be observed (Friml *et al*., 2003). Expression of the *DR5* marker shifts basally into the uppermost suspensor cells and its activity in the pro-embryo ceases. This event synchronically occurs with with a reversal in the PIN protein polarity. From an apparently random distribution, PIN1 becomes basally localized in the pro-vascular cells facing the hypophyseal cell, whereas PIN7 shifts from the apical to the basal membrane of the suspensor cells (Steinmann *et al*., 1999; Friml *et al*., 2003; Fig. 1B). Concomitantly, the hypophyseal cell boundary becomes marked by the presence of a third PIN family member, PIN4, presumably supporting the action of the by PIN1 facilitated auxin transport (Friml *et al*., 2002a; Friml *et al*., 2003; Fig. 1B). PIN7-mediated efflux of auxin in the suspensor might operate at a lower rate than the auxin transport by PIN1 and PIN4, with the observed reversal of the auxin gradient as a consequence (Friml *et al*., 2003). Positioning of the auxin maximum in *pin4* mutant embryos is delayed and less restricted to the basal domain than that in the wild type (Friml *et al*., 2002a). *pin4* mutants display premature divisions of the hypophysis derivatives at the globular stage and occasional supernumerary cell divisions in the QC region at later stages. These aberrant divisions correlate with an expanded expression domain of the QC-specific enhancer trap line *QC25*, suggesting that cell fate is not properly specified in the root meristem of *pin4* mutants because of impaired positioning of the basal auxin maximum (Friml *et al*., 2002a).

The importance of basal auxin accumulation for the hypophysis fate determination is further supported by genetic studies on molecular components involved in the regulation of polar PIN delivery (Friml *et al*., 2004; Michniewicz *et al*., 2007). Overexpression of the protein serine/threonine kinase PINOID (Christensen *et al*., 2000; Benjamins *et al*., 2001) in the early globular embryo prohibits the normal basal-to-apical shift of the PIN1 localization and, hence, averts the appearance of a basal auxin maximum. Consequently, the hypophysis is misspecified, as demonstrated by its aberrant transverse division and absence of a seedling root (Friml *et al*., 2004). Loss of function of the A regulatory subunits of the protein phosphatase 2A (PP2A) also leads to a basal-to-apical PIN polarity shift in developing embryos, resulting in unfocused *DR5* expression at the basal end and uncoordinated divisions of the presumptive hypophysis (Michniewicz *et al*., 2007). Biochemical studies demonstrate PP2A and PID act antagonistically on reversible phosphorylation of PIN proteins, thereby regulating the apical-to-basal targeting of these auxin efflux carriers (Michniewicz *et al*., 2007).

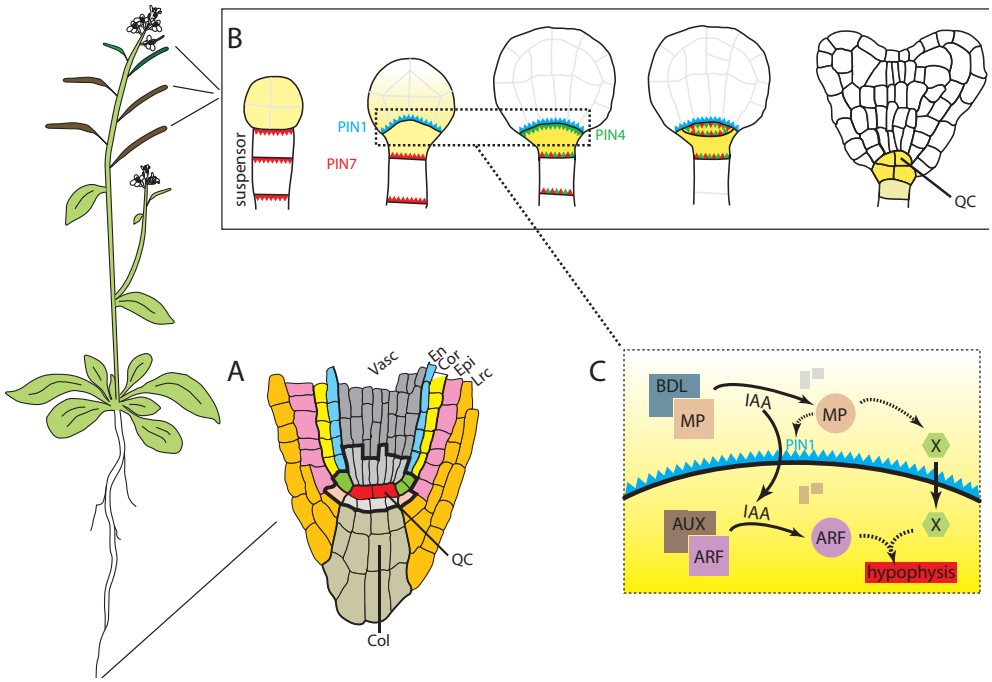

Figure 1. Root development. **A**. Schematic representation of the *Arabidopsis* root. **B**. Root specification during early embryogenesis. Focus is on the instructive elements in root specification. Colored serrated lines at the border of cells indicate respective PIN transport facilitators. **C**. Hypophysis specification involving auxin (IAA) and its signal transducers BDL and MP. Abbreviations: Col, columella; Cor, cortex; En, endodermis; Epi, epidermis; Lrc, lateral root cap; Vasc, vascular tissue;. Yellow indicates high levels of auxin accumulation. For detailed description, see text.

Specification of the hypophysis relies heavily on the interacting BDL and MP proteins. In *bdl* and *mp* mutants, the presumptive hypophysis fails to undergo the asymmetric division that generates the precursors of the QC and columella stem cells (Berleth and Jürgens, 1993; Hamann *et al*., 1999). Interestingly, *MP* and *BDL* transcripts accumulate in the pro-embryo, but the basal part of the early embryo does not express either mRNA. After the asymmetric division of the hypophysis, these transcripts start to accumulate in the lens-shaped daughter cell (Hamann *et al*., 2002). Since at early stages MP nor BDL proteins move in the presumptive hypophysis, they probably act non-cell-autonomously in hypophysis specification through a secondary signal (Hamann *et al*., 2002; Weijers *et al*., 2006; Fig. 1C). A potential candidate to act as such a messenger might be auxin itself; after all, auxin accumulates in the uppermost suspensor cell at the time of its specification to become the hypophysis (Friml *et al*., 2003). Moreover, both *PIN1* expression levels and the basal auxin translocation toward the hypophysis appear to depend on BDL activity, putting auxin transport and part of its machinery downstream of the MP and BDL action. Exogenous application of the synthetic auxin 2,4-D to *mp* and *bdl* mutants is not sufficient to restore the hypophysis specification, signifying that MP/

BDL-dependent factors exist other than auxin that act on this specification event. However, the 2,4-D concentration used in the attempt to rescue the *mp* and *bdl* root phenotypes has also been reported to interfere with normal development (Friml *et al*., 2003). It would be interesting to see whether auxin specifically produced in, or supplied to, the hypophyseal cell could complement the *mp* and *bdl* hypophyseal cell defect. Intriguingly, ectopic expression of a stabilized *bdl* mutant in the hypophysis derivatives at heart stage did not inflict embryonic root defects, suggesting that BDL-dependent MP action is only required for hypophysis specification and plays no role in subsequent embryonic root formation (Weijers *et al*., 2006).

Although the *orc* mutant has been identified in a genetic screen for post-embryonic root patterning defects, the first phenotypic abnormalities have been traced back to late globular embryos, where divisions of the hypophyseal cell are either absent or irregular (Willemsen *et al*., 2003). Accordingly, ectopic DR5 activity perturbs the distribution of auxin in basal *orc* embryo domains. Map-based cloning of the *orc* mutation has identified a point mutation in *STEROL METHYLTRANSFERASE 1* (*SMT1*), encoding a protein required for sterol homeostasis, indicating that a balanced sterol composition is a major requirement for proper auxin distribution during embryogenesis (Diener *et al*., 2000; Willemsen *et al*., 2003).

Together, the data establish that an auxin maximum at the basal pole is important for correct hypophysis specification and subsequent root meristem formation. In addition, specification of the hypophysis might also rely on another, MP/BDL-dependent non-cell autonomous signal, suggesting that, rather than specifying the hypophysis fate directly, auxin induces a primary response in the adjacent pro-embryo cells and is then transmitted basally as part of a secondary signal (Weijers *et al*., 2006; Fig. 1C).

3. Root stem cell niche specification

At late globular stage, the hypophysis has divided asymmetrically, producing a basal cell from which the root cap cells originate and a smaller lens-shaped apical cell that generates the four mitotically inactive QC cells (Jürgens and Mayer, 1994; Scheres *et al*., 1994; Fig. 1B). At heart stage, the QC is assumed to recruit the immediately adjacent cells to become the root stem cells. The QC and surrounding stem cells together form the root stem cell niche, a specialized microenvironment conditioned to maintain stem cells. At late heart stage, asymmetric periclinal division in the lowest protoderm cells generate epidermis and lateral root cap, marking the first stem cell division within the newly formed root stem cell niche (Scheres *et al*., 1994). At mid torpedo stage, a first round of asymmetric divisions of the columella stem cells produces a second layer of columella root cap cells and the ground tissue stem cell daughters generated the first endodermal cells (Jürgens and Mayer, 1994).

3.1 A role for AP2 transcription factors

From the early globular stage on, an auxin maximum is located at the basal tip of the developing embryo that is maintained throughout post-embryonic root development (Sabatini *et al*., 1999; Friml *et al*., 2003; Fig. 1B). In post-embryonic roots, a lateral shift of the endogenous auxin maximum induced by auxin transport inhibitors respecifies the root stem cell niche fate in ground tissue layers. Former endodermal cells express the QC-specific marker *QC46* and the adjacent cortex cells display the columella stem cell marker *J2341* and divide. Exogenous application of auxin induces cell fate changes similar to those observed upon polar auxin transport inhibition, suggesting that an auxin maximum functions as a positional cue for the stem cell niche (Sabatini *et al*., 1999).

In the past years, potential effector genes of the instructive auxin maximum have been identified. *PLETHORA1 (PLT1)* and *PLT2* encode auxin-inducible members of the AP2 domain transcription factor family and are redundantly required for the embryonic specification of the stem cell-organizing QC (Aida *et al*., 2004). *plt1plt2* double mutants show aberrant divisions of the lens-shaped cell and fail to express the *QC25* and *QC46* markers throughout embryogenesis. Exogenous application of auxin fails to rescue the stem cell defects in *plt1plt2* mutants, indicating that the required PLT activity for root stem cell niche formation cannot be bypassed by auxin. At heart stage, expression of the *PLT* genes is restricted to the basal stem cell niche, in which MP and its close homolog NONPHOTOTROPIC HYPOCOTYL 4 (NPH4/ARF7; Harper *et al*., 2000) act redundantly to maintain the *PLT* expression (Aida *et al*., 2004). Strikingly, in *pin2pin3pin4pin7* quadruple mutants, the *PLT1* expression expands throughout the whole embryo. When explanted, these embryos develop reduced cotyledons and root hairs emerge at more apical positions on the seedling. Conversely, in *pin1pin3pin4pin7* quadruple mutants, the expanded expression of the shoot meristem identity gene *WUSCHEL (WUS)* leads to explants with arrested root growth and an expanded shoot domain, implying that directional auxin transport conducted by the PIN proteins regulates the patterning of embryonic stem cell domains (Blilou *et al*. 2005). In turn, the *PIN4* transcript has been found to be severely reduced in *plt1plt2* mutants, as well as the expression of *PIN3* and *PIN7* in the post-embryonic elongation zone. All together, these findings suggest a feedback loop for embryonic root primordium formation and stabilization: PIN proteins restrict *PLT* expression in the basal embryo domain to initiate embryonic root specification, and, in turn, PLT activity regulates *PIN* transcription to stabilize the position of the root primordium (Blilou *et al*., 2005).

Recently, two additional *PLT* family members, *PLT3* and *BABY BOOM (BBM)*, have been identified that act in concert with *PLT1* and *PLT2*, contributing to embryonic root development and stem cell maintenance (Galinha *et al*., 2007).

From the heart stage of embryogenesis onward, the expression domain of *PLT3* and *BBM* largely overlaps with that of *PLT1* and *PLT2*, with the strongest expression in the basal stem cell niche. Removal of wild-type copies of *PLT3* and/or *BBM* in a *plt1plt2* double mutant background increases the patterning defects in the root pole development. These defects culminate in root- and hypocotyl-less seedlings in the progeny of self-fertilized $plt1^{-/-}plt2^{+/-}plt3^{-/-}bmm^{-/-}$, indicating that the *PLT* activities are largely additive and dosage dependent. Aberrant development starts at the early globular stage as revealed by transverse divisions of the hypophysis. Apical embryo development, however, seems largely unaffected in triple mutants. Together with homeotic fate transformation of the shoot meristem toward root fate upon ectopic *PLT2* expression, these results suggest that *PLT* genes act as master regulators for root development (Galinha *et al*., 2007).

3.2 A role for GRAS transcription factors

Other transcription factors involved in stem cell niche specification at early heart stage are represented by members of the GRAS family, *SHORT-ROOT* (*SHR*) and *SCARECROW* (*SCR*). *shr* and *scr* mutants display a disorganized QC/columella region and roots that cease growth prematurely (Benfey *et al*., 1993; Scheres *et al*., 1995; Di Laurenzio *et al*., 1996; Helariutta *et al*., 2000). Moreover, expression of *QC25* and *QC46* is perturbed in both mutants and columella stem cells fail to be maintained, indicating a role for SHR and SCR in specification of the QC and maintenance of a functional stem cell niche (Sabatini *et al*., 2003). The earliest detectable *SCR* expression is in the hypophyseal cell and precedes its division to generate the QC and the columella lineages (Wysocka-Diller *et al*., 2000). At later developmental stages, *SCR* expression gets restricted to the endodermal cell layer, cortex/endodermis stem cell, and the QC (Fig. 2A). In *shr* mutants, expression of *SCR* is severely reduced, indicative that SHR regulates the *SCR* expression (Helariutta *et al*., 2000). Recently, Levesque *et al*. (2006) have identified *SCR* as a direct target gene that is positively regulated by SHR. *SHR* is not transcribed in the SCR domain but in the adjacent provascular cells from where regulated and targeted movement transfers the protein into the adjacent cell layers to promote *SCR* transcription (Nakajima *et al*., 2001; Gallagher *et al*., 2004; Fig. 2A). In *scr* mutants, recuperation of the *SCR* expression in the QC is sufficient for cell-autonomous rescue of the QC identity, maintenance of the surrounding stem cells; and root growth. In contrast, ectopic expression of *SCR* only in the stem cells is insufficient for their maintenance and to restore the QC identity, suggesting that the SCR activity enables the QC to maintain the surrounding stem cells in a non cell-autonomous fashion (Sabatini *et al*., 2003). These observations are in concordance with earlier studies in which the QC has been identified as a negative regulator of differentiation of adjacent stem cells (van den

Berg *et al*., 1997). Re-expression of *SCR* in the QC of *shr* mutants is not sufficient to restore QC and stem cell identity, hinting at a more elaborate role for SHR in QC function and stem cell maintenance than its sole requirement for *SCR* transcription (Sabatini *et al*., 2003). In fact, recent findings indicate that SHR and SCR proteins interact and share a common set of transcriptional target genes (Cui *et al*., 2007, see below). Most likely, the regulatory activity of the SCR/SHR protein complex is required for the proper QC function.

In the QC, SCR is required for maintaining its own expression and the nuclear localization of SHR (Helariutta *et al*., 2000; Cui *et al*., 2007). An additional factor regulating the nuclear localization of SHR has been identified recently as a zinc finger transcription factor, designated JACKDAW (JKD; Welch *et al*., 2007). In *jkd* mutants, the QC is misspecified and stem cell activity is compromised, resulting in plants with reduced root length and meristem cell numbers. Moreover, *SCR* expression is absent from the QC and SHR predominantly localizes to the cytoplasm. In *scr* mutants, a similar localization pattern for SHR has been observed, suggesting that JKD controls the nuclear localization of SHR mainly through its effect on the maintenance of *SCR* expression (Welch *et al*., 2007).

3.3 Combinatorial gene activity positions the stem cell niche

Overexpression of *PLT* during embryogenesis ectopically accumulates QC and associated root stem cells and, in most extreme cases, leads to a complete transformation of the embryo toward root identity (Aida *et al*., 2004). In accordance with ectopic auxin application studies (Sabatini *et al*., 1999; see above), ectopic QC cells in the *PLT* overexpressors appear to originate from cells adjacent to the stele, suggesting that stele/endodermis-residing factors are necessary, in concert with *PLT*, to specify the root stem cell niche. *SHR* and *SCR* are likely candidates because both are expressed in the stele/endodermis and are required for QC specification and maintenance. In addition, transcription of *PLT1* is not affected in *shr* and *scr* mutants and *PLT* activity is not required for *SHR* or *SCR* expression, indicative of parallel inputs. Accordingly, in *plt1,plt2,shr* and *plt1,plt2,scr* triple mutant combinations, cells in the root meristem differentiate earlier than any of the *shr* or *scr* single or *plt1,plt2* double mutants (Aida *et al*., 2004). Moreover, although the lack of expression of the QC markers *QC25* and *QC46* in *shr, scr* and *plt1,plt2* mutants suggests that both pathways share a set of target genes, the *PLT* and *SHR/SCR* pathways do not fully converge on a same set of target genes because the expression of *QC184* solely depends on the *PLT* activity (Sabatini *et al*., 2003, Aida *et al*., 2004). All together, these data support a model in which the *PLT* and *SHR/SCR* signaling pathways commonly regulate the root stem cell niche specification, providing spatial cues and cell fate instructions through the overlap of their expression domains (Aida *et al*.,

2004; Fig. 2A).

Although genetic studies performed in the developing embryo would be most informative when examining stem cell niche specification, some of the molecular mechanisms of pattern formation and niche specification might also be learned from regeneration experiments in the post-embryonic root. Laser-induced ablation of QC cells rapidly respecifies the QC and root cap in the distal provascular tissue (van den Berg *et al*., 1995), whereas the laser-induced wound probably disrupts the acropetal auxin flow toward the root tip, resulting in a proximal shift of the auxin response maximum as fast as 3 hours after ablation (Sabatini *et al*., 1999; Xu *et al*., 2006). In response, the *PLT1* expression domain shifts accordingly to the newly formed maximum. Later, *SHR* and *SCR* also reallocate their expression domains proximally, resulting in a respecified QC within 2 days after ablation. The induction of the new cell fates relies on the newly established expression domains and combinatorial activity of *PLT*, *SHR*, and *SCR*, because regeneration does not occur in these mutant backgrounds. Furthermore, wild-type *PIN4* expression and polarity is re-established only after *PLT*, *SHR* and *SCR* have adopted their new expression domains. Thus, upon ablation, the formation of a new proximal auxin response maximum first induces cell fate changes mediated by the root patterning genes and, only consequently, changes in polarity of the auxin flow facilitated by the PIN proteins (Xu *et al*., 2006).

These findings support the current model of embryonic root stem cell niche formation. In response to a PIN-mediated auxin maximum, the *PLT* patterning genes become restricted to define the stem cell region in concert with *SHR* and *SCR* and, in turn, control root-specific *PIN* expression to stabilize the maximum (Blilou *et al*., 2005; Xu *et al*., 2006; Galinha *et al*., 2007).

4 Radial patterning

The radial organization starts within a group of cells derived from the apical cell at the octant stage of embryogenesis. A first set of periclinal divisions separates three founder cells for the protoderm, ground tissue, and procambium whose derivatives will later form the epidermis/lateral root cap, endodermis/cortex, and vascular bundle in the root.

The vasculature is laid down at the late globular stage when the inner four procambium cells perform a round of periclinal divisions that separate the pericycle from the central provascular tissue (Scheres *et al*., 1995). Through subsequent periclinal divisions, the central cells generate the xylem and phloem lineage founder cells that will ultimately produce the corresponding conductive vascular bundles.

At late heart stage, the most basal protoderm cells carry out an asymmetric periclinal division generating two daughter cells with separate fate (Dolan *et al*., 1993; Scheres *et al*., 1994). Consecutive anticlinal divisions of both daughter cells

will produce one file of lateral root cap and one epidermal cell layer. Recently, the *FEZ* and *SOMBRERO* genes have been identified from a marker-based screen that seem specifically involved in orienting the division of this asymmetric epidermis/lateral root cap stem cell division (Willemsen et al., 2008). The developing epidermal cells will differentiate either as root-hair cells or non-hair cells, depending on genetic determinants and positional information (Dolan *et al*., 1993; see Chapter 3).

4.1 Epidermis fate initiation and receptor signaling

The onset of epidermis cell differentiation is marked by the asymmetric segregation of the homeodomain-encoding genes *Arabidopsis thaliana MERISTEM LAYER1* (*ATML1*) and *PROTODERMAL FACTOR2* (*PDF2*). Expression of ATML1 and PDF2 initiates at the quadrant-stage embryo and segregates to the L1- or protoderm layer during early globular stage (Lu et al., 1996; Abe et al., 2003). *atml1pdf2* double mutation results in severe defects in shoot epidermal cell differentiation, but seem not to affect the anatomy of the root apical meristem and root growth (Abe et al., 2003).

Recently, the redundant leucine-rich repeat receptor kinases *RECEPTOR-LIKE PROTEIN KINASE1* (*RPK1*) and *TOADSTOOL2* (*TOAD2*) have been shown to be critical for radial patterning (Nodine *et al*., 2007). *rpk1toad2* double mutants are embryo lethal and arrest as mushroom-shaped embryos at heart stage. Phenotypic defects are first observed at the early globular stage, correlating with the overlapping expression of both proteins and consist of bloated central protoderm cells. These cells initially express the protoderm marker *ML1*, but fail to maintain its expression as the phenotype becomes apparent. In addition, the vascular primordium markers *ZWILLE/PINHEAD* and *SHR* are ectopically expressed in ground tissue and protoderm, whereas the ground tissue marker *SCR* is absent. Interestingly, also the correct asymmetric division of the hypophysis is impaired, failing to produce the lens-shaped cell. However, frequent failure to express *SCR* and the ectopic provascular marker indicates that defects in hypophysis specification cause the aberrant asymmetric division. These results indicate that *RPK1/TOAD2* signaling is required to maintain the protoderm fate, to restrict provascular fate and to specify hypophysis and ground tissue either directly or indirectly due to misspecification of the surrounding tissues (Nodine *et al*., 2007). Up and downstream effectors are needed to separate cause from effect.

4.2 Ground tissue patterning

Subsequent asymmetric periclinal divisions of the ground tissue founder cell generate the endodermis and cortex tissues in early heart stage embryos (Scheres

et al., 1994). *SHR* and *SCR*, besides their function as root stem cell regulators, are also important determinants of this radial pattern formation process (Benfey *et al*., 1993; Scheres *et al*., 1995; Di Laurenzio *et al*., 1996; Helariutta *et al*., 2000; Fig. 2B). In *shr* and *scr* mutants, only a single layer of ground tissue is present between epidermis and pericycle as a result of increased recalcitrance of the ground tissue precursor to divide periclinally. Whereas the single layer of ground tissue has a mixed cortex/endodermis identity in *scr* mutants (Di Laurenzio *et al*., 1996; Heidstra *et al*., 2004), it expresses cortex fate markers solely in *shr* mutants, suggesting a role for SHR not only in promoting the asymmetric stem cell division but also the endodermis identity (Benfey *et al*., 1993). The different functions of *SHR* in radial pattern formation and endodermis specification, but also in stem cell maintenance, might be reflected by domain-specific expression or function of direct downstream target genes. An extensive study to identify downstream target genes predicted eight candidates positively regulated by SHR of which four genes were able to bind SHR to their promoter sequences *in vivo* (Levesque *et al*., 2006). Two of these genes, *MAGPIE* (*MGP*) and *NUTCRACKER* (*NUC*), encode closely related C2H2 zinc finger transcription factors that are expressed in the cortex/endodermis stem cell and the lower endodermal lineage. Therefore, *MGP* and *NUC* could act redundantly in stem cell fate regulation and/or radial patterning. A third target, the metabolic enzyme tropine reductase (TRI), is mostly produced in the endodermis, suggesting a possible role in endodermis fate establishment and/or radial patterning. Finally, SHR has been found to directly regulate *SCR* transcription through binding to the *SCR* promoter region (Levesque *et al*., 2006). Given that SCR is cell autonomously required for QC specification and asymmetric ground tissue cell division (Sabatini *et al*., 2003; Heidstra *et al*., 2004; see below), SHR functions in these two processes partly through direct regulation of *SCR* (Levesque *et al*. (2006). Interestingly, a number of transcriptional targets of SHR also appear to be direct targets of SCR, including SCR itself that can bind to its own promoter (Cui *et al*., 2007). In a *scr* background, binding of SHR to the promoter of some of its targets is abolished, indicating functional interdependence between these two transcriptional regulators. A molecular basis for this interdependence is provided by the finding that SCR physically interacts with SHR in yeast two-hybrid and reciprocal pull-down experiments (Cui *et al*., 2007).

Clonal deletion of *SCR* from the ground tissue stem cells results in the single layer of ground tissue typical for the *scr* mutant phenotype, strongly hinting at a strict cell-autonomous requirement of SCR for the periclinal division of the endodermis/cortex stem cell daughter (Heidstra *et al*., 2004). Periclinally divided ground tissue clones that segregate activated *SCR* expression to the "outer" cells continue to express endodermal markers in the "inner" cells that lack *SCR* expression. Moreover, endodermis and cortex-specific markers are maintained in their respective

ground tissue layer upon clonal *SCR* deletion. These results indicate that SCR is required only transiently for stable and immediate separation of cell fates, possibly by respecification of the chromatin state at mitosis (Heidstra *et al*., 2004).

Although both SHR and SCR are present in the complete endodermis cell layer, the asymmetric periclinal division is restricted to the cortex/endodermis stem cell daughter (Fig. 2B), suggesting that the presence of stem cell-specific factors aid the process of asymmetric division and/or the involvement of extrinsic "top-down" signals from mature ground tissue cells to reinforce the asymmetric division. The latter possibility has been deduced from laser ablation experiments that revealed the inability of ground tissue stem cells to perform asymmetric divisions after being isolated from their mature daughter cells (van den Berg *et al*., 1995). However, clonal induction of *SCR* in the *scr* mutant revealed that all ground tissue cells are competent to perform the periclinal division, including the segregation of both fates, in the absence of mature endodermis and cortex acting as a patterning template. Together with the strict cell-autonomous action of SCR, the need for top-down signaling is ruled out to pattern the ground tissue (Heidstra *et al*., 2004). A possible reason that the stem cell daughter cells do not divide periclinally after laser ablation of their mature daughter cells, might be linked to non-cell-autonomous signaling from the QC that prevents progression of stem cell differentiation (van den Berg *et al*., 1997; Sabatini *et al*., 2003; Heidstra *et al*., 2004; Fig. 2B).

Movement of SHR to the endodermal lineage requires the protein to be localized cytoplasmically in the stele. However, cytoplasmic localization does not automatically imply movement as demonstrated by missense mutations that disrupt nuclear localization, but also movement of SHR, indicating that SHR transport is regulated and targeted (Gallagher *et al*., 2004; Fig. 2B). Moreover, ectopic expression of a complementing GFP-tagged version of SHR was not able to move from phloem companion cells or epidermal cells, suggesting a need for vascular-specific factors to enable SHR movement (Sena *et al*., 2004). When expressed from the *SCR* promoter, SHR induces supernumerary ground tissue layers with endodermis characteristics, substantiating a model in which restricted *SHR* expression in the stele and limited SHR movement into only the adjacent endodermis prevent continued activation of *SCR* and, subsequently, additional asymmetric periclinal ground tissue divisions (Nakajima *et al*., 2001). But how is the SHR movement restricted to only one cell layer? Rare periclinal ground tissue divisions in *scr* mutants maintain GFP:SHR in both layers, suggesting that SCR is required for the asymmetry of the periclinal ground tissue division and fate separation by restricting SHR movement to the endodermis only (Heidstra *et al*., 2004; Fig. 2B). In addition, movement of GFP:SHR in a *scr* mutant background has only been observed from the epidermis (Sena *et al*., 2004). Recent findings that SHR and SCR are bound in a complex indicate that indeed SCR sequesters SHR into the nucleus of endodermal cells, thus preventing its movement

(Cui *et al*., 2007), as illustrated in *SCR* RNAi plants in which residual SCR cannot seize all incoming SHR proteins and develop additional endodermal layers (Cui *et al*., 2007).

The SHR targets *MGP* and *NUC* have been isolated also independently in a screen for ground tissue expressed genes together with a third zinc finger family member *JKD* (Welch *et al*., 2007). *JKD* is expressed in the QC, cortex/endodermis stem cell, and, to a lesser extent, in the endodermal lineage. Initiation of *JKD* expression at early globular stage does not depend on *SHR* or *SCR*, but both factors are required post-embryonically for *JKD* maintenance. *mgp* and *nuc* single mutants have no apparent phenotype, suggesting a profound genetic redundancy. *jkd* mutants, besides the QC defects discussed above, display ectopically periclinal divisions in the cortex producing an additional layer with endodermis fate. These extra divisions have not been observed in *jkdshr* and *jkdscr* double mutants, indicating that *JKD* acts in the ground tissue by modifying the activity of SHR and SCR. *MGP* RNAi lines in wild type reveal no phenotype, but combined with *jkd* homozygotes largely complement the ground tissue phenotype, suggesting that MGP promotes cell division. *In planta*, protein-protein interaction studies among SHR, SCR, JKD, and MGP indicate that these proteins are capable of nuclear complex formation (Welch *et al*., 2007), substantiating an elaborate model for the molecular mechanism that controls radial ground tissue patterning. MGP redundantly facilitates the asymmetric cell division by binding to the SHR/SCR complex and JKD inhibits this activity by competing for binding in the same complex.

As the root ages, a new layer of ground tissue can be formed distant from the QC that rapidly takes on the cortex identity (Baum *et al*., 2002; Paquette and Benfey, 2005). In *scr* mutants, formation of this extra cortex layer, termed “middle cortex”, is observed at a much earlier time point than in the wild type. This phenotype can even be intensified by reducing gibberellic acid (GA) levels in the *scr* background, either genetically or chemically, indicating that both SCR and GA act independently as negative regulators of middle cortex initiation (Paquette and Benfey, 2005). In contrast to *scr*, the ground tissue in *shr* never develops an extra cortical layer and seems insensitive to reduced GA levels. Thus, the maturation of the ground tissue in the root is promoted by a SHR-dependent mechanism that is independently regulated by SCR and GA (Paquette and Benfey, 2005).

1.5 Stem cell maintenance

Following germination, new cells are continuously added by repetitive asymmetric divisions of stem cells in the heart of the root meristem to ensure perpetuation of, and elaboration on, the organization set during embryogenesis. Thereupon, stem cell daughters undergo additional divisions in the meristematic zones before rapid

expansion and differentiation. To guarantee stem cell action for extensive periods of time, the balance between stem cell proliferation and differentiation must be tightly regulated.

5.1 Conserved factors in root and shoot organizer signaling

Laser-induced cell ablation experiments have identified the QC as a source of short-range signals maintaining the immediately adjacent cells as root stem cells (van den Berg *et al*., 1997). A similar mechanism operates in the shoot meristem where an organizing center (OC) of slowly dividing cells is essential to maintain the adjacent stem cell pool. The OC is defined and maintained by expression of the *WUS* homeobox gene (Laux *et al*., 1996; Mayer *et al*., 1998). *WUS* regulates non-autonomously regulates the expression of the secreted protein CLAVATA3 (CLV3) in the stem cells that, in turn, interacts with the CLV1 receptor probably in a complex with CLV2 to limit the *WUS* domain expression and size of the OC (Fletcher *et al*., 1999; Brand *et al*., 2000; Schoof *et al*., 2000, Ogawa *et al*., 2008). This negative feedback loop between OC and stem cells provides a robust mechanism to balance the stem cell population in the shoot meristem. Whether a similar regulatory mechanism controls the root stem cell population is uncertain. However, some signaling components might be functionally conserved between both meristems.

QC-specific expression of *WOX5* is non-autonomously required for distal root stem cell maintenance similar to the role of *WUS* in the shoot (Sarkar *et al*., 2007; Fig. 2A). Loss of *WOX5* function results in differentiation of the columella stem cells, while overexpression of *WOX5* generates an indefinite number of columella stem cells by blocking the differentiation of their daughters or by reverting the fate of the differentiated columella root cap cells. Whereas stem cell maintenance normally depends on QC signaling, laser-induced ablation of the QC does not lead to differentiation of the additional columella stem cells (van den Berg *et al*., 1997; Sarkar *et al*., 2007), suggesting that the WOX5 protein itself moves toward stem cells as the long postulated short-range factor (van den Berg *et al*., 1997). Alternatively, ectopic *WOX5* expression might activate downstream signals that normally arise in the organizer cells that now enable self-maintenance of the stem cell population. Expression of *WOX5* is predominantly regulated by the *SHR*/*SCR* signaling pathway, identifying *WOX5* as one of its downstream effectors in stem cell maintenance. Interestingly, promoter swapping experiments have demonstrated that both *WUS* and *WOX5* genes are interchangeable in stem cell control. *WOX5* expression under the control of the *WUS* promoter can rescue the stem cell defects in the *wus* mutant and, vice versa, QC-specific expression of *WUS* can compensate for the loss of *WOX5* function, demonstrating that some of the central regulators of stem cell identity in the root and shoot meristems are equivalent (Sarkar *et al*., 2007; Fig. 2A).

Additionally, ectopic expression of CLV3-like peptides (CLEs) has been shown to affect root meristem maintenance in a *CLV2*-dependent manner (Fiers *et al*., 2005). Whereas *CLV3* overexpression inhibits stem cell proliferation in the shoot (Brand *et al*., 2000), *CLE* overexpression in roots does not seem to interfere primarily with the QC identity nor with stem cell maintenance, but rather to modulate the activity of the stem cell daughters that populate the meristem (Casamitjana-Martínez *et al*., 2003; Hobe *et al*., 2003; Fiers *et al*., 2004). However, it must be noted that the effect on proximal stem cell division rates has not been investigated. The functional product of genes from the *CLE* family has been identified *in planta* as a peptide of only 12 amino acids (Ito *et al*., 2006; Kondo *et al*., 2006), generated by the post-translational processing of *CLE* gene products. In a mutagenesis screen for suppressors of the *CLE19* overexpression phenotype, *sol1* and *sol2* completely rescued the root length and meristem defects up to 1 week after germination. *SOL1* encodes a putative Zn^{2+} carboxypeptidase, suggesting a role in the processing of CLE peptides. The *sol1* mutant, however, displays no abnormalities during plant development. These findings suggest that a CLV-like pathway might control the root meristem maintenance (Casamitjana-Martínez *et al*., 2003). The signals emanating from the shoot and root organizers required to sustain stem cells in their undifferentiated state remain unidentified.

The *halted root* (*hlr*) mutant has a reduced meristem activity after germination, resulting in retarded root and shoot growth (Ueda *et al*., 2004). Although the root meristem is properly specified during embryogenesis, *SCR* expression in the QC and cortex/endodermis stem cells is rapidly lost during post-embryonic development. *HLR* has been found to encode a subunit of the 26S proteasome (Ueda *et al*., 2004), indicating that the proteasome machinery is needed for stable expression of meristem regulators within the plant stem cell niches.

5.2 Stem cell regulation through cell cycle control

Recently, the *RETINOBLASTOMA-RELATED* (*RBR*) gene has been identified as a key component in root stem cell regulation (Wildwater *et al*., 2005). RNAi-induced reduction of *RBR* transcript levels in the root meristem results in excessive stem cell accumulation of all cell types. The rates of cell division within the stem cell pool, however, are not affected, indicating that the increase in stem cell numbers observed in *RBR* RNAi lines is due to prolonged maintenance of the stem cell identity. In agreement with the RNAi phenotype, overexpression of *RBR* primarily affects the stem cells, leading to a rapid loss of their undifferentiated state. As in wild type, laser-induced ablation of the QC in *RBR* RNAi roots leads to rapid differentiation of the columella stem cells, showing that the supernumerary stem cells are still under QC control. Interestingly, this experiment demonstrates the ability of the QC to

signal over multiple cell layers to maintain stem cells, equivalent with the OC in the shoot meristem. Reduction of *RBR* can compensate for the loss of stem cell activity in a *scr* mutant background, but not in the *shr* or *plt1plt2* mutants, implying that the role of *SCR* in stem cell control is to down-regulate the RBR pathway in the QC, thereby promoting stem cell maintenance in a non-cell autonomous manner. After all, if a QC-emitted signal was required to suppress RBR in the surrounding stem cells, then stem cell maintenance should be independent of the QC an *RBR* RNAi background (Wildwater *et al*., 2005). In mammals, the RETINOBLASTOMA (RB) protein acts as a tumor suppressor and cell cycle regulator. RB inhibits progression of the cell cycle by formation of a repressive complex with the cell cycle-promoting E2F transcription factors (for a review see Weinberg, 1995). Phosphorylation of RBR by the upstream acting D-type cyclin (CYCD)-cyclin-dependent kinase (CDK) complexes releases the inhibition on E2F action, allowing the cell cycle to progress. CDK/CYCD action is predicted to be inhibited by Kip-related proteins (KRPs) (De Veylder *et al*., 2001). Overexpression of the *Arabidopsis* homologs of these cell cycle components resulted in an accumulation (CYCD3 and E2Fa) or a loss (KRP2) of stem cells, in accordance with their postulated roles in the plant RBR pathway (Wildwater *et al*., 2005), strongly suggesting that RBR controls the population of stem cells in response to D-type cyclins and through the modulation of E2F action.

5.3 Auxin and stem cell regulation

Accumulating evidence points toward a role for the stable basal auxin maximum in stem cell maintenance. In *PID* overexpressors, quantified auxin levels in the root meristem are significantly decreased as a result of mislocalization of PIN proteins (Friml *et al*., 2004). In concurrence, loss of stem cells is followed by terminal differentiation of the meristem. Interestingly, the *35S::PID*-mediated stem cell differentiation is delayed in *pin2* and *pin4* mutant backgrounds. In these *pin* mutants, auxin accumulates at the root tip presumably as a consequence of the interrupted auxin flow (Friml *et al*., 2002a; Ottenschläger *et al*., 2003), suggesting that increased auxin levels at the root tip in *35S::PID* plants can partially restore the stem cell function. In accordance, treatment of *35S::PID* plants with NPA restores the *DR5* marker exprression and prevents meristem differentiation (Friml *et al*., 2004).

Alternative evidence comes from the characterization of the redundant *ARF10* and *ARF16* auxin response factors that have been found to restrict the distal stem cell fate and to promote columella cell differentiation (Wang *et al*., 2005). The expression of *ARF16* is controlled independently by auxin and *microRNA160* (*miR160*), generating its columella and root cap-specific expression pattern. Whereas no root growth phenotype has been reported for the *arf10* and *arf16* single loss-of-function mutants (Okushima *et al*., 2005), the *arf10arf16* double mutant displays

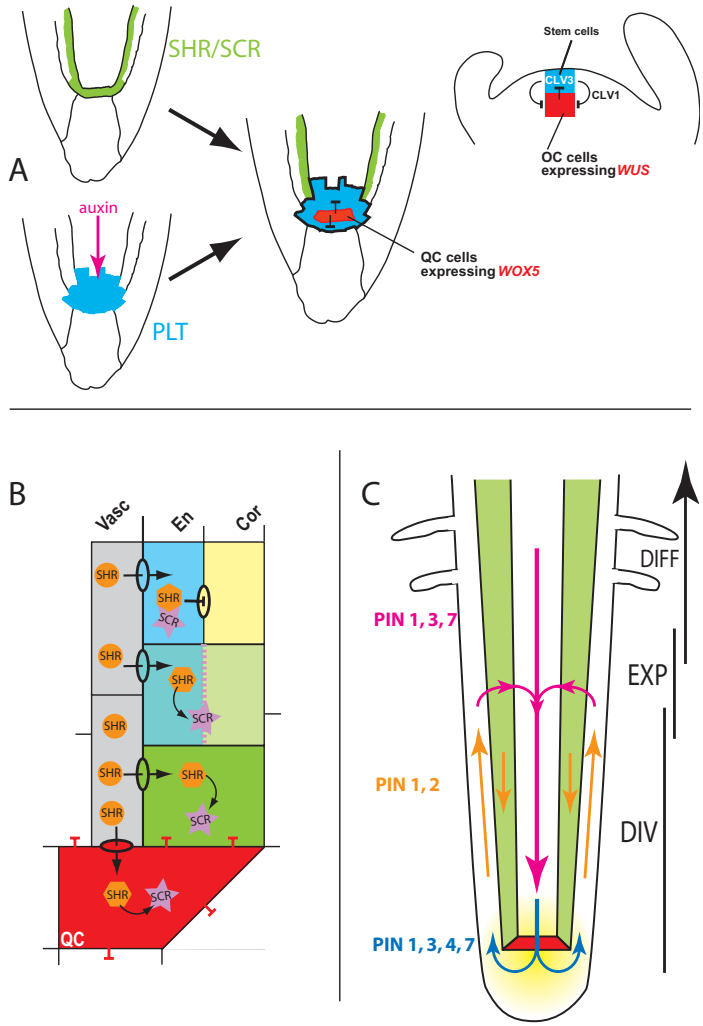

Figure 2. Genes controlling different aspects of root development. A. Combinatorial code of root stem cell niche specification. Peak levels of PLT expression are indicated in blue and SHR and SCR protein expression in green. WUS and WOX5 represent conserved gene activities for stem cell maintenance. **B**. Asymmetric ground tissue division. **C**. Interpretation of PLT proteins by the PIN protein-mediated auxin gradient, determining the zones of cell division, expansion, and differentiation. Abbreviations: Cor, cortex; DIFF, cell differentiation, DIV, zone of cell division (meristem);.En, endodermis; EXP, cell expansion; QC, quiescent center; SCN, stem cell niche; Vasc, vascular tissue; For detailed description, see text.

supernumerary root cap layers containing cells with mixed distal, proximal, and QC cell fates. Consistent with the *arf10arf16* loss-of-function phenotype, *miR160*-uncoupled overexpression of *ARF16* confers a loss of columella stem cells and differentiation of the proximal meristem (Wang *et al*., 2005). It would be interesting to determine the interplay between QC signaling and ARF10/ARF16 in distal stem cell maintenance. Possibly, the QC acts non-cell-autonomously to prevent ARF-mediated differentiation of the columella stem cells.

5.4 Chromatin and stem cell maintenance

During the last decade, examples in various model organisms point toward an active role of chromatin-remodeling factors in maintaining the balance between stem cell identity and the path to differentiation (Meshorer and Misteli *et al*., 2006). Also in *Arabidopsis*, chromatin-modifying proteins have been linked to meristem stability and stem cell maintenance. The *fasciata1* (*fas1*) and *fas2* mutants display severe defects in root and shoot meristem organization, leading to reduced root growth and aberrant shoot development (Leyser and Furner, 1992; Kaya *et al*., 2001). The premature termination of the root meristem in *fas* mutants is accelerated by the reduced activity of the QC and stem cells. Columella stem cells are quickly lost after germination, as indicated by accumulation of starch granules, and lack of *SCR* expression from the QC (Kaya *et al*., 2001). Formation of the embryonic root, however, is not affected. These developmental defects are also observed in the *brushy1* (*bru1*)/*mgoun3* (*mgo3*)/*tonsoku* (*tsk*) mutant (Guyomarc'h *et al*., 2004; Suzuki *et al*., 2004; Takeda *et al*., 2004), suggesting that the *FAS* and *BRU1/MGO3/TSK* genes are specifically required for post-embryonic meristem maintenance by regulating stem cell activity, at least partially, through their role in preserving the QC integrity. *FAS1* and *FAS2* encode two subunits of the *Arabidopsis* chromatin assembly factor 1 (CAF-1) complex, a histone chaperone complex thought to be involved in the maintenance of epigenetic information in chromatin during mitosis (Kaya *et al*., 2001). The genetic relationship between the CAF-1 complex and the BRU1/MGO3/TSK protein that is hypothesized to be part of a nuclear protein complex is unclear, although epistatic analysis suggests that they might have common targets in some developmental aspects (Guyomarc'h *et al*., 2004; Takeda *et al*., 2004). Two putative histone chaperones, NAP1-RELATED PROTEIN1 (NRP1) and NRP2, have recently been described as required for maintaining post-embryonic root growth (Zhu *et al*., 2006). Although the initial development of *npr1npr2* double mutants seems normal, the root ceases to grow 7 days after germination due to a lack of cell division. In contrast with *fas* and *bru1*/*mgo3*/*tsk*, the *nrp1nrp2* double mutant has a root-specific phenotype, suggesting a more restricted role for NRP1 and NRP2. In *tebichi* (*teb*) mutants, developmental root meristem have defects similar to those of the *bru1*/*mgo3*/*tsk* and *fas* mutants (Inagaki *et al*., 2006). The *TEB* gene encodes a protein that contains both helicase and DNA polymerase domains. As in *fas2*, cells expressing *CYCB1;1*-β-glucuronidase (*GUS*) accumulate in the *teb* and *bru1*/*mgo3*/*tsk* mutants, suggesting that the normal cell cycle progression is impaired. The disorganization and premature differentiation of the root meristem in these mutants might thus be connected with a defect in cell cycle progression at the G2-to-M transition (Suzuki *et al*., 2005; Inagaki *et al*., 2006). These mutants also exhibit shoot meristem maintenance defects, indicating a common role for these chromatin-

stabilizing factors in stem cell/meristem maintenance, possibly by maintaining the expression state of the meristem regulatory genes. Additionally, components of the CAF complex have been shown to bind RBR in plants (Ach *et al*., 1997; Kaya *et al*., 2001), opening up the possibility that RBR mediates stem cell control through the regulation of the chromatin state. This hypothesis is supported by recent results that indicate that known chromatin factor mutants act as enhancers of the *RBR* RNAi phenotype (N. Kornet and B. Scheres, 2009).

6 Meristem maintenance and root zonation

Once a daughter stem cell has been created through an asymmetric division of the stem cell, it must acquire not only radial tissue-specific properties (e.g., cortex, epidermis, or endodermis fate), but also be informed how to conduct itself according to its position on the apical-basal axis. Proliferation of stem cell daughters within the meristematic zone produces a reservoir of cells that, once pushed into the elongation zone, will elongate to ensure steady root growth. It is of crucial importance that cells within and between meristematic and elongation zone act in a coordinated fashion because an imbalance between cell division and rate of cell elongation zone will eventually result in retarded root growth. Auxin has been linked to both these processes because cell expansion and division can be stimulated upon external application of auxin (for a review, see Srivastava, 2002).

6.1 Auxin transport dictates root zonation

During root growth, a continuous flow of auxin produced by the shoot apex is transported toward the root tip in a PIN-dependent manner. Auxin seems to circulate through the meristem by basipetal transport and lateral redistribution as suggested by the polar localization of the PIN proteins produced in the root (Fig. 2C). PIN1 mainly resides at the basal membrane of the vascular cells (Gälweiler *et al*., 1998; Blilou *et al*., 2005). PIN2 localizes apically in epidermal and lateral root cap cells and predominantly basally in cortical cells (Müller *et al*., 1998; Blilou *et al*., 2005). PIN3 is active tier two and three of the columella without any pronounced polarity. In the elongation zone, however, PIN3 has been found at the basal side of vascular cells and the lateral side of pericycle cells (Friml *et al*., 2002b; Blilou *et al*., 2005). PIN4 is detected in and around the QC and localizes basally in provascular cells (Friml *et al*., 2002a; Blilou *et al*., 2005). PIN7 resides at lateral and basal membranes of provascular cells in the meristem and elongation zone, whereas in the columella it coincides with the PIN3 domain (Blilou *et al*., 2005). Mediated by combined PIN1, PIN3, and PIN7 action, auxin is transported basally through the provasculature toward the root cap, where PIN3, PIN4, and PIN7 action maintain

the position of the auxin maximum and redistribute auxin to the lateral periphery. PIN2 facilitates acropetal transport through the epidermis to the elongation zone where the auxin can be reloaded into the provascular system facilitated by PIN1, PIN2, PIN3, and PIN7 (Blilou *et al*., 2005; Fig. 2C). Consistent with this model of auxin circulation, ectopic induction of auxin biosynthesis in the QC results in enhanced auxin responses, measured by *DR5* reporter expression, appearing first in the columella area, subsequently in the lateral root cap and epidermis, and finally in the provascular strand (Blilou *et al*., 2005). The significance of this auxin reflux loop on cell division and cell expansion is evident from the analysis of *pin* mutant root tips. Whereas *pin* single mutants have root and meristem lengths close to those of the wild type, *pin1,pin2* double and all triple and quadruple mutants containing *pin2* display a more than additive reduction in root and meristem sizes (Blilou *et al*., 2005). Importantly, these defects can be rescued by exogenous auxin application, suggesting that basipetal auxin transport to the meristematic cells plays a critical role in meristem length regulation, predominantly through the control of cell division. In the elongation zone, the final cell size is reduced in several *pin* mutants, while the meristematic cell size corresponds to that of the wild type (Blilou *et al*., 2005). These data indicate that modulating auxin (re)distribution through *PIN* gene control can regulate both cell division and cell elongation in the root meristem (Fig. 2C).

Exactly how robust this system of an efflux-driven auxin gradient is has been demonstrated in a model that describes diffusion and PIN-facilitated auxin transport in and across cells within a virtual root system (Grieneisen *et al*., 2007). In this model, a wide range of parameters has been tested for their effect on global auxin distribution, including membrane permeability, auxin production and decay, drastic alterations in influx and efflux (such as root cut and tissue ablation), and cell division and expansion. None of these *in silico* experiments influenced auxin distribution within the root; an observation that was supported by equivalent *in planta* experiments. However, when auxin reflux is prohibited by eliminating all laterally oriented PINs, the maximum quickly dissolves as auxin is directed upward and unable to feed back into the system, suggesting that the presence of the auxin maximum and gradient is solely the consequence of the global PIN topology within the root. Moreover, when cells are given the simple instructions to divide at high auxin levels and elongate at low concentrations, a self-organizing root system develops in time with similar zonation and auxin distribution properties as seen in wild-type roots. Thus, the graded auxin distribution, enforced by PIN-mediated transport, is sufficient to explain the maintenance and growth of the meristematic and elongation zone without the need for additional regulatory processes (Grieneisen *et al*., 2007, Fig. 2C). The explanatory power of this model can now be exploited to assess auxin transport-regulated development and make precise predictions on the phenotypic outcome of specific genetic and cell biological manipulations regarding

root development.

6.2 Dose-dependent regulation of root zonation

How the graded distribution of auxin dictates a range of distinct cellular behaviors is currently unknown, but experimental data suggest an important role for the PLT family of transcription factors. As mentioned above, a combination of four *PLT* genes are redundantly required for root development and stem cell maintenance. In the post-embryonic root, PLT protein fusions display graded distribution along the meristem with maximum expression in the stem cell niche that extends into the meristematic zone and, for PLT2 and PLT3 fusions, into the elongation zone (Galinha *et al*., 2007; Fig. 2C).

When produced in restricted domains, the PLT proteins can only compensate partially for the loss of *plt1plt2*, indicating that the PLT concentration gradient instructs a different output in different regions. Expression of *PLT2* in the proximal part of the meristem, for example, increases meristem prolongation but fails to maintain the stem cell niche in *plt1plt2* mutants. Steepening the slope of the *PLT2* expression gradient in the *plt1plt2* background rescues the stem cells, but root and meristem sizes are severely decreased when compared to the almost complete complementation when expressed from its full promoter. Moreover, inducible overexpression of *PLT2* strengthens this hypothesis as it shifts the meristem boundary upward, promoting continuous growth of the meristematic zone and inhibiting cell expansion at the elongation zone (Galinha *et al*., 2007).

Interestingly, the stem cell area in *PLT2* overexpression plants is not altered, suggesting a limiting factor is constricting the high PLT2 dosage effects on the stem cell niche. This factor has been found to be *RBR*, mentioned above as a key component in root stem cell regulation independently from *PLT* (Wildwater *et al*., 2005). Overexpression of *PLT2* in *RBR* RNAi background results in an expansion of dividing cells in the root cap area and ectopic stem cell-like divisions in the proximal meristem (Galinha *et al*., 2007).

Together, these observations reveal that the PLT protein gradients define three outputs in the growing root system. High levels of PLT activity promote stem cell identity and maintenance and low levels mitotic activity of stem cell daughters; further reduction in levels is required for cell differentiation at the elongation zone (Galinha *et al*., 2007). Although a direct molecular link between auxin action and *PLT* gene activation is lacking, proper auxin distribution and response systems are essential for correct *PLT* gene transcription (Aida *et al*., 2004; Blilou *et al*., 2005), opening up the possibility that the PLT protein gradient dictates zonation of the root as a read-out of the stable auxin maximum and predicted associated auxin gradient (Grieneisen *et al*., 2007, Fig. 2C).

7 Meristem activation, root growth and cell division

Reactivation of cell division in the root apical meristem at germination and onset of growth are essential for post-embryonic development. Germination is triggered by water uptake by the quiescent dry seed and is generally considered to be complete when the radicle penetrates the seed coat. The earliest signs of germination are the resumption of metabolic processes, followed by directional cell expansion, and eventually the activation of cell division in the apical meristems (reviewed in Koorneef *et al*., 2002). Once activated, an essential part of the coordinated root growth is the integration of various informative signals into a univocal response often requiring extensive cross-talk between distinct hormone signaling pathways.

7.1 Redox homeostasis controls meristem activation

In the *root meristemless1* (*rml1*) mutant, the embryonic root develops normally, but a failure to initiate cell division during germination results in seedlings with an extremely short root unable to establish and maintain an active meristematic zone of cell division (Cheng *et al*., 1995; Vernoux *et al*., 2000). *RML1* has been found to be allelic to *CADMIUM SENSITIVE2*, encoding the first enzyme in the glutathione (GSH) biosynthesis pathway, γ-glutamylcysteine synthetase (Cobbett *et al*., 1998; Vernoux *et al*., 2000). Treatment of *rml1* mutants with applied GSH is sufficient to restore post-embryonic root development, indicating that the absence of cell division in the *rml1* root results from GSH depletion (Vernoux *et al*., 2000). In wild-type plants, inhibiting GSH biosynthesis has an effect on the mitotic root growth similar to that in *rml1*, while exogenous application increases the number of meristematic cells going through the mitotic cycle (Sánchez-Fernández *et al*., 1997; Vernoux *et al*., 2000). Accordingly, treatment of cultured tobacco (*Nicotiana tabacum*) cells with the GSH biosynthesis inhibitor, buthionine sulfoximine, traps the cells in G1 phase (Vernoux *et al*., 2000). A role for endogenous GSH in the control of cell proliferation is provided by the mapping of GSH levels in the root meristem. Low levels of GSH are associated with the mitotically inactive QC compared to the surrounding dividing stem cells (Sánchez-Fernández *et al*., 1997; Jiang *et al*., 2003). The glutathione redox couple GSH (reduced form) and GSSG (oxidized form) act as a homeostatic redox buffer (for a review, see Meyer and Hell, 2005). In recent years it has become evident that the intracellular redox state plays a critical role in regulating cell proliferation, possibly by controlling key components of the G1-to-S transition (den Boer and Murray, 2000; Jiang and Feldman, 2005 and references therein). Therefore, the impairment of GSH production in *rml1* mutants leads presumably to an overall changed redox state in the root, arresting the cells in the G1/S phase (Vernoux *et al*., 2000). Exactly how redox homeostasis and cell

cycle regulation interconnect at the molecular level is unclear. Interestingly, auxin has been linked to changes in intracellular redox state via its correlation with the production of reactive oxygen species. Possibly, auxin contributes to the quiescence of the QC cells by maintaining these cells in the G1/S phase through redox control (Jiang *et al*., 2003; Jiang and Feldman, 2005).

At the moment, it is still debated whether cell cycle progression is activated before or after radicle protrusion, but early activated core cell cycle genes, such as CYCD, and the formation of a proper microtubule network certainly play key roles in regulating the extent of cell division (Barrôco *et al*., 2005; Masubelele *et al*., 2005).

7.2 Hormonal control of root growth and cell division

Low concentrations of brassinosteroids (BR) have been shown to promote root growth, whereas high BR levels have a negative effect on root length. In accordance, BR-deficient mutants develop significantly shorter roots than wild-type plants (Müssig *et al*., 2003). Recent molecular genetic advances have identified a key component mediating the interaction between BR levels and auxin signaling in root growth. The natural loss-of-function allele *brevis radix* (*brx*) negatively affects cell division and cell elongation in the root tip (Mouchel *et al*., 2004). The *brx* phenotype results from a root-specific deficiency of BR, due to reduced expression of the rate-limiting enzyme in BR biosynthesis CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARF (CPD), Mouchel *et al*., 2006). In *brx* mutants, the auxin response is severely reduced, but can largely be restored by exogenous brassinolide application, suggesting that BR levels in the root influences auxin-induced transcriptional responses (Mouchel *et al*., 2006). Expression of *BRX* is strongly induced by auxin and repressed by BR, connecting the production of BR and auxin responses through a *BRX*-mediated feedback loop with root growth. Moreover, BR treatment has been shown to enhance polar auxin accumulation in the root (Li *et al*., 2005). The observation that modification of endogenous BR levels alter *PIN* transcript levels hint at a direct link between BR levels and polar auxin transport activities during developmental processes (Li *et al*., 2005).

Furthermore, giberillic acid (GA) has been identified as a regulator of root growth, as demonstrated by the reduced primary root growth of GA-deficient seedlings (Fu and Harberd, 2003). The interaction between GA and auxin has been studied in detail, revealing at least two levels of regulation. In roots, auxin signaling has been shown to enhance the GA-induced degradation of the GRAS family member RGA, a repressor of GA signaling and root growth (Fu and Harberd, 2003). Additionally, auxins have been reported to regulate GA metabolism by direct upregulation of enzymes performing rate-limiting steps in GA biosynthesis (Frigerio

et al., 2006). The inhibitory effect of ethylene on root growth is, at least partially, also mediated by RGA (Achard *et al*., 2003). Together, these results indicate that the protein RGA plays an important integrative role in the hormone response network for root growth.

Another integrator of hormonal pathways and developmental processes in root growth is the 36-amino acid peptide POLARIS (PLS), linking auxin homeostasis to ethylene signaling (Casson *et al*., 2002; Chilley *et al*., 2006). Mutant *pls* seedlings have thick, short roots and exhibit an enhanced ethylene signaling phenotype known as the triple response. Moreover, acropetal transport of auxin is reduced in *pls* seedlings as well as the free auxin levels in the root tip. All these phenotypic defects could be suppressed by pharmacological or genetic inhibition of ethylene signaling, indicating that PLS-mediated ethylene signaling has a negative impact on auxin transport and accumulation. Transcription of *PLS* is rapidly upregulated by auxin and negatively by ethylene, suggesting a self-reinforcing mechanism in which auxin stimulates PLS activity to suppress the growth-inhibitory effects of ethylene signaling in the root tip (Chilley *et al*., 2006). In contrast, various studies have uncovered a positive correlation between ethylene responses and auxin biosynthesis. Three independent complementary studies of ethylene-auxin crosstalk in root growth indicate that the growth inhibitory effect of ethylene is in fact mediated predominantly through auxin (Růžička *et al*., 2007; Stepanova *et al*., 2007; Swarup *et al*., 2007). Synthesis of auxin in the root tip is enhanced in response to ethylene and distributed basipetally in a manner dependent on PIN2 and AUX1 to the cells of the elongation zone where it accumulates to induce local auxin responses that inhibit cell elongation and overall cell growth. Disruption of auxin biosynthesis by the loss-of-function mutants of the WEAK ETHYLENE INSENSITIVE2/ANTHRANILATE SYNTHASE a1 (WEI2/ASA1) and WEI7/ANTHRANILATE SYNTHASE b1 (ASB1) genes that encode subunits of a rate-limiting enzyme of the auxin biosynthesis, prevent the provoked auxin increase upon ethylene treatment, hence fully suppress the ethylene-mediated inhibition of root growth (Stepanova *et al*., 2005). These seemingly contradictory data on ethylene-auxin crosstalk illustrate once more the complex order of crosstalk interactions between hormonal pathways (Chilley *et al*., 2006). Recently, ethylene signaling has been linked with promoting cell division of the QC (Ortega-Martínez *et al*., 2007), although it remains unclear whether these effects are mediated by auxin.

The enlarged root meristem of cytokinin-deficient plants indicates that cytokinins have a negative regulatory function in root growth (Werner *et al*., 2003). Rather than controlling cell division rates, cytokinins are thought to regulate the number of mitotic cells in the meristem (Beemster and Baskin, 2000; Werner *et al*., 2003). By selectively reducing endogenous cytokinin levels in the vascular tissue at the meristem transition zone (TZ), Dello loio *et al*. (2007) pinpointed this region as the site of cytokinin action to control the root meristem size. Reduction

of cytokinin levels in other parts of the root meristem had no affect on root growth. Interestingly, proper auxin distribution in the meristem was shown to be necessary for mediating the effects of cytokinin on meristem size control (Dello loio *et al*., 2007). From these data, it is clear that hormones play an essential role in regulating root growth, although the molecular effectors are scarcely known. Where hormones account for the input from different developmental and environmental controls, it is the cross-talk between the various signaling pathways that fine-tunes decisions on cell division, meristem size, and elongation.

8 Concluding remarks

A precondition for embryonic root initiation is the proper specification of the apical embryo domain, a process that is guided by auxin accumulation in the pro-embryo. In response to the increase in auxin levels, BDL-dependent action of MP non-cell-autonomously promotes specification of the hypophysis by enabling basally orientated auxin flow and, presumably, another unidentified signal. It would be interesting to see what the specific auxin response in the hypophysis includes. One possibility is that the changes in auxin response focus and maintain expression of the *PLT* root determinants.

Computational modeling of auxin distribution in the root predicts an auxin maximum and highly stable gradient with morphogenic properties. How the auxin gradient is translated into defined cellular outputs is presently unclear, though the *PLT* genes are likely candidates to be involved. The functional concentration gradient of the *PLT* genes overlaps with the auxin gradient and requires the auxin response machinery. Analysis of downstream *PLT* targets will be needed to assess how much of the response to graded activity is due to additive concentration effects on the same targets and to differences in target specificity.

The root stem cells are specified and maintained by the combinatorial action of *PLT* and *SHR*/*SCR* genes. Although these pathways act largely independently from one another, it is conceivable that they convey downstream a set of stem cell-promoting target genes. Most probably, target genes control subsets of the processes controlled by their activators, resulting in subtle phenotypes, as illustrated by the *wox5* mutant acting downstream in the *SHR*/*SCR* pathway in which the lack of columella stem cells does not affect root growth and would, therefore, be easily missed in forward genetics screens. In addition, the identification of such target genes might be hampered by their genetic or functional redundancy, as shown by the lack of phenotypes in a knock-out analysis of QC-enriched genes (Nawy *et al*., 2005).

The interesting findings that (1) the QC can signal over multiple layers when *RBR* levels are low and (2) *WOX5* expression in the QC is required for root stem cell

maintenance, point out that common mechanisms and genes operate in both root and shoot stem cell niches. These results might indicate that a common origin for the stem cell niche predates the shoot/root separation.

It is becoming more and more clear that *Arabidopsis* root development is also controlled by microRNAs and chromatin remodeling factors; such factors have now been shown to regulate stem-cell identity and future research will have to focus on linking these regulation levels to the existing patterning genes.

Plant hormones provoke many responses after application that could, until recently, not be explained at the molecular level. This situation has changed with the identification of the transport machinery, receptors and their interactors, response factors and other downstream effectors of the main classes of plant hormones. These discoveries will further help to understand the cross-talk between the different hormones in development.

The understanding of *Arabidopsis* root development has taken a giant leap since the early nineties, when the root became appreciated as a model to study basic issues in developmental biology. Many of the fundamental questions from that era have now been answered: the clonal origin of the root and root morphology have been described in detail and major players in the genetic control of root initiation, stem cell maintenance, and root growth have been identified. Now is the time to create links that connect the available data and generate interactive gene and protein networks. With the advent of genomics and related 'omics' approaches, a vast amount of data is generated that might help in this process.

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Chapter 2

Conserved factors regulate signaling in *Arabidopsis thaliana* shoot and root stem cell organizers

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Abstract

Throughout the lifespan of a plant, which in some cases can last more than one thousand years, the stem cell niches in the root and shoot apical meristems provide cells for the formation of complete root and shoot systems, respectively. Both niches are superficially different and it has remained unclear whether common regulatory mechanisms exist. Here we address whether root and shoot meristems use related factors for stem cell maintenance. In the root niche the quiescent centre cells, surrounded by the stem cells, express the homeobox gene *WOX5* (*WUSCHEL-RELATED HOMEOBOX 5*; Haecker et al., 2004), a homologue of the *WUSCHEL* (*WUS*) gene that non-cell-autonomously maintains stem cells in the shoot meristem (Mayer et al., 1998). Loss of *WOX5* function in the root meristem stem cell niche causes terminal differentiation in distal stem cells and, redundantly with other regulators, also provokes differentiation of the proximal meristem. Conversely, gain of *WOX5* function blocks differentiation of distal stem cell descendents that normally differentiate. Importantly, both *WOX5* and *WUS* maintain stem cells in either a root or shoot context. Together, our data indicate that stem cell maintenance signaling in both meristems employs related regulators.

Introduction

Higher organisms evolved the ability to keep founder cells undifferentiated and pluripotent by signals provided in specialized stem cell niches (Spradling et al., 2001). In the model plant *Arabidopsis thaliana*, root and shoot meristem stem cell niches are organized differently compared with each other (Laux, 2003). In the root meristem, the stem cells surround a small group of rarely dividing cells, termed the quiescent centre (Fig. 1a), and give rise to distal (columella), lateral (lateral root cap and epidermis) and proximal (cortex, endodermis and stele) cell types. Ablation studies show that short range signals from the quiescent centre keep only the directly abutting stem cells undifferentiated (van den Berg et al., 1997), similar to most animal stem cell niches studied so far (Spradling et al., 2001). In contrast, in the shoot meristem, a zone of three stem cell layers is maintained by an underlying organizing centre (Mayer et al., 1998) (Supplementary Fig. 1a), which regulates the stem cell pool as a whole. In addition, the regulatory genes described so far for root and shoot stem cell niches are different (Vernoux, et al., 2005).In the shoot meristem, a feedback mechanism between the organizing centre and stem cells dynamically regulates maintenance of the stem cell pool: *WUS* activity in the organizing centre keeps stem cells undifferentiated and induces expression of the signal peptide CLAVATA3 (CLV3), which in turn restricts the size of the *WUS* expression domain (Supplementary Fig. 1a; Schoof et al., 2000; Brand et al., 2000). We asked whether a related stem cell maintenance mechanism might operate in the root by analysing the *WOX* family for functions in the root meristem (Haecker et al., 2004).

Results

Expression of *WOX5* messenger RNA (Haecker et al., 2004) and a *WOX5-GUS* reporter gene initiates in the embryonic cell lineage that gives rise to the quiescent centre (Supplementary Fig. 1b), and persists in the quiescent centre (Fig. 1b, and Supplementary Fig. 1c) during postembryonic root growth. This expression pattern is strikingly similar to that of *WUS* in the shoot meristem (Mayer et al., 1998; Supplementary Fig. 2), raising the question of whether both genes might have similar functions in the respective stem cell niches.

In seedlings of the putative null allele *wox5-1* (Supplementary Fig. 1d,e), the cells at the quiescent centre position have abnormal shape and are enlarged in comparison to wild type (Fig. 1c,d, arrows, and Supplementary Table 1). Notably, adjacent columella stem cells are even larger, suggesting that they have undergone differentiation (Fig. 1d, bottom arrow). No other abnormality was observed in *wox5-1* mutants. Complementation of the mutant defects by a *WOX5* complementary DNA construct confirmed that the *wox5-1* mutation causes the observed phenotype (Supplementary

Fig. 3a).

Of several quiescent-centre-specific reporter genes (Sabatini et al., 1999), only *QC184* is not expressed in *wox5-1* and expression is lacking as early as the heart embryo stage (Fig. 2a,b,d,e, and Supplementary Table 2), indicating that *WOX5* functions in root development from early embryogenesis. In contrast, *QC25*, *QC46* and *WOX5-GUS* are expressed in slightly expanded domains compared to wild type (Fig. 2c,f; Supplementary Fig. 3c-h, and Supplementary Table 2). These data indicate that *WOX5* is required for some aspects of quiescent-centre-specific gene expression, but seems not to be a major factor in quiescent centre specification.

We asked whether in *wox5-1* roots the distal stem cells might have undergone premature differentiation, which in the columella can be visualized by the accumulation of starch-granule-containing organelles involved in gravitropic sensing. Indeed, in *wox5-1* roots, the cells at the stem cell position, but not the quiescent centre, accumulate starch granules (Fig. 2e,f, and Supplementary Table 2). In contrast, the proximal meristem appears normal. These findings indicate that *WOX5* gene expression in the quiescent centre is required to non-cell-autonomously maintain the distal stem cells as undifferentiated, similar to the role of *WUS* in the shoot (Mayer et al., 1998).

We then investigated whether *WOX5* activity is also sufficient to repress columella cell differentiation, by ubiquitously expressing a dexamethasone (DEX)-inducible *WOX5* gene. DEX induction results in small cells that lack starch granules in the place of differentiated columella and lateral root cap cells (Fig. 2h,

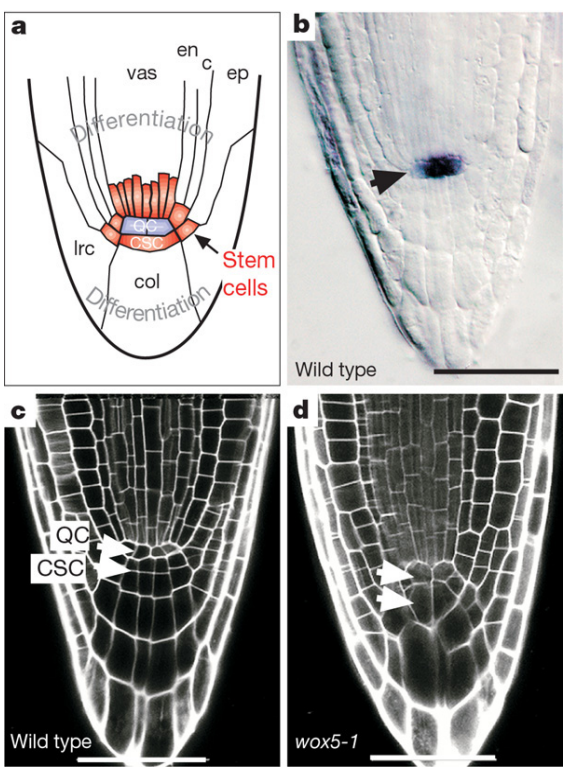

Figure 1. WOX5 expression in the quiescent centre is required for root meristem development. a, Schematic of *A. thaliana* root meristem with columella (col), columella stem cells (CSC), quiescent centre (QC), lateral root cap (lrc), epidermis (ep), cortex (c), endodermis (en) and vascular bundle (vas), redrawn with permission from Laux, 2003. **b**, *WOX5* mRNA expression in the quiescent centre. **c–d**, *wox5-1* roots display enlarged cells at the quiescent centre and columella stem cell (CSC) positions. Scale bars, 50 µm.

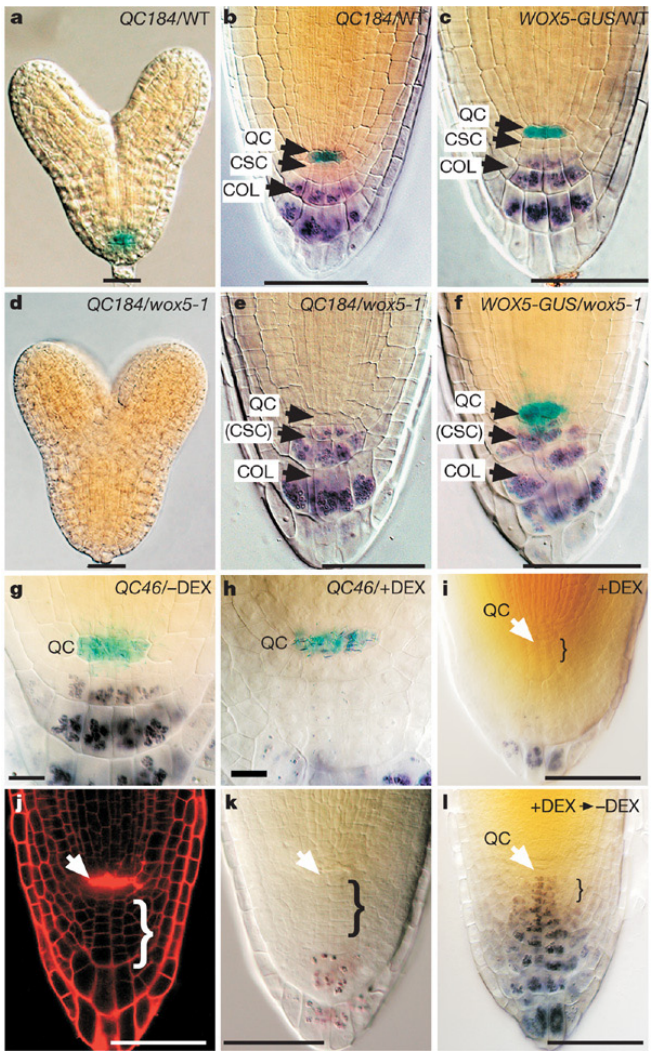

Figure 2: WOX5 represses differentiation in the columella. **a**–**f**, *wox5-1* heart-torpedo stage embryos (**d**) and root tips (**e**) lack *QC184* expression, express *WOX5-GUS* (**f**) in an enlarged domain and accumulate starch at the CSC position (**e**, **f**). **g**, **h**, *35S-WOX5* expression gives small cells in the columella, which lack *QC46* expression and starch grains (**h**). **j**–**l**, Quiescent centre ablation (arrow) does not induce differentiation (**j**, **k**), whereas transfer to non-inducing medium does (**l**; control, **i**). Nomarski (**a**–**i**, **k**, **l**) and confocal (**j**) median longitudinal images are shown. GUS staining, blue; starch, violet; waved brackets, extra small cells; COL, columella; CSC, columella stem cells; QC, quiescent centre; DEX, dexamethasone. Scale bars, 20 µm (**a**, **d**), 50 µm (**b**, **c**, **e**, **f**, **i**–**l**) and 10 µm (**g**, **h**).

i; Supplementary Fig. 4a, and Supplementary Table 1), indicating that they have not undergone normal differentiation. Unlike quiescent centre cells, the small cells divide, as shown by the continuously increasing cell number and expression of the cell cycle marker *CYCB1-GUS* (Supplementary Fig. 4b) and quiescent centre markers are not induced (Fig. 2h). Consistent with a loss of differentiated columella cells, *35S[GVG]-WOX5* (*35S-WOX5*; Supplementary Methods) roots display reduced gravitropism (Supplementary Fig. 4c). After withdrawal of DEX, however, the small cells readily accumulate starch grains typical for differentiated columella cells (Fig. 2l). Normally, laser ablation of the quiescent centre causes differentiation of stem cells (van den Berg et al., 1997; Wildwater et al., 2005), but this did not have any effect in *35S-WOX5* (Fig. 2j, k). Thus, ectopic *WOX5* expression is sufficient to block differentiation of columella stem cell daughters without the requirement of any further quiescent centre signaling.

Previous studies highlighted auxin-dependent transcription of the *PLETHORA*

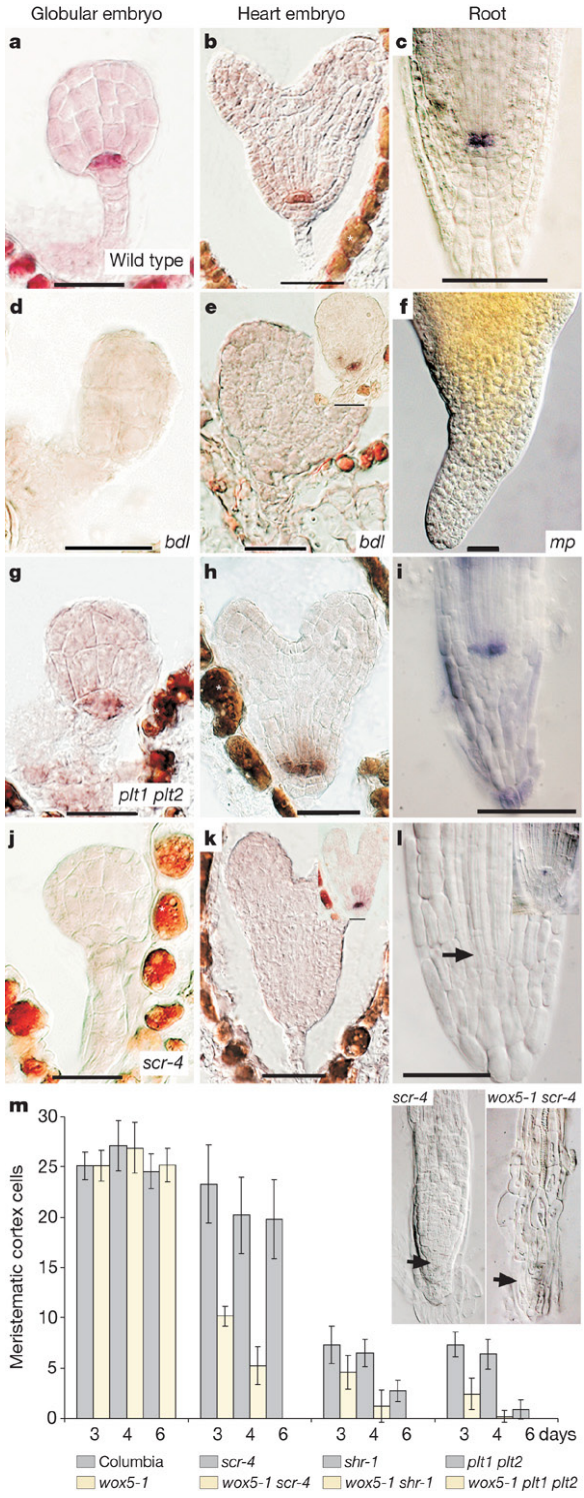

Figure 3: *WOX5* interaction with *SHR/SCR* and auxin pathways. a–l, Hybridization with *WOX5* antisense probe. *WOX5* expression was detected in wild type (**a**–**c**), absent in *bdl* and *mp* (**d**–**f**, inset shows sporadic exceptions), deregulated in *plt1 plt2* (**g**–**i**), and undetectable in *scr-4* (**j**–**l**, insets show sporadic exceptions). **m**, *wox5-1* enhances differentiation in the proximal meristem in *scr-4* (inset), *shr-1* and *plt1 plt2* mutants. Error bars, s.d. Longitudinal median histological (**a**, **b**, **d**, **e**, **g**, **h**, **j**, **k**) and optical (**c**, **f**, **i**, **l**, **m**) sections are shown. Four-day-old roots (**c**, **i**, **l**) or middle stage embryos (**f**) were used. Hybridization signal is red–blue; asterisk, reddish color is staining independent of hybridization reaction. Arrows, quiescent centre position. Scale bars, 25 μm (**a**, **b**, **d**–**f**, **g**, **h**, **j**, **k**) and 50 μm (**c**, **i**, **l**).

(*PLT*) genes and *SHORTROOT/SCARECROW* (*SHR/SCR*) functions as essential components in root meristem maintenance and we asked how *WOX5* interacts with these pathways (Sabatini et al., 1999; Sabatini et al., 2003; Aida et al., 2004; Weijers et al., 2006). In mutants for the auxin response regulators *BODENLOS* (*BDL)* or *MONOPTEROS* (*MP;* Fig. 3d-f; and Supplementary Table 3), *WOX5* expression is rarely detected, consistent with the notion that *BDL*/*MP*-mediated auxin signaling is required for the embryonic initiation of the quiescent centre (Weijers et al., 2006; Berleth and Jürgens, 1993). Auxin accumulation in the quiescent centre region provides patterning information to the root meristem mediated through *PLT* genes (Sabatini et al., 1999; Aida et al., 2004; Blilou et al., 2005).

In *wox5-1* mutants, auxin accumulation seems normal based on the expression of the auxin response reporter gene *DR5-GUS* (Supplementary Fig 5a, b). In *plt1 plt2* double mutants, *WOX5* expression is occasionally slightly expanded (Fig. 3g-i, and Supplementary Table 3), whereas *PLT1* expression is normal in *wox5-1* (Supplementary Fig. 5c, d and Supplementary Table 2).

The SHR protein moves from provascular cells into the quiescent centre and activates transcription of the *SCR* gene (Nakajima et al., 2001), which is required to specify quiescent centre identity and to maintain stem cells (Sabatini et al., 2003). *WOX5* expression is reduced or undetectable in *shr* and *scr* mutants (Fig. 3j-l; Supplementary Fig. 5g-i, and Supplementary Table 3), whereas an *SCR* reporter gene is correctly expressed in *wox5-1* roots (Supplementary Fig. 5e, f, and Supplementary Table 2). Collectively, our data indicate that *WOX5* expression depends on the induction in the root pole by *MP*-mediated auxin signaling and on *SHR/SCR* activity, whereas *PLT1* and *PLT2* have only a minor role in confining *WOX5* expression to the quiescent centre.

The requirement of *WOX5* for only columella stem cell maintenance suggests unidirectionality of this signaling pathway in stem cell control, similar to *WUS*-mediated stem cell control in the shoot stem cell niche. We investigated whether effects of the *wox5-1* mutation on the proximal meristem might be masked by redundant functions. Indeed, in *scr-4*, *shr-1* single mutants and *plt1 plt2* double mutants, the *wox5-1* mutation promotes differentiation in the proximal region of the root meristem in addition to its effects on distal stem cells (Fig. 3m, and Supplementary Fig. 5j, k). This suggests that *WOX5* gene expression in the quiescent centre redundantly contributes to proximal stem cell activity or alternatively that *WOX5* has a stem-cell-independent function in the proximal meristem, as has been reported for its upstream regulator SCR (Sabatini et al., 2003; Supplementary Fig. 7).

The striking similarities of *WOX5* and *WUS* expression patterns and functions in root and shoot stem cell niche organizers raised the question of whether both genes are functionally equivalent. Indeed, expression of the *WUS* cDNA from the *WOX5* promoter completely restores quiescent centre and stem cells in the *wox5-1* root meristem (Fig. 4a, b, and Supplementary Fig 6a, b). Interestingly, ectopic shoot tissue was never observed when *WUS* was expressed in the quiescent centre. This contrasts to previous studies expressing *WUS* in a broader range of cell types (Gallois et al., 2004) and favors the interpretation that organizer signaling only maintains stem cells as undifferentiated, whereas the fate of differentiating daughters is determined by the tissue context. Conversely, expression of a *WUS-WOX5* transgene rescues premature termination of inflorescence meristems and occasionally of floral meristems in

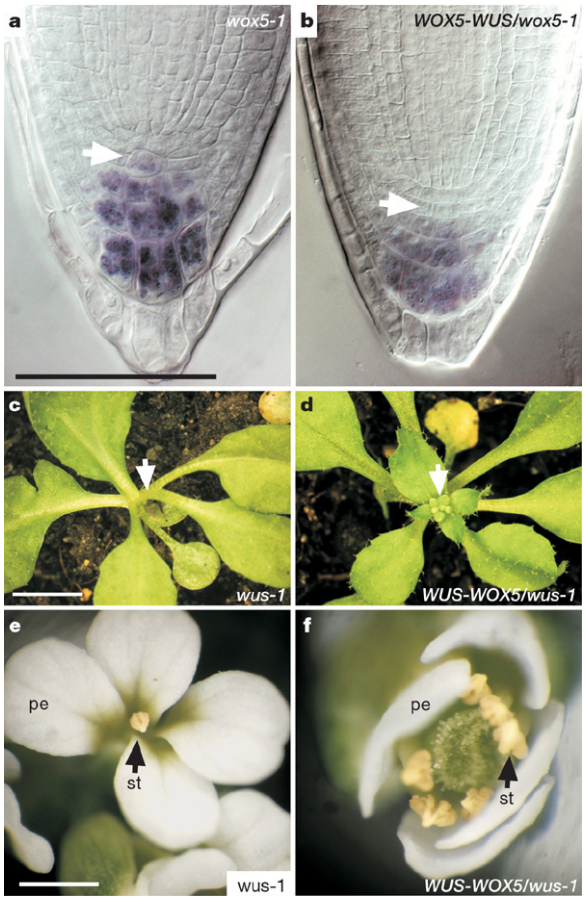

Figure 4: WOX5 and WUS are interchangeable in stem cell control. **a**, **b**, *WOX5-WUS* expression restores quiescent centre and columella stem cells (white arrows) in *wox5-1*. Violet, starch grains. **c–f**, Rescue of the shoot meristem stem cell niche. *wus-1* shoot (**c**) and floral meristem (**e**) fail to maintain stem cells and terminate prematurely. *WUS-WOX5* expression restores an indeterminate inflorescence meristem (**d**) and complete flowers (**f**). Twenty-six-day-old plants (**c**, **d**) and flowers (**e**, **f**) are shown. White arrows indicate shoot apex (**c**, **d**). pe, petal; st, stamen. Scale bars, 1 cm (**c**, **d**), 1 mm (**e**, **f**) and 50 µm (**a**, **b**).

the putative null allele *wus-1* (Fig. 4c-f, and Supplementary Fig. 6d-g). The rescued *wus-1* mutants resemble the weak *wus-6/jam* allele (Hamada et al., 2000), suggesting that the *WUS-WOX5* transgene complements *WUS* function but provides a reduced level of organizing centre signaling. Defective ovule development in *wus* mutants is not rescued by *WUS-WOX5* (Supplementary Fig. 6c), indicating that both regulators are interchangeable in only stem cell maintenance. Significantly, the early embryo genes *WOX8* and *WOX9* are not able to rescue *wus-1* defects (H. Breuninger and T.L., unpublished observations), showing that the ability to regulate stem cell maintenance is not a general property of WOX proteins.

Discussion

The extent of mechanistic similarity and evolutionary relationship between root and shoot stem cell niches has remained unclear thus far. Here we show that the organizers of both niches employ related regulators to provide stem cell maintaining signals. However, there also seem to be differences between both niches. For example, *CLV3* homologues promote meristem differentiation but do not influence stem cell maintenance in the root on overexpression and it is thus unknown whether roots, similar to shoots, employ a *WUS–CLV3* related feedback mechanism (Casamitjana-Martinez et al., 2003; Hobe et al., 2003).

Our results suggest that *WOX5* in the root stem cell niche has a more direct function in stem cell signaling, rather than in specifying quiescent centre identity (Supplementary Fig. 7). The nature of the signals that are induced by *WUS*/*WOX5* expression in the stem cell organizers is presently unresolved. WOX5 protein itself or a downstream factor might move to stem cells as the long-postulated short-range factor (van den Berg et al., 1997). To date, WOX5 protein has not been robustly detected, presumably owing to very low expression levels. However, on the basis of localization of a functional WUS–GUS fusion protein, movement of WUS protein out of the organizing centre seems not to be necessary for its function in shoot meristem stem cell maintenance, indicating that factors downstream of *WUS* act as signals (Supplementary Fig. 6h, i).

Palaeobotanical evidence for early root structures is controversial, but the current view is that roots have evolved independently at least twice in Lycophytina and Euphyllophytina (Kenrick and Crane, 1997a; Kenrick and Crane, 1997b). It remains to be shown whether *WUS* and *WOX5* shared an ancestral function in stem cell control since root and shoot separation, or whether they have been recruited for this function after the diversification of stem cell niches.

Methods Summary

Plant work

wox5-1 (SALK038262) and *wox5-3* (SALK147644) are transfer (T)-DNA insertion alleles in the Columbia (Col) accession and were obtained from the *Arabidopsis* Biological Resource Center (ABRC, USA) and the Nottingham *Arabidopsis* Stock Center (NASC, UK). *7xDR5-GUS* (Ulmasov et al., 1997) was kindly provided by J. Marfett, and mp^{U51}(Berleth and Jürgens, 1993) by G. Jürgens (Tübingen). Root length and meristem size were measured as described (Wildwater et al., 2005). The number of meristematic cells was obtained by counting cortex cells showing no signs of rapid elongation. Quiescent centre laser ablations were performed in 4-day-old roots using a Leica SP2 inverted confocal laser scanning microscope (van den Berg et al., 1997). Roots were stained for amyloplasts using Lugol (Sigma) 24-h after ablation (Willemsen et al., 1998). For confocal microscopy, root tips were mounted in 10 μg ml^{-1} propidium iodide solution. Microscope settings were as described (van den Berg et al., 1997).

Expression analysis

Starch granules (Willemsen et al., 1998) and β-glucuronidase activity (Schoof et al., 2000) were visualized as described. *35S-WOX5* seedlings were cultured on standard 0.5x MS medium supplemented with or without 1 μM dexamethasone. *In situ* hybridization of sections, whole-mount embryos, and 4-day-old seedlings were performed as described (Haecker et al., 2004; Willemsen et al., 1998). The *PLT1* riboprobe has been described previously (Aida et al., 2004). The *WOX5* riboprobe was prepared from a cDNA without the homeobox to avoid cross-hybridization with related mRNAs.

Further experimental details are provided as supplementary information.

Acknowledgements

We are grateful to E. van der Graaff for the data shown in Supplementary Fig. 4c, and e. Tucker, T. Demlow and B. Geiges for experimental help. We thank G. Jürgens for *mp* seeds, E. Kiegle and J. Haseloff for mGFP5ER and UAS vectors, M. Terlou for software development for root and meristem measurements, P. Graf for photography and E. Tucker and M. Tucker for comments on the manuscript. This work was sponsored by grants from the European Union (REGIA) and the Deutsche Forschungsgemeinschaft (to T.L.), a Netherlands Genomics Initiative grant (to R.H.), and grants from the Japan Society for the Promotion of Science and the Novartis Foundation (to K.N.).

Sequence of *WOX5* mRNA is available in GenBank under accession numbers NM_111961 and AY251398.

Supplemental data to:

Conserved factors regulate signaling in *Arabidopsis thaliana* shoot and root stem cell organizers

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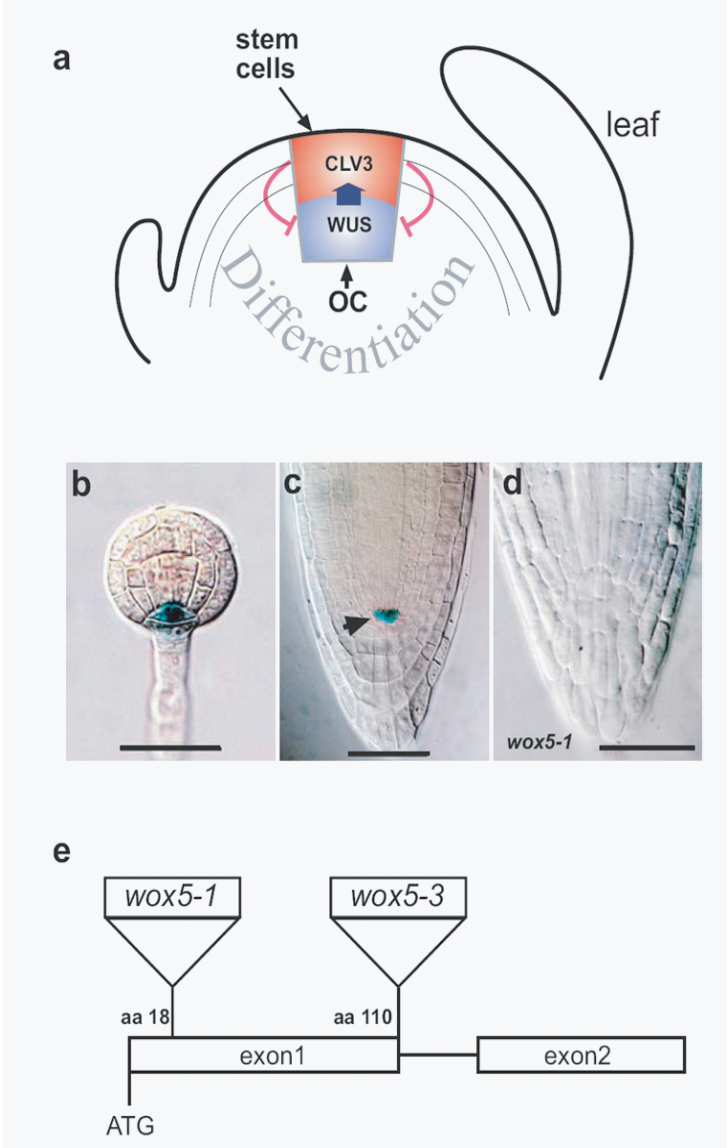
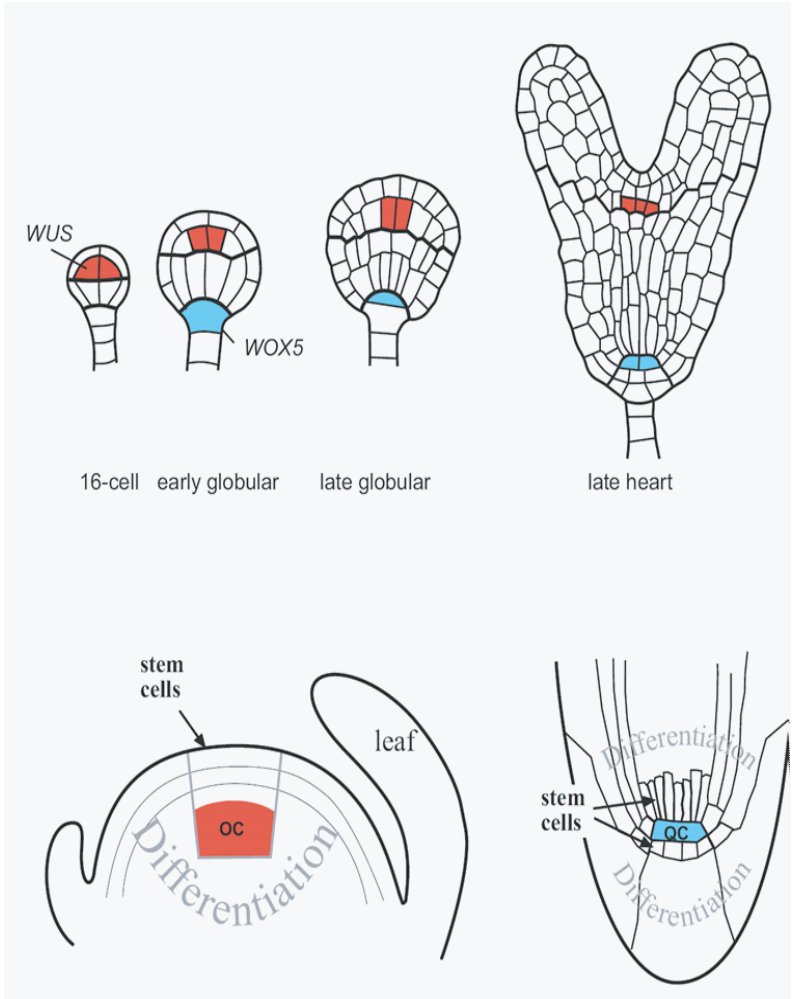
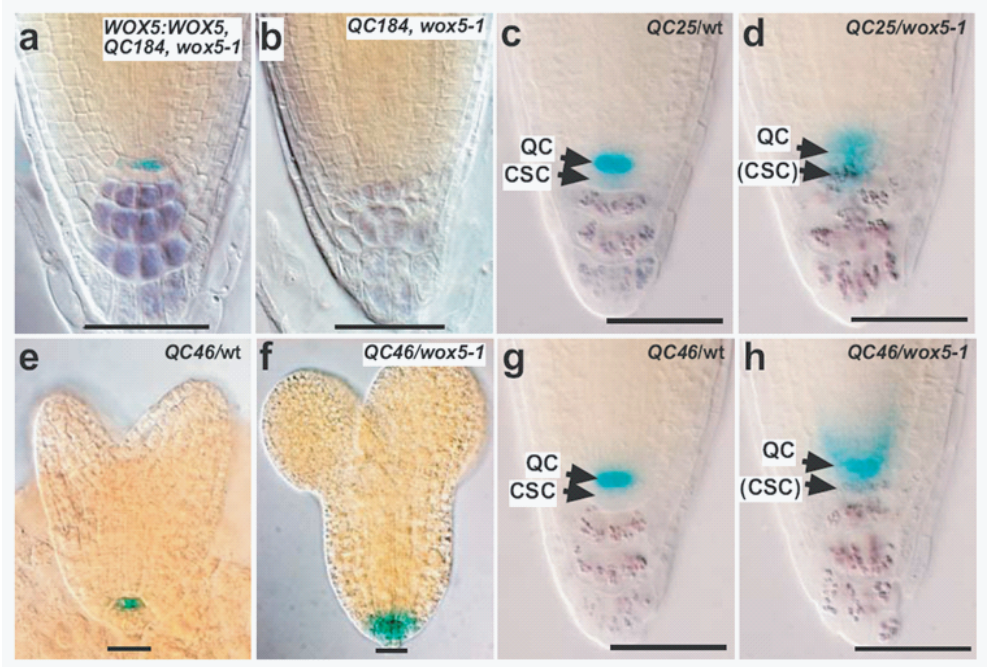


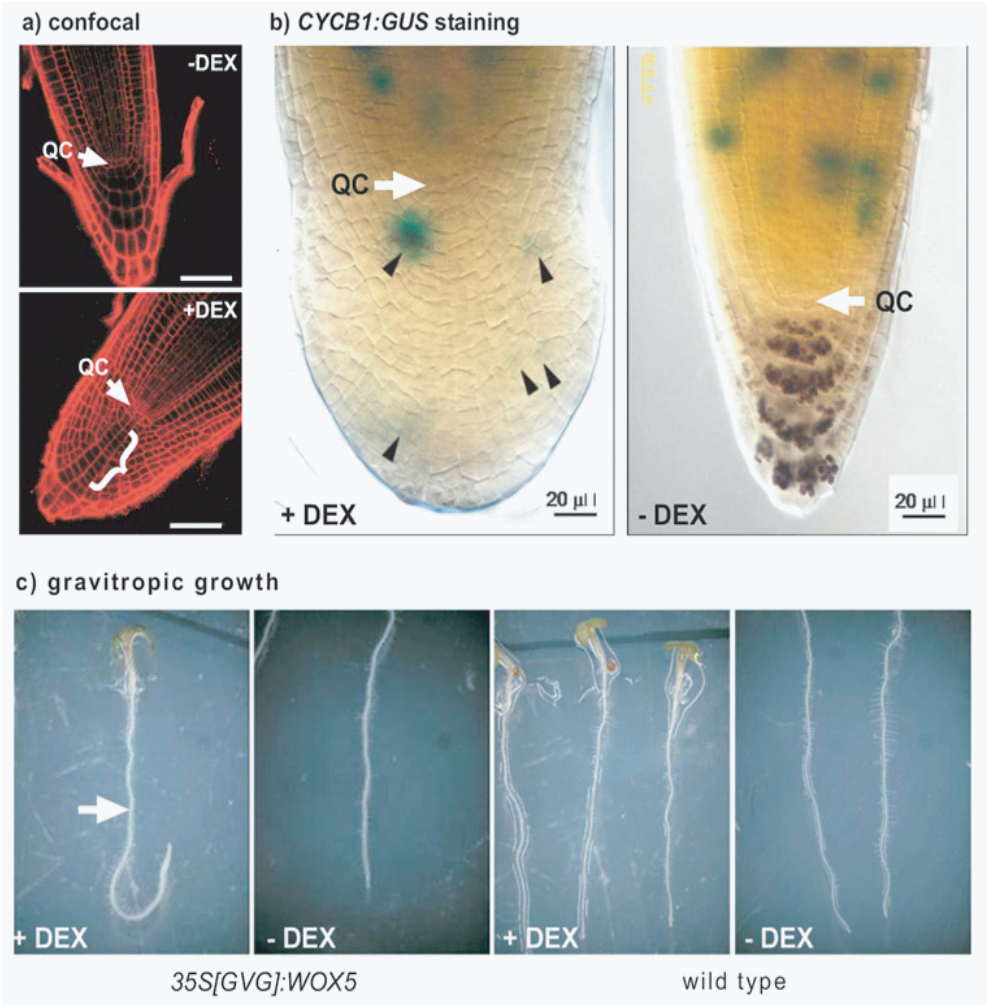
Supplementary Figure 1 – Shoot meristem organization, *WOX5* expression and mutant alleles. a, Schematic representation of the shoot meristem stem cell niche with WUS and CLV3 expression domains in the organizing center (OC) and at the stem cell position, respectively. Adopted from Laux (2003) **b-c**, WOX5 is expressed throughout QC development. A WOX5:GUS gene that mimics endogenous mRNA expression is expressed in the embryonic QC cell lineage (**b**) and during postembryonic development (**c**). **d**, In *wox5-1* roots, *WOX5* mRNA is not detectable and QC cells and distal stem cells have enlarged in comparison to wild type. **e**, Schematic representation of the WOX5 gene with the position of the insertions in *wox5-1* and *wox5-3*. Amino acid residue positions of insertions are indicated (aa). Scale bars: 50 µm.



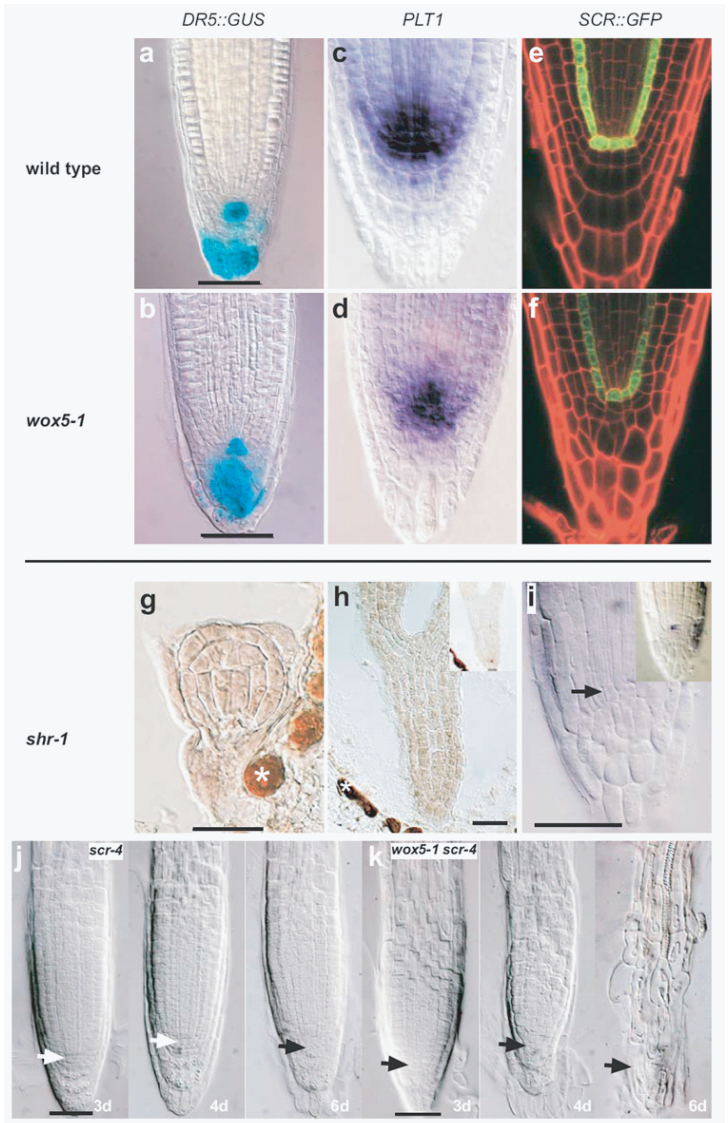
Supplementary Figure 2 – Comparison of *WOX5* and *WUS* expression patterns. *WUS* expression (red) initiates in 4 apical subepidermal cells of the 16-cell embryo, is continued during several rounds of asymmetric divisions in the cell lineage that will form the OC, and remains restricted to the OC in the active shoot meristem. WOX5 expression (blue) is initiated in the hypopheseal cell of the early globular embryo, is continued in the lense shaped cell that will give rise to the QC during an asymmetric division, and is restricted to the QC in the active root meristem.



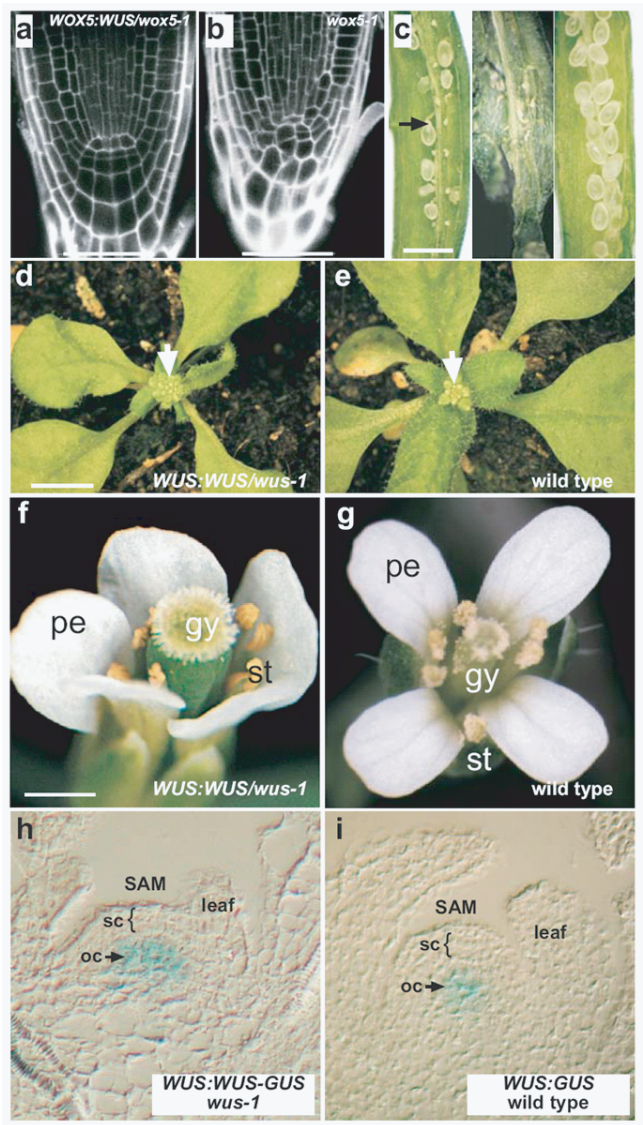
Supplementary Figure 3 – WOX5 is required for stem cell identity in the root meristem. a,b, Complementation of *wox5-1* mutant by WOX5 cDNA expressed from the WOX5 promoter. Expression of QC marker QC184 (light blue) and lugol staining of starch grains (violet) are shown. **c-h**, Median longitudinal optical sections through 4 day-old root tips (**c,d,g,h**) and heart-torpedo stage embryos (**e,f**) show that *wox5-1* roots express the QC identity marker *QC25* (**d**) and *QC46* (**f,h**), in larger domains than in wild type (**c,e,g**). The columella stem cells (CSC) in the wild type root meristem are localized subjacent to the QC and do not contain starch grains (that stain with lugol) in contrast to their differentiated daughter cells (**c,g**). In *wox5-1* roots, the cells at the stem cell position have undergone differentiation and stain with lugol (**d,h**). GUS staining gives a bright blue color; amyloplast (lugol) staining a reddish/violet color. Scale bars: 50 µm (**a-d,g,h**); 20 µm (**e,f**).



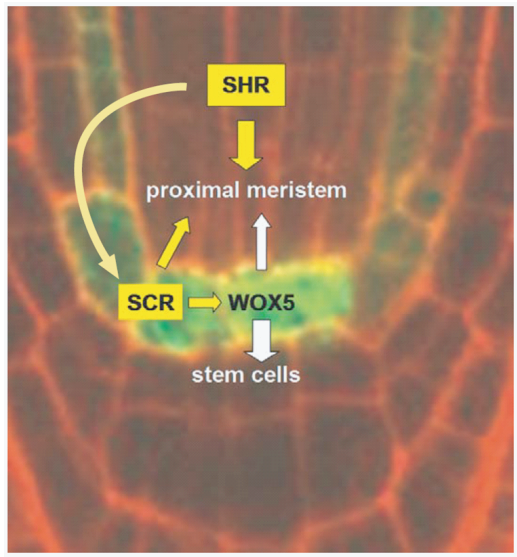
Supplementary Figure 4 – Cell division and gravitropic response of 35S(GVG):WOX5 plants. a, induced 35S(GVG):WOX5 expression results in extra layers of small undifferentiated cells in the root tip (+DEX, waved bracket), which does not occur in the non-induced control (-DEX). **b**, expression of CYCB1:GUS, a marker for G2-M transition, in a DEX-treated 35S(GVG):WOX5 root is detected in the small cells (arrowheads) several layers distal from the QC (arrows in left panel), which is never observed in differentiated large wild type columella cells (right panel). Both images show normal expression of the marker gene in the proximal meristem. Note also the absence of starch grains (stained dark brown with lugol in non-induced root) in columella of the DEX induced root. **c**, DEX-treated 35S(GVG):WOX5 roots exhibit agravitropic growth, not observed in the absence of DEX induction or wild type seedlings under the same conditions. Arrow indicates approximate length of the root at the time of transfer to DEX medium. Scale bars: 50 µm (**a**); 20 µm (**b**).



Supplementary Figure 5 – WOX5 interactions with known pathways. a-f, Expression of DR5::GUS (**a,b**), *PLT1* mRNA (**c,d**), and pSCR:GFP (**e,f**) root regional markers are not altered in the *wox5-1* mutant. **g-i**, in *shr-1* mutants, *WOX5* expression is undetectable, except for sporadic cases of reduced expression (insets in **h,i**). Longitudinal median histological section of globular embryo (**g**) and torpedo stage embryo (**h**) are shown after in situ hybridization with a *WOX5* antisense probe. Optical longitudinal median sections through 4-day-old root tip is shown after whole mount in situ hybridization with *WOX5* antisense probe (**i**). Hybridization signal is recognized as a blue/brown color (insets **h,i**). *, staining independent of hybridization reaction. **j-k**, the *wox5-1* mutant enhances differentiation in the proximal meristem in combination with *scr-4*. Arrows indicate the QC position. Scale bars: 50 µm (**a-f,i,j,k**); 20 µm (**g,h**).



Supplementary Figure 6 - Functional WOX5/WUS comparison. a,b, Expression of WUS from the WOX5 promoter restores the QC and columella stem cells in *wox5-1* roots. Confocal images are shown. **c-g**, Rescue experiments in the shoot meristem stem cell niche. Ovule development is rescued only by *pWUS:WUS* (**c**, left, arrow indicates rescued ovule), but not by *pWUS:WOX5* (**c**, middle) expression. Right panel (**c**) shows a wild type silique. *WUS:WUS* control expression (**d,f**) in a homozygous *wus-1* background rescues stem cell maintenance, giving rise to an indeterminate inflorescence meristem (**d**) and normal flowers (**f**) as observed in wild type (**e,g**). 26 day-old plants (**d,e**) and flowers (**f,g**) are shown. White arrows indicate shoot apex. **h,i**, A functional WUS-GUS fusion protein expressed from the *WUS* promoter rescues the *wus-1* mutant and remains localized in the centrally located organizing center (oc) of the shoot apical meristem (SAM), but is not detected in the uppermost three stem cell (SC) layers (**h**), indistinguishably from the GUS protein expressed from the *WUS* promoter (**i**). gy, gynoceum; pe, petal; st, stamen. Scale bars: 1 cm (**d,e**); 1 mm (**c,f,g**); 50 µm (**a,b**).



Supplementary Figure 7 – *SHR/SCR-WOX5* interactions in root stem cell maintenance. *WOX5* gene expression in the QC is required to maintain the underlying distal stem cells and, redundantly, the proximal meristem undifferentiated. SHR/SCR signaling affects root meristem maintenance both through *WOX5* and independently of *WOX5*.

Methods and Materials

PCR Based Genotyping

WOX5 alleles were genotyped using gene specific primers F11B5' (5'-TAGATGGAACAGAAGCCTAGATAGGTTAGGA-3') and F11B3' (5'-TCTGTGATGCAAATAGAACTATTCGTTAATG-3'), and the T-DNA left border specific primer SalkLBa1 (5'-TGGTTCACGTAGTGGGCCATCG-3'). The *wus-1* mutation was identified by PCR as described previously (Gross-Hardt et al., 2002).

Transgenic work

For the *WOX5:NLSGUS* (AKS34) construct, 4.6 kb of the 5' upstream region of *WOX5* cDNA was amplified from Landsberg *erecta* (L*er*) genomic DNA with WOX5P-S1 (5'-AACTGCAGAAAGACTTTTATCTACCAAC-3') and WOX5P-As (5'-AACTGCAGTTCAGATGTAAAGTCCTC-3') primers, subcloned into pGEM-T (Promega), digested with PstI and cloned into PstI digested MT255 (a pBar-NLSGUS vector) producing AKS34. The *WOX5* inducible ectopic expression plants contain the construct with *UAS:mGFP5ER* (kindly provided by J. Haseloff) and *35S:GAL4:VP16:GR* (*GVG*; Aoyama and Chua, 1997) and the construct with *UAS:WOX5* cDNA. Both constructs were transformed separately into wild-type *Arabidopsis* (accession *Col*) and the resulting lines were crossed to generate *35S[GVG]:WOX5* plants.

To generate *WOX5:WOX5* (AKS82), *WOX5* cDNA was amplified from Ler cDNA library with F11B9-Up (5'-TCTCGAGGGATATATACACAGGCCCTAAAACGT-3') and F11B9-DOW (5'-TGGATCCTTGTTATTGTGGTCAATGACTTAAAGAAA-3'), and cloned into pGEM-T producing AKS16. *WOX5* cDNA amplified from AKS16 with WOX5-S-BamHI (5'-CGGGATCCCGATATATACACAGGCCC-3') and F11B9-DOW primers was cloned into the pGEM-T vector (Promega; producing AKS81), re-isolated as a BamHI fragment, and cloned behind the *WOX5* promoter. The *WOX5:WUS* (AKS84) construct was generated by isolating BamHI digested *WUS* cDNA from MT141 (Lenhard et al., 2002) and cloned behind the *WOX5* promoter into AKS70 using a BamHI restriction site. Unless otherwise described, all binary construct were in a *pGPTV-BAR* vector (Becker et al., 1992). To express *WOX5* in the shoot meristem stem cell niche, *WOX5* cDNA was cloned behind the pOp promoter (Moore et al., 1998) and both, *pOp:WOX5* and *pOp:WUS* were expressed by a *WUS:LhG4* driver construct as described previously (Gross-Hardt et al., 2002; Schoof et al., 2000).

Chapter 3

PLETHORA proteins as dose-dependent master regulators of *Arabidopsis* root development

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Abstract

Factors with a graded distribution can program fields of cells in a dose-dependent manner (Tabata and Takei, 2004; Gurdon and Bourillot, 2001), but no evidence has hitherto surfaced for such mechanisms in plants. In the *Arabidopsis thaliana* root, two *PLETHORA* (*PLT*) genes encoding AP2-domain transcription factors have been shown to maintain the activity of stem cells (Aida et al., 2004). Here we show that a clade of four PLT homologues is necessary for root formation. Promoter activity and protein fusions of PLT homologues display gradient distributions with maxima in the stem cell area. PLT activities are largely additive and dosage dependent. High levels of PLT activity promote stem cell identity and maintenance; lower levels promote mitotic activity of stem cell daughters; and further reduction in levels is required for cell differentiation. Our findings indicate that PLT protein dosage is translated into distinct cellular responses.

Introduction

During animal development, instructive molecules acquire a graded distribution and induce distinct cellular responses in a concentration-dependent manner. Whether similar mechanisms occur in plants has been controversial; dosage-sensitive action of plant hormones has been inferred only after external application (Skoog and Miller, 1957). Plant stem cell regions, which supply cells for the growing root and shoot systems (Weigel and Jürgens, 2002), are potential sites of action for instructive gradients. Stem cells are maintained in local micro-environments, which are similar to animal stem cell niches (Spradling et al., 2001). Stem cell daughters undergo additional divisions in transit-amplifying cell compartments called meristems; when cells leave the meristem they rapidly expand and differentiate. The *PLETHORA1* (*PLT1*, At3g20840) and *PLT2* (At1g51190) genes encode AP2-domain transcription factor family members essential for defining the root stem cell niche (Aida et al., 2004). *plt1*;*plt2* mutants display stem cell loss, loss of transit-amplifying cells and reduced cell expansion. PLT1 and PLT2 expression strongly correlates with a transcriptional response maximum to the plant hormone auxin in the root tip (Aida et al., 2004; Xu et al., 2007) and this maximum has been shown to have profound organizing activity (Sabatini et al., 1999); a property often associated with sources of instructive gradients. Here, we reveal that the *PLT* gene family controls distinct aspects of root development in a dose-dependent manner through PLT expression gradients that culminate in the stem cell niche.

Results

The proteins encoded by At5g10510/*AINTEGUMENTA-LIKE6*(*AIL6*)/*PLT3* and At5g17430/*BABY BOOM*(*BBM*) group with PLT1 and PLT2 in the AP2/ERF transcription factor family (Supplementary Fig. 1; Nole-Wilson et al., 2005), and these candidate redundant factors are predicted to be expressed in the root (Birnbaum et al., 2003).

From the heart-stage of embryogenesis onward, *PLT3* is expressed in provascular cells, the quiescent centre and columella progenitor cells (Fig. 1a). Post-embryonically, *PLT3* messenger RNA accumulates in the root stem cell niche with the strongest signal in the columella stem cell layer (Fig. 1b), in contrast to the predominant quiescent-centre-localization of *PLT1* and *PLT2* transcript (Aida et al., 2004). At the heart-stage of embryo development, *BBM* is expressed in provascular cells and in the lens-shaped quiescent centre progenitor cell (Fig. 1c). Post-embryonically, *BBM* transcript accumulates in the quiescent centre and columella stem cells—in a similar manner to the *PLT* mRNAs—and in provascular cells of the proximal meristem (Fig. 1d).

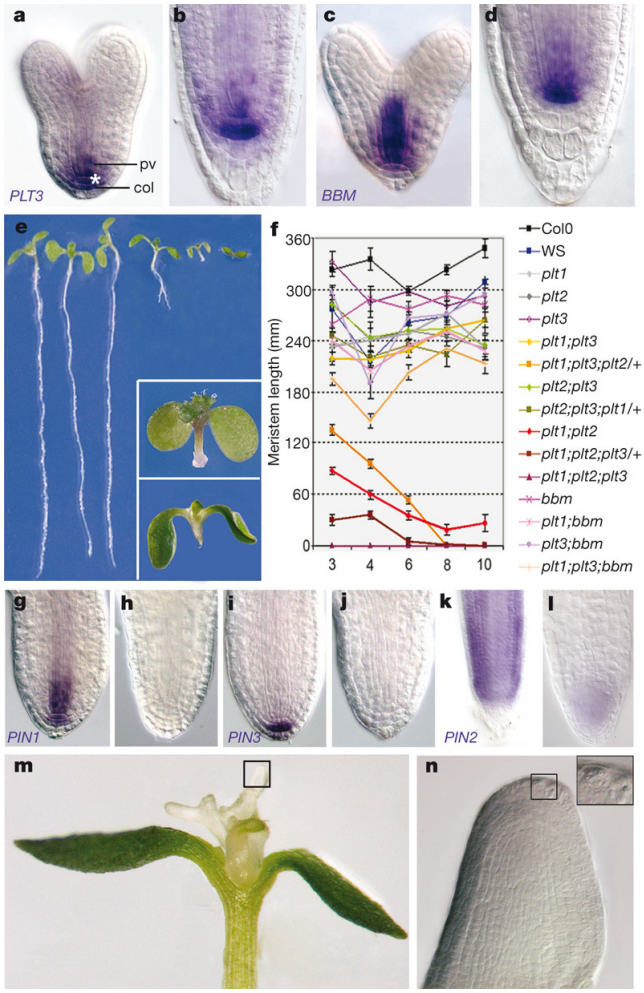


Figure 1: Four *PLT* genes promote root formation. a–d, *In situ* hybridization with *PLT3*- (**a, b**) and *BBM*- (**c, d**) specific probes in wild-type embryos at heart-stage (**a, c**), and in roots of 3 d.p.g. wild-type plants (**b, d**). Asterisk, quiescent centre; pv, provasculature; col, columella. **e**, Seedlings 10 d.p.g., from left to right: wild type, *plt3*$^{-/-}$, *bbm-1*$^{-/-}$, *plt1*$^{-/-}$*plt2*$^{-/-}$, *plt1*$^{-/-}$*plt2*$^{-/-}$*plt3*$^{-/-}$ and a *plt1*$^{-/-}$*plt2*$^{+/-}$*plt3*$^{-/-}$*bbm-1*$^{-/-}$ segregant. Insets show magnification of *plt1*$^{-/-}$*plt2*$^{-/-}$*plt3*$^{-/-}$ mutant (upper) and *plt1*$^{-/-}$*plt2*$^{+/-}$*bbm-1*$^{-/-}$ segregant (lower). **f**, Meristem size in wild type (Col0 and WS) and *plt* mutants at the indicated d.p.g. For each data point, n = 10 to 50; error bars, s.e.m. **g–l**, *In situ* hybridization using PIN probes on wild-type (g, i) and *plt1*$^{-/-}$*plt2*$^{-/-}$*plt3*$^{-/-}$ (h, j) torpedo-stage embryos and wild-type (**k**) and *plt1*$^{-/-}$*plt2*$^{-/-}$*plt3*$^{-/-}$ mutant (**l**) 2 d.p.g. seedlings. **m**, Shoot of 9 d.p.g. *35S-PLT2–GR* plant 6 days after dexamethasone application. **n**, Magnification reveals cellular organization of ectopic root including columella starch granules.

The *plt3-1* mutant allele carries a T-DNA insertion interrupting the first AP2 domain (Supplementary Fig. 2). No transcript was detected by PCR with reverse transcription (RT–PCR) or by *in situ* hybridization on *plt3-1* seedlings (data not shown), suggesting that *plt3-1* is a null allele. Homozygous *plt3* single mutants have slightly shorter roots and meristems compared to wild type, but *plt1*$^{-/-}$*plt2*$^{-/-}$*plt3*$^{-/-}$ triple homozygotes are rootless (Fig. 1e, upper inset). Progeny from *plt1*$^{-/-}$*plt2*$^{-/-}$*plt3*$^{+/-}$, *plt1*$^{-/-}$*plt2*$^{+/-}$*plt3*$^{-/-}$ and *plt1*$^{+/-}$*plt2*$^{-/-}$*plt3*$^{-/-}$ plants segregate ~25% rootless triple mutants (Supplementary Table 2), demonstrating linkage between the rootless phenotype and the three *PLT* genes. The embryonic root pole of triple homozygous seedlings is fully differentiated at 3 days post germination (d.p.g.) and adventitious root primordia arrest at 6 d.p.g. (Supplementary Fig. 3). Mature *plt1*$^{-/-}$*plt2*$^{-/-}$ embryos have only subtle defects in the cellular organization of the distal-most region Aida et al., 2004; Supplementary Fig. 4), but *plt1*$^{+/-}$*plt2*$^{-/-}$*plt3*$^{-/-}$ parents yield ~25% embryos with aberrant root poles that lack a lateral root cap cell layer

(Supplementary Fig. 4).

We previously showed that *plt1*$^{-/-}$*plt2*$^{-/-}$ mutants have strongly reduced transcription of the *PIN4* gene, which encodes an auxin efflux facilitator (Blilou et al., 2005). In triple mutant embryos from *plt1*$^{+/-}$*plt2*$^{-/-}$*plt3*$^{-/-}$ parents, *PIN1* and *PIN3* mRNAs are strongly reduced (Fig. 1g–j and Supplementary Table 1). Post-embryonic *PIN2* mRNA is strongly reduced in triple mutant roots before differentiation (Fig. 1k, l). Therefore, PLT1, PLT2 and PLT3 redundantly control expression of multiple *PIN* genes in the embryonic and postembryonic root.

bbm-1 and *bbm-2* mutant alleles carry T-DNA insertions before and in the beginning of the first AP2 domain, respectively (Supplementary Fig. 2). Truncated transcripts are detected by RT–PCR and may be translated, but genetic interactions (described below) suggest that the insertions cause loss-of-function effects. *plt3*$^{-/-}$*bbm*$^{-/-}$ double mutants have a shorter root and root meristem than either single mutant (Fig. 1f, and Supplementary Fig. 3).

Intriguingly, the progeny of plants segregating different *plt* and *bbm* allele combinations lack root and hypocotyl (Fig. 1e, lower inset) at significant frequencies (Supplementary Table 2), reaching ~10% of the progeny of selfed *plt1*$^{-/-}$*plt2*$^{+/-}$*plt3*$^{-/-}$ *bbm-2*$^{-/-}$. These defects initiate in the early basal embryo (Supplementary Fig. 5) and resemble those in mutants of the auxin response factor *MONOPTEROS* (Hardtke and Berleth, 1998) and the auxin perception machinery (Hellmann et al., 2003; Dharmasiri et al., 2005). *PLT* genes do not seem to strongly perturb early global auxin-dependent patterning processes, as suggested by essentially normal cotyledon vasculature in the triple mutant (Supplementary Fig. 4). Segregation of *plt2* in a homozygous *bbm* background and vice versa yields ~25% early arrested embryos, and homozygous double mutants could not be recovered, indicating a redundant function in early embryogenesis (data not shown).

Ectopic root structures are initiated by constitutive embryonic expression of *PLT* genes Aida et al., 2004) and after induction of *BBM* expression (Srinivasan et al., 2007). To test whether PLT induction induces a developmental switch to root development, we expressed a PLT2–GR fusion protein that complements *plt1*$^{-/-}$*plt2*$^{-/-}$ after dexamethasone (Dex) induction, when driven by its own promoter (Supplementary Fig. 6). When *35S-PLT2–GR* is activated by application of Dex, roots are produced from the shoot apex (Fig. 1m, n). Our gain- and loss-of-function experiments indicate that *PLT* genes are master switches for root development.

plt1$^{-/-}$*plt2*$^{+/-}$*plt3*$^{-/-}$ mutants have intermediate root and meristem size between *plt1*$^{-/-}$ *plt3*$^{-/-}$ and *plt1*$^{-/-}$*plt2*$^{-/-}$*plt3*$^{-/-}$ (Fig. 1e, f, and Supplementary Fig. 3) and 50% of *plt1*$^{-/-}$*plt2*$^{+/-}$*plt3*$^{-/-}$*bbm*$^{-/-}$ seedlings have shorter roots than *plt1*$^{-/-}$*plt3*$^{-/-}$*bbm*$^{-/-}$, whereas 50%

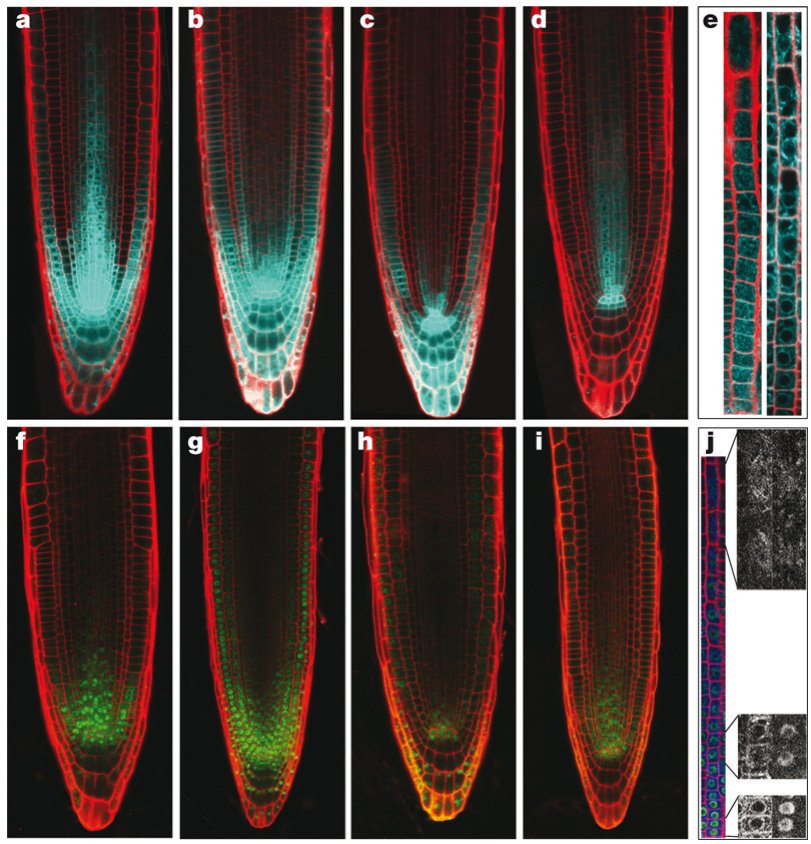


Figure 2: *PLT* promoter activity and PLT protein fusions display gradients. **a–d**, CFP reporter driven by full-size promoters of *PLT1* (**a**) *PLT2* (**b**) *PLT3* (**c**) and *BBM* (**d**). **e**, Epidermal gradient of *PLT2* (left) but not *RCH2* (right) promoter. **f–i**, YFP reporter fused in-frame to genomic fragments of *PLT1* (**f**), *PLT2* (**g**), *PLT3* (**h**) and *BBM* (**i**). **j**, Co-localization in one plant of *PLT2* transcriptional (CFP, left magnification) and translational (YFP, right magnification) fusion viewed in different regions using separate channels.

have no primary root (Supplementary Table 2). *plt3* alleles are also semi-dominant, because growth and meristem maintenance defects in $plt1^{-/-}plt2^{-/-}plt3^{+/-}$ seedlings are intermediate between $plt1^{-/-}plt2^{-/-}$ and $plt1^{-/-}plt2^{-/-}plt3^{-/-}$ (Fig. 1f, and Supplementary Fig. 3). The semi-dominance of *plt2* and *plt3* loss-of-function alleles indicates dose-dependent activity.

To test whether *PLT* genes equally contribute to PLT 'dosage', we transformed $plt1^{-/-}plt2^{-/-}$ double mutants with *PLT1*, *PLT2*, *PLT3* and *BBM* genes fused to the yellow fluorescent protein gene *YFP* and driven by the full *PLT2* promoter. In independent lines with similar overall YFP levels, *PLT1* and *PLT2* fully complemented and *PLT3* and *BBM* partially complemented root growth in the double mutant. All PLT proteins rescued columella stem cell activity (Supplementary Figs 6 and 7). Thus, total PLT levels and to some extent intrinsic differences in PLT protein activity contribute to

root growth and stem cell maintenance.

Transgenic lines carrying complete promoters of the *PLT* genes fused to the cyan fluorescent protein gene *CFP* reveal highest promoter activity in the stem cell niche, consistent with mRNA levels, but they also show graded activity in the proximal meristem (Fig. 2a–d). Gradients can be observed in epidermal surface views, excluding quenching effects, and they are specific to *PLT* promoters (Fig. 2e). To analyse whether this promoter activity drives a PLT protein gradient, we combined the PLT–YFP fusions with their corresponding full promoters. *PLT1* and *PLT2* gene fusions complemented *plt1*$^{-/-}$*plt2*$^{-/-}$mutants (Supplementary Figs 6 and 7, and data not shown).

All PLT protein fusions revealed conspicuous gradients that extend into the transit-amplifying cells and, for the PLT2 and PLT3 fusions, into the elongation zone (Fig. 2f–i). The promoter and protein gradients fully match when combined in one plant (Fig. 2j). We previously reported accumulation of *PLT* transcripts in the stem cell area (Aida et al., 2004), but, after extended staining, *PLT1 in situ* hybridizations also reveal a broader expression domain (Supplementary Fig. 8). We concluded that *PLT* promoter activity leads to protein gradients with maximum expression in the stem cell niche. PLT1 and PLT2 expression maxima broadly encompass the niche, whereas PLT3 and BBM are more restricted.

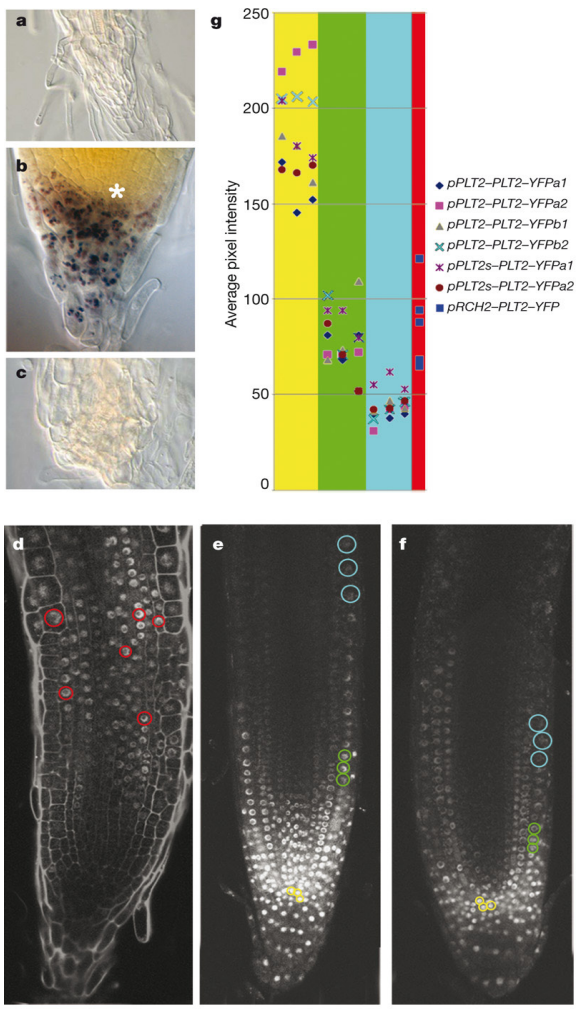
We asked whether differences in PLT expression domains affect the ability of PLT proteins to compensate for redundant partners. Indeed, *PLT1* and *PLT2* only partially complement a *plt1*$^{-/-}$*plt2*$^{-/-}$ mutant when driven by the *BBM* promoter (Supplementary Figs 6 and 7).

Our experiments suggested that the PLT protein concentration gradient instructs different outputs in different regions, even though each gene slightly differs in activity and expression profile. We therefore tested whether altering the level or shape of the PLT2 gradient affects the position of developmental boundaries. We expressed the PLT2–YFP fusion in *plt1*$^{-/-}$*plt2*$^{-/-}$ mutants under the *RCH2* promoter, which has low activity in the stem cell area but is active in meristematic and elongating cells at a level comparable to that of the *PLT2* promoter (Fig. 3d–g). *RCH2-PLT2–YFP* prolongs transit-amplifying cell divisions but fails to maintain stem cells at 7 d.p.g. (Fig. 3b, d). The transit-amplifying cell pool is lost at 12 d.p.g. (Fig. 3c). We concluded that intermediate PLT levels in the meristem promote transient cell cycling.

To validate that meristem size is controlled by a PLT gradient, we analysed *plt1*$^{-/-}$ *plt2*$^{-/-}$ mutants complemented with the PLT2–YFP construct driven by a truncated 1.3-kb *PLT2* promoter fragment (*pPLT2s*). This truncated promoter drives significant expression in the stem cell area but the gradient declines more rapidly (Fig. 3e, f).

Accordingly, stem cells are rescued but root and meristem sizes are ~50% smaller (Supplementary Fig. 7). The amount of YFP signal per mid-nuclear section in the stem cell zone, halfway the meristem, and in the first expanding cells, provides three clearly separated intensity ranges that match with zonation in the full- and truncated-promoter driven gradients (Fig. 3g), suggesting that the PLT2 gradient defines meristem zonation.

A dose-dependent gradient model predicts that PLT overexpression shifts the meristem boundary. Indeed, Dex induction of *35S-PLT2–GR* plants promotes continuous growth of the transit-amplifying cell pool and meristem size increases (Fig. 4a–c). Ink toner marks marking the elongation zone boundaries at the time of induction reveal that PLT overexpression sustains cell division only in cells that are still cycling and inhibits cell expansion in the elongation zone. These data reinforce the idea that distinct PLT levels dictate cell proliferation and mitotic exit.



The auxin response marker *DR5-GUS* (Sabatini et al., 1999) and *PIN3* transcription do not change in *35S-PLT2–GR* plants just before the onset of meristem size

Figure 3: PLT expression regulates stem cell maintenance and meristem boundary. a–d, Meristem prolongation but not stem cell rescue in *RCH2-PLT2–YFP* plants. Nomarski optics image of root tip of 7 d.p.g. *plt1;plt2* (**a**), and of *plt1;plt2 RCH2-PLT2–YFP* at 7 d.p.g. (**b**) and 12 d.p.g. (**c**). Starch granule staining (brown) shows no rescue of columella stem cells below the quiescent centre. Confocal view of 7 d.p.g. *plt1;plt2 RCH2-PLT2–YFP* root (**d**) shows that the meristem is rescued and reveals no expression of PLT2–YFP in the stem cell area. Asterisk in **b**, quiescent centre. **e**, **f**, Promoter truncation shifts the meristem boundary. CLSM views at identical pinhole and laser settings for *RCH2-PLT2–YFP* (**d**), *pPLT2-PLT2–YFP* (**e**) and *pPLT2s-PLT2–YFP* (**f**). **g**, Quantification of fluorescence per nucleus in *pRCH2-PLT2–YFP* transient meristem (red circles in **d,** and red graph areas), and in stem cells (yellow in **e**, **f** and graph area), mid-meristem (green in **e**, **f** and graph area) and first elongating cells (blue in **e**, **f** and graph area) of *pPLT2-PLT2–YFP* and pPLT2s-PLT2–YFP (a and b indicate independent transformants, 1 and 2 indicate different roots).

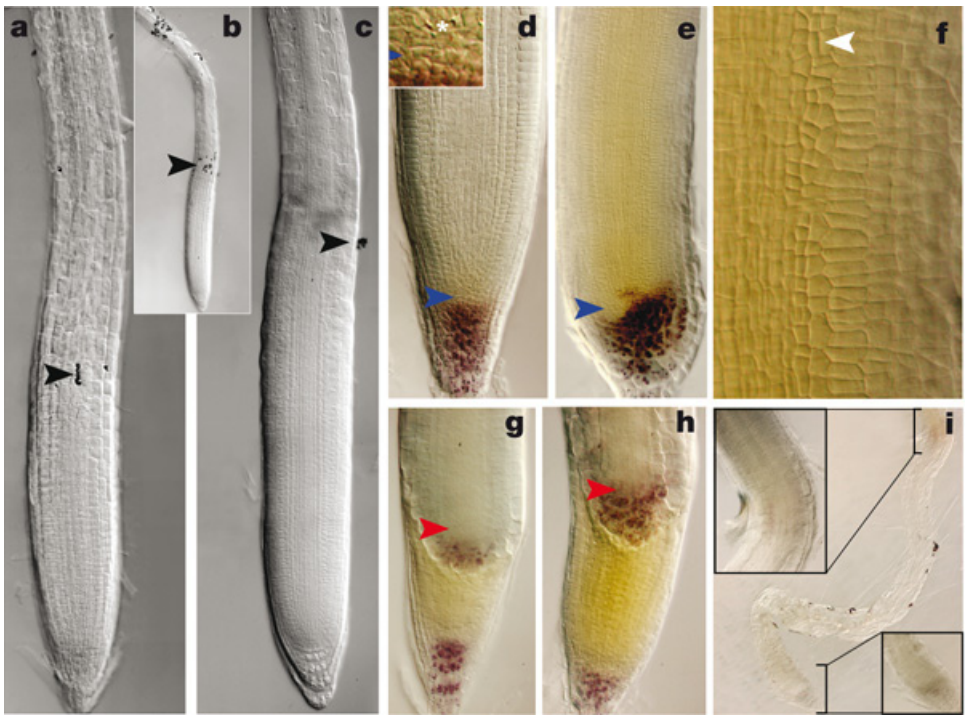


Figure 4: Inducible expansion of meristem and stem cell area with PLT2–GR fusions. a–c, *35S-PLT2–GR* 7 d.p.g. without Dex (**a**) and 1 d after 5 µM Dex application (**b**, **c**). Overview shows positioning of ink toner particles that mark the meristem boundary (black arrowhead) and upper elongation zone boundary at the onset of induction (**b**); the elongation zone boundary is defined as the position where cortical cells rapidly expand. Induced PLT2–GR roots reveal cell division below the meristem boundary and incomplete cell elongation (**c**). **d–f**, *35S-PLT2–GR;pRCH1-RBR* RNAi plants: 10 d.p.g. without Dex revealing the two *RBRi*-induced stem cell layers below the quiescent centre (blue arrowhead, inset), asterisk indicates the quiescent centre (**d**); with 3 d of Dex application, revealing excessive root cap stem cells (blue arrowhead) and periclinal divisions in the proximal meristem (**e**); magnification with ectopic periclinal divisions (**f** , white arrowhead). **g–i**, Duplication of the stem cell area (red arrowheads) and distal cell types (brown starch granules) in ~10% of 8 d.p.g. *35S-PLT2–GR*, *pRCH1-RBRi* plants after Dex application. Early (**g**), mid- (**h**) and late (**i**) stages of ectopic stem cell centre; note the prolonged activity of both stem cell centres (**i**, inset).

expansion, but only at later stages, indicating that PLT-induced expansion of the division zone is not caused by rapid changes in *PIN* expression (Supplementary Fig. 9).

Notably, the stem cell area in PLT2–GR plants is not altered after induction (Fig. 4c). The *RETINOBLASTOMA* (*RBR*) pathway was recently identified as an independent stem cell input (Wildwater et al., 2005), so we reasoned that this pathway might still limit stem cell pool size in the presence of higher PLT levels. Therefore, we combined a root-specific RNA interference (RNAi)-mediated silencing construct (*RCH1-RBRi;* Wildwater et al., 2005) with *35S-PLT2–GR*. After induction with Dex in the double transgenic, root meristem size increases as in *35S-PLT2–GR*, but clusters of dividing cells in the root cap area expand beyond that seen in *RCH1-RBRi*

alone (Fig. 4d, e). Moreover, periclinal divisions normally associated with stem cells occur throughout the proximal area (Fig. 4f). These data suggested that the high expression region of the PLT gradient can be instructive for stem cell fate. Dramatic support for this notion is provided by duplications of the distal stem cell area in ~10% of *RCH1-RBRi*;*35S-PLT2–GR* root meristem zones (Fig. 4g–i). We concluded that high PLT levels define the stem cell domain, confirming PLT dosage-dependent stem cell specification. This effect is normally limited by RBR. Low RBR levels in the *RCH1-RBRi* transgenic display limited expansion of the stem cell domain (Wildwater et al., 2005) because the PLT levels dictated by the gradient are limiting.

Discussion

Our data indicate that PLT protein gradients define three outputs in the growing root primordium: stem cell programming, mitotic activity and exit to differentiation. Analysis of PLT target genes will be required to assess how much of the response to graded activity is due to additive concentration effects on the same targets and to differences in target specificity.

Although the molecular link between auxin action and *PLT* gene activation may not be direct (Aida et al., 2004), auxin distribution and response systems are essential for correct *PLT* gene transcription. This raises the possibility that PLT proteins promote stem cells and transit-amplifying cells as a graded read-out of auxin distribution. In an accompanying paper, we provide evidence that PIN-mediated polar auxin transport establishes a dynamic gradient spanning the root meristem (Grieneisen et al., 2007). Hence it is tempting to speculate that an auxin gradient underlies the observed PLT gradients. Classical morphogen systems were conceptualized as independent from the response system. However, several gradients in animal development involve complicated dynamics (for example, O'Conner et al., 2006) and the static concept of positional information is being challenged (Jaeger et al., 2006). We show that PIN polar auxin transport facilitator expression that is essential for correct auxin distribution is regulated by PLT activity, which is a clear example of entanglement between positional information and its response system.

Methods Summary

Plant work

plt1-4 and *plt2-2* alleles were described in Aida et al. (2004), *plt3-1*, and *bbm-1* and *bbm-2* are salk T-DNA insertion lines 127417, 097021 and 067917, respectively, provided by the Signal Insertion Mutant Library (http://signal.salk.edu/). The T-DNA insertion in *PLT3* was confirmed by genotyping. The *plt1*;*plt2*;*plt3* triple mutant was generated by crossing *plt3-1* to *plt1-4*;*plt2-2*. *bbm-1* and *bbm-2* were crossed to *plt1-4*;*plt2-2* and *plt3-1* and allelic combinations were selected from F_2 populations. The T-DNA insertion site on *bbm-1* and *bbm-2* lines was verified by genotyping. Primers for genotyping are indicated in Supplementary Table 3. Promoter and genomic sequences were amplified from Col-0 genomic DNA using the primer combinations listed in Supplementary Table 3. Promoter fragments were fused to the endoplasmic reticulum targeted CFP coding sequence in a pGreenII vector (Hellens et al., 2000). For translational fusions, *PLT* genomic sequences were fused at the 3' end to either the *YFP* coding sequence or the carboxy-terminal-encoding region of the rat glucocorticoid (GR) receptor (Aoyama and Chua, 1997) and placed under the control of particular promoters (amplified regions are described in Supplementary Table 3). Promoter swaps were performed by fusing 5.8 kb of *PLT2* and 4.2 kb of *BBM* promoter fragments to the *YFP*-fused *PLT* genomic sequences. Transgenic plants were generated by transforming Col-0 wild-type or *plt1-4*;*plt2-2* plants, as described (Clough and Bent, 1998).

Phenotype analysis and microscopy

Light microscopy (Willemsen et al., 1998), confocal microscopy and aniline blue staining (Bougourd et al., 2000) of mature embryos was performed as described. Root length was measured, as before (Aida et al., 2004). Meristem cell length was measured using ImageJ (v.1.36) and mature cortical cell length as well as fluorescence levels were determined using Zeiss LSM Pascal (3.2SP2) software.

In situ hybridization

Whole-mount RNA *in situ* hybridization was performed as described (Blilou et al., 2005). The *PLT3* and *BBM* riboprobes, specific for non-conserved sequences downstream of the AP2 repeats, were prepared from templates amplified from complementary DNA (for primers, see Supplementary Table 3). The *PLT1* probe is as in Aida et al. (2004); the *PIN1*, *PIN2* and *PIN3* probes are as in Friml et al. (2002).

Further experimental details are provided as supplementary information

Acknowledgements

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Supplemental data to:

PLETHORA proteins as dose-dependent master regulators of *Arabidopsis* root development

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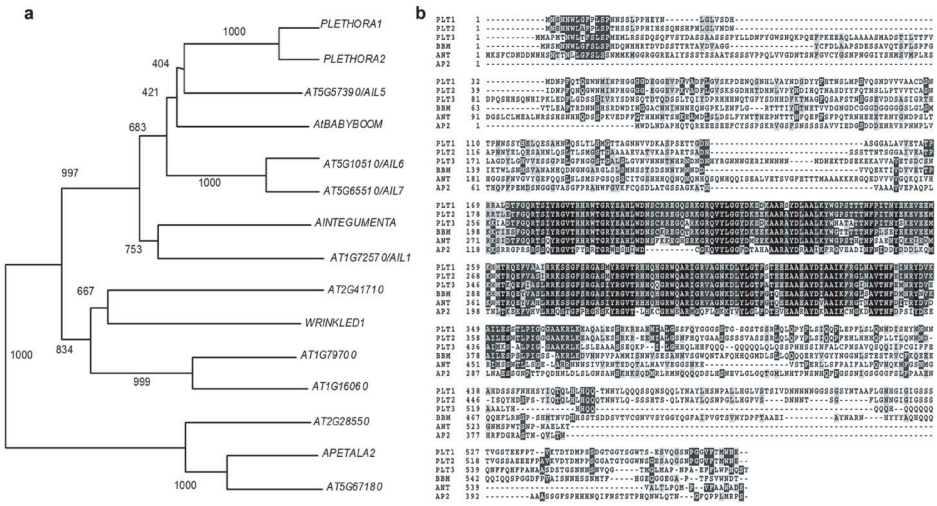
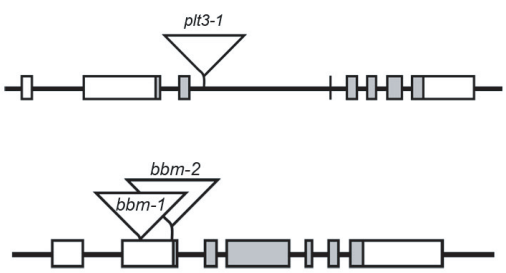
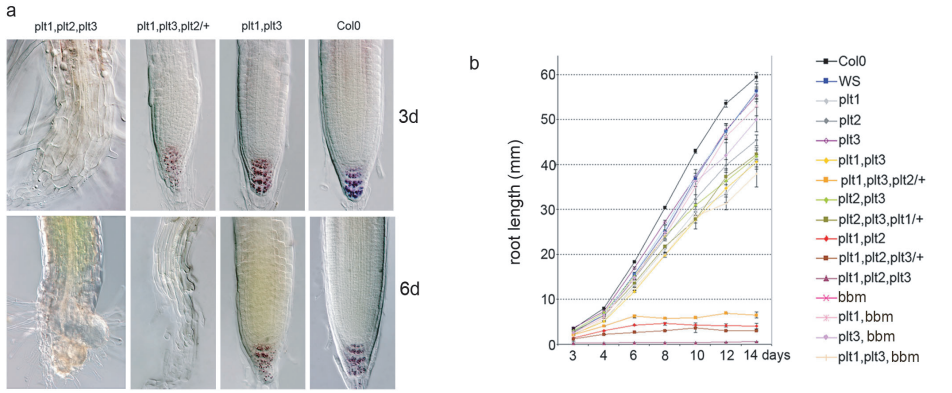


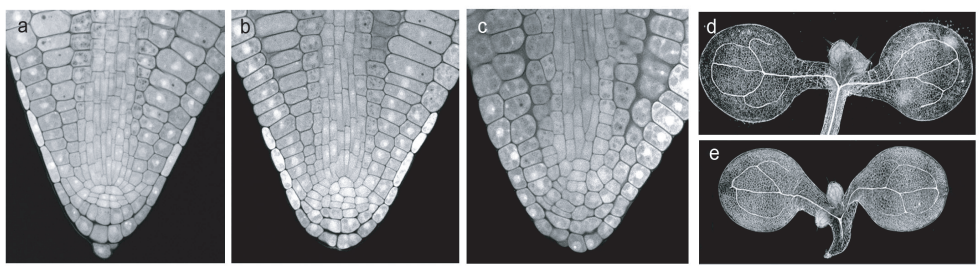
Supplementary Figure 1 – Sequence alignment and phylogenetic tree of PLT homologues. The protein sequences of *Arabidopsis* AP2/ERF genes were deduced from coding sequences found in the *Arabidopsis* genome (TAIR) database. PLT3 and AtBBM sequences were confirmed by sequencing the cDNA. (**a**) Phylogenetic tree using complete protein sequences constructed with ClustalX1.81. The tree was made with 1000 bootstrap trials, with correction for gaps in sequences. (**b**) Alignments of amino acid sequence of PLT1, PLT2, PLT3/AIL6, AtBBM, ANT and AP2.



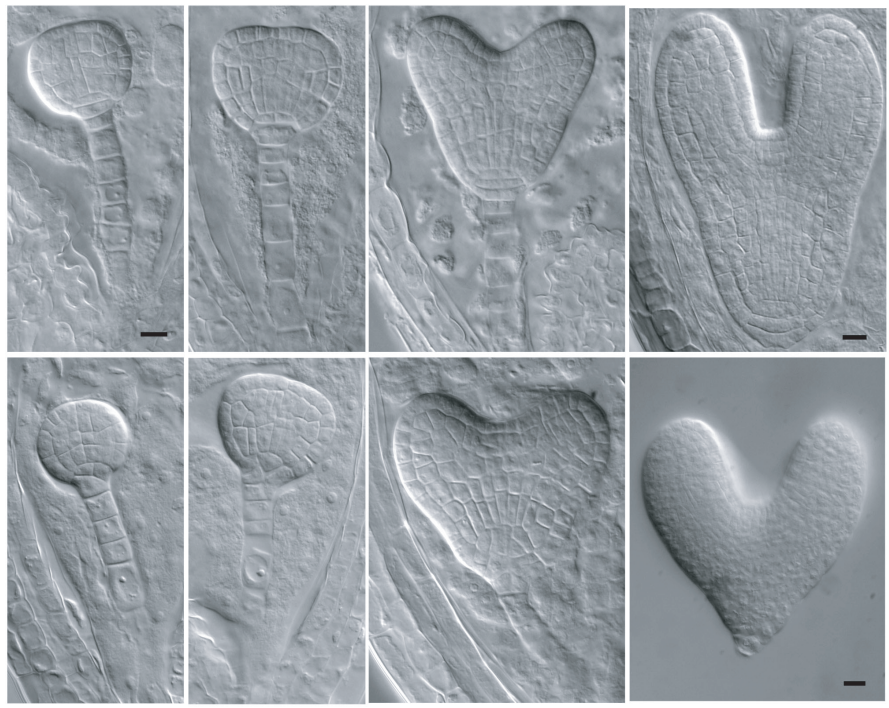
Supplementary Figure 2 – Location of T-DNA insertion sites in *plt3* and *bbm* alleles. The *plt3-1* insertion site was determined to be 1695 bp from the translation start and mapped with primers that border the insertion site (PLT3R and PLT3L) and with a T-DNA specific primer (LBb1) plus PLT3R. The *bbm*-1 and *bbm*-2 insertion sites were at position 792 bp and 918 bp, respectively, and were verified using two PCR reactions: a reaction with primers that border the insertion site (*bbm-1*, BBM1R and BBM1L; *bbm-2*, BBM2R and BBM2L) and a PCR reaction with a T-DNA specific primer (LBb1) and BBM1L or BBM2R, for *bbm-1* or *bbm-2*, respectively. Boxes, exons; gray boxes, conserved AP2 repeat; triangle shows insertion site of T-DNA. Primer sequences listed in Table S3.



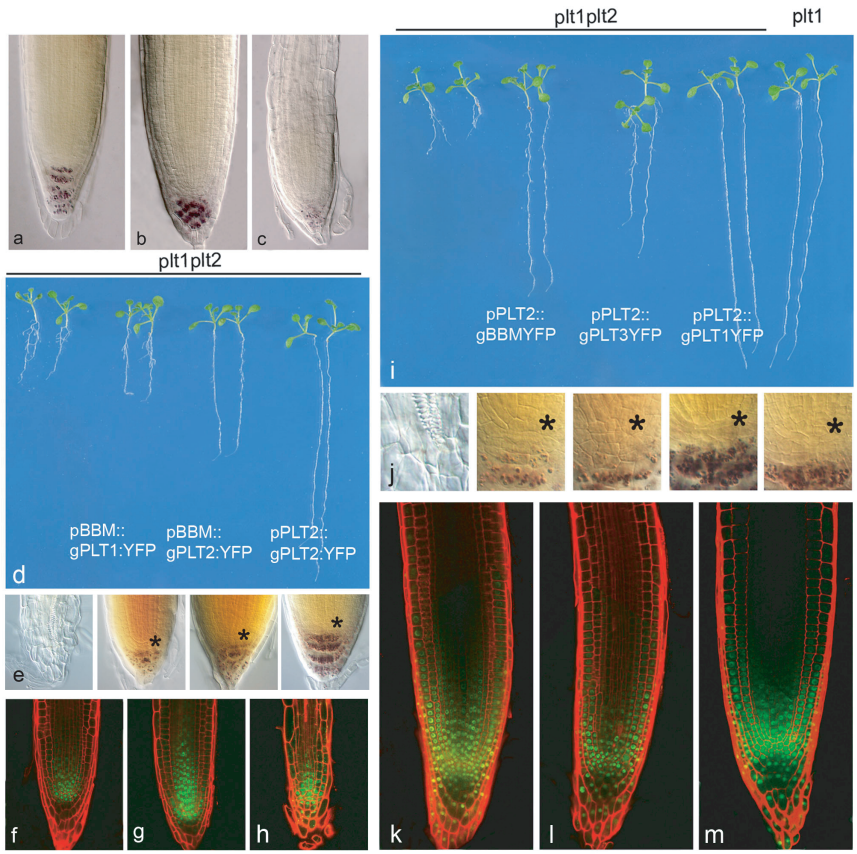
Supplementary Figure 3 – PLT dosage effect on root development. (**a**) Nomarski optics images of 3 and 6 dpg seedlings of indicated genotypes reveal dosage effect. Starch granules in columella cells stain brown. (**b**) Root length in wild-type and *plt* mutants at indicated dpg. For each data point, n=10 to 50, bars: std error.



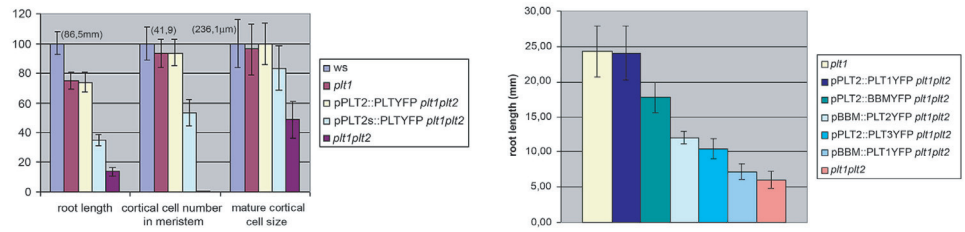
Supplementary Figure 4 – Embryo and seedling phenotypes of *plt* mutants. (**a-c**) Root pole of mature embryos stained with aniline-blue. Wild-type (**a**), *plt1*$^{-/-}$*plt2*$^{-/-}$ (**b**), and *plt1*$^{-/-}$*plt2*$^{-/-}$*plt3*$^{-/-}$ (**c**). (**d,e**) vasculature in cleared wt (**d**) and *plt1*$^{-/-}$*plt2*$^{+/-}$*plt3*$^{-/-}$*bbm-2*$^{-/-}$ (**e**) seedlings.



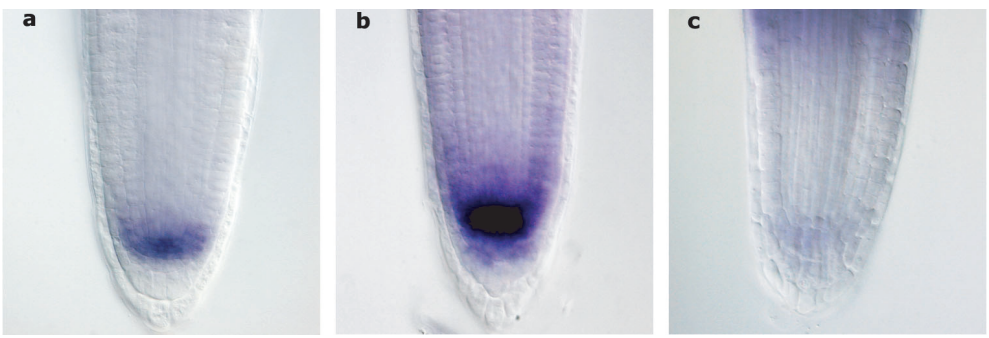
Supplementary figure 5 – Basal embryonic phenotype in progeny of *plt1*$^{-/-}$*plt2*$^{+/-}$*plt3*$^{-/-}$*bbm-2*$^{-/-}$ parents. Upper row from left to right: wildtype-like sibling embryos at early and late globular, heart and torpedo stages. Lower row: mutants of comparable stages. Frequency of aberrant divisions at early heart stage was 15% (n=64). Scale bars: 10 μm.



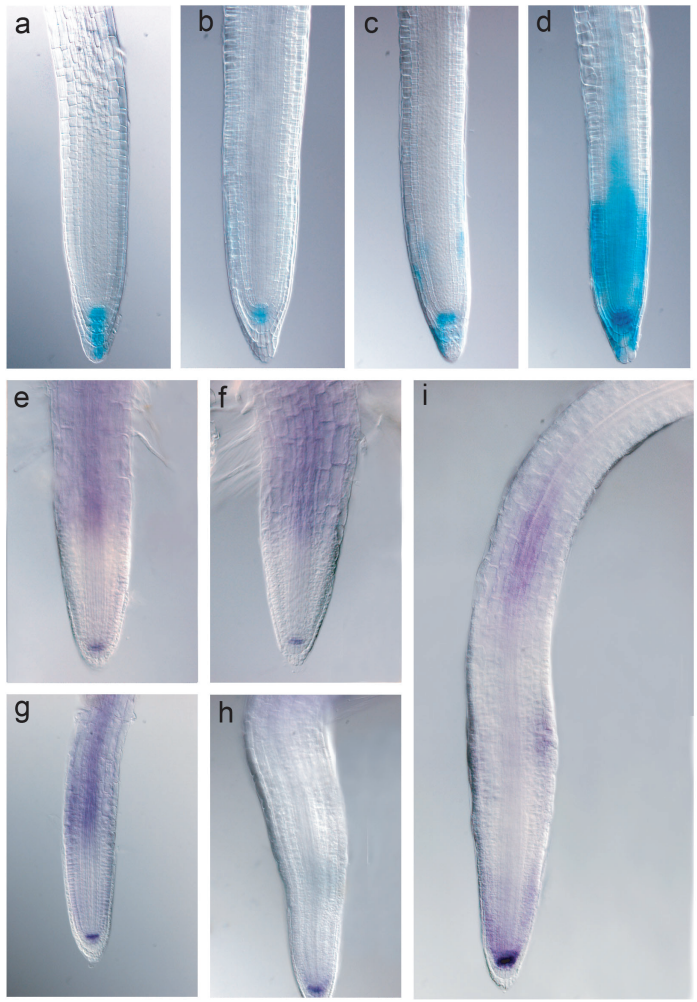
Supplementary Figure 6 – Complementation analysis of PLT fusion proteins. (**a-c**) pPLT2::PLT2:GR complementation, 4 dpg seedlings, (**a**) *plt1*$^{-/-}$; (**b**) *plt1*$^{-/-}$*plt2*$^{-/-}$ pPLT2::gPLT2:GR induced with 5 µM dex; (**c**) *plt1*$^{-/-}$*plt2*$^{-/-}$ pPLT2::gPLT2:GR without dex. (**d-h**) Complementation analysis of *plt1*$^{-/-}$*plt2*$^{-/-}$ with *PLT* genomic regions driven by the *BBM* promoter. (**d**) Root lengths at 9 dpg; (**e**) columella stem cell maintenance at 7 dpg. (**f-h**) Confocal images of pBBM::gPLT1:YFP (**f**), pBBM::gPLT2:YFP (**g**) and pBBM::gBBM:YFP (**h**, no complementation) in *plt1*$^{-/-}$*plt2*$^{-/-}$ 7 dpg seedlings. (**i-m**) Complementation analysis of *plt1*$^{-/-}$*plt2*$^{-/-}$ with *PLT* genomic regions driven by the *PLT2* promoter. (**i**) Root length at 9 dpg; (**j**) stem cell niche at 7 dpg; Confocal images of pPLT2::gBBM:YFP (**k**), pPLT2::gPLT3:YFP (**l**) and pPLT2::gPLT1:YFP (**m**) in *plt1*$^{-/-}$*plt2*$^{-/-}$ 7 dpg seedlings. Asterixes, QC.



Supplementary Figure 7 – Complementation assay using full and partial *PLT2* promoter fragments and promoter swaps. Left: pPLT2 is the long promoter fragment, pPLT2s is a weak expression line with the short PLT2 promoter fragment. Differences with *plt1*$^{-/-}$*plt2*$^{-/-}$ mutant indicate complementation. Values were calculated as the percentage of the indicated 9 dpg wildtype values. For each data point, n=25, bars: std deviation of the mean. Right: Root length of 7 dpg transgenic *plt1*$^{-/-}$*plt2*$^{-/-}$ plants expressing promoter swaps. For each data point, n=25, bars: std deviation of the mean.



Supplementary Figure 8 – A *PLT1* mRNA gradient. *PLT1 in situ* hybridization of 2 dpg roots of wild-type Col-0 (**a** and **b**) and *plt1-1*$^{-/-}$ null allele (**c**), stained for 2 hours (**a**) and 8 hours (**b** and **c**). Region above meristem shows background staining. Notably, promoter and protein fusions show expression in all columella tiers whereas transcripts are restricted to tiers 1 and 2. Based on observations of unrelated probes and gene fusions expressed in this area, we attribute this discrepancy to altered probe penetration in columella cells.



Supplementary Figure 9 – Auxin response and *PIN* transcription upon PLT overexpression. (**a-d**) DR5::GUS expression in 3dpg 24 hr Dex treated 35S::PLT2:GR roots that have clearly expanded meristems. No Dex (**a**); 24 hrs Dex with unchanged GUS expression (15%; n=53); with mild ectopic expression in proximal meristem (72%); with strong ectopic expression (13%). (**e-i**) *PIN3* in situ hybridization in 35S::PLT2:GR roots treated with Dex or mock at 2dpg. 12 hr no Dex (**e**); 12 hr Dex (**f**); 24 hr no Dex (**g**); 24 hr Dex with change in vascular *PIN3* (92%; n=24) (**h**); 24 hr Dex without change in vascular *PIN3* (8%).

Supplementary Table 1 – quantification of staining patterns after PIN1 and PIN3 in situ hybridization in embryos from self-fertilised plants of the indicated genotypes. Embryos were at heart to bent cotyledon stages of development.

Genotype of parent plant	PIN1 probe		PIN3 probe*		
	stained	n	not stained	strongly stained	n
experiment 1					
wt (Col-0)	89%	28	2%	54%	72
plt2$^{-/-}$plt3$^{-/-}$	62%	42	10%	39%	75
plt1$^{+/-}$plt2$^{-/-}$plt3$^{-/-}$	46%	59	25%	13%	178
experiment 2					
wt (Col-0)	85%	46	6%	54%	50
wt (WS)	93%	14	0%	85%	20
plt1$^{-/-}$plt2$^{-/-}$	47%	17	20%	28%	21
plt1$^{-/-}$plt3$^{-/-}$	85%	41	0%	56%	23
plt2$^{-/-}$plt3$^{-/-}$	71%	14	10%	60%	20
plt1$^{-/-}$plt2$^{+/-}$plt3$^{-/-}$	61%	57	15%	39%	74
plt1$^{+/-}$plt2$^{-/-}$plt3$^{-/-}$	59%	39	23%	41%	115

*: only staining in columella was quantified

Supplementary Table 2- Seedling phenotype in different allelic combinations of *plt/bbm* mutants. Frequency of basal defects were scored in the progeny of self-fertilised plants of the indicated genotype. *plt1,plt3,bbm-1,plt2/+* seedlings display diverse defects that can be grouped in three phenotypic classes of different penetrance Used mutant alleles: *plt1-4, plt2-2, plt3-1*.

genotype of parent plant	n	no root/ hypocotyl	no root	short root[#]
wt (Col-0)	869	0	0	-
wt (WS)	620	0	0	-
plt1	510	0	0	-
plt2	741	0	0.40%	-
bbm-1	774	0	0.13%	-
bbm-2	880	0	2.3%	-
plt1,bbm-1	654	0	0.31%	-
plt1,bbm-2	169	0.59%	0	-
plt1,bbm-1,plt2/+	935	0.64%	0.11%	-
plt1,bbm-2,plt2/+	776	2.1%	0.51%	-
plt2,bbm-2/+	984	0	0.31%	-
bbm-2,plt2/+	861	0	0.12%	-
plt1,plt2	2233	0.05%	0.09%	-
plt1,plt2,bbm-1/+	768	3.4%	1.3%	-
plt1,plt2,bbm-2/+	770	0.52%	0.52%	-
plt3	1650	0	0.85%	-
plt1plt3	504	0	1.2%	-
plt2,plt3	731	0	0	-
plt1,plt3,plt2/+	742	0	**27%**	-
plt2,plt3,plt1/+	623	0	**28%**	-
plt1,plt2,plt3/+	553	0	**25%**	-
plt1,plt3,bbm-1	200	0	1%	2.5%
plt3,bbm,plt2/+	261	0	0.77%	2.3%
plt1,plt3,bbm-1,plt2/+	303	**6.6%**	**25%**	**38%**
plt1,plt3,bbm-1,plt2/+ siblings*:		**9.5%**	**35.7%**	**54.8%**

[#]: seedlings with shorter root than *plt1,plt2* mutants that terminate growth at 6 dpg.

-: not determined, see figure 2 for root length.

*: heterozygous siblings (68% of population).

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Supplementary Table 3. Primer sequences used for cloning, mapping, genotyping and riboprobe amplification.

fragment	abbrev	fragm size	Forward primer	Reverse primer
PLT1 promoter	pPLT1	4.5 kb	pPLT1-Fa (GGGGACCACTTTGTACAAGAAAGCTGG GTTAGTGTCGTTCCAAACTGAAAACGTTG)	pPLT1-Ra (GGGGACTGCTTTTTTGTACAAACTTGT AAAGCCAAGCCAGTTGTTAGAA)
PLT2 long promoter	pPLT2L	5.8 kb	pPLT2-Fa (GGGGACAACTTTGTATAGAAAAGTTGTT TCAACTCTCGTTGCATTGACT)	pPLT2-Ra (GGGGACTGCTTTTTTGTACAAACTTGT CGCGAGCCAGTTGTTAGAAT)
PLT2 short promoter	pPLT2S	1.3 kb	pPLT2-Fj (GGGGACAACTTTGTATAGAAAAGTTGTT GAGAGGGAATTAGGGTTTGGAC)	pPLT2-Ra (GGGGACTGCTTTTTTGTACAAACTTGT CGCGAGCCAGTTGTTAGAAT)
PLT3 promoter	pPLT3	4.6 kb	pPLT3-Fa (GGGGACAACTTTGTATAGAAAAGTTGTT CTTATTTGCATGGGATCGTCTT)	pPLT3-Ra (GGGGACTGCTTTTTTGTACAAACTTGT CATCTCCATTGGTGACAGAGAA)
BBM promoter	pBBM	4.2 kb	pBBM-Fa (GGGGACAACTTTGTATAGAAAAGTTGTT GAAAGCTTACGATTACAGAGACCAAAAGGGG)	pBBM-Ra (GGGGACTGCTTTTTTGTACAAACTTGT CATATAATATTCTAACTACTCCTTGTGATA)
RCH2 promoter	pRCH2	2.3 kb	pRCH2-Fa (GGGGACAACTTTGTATAGAAAAGTTGTT TCGAGGCAAAGACCCTTGAAACAAG)	pRCH2-Ra (GGGGACTGCTTTTTTGTACAAACTTGT TAGGAAGAGAACATAAGAGGGTTTAAG)
PLT1 genomic region	gPLT1	2.6 kb	gPLT1-Fa (GGGGACAAGTTTGTACAAAAAAGCAGGC TTTATGAATTCTAACAACTGGCTTGG)	gPLT1-Ra (GGGGACCACTTTGTACAAGAAAGCTGG GTTCTCATTCCACATAGTGAAAACACCA)
PLT2 genomic region	gPLT2	2.8 kb	gPLT2-Fa (GGGGACAAGTTTGTACAAAAAAGCAGGC TTTATGAATTCTAACAACTGGCTCGCGTT)	gPLT2-Ra (GGGGACCACTTTGTACAAGAAAGCTGG GTTTTCATTCCACATCGTGAAAACACCTC)
PLT3 genomic region	gPLT3	4.0 kb	gPLT3-Fa (GGGGACAAGTTTGTACAAAAAAGCAGGC TTTATGGAGATGTTGAGGTCATCTGATCA)	gPLT3-Ra (GGGGACCACTTTGTACAAGAAAGCTGG GTTGTAAGACTGATTAGGCCAGAGGAAG AACTCAGC)
BBM genomic region	gBBM	3.0 kb	gBBM-Fa (GGGGACAAGTTTGTACAAAAAAGCAGGC TTTATGAACTCGATGAATAACTGGTTAGGC)	gBBM-Ra (GGGGACCACTTTGTACAAGAAAGCTGG GTTAGTGTCGTTCCAAACTGAAAACGTTG)
PLT3 riboprobe		400 bp	PLT3C-F (ATCGGTGGTGCAGCTAAACG)	PLT3C-R (AAGAACTCAGCCGGATTTGG)
BBM riboprobe		550 bp	BBMC-F (AATCCGGTTCCAGCTATGATG)	BBMC-R (TCCAAACTGAAAACGTTGGAG)
plt3-1 genotyping		921bp	PLT3L (TTGTGATTTGCCATTGACTAAAGGT)	PLT3R (GAAAACAGTCCAATGGTCTCACATC)
bbm-1 genotyping		911bp	BBM1L (CACTCTCTCTCAACCGAACCATTAG)	BBM1R (CATCTTTTCTTTCTCCTTTCGGTGT)
bbm-2 genotyping		963bp	BBM2L (ACTTTAGTGCGGCTAAATCGTAAGC)	BBM2R (CAATAACGAACAAAATGGACCAAAG)

Chapter 4

Dosage-dependent activation of PLT2 targets establishes a feed-forward network that regulates *Arabidopsis* root development

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Abstract

In the *Arabidopsis* root, the auxin responsive PLETHORA (PLT)-family of AP2-domain transcription factors is thought to specify cell identity and division potential in a concentration-dependent manner. Therefore, the quantitative information of the PLT protein gradient has to be interpreted as threshold responses in which all-or-none changes in target gene expression allow the selection of discrete cell identities in the developing root. Here we present a whole-genome approach to identify the immediate transcriptional targets of PLT2 that contribute to distinct cell fate specification. Combining multiple microarray experiments we identified 100 target genes that are positively regulated by PLT2 in the presence of cycloheximide, an inhibitor of protein translation. These, presented specific leads to embryonic root development, cell wall modifications, cytoskeleton organization, and cell cycle regulation. Moreover, our results show that PLT2 can directly induce genes with non-overlapping expression patterns in the root and, in at least one case, in a dose-dependent manner. Most strikingly, identification of the auxin transport facilitators, auxin biosynthesis and auxin signaling components as direct PLT2 targets indicate a multi-level regulatory feedback into the morphogenic auxin gradient that specifies root patterning.

Introduction

Signaling molecules that are distributed in a concentration gradient along a field of cells can directly induce distinct cellular responses in a dose-dependent manner (reviewed in Tabata and Takei, 2004). As the fate of each cell in the field depends on the concentration of the signaling molecule, the gradient prefigures the pattern of development. This concept of regulation has been extensively studied in animal biology and has culminated in the identification of the classical "morphogens", such as Biciod, which establishes anteroposterior polarity in the developing *Drosophila* embryo (Driever and Nusslein-Volhard, 1988a, 1988b), bone morphogenic proteins (BMPs) that control dorsal-ventral patterning in many species (reviewed in De Robertis and Curoda, 2004) and members of the Hedgehog family that control *Drosophila* appendage development (Strigini and Cohen, 1997). Traditionally, this way of transducing positional information is viewed as a unidirectional process in which establishment and interpretation of the positional values are independent of each other. However, this classical model of the morphogen concept has encountered some resistance recently as it fails to account for the observed dynamic and regulative properties of gradient-based morphogenetic fields (Jaeger et al., 2008). In particular, the robustness (consistent pattern development in presence of perturbations or fluctuations) and scaling abilities (adjusting the pattern of cell fates in a developing tissue with the overall size of the tissue) of the system cannot be addressed convincingly by the classical morphogen theory. In recent years, accumulating experimental evidence has made clear that the establishment of the morphogen gradient is highly entangled with its interpretation by the target tissue, often through complex regulatory feedback mechanisms (reviewed in Jaeger et al., 2008). In plants, indisputable evidence for morphogen action is lacking. However, a recently elucidated mechanism controlling root growth and development presents strong analogies to contemporary morphogen theory.

In the *Arabidopsis thaliana* root, PIN facilitated transport of the phytohormone auxin establishes a basal response maximum that converges on the stem cell area (Sabatini et al., 1999; Blilou et al., 2005). Computational modeling of auxin fluxes within the root predicts that a meristem spanning auxin gradient is inherently associated with this maximum (Grieneisen et al., 2007). At the maximum, auxin is required for cell fate specification and, more distally, for cell division and cell expansion (Sabatini et al., 1999; Blilou et al., 2005). The positional information provided by the presumed auxin gradient is suggested to be translated into expression of the auxin responsive *PLETHORA* (*PLT*) gene-family of AP2-domain transcription factors, who's cumulative transcription patterns match the auxin gradient (Aida et al., 2004; Galinha et al., 2007; Grieneisen et al., 2007). Activity of the PLT proteins is mainly additive and defines at least three different cellular outputs in a

dose-dependent manner. High levels of PLT promote stem cell identity, moderate levels command cell proliferation, and low levels allow for mitotic exit, expansion and differentiation. In order to control these separate aspects of root morphology, the quantitative information of the PLT gradient has to be interpreted as threshold responses in which changes in target gene expression allow the selection of discrete cell identities and/or differentiation states, in the developing root.

In this chapter, we describe the use of whole-genome microarray analysis to identify the immediate transcriptional targets of PLT2 that program distinct cell fates in the root. Our results indicate two important aspects of PLT2 target regulation: (1) PLT2 can directly induce genes with distinct and non-overlapping expression patterns in an apparent dose-dependent manner and (2) the varied identity of downstream targets suggests that PLT2 controls diversified developmental pathways associated with cell proliferation and growth. Interestingly, the identification of auxin transport facilitators, biosynthetic regulators and signaling components as direct PLT2 targets indicate a multi-level regulatory feedback into the auxin gradient that specifies root patterning.

Results

Microarray profiling identifies direct targets of PLT2

Analogous to previous studies successfully identifying downstream targets of floral homeotic genes (Gomez-Mena et al., 2005; William et al., 2004; Ito et al., 2004), we used the constitutive 35S promoter to drive PLT2:GR throughout the seedling for comprehensive target gene induction. Initial phenotypic effects of ectopic PLT2:GR activation in young seedlings are the expansion of the root meristem area and the conversion of shoot derived organs towards root fate (Galinha et al., 2007). Continuous dexamethasone (Dex) application or germination of 35S::PLT2:GR seedlings on Dex containing medium result in a dramatic apical to basal fate change, transforming all "above ground" structures into transparent root-like tissue (Supplementary Fig. 1). These induction experiments indicate that the transcriptional program to initiate root formation can be activated solely by PLT2 and enforced on all plant tissues in a Dex-dependent manner. This allows us to collect whole seedling material for subsequent profiling analysis.

To monitor downstream events upon PLT2 induction, we activated PLT2:GR in three day old seedlings and compared the transcriptional profile with those in which PLT2:GR was not activated (mock-treated). We treated the seedlings for 2 hrs, 4 hrs and 8 hrs with Dex before profiling their transcriptome using Arabidopsis whole-genome ATH1 microarrays (Affymetrix) to extract the sequence of transcriptional events. In order to identify direct transcriptional targets of PLT2, we included two

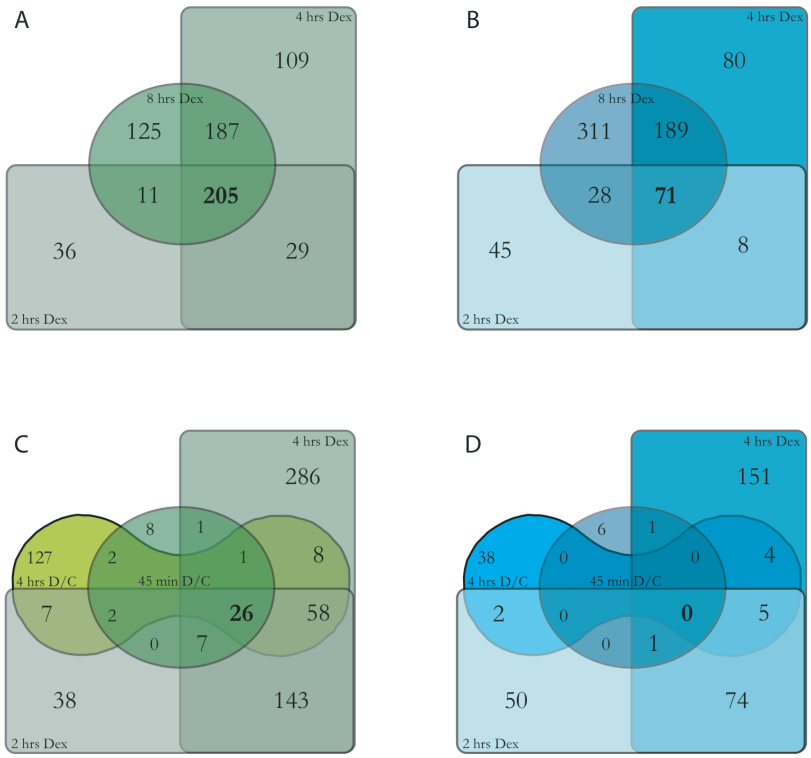

Figure 1: **Identification of PLT2 targets.** (**A-D**) Edward's Venn diagram illustrates the overlap between differentially expressed (DE) genes in induced 35SPLT2GR seedlings at different time points. DE genes were selected based on a $\log_2$ ratio of ≤ -1 or ≥ 1 and q-values less the 0.05. In case of 45 min. Dex/Cyc treatment, DE genes were chosen with $\log_2$ ratio of ≤ -0.59 or ≥ 0.59 and q-values less the 0.2. (**A**) Overlap between genes upregulated after 2, 4, and 8 hrs of Dex induction. (**B**) Overlap between genes upregulated after 2 and 4 hrs of Dex induction and 45 min. and 4 hrs of combined Dex/Cyc treatment. Genes from the intersections containing 1, 7, 8, 26, and 58 gene(s) are considered candidate direct targets. (**C**) Overlap between genes downregulated after 2, 4, and 8 hrs of Dex induction. (**D**) Overlap between genes downregulated after 2 and 4 hrs of Dex induction and 45 min. and 4 hrs of combined Dex/Cyc treatment. Genes from the intersections containing 1, 4, and 5 gene(s) are considered candidate direct targets. Common responsive genes in all experiments are indicated in bold.

time points of Dex treatment in the presence of the protein synthesis inhibitor cycloheximide (Cyc); 45 minutes and 4 hrs treatments. This allows for the transcription of immediate targets yet blocks those genes that are further down the pathway by preventing translation and thus action of intermediary factors.

To assess differentially expressed genes, we used Limma in conjunction with the affy package for Affymetrix data in R (www.r-project.org) to analyze the obtained microarray data (Smyth, 2005). These computations revealed with statistical confidence (FDR <0.005) the immediate transcriptional snowball effect initiated by PLT2:GR activation. After 2 hrs Dex treatment, a total of 281 genes have been

upregulated and 152 downregulated more than two-fold; at 4 hrs these number have increased to 530 upregulated (overlap 2 hrs time point 234 genes) and 348 downregulated genes (79 genes overlap with 2 hrs; Fig. 1a, 1b). At this point, a numerical equilibrium appears to have been reached for the positively regulated genes since the 8 hrs Dex treatment does not result in an absolute increase of upregulated genes (528 in total; 392 overlapping with 4 hrs treatment). The quantity of downregulated genes still increases in time, topping off at 599 genes after 8 hrs of steroid treatment (overlap with 4 hrs of 260 genes). An analogous microarray study has shown that Dex treatment leaves transcription levels in wildtype seedlings largely unchanged (Hanson et al., 2008). It is therefore plausible that the observed effect on target gene expression is solely the result of transcriptional regulation by Dex activated PLT2:GR.

In the Dex/Cyc treated 35S::PLT2:GR seedlings a total of 47 genes were found upregulated after 45 min. of PLT2 activation (Fig. 1c). Notably, we identified only 8 genes that were downregulated after 45 min. Dex/Cyc treatment suggesting that the direct action of PLT2 is mostly of an activating nature (Fig. 1d). Alternatively or in addition, the presence of Cyc might stabilize mRNA turnover thereby masking initial repressive PLT2 activity. After 4 hrs of combined Dex/Cyc-treatment, 231 genes were found upregulated (29 genes overlapping with 45 min.) and 49 genes downregulated (no overlap with 45 min.).

From the upregulated targets in all microarray datasets we identified an overlapping set of 26 highly significant genes that are good candidates for direct targets of PLT2 (Fig. 1c). These genes are rapidly induced after PLT2 activation, even when inhibiting protein synthesis, and remain expressed at elevated levels up to 8 hrs after induction. An additional 74 genes do not meet these criteria; yet can be considered as candidate direct targets. They either require at least two or four hours of Dex induction (58 and 8 genes resp., Fig. 1c), or are upregulated in all experiments except after 4 hrs of combined Dex/Cyc treatment or 2 hrs Dex (7 and 1 gene(s) resp., Fig. 1c). The 100 candidates are listed in Table 1 according to their level of induction after 45 min. Dex/Cyc PLT2:GR activation. A comparable strategy to identify candidate targets that are negatively regulated by PLT2 returned 10 genes (Fig. 1d, and Supplementary Table 1). We focused our subsequent analysis mainly on the upregulated candidate targets.

To validate our statistical interpretation of the conducted microarray experiment, we performed an independent 45 min. Dex/Cyc-induction experiment and analyzed the transcriptional response of a subset of candidate targets by quantitative real-time PCR (qRT-PCR). For all 25 genes tested, levels of gene expression were consistently upregulated more than 1.5-fold in three independent experiments after

Table 1: List of positively regulated candidate direct target genes of PLT2, ranked according to their inductive fold-change at 45 min. of combined Dex/Cyc treatment. Asterisks indicate those genes with a q-value equal to or less than 0.2 within the 45 min. Dex/Cyc experiment; q-value for all genes in other experiments ≤0.05. Genes with an exclamation mark have also been identified as direct targets of BBM (Passarinho et al. 2008).

Gene	Description	45 min. Dex/Cyc	4 hrs. Dex/Cyc	2 hrs. Dex	4 hrs. Dex	8 hrs. Dex	Reference
AT4G14690 !	chlorophyll A-B binding family protein / early light-induced protein (ELIP2)	17.5*	16.4	23.3	36.0	55.5	Tzvetkova-Chevolleau et al., 2007
AT2G03830 !	expressed protein	9.0*	12.0	11.9	17.3	15.9	
AT3G01840	protein kinase family protein	5.8	1.6	9.3	10.0	9.0	
AT1G56680	glycoside hydrolase family 19 protein	5.7*	9.0	16.1	44.7	53.3	
AT3G62760	glutathione S-transferase (AtGSTF13)	5.5*	7.0	21.2	36.4	50.5	
AT5G60630	expressed protein	4.5*	7.7	9.5	6.5	5.3	
AT5G56790	protein kinase family protein	4.0*	2.3	5.2	6.3	5.8	
AT5G60890	Myb-like transcription factor (ATR1/ MYB34)	3.7*	6.1	7.3	7.6	7.0	Celenza et al., 2005
AT1G55760	BTB/POZ domain-containing protein	3.4*	2.0	4.2	4.0	3.5	
AT4G28950	Rac-like GTP-binding protein (ARAC7/ ROP9)	3.4*	7.6	7.6	7.2	5.5	
AT1G75580	auxin-responsive protein	3.3*	4.2	3.2	3.3	3.0	
AT3G15720	glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein	3.2*	2.1	4.7	5.7	8.4	
AT4G37430	cytochrome P450 81F1 (CYP81F1) (CYP91A2)	3.2*	2.3	10.6	11.7	11.5	
AT1G70420	expressed protein	3.1	1.3	3.9	2.6	2.7	
AT5G44460	calcium-binding protein	3.0*	3.2	3.8	3.3	2.4	
AT5G47440 !	expressed protein	2.9*	3.6	3.2	3.4	5.4	
AT5G02550 !	expressed protein	2.8	8.1	3.4	3.7	5.4	
AT1G21090	hydroxyproline-rich glycoprotein family protein	2.7*	2.7	4.1	4.1	3.9	
AT5G28640	SSXT protein-related / glycine-rich protein ANGUSTIFOLIA3 (AN3)	2.6	2.0	3.7	3.7	4.3	Horiguchi et al., 2005
AT4G36830	GNS1/SUR4 membrane family protein	2.6*	4.8	2.9	5.4	4.7	
AT2G34020 !	similar to calcium-binding EF hand family protein	2.5	6.0	4.0	5.6	4.4	
AT5G26731	expressed protein	2.5	4.1	5.5	3.5	2.9	
AT1G35612	expressed protein	2.4*	5.2	2.1	2.4	1.9	
AT5G58750 !	wound-responsive protein-related	2.4*	4.2	9.4	9.1	9.6	
AT1G50570	C2 domain-containing protein	2.4*	2.6	2.4	2.3	2.4	
AT4G34970 !	actin-depolymerizing factor	2.4	49.9	9.2	11.8	18.0	
AT5G47060	senescence-associated protein-related	2.4	1.6	4.2	4.6	3.8	
AT3G50870	zinc finger (GATA type) family protein MONOPOLE (MNP)	2.3*	3.7	2.1	2.9	3.7	
AT5G13290	protein kinase family protein (CORYNE)	2.3	1.8	2.5	2.3	2.6	Müller et al., 2008
AT1G06980	expressed protein	2.2	2.8	1.6	2.2	2.1	
AT5G14690	expressed protein	2.2	2.1	3.1	6.5	7.7	
AT3G02210	phytochelatin synthetase family protein / COBRA cell expansion protein (COBL1)	2.2	3.0	5.2	5.0	6.2	
AT3G24240	leucine-rich repeat transmembrane protein kinase (RCH2)	2.2	4.2	3.5	2.7	2.4	Casamitjana Martinez et al., 2003
AT2G21650	myb family transcription factor (MEE3)	2.2	2.3	14.2	17.5	22.3	Pagnussat et al., 2005
AT2G01210	leucine-rich repeat transmembrane protein kinase	2.2*	2.4	3.4	3.4	2.8	
AT4G02290 !	glycosyl hydrolase family 9 protein (ATGH9B13)	2.1*	8.7	3.4	7.3	7.4	
AT1G12860	basic helix-loop-helix (bHLH) family protein (SCRM2)	2.1	3.0	1.7	2.1	2.0	Kanaoka et al., 2008

Table 1- continued from previous page

Gene	Description	45 min. Dex/Cyc	4 hrs. Dex/Cyc	2 hrs. Dex	4 hrs. Dex	8 hrs. Dex	Reference
AT2G38400 !	alanine:glyoxylate aminotransferase 2 homolog (AGT3)	2.0	6.8	2.8	2.3	1.7	
AT5G17640	expressed protein	2.0	1.7	2.6	3.0	3.2	
AT5G53290 !	ERF (ethylene response factor) subfamily B-5 of ERF/AP2 transcription factor family.	1.9	5.4	6.8	8.6	8.9	Rashotte et al., 2005
AT3G57010	strictosidine synthase family protein	1.9*	3.0	4.6	3.7	3.0	
AT1G68430	expressed protein	1.9	3.2	4.8	2.6	2.5	
AT3G44735	phytosulfokine 3 precursor (PSK3)	1.9	1.7	2.9	3.0	2.2	
AT5G59130	subtilase family protein	1.9	2.3	3.7	3.6	4.3	
AT3G14230	ERF subfamily B-2 of ERF/AP2 transcription factor family (RAP2.2).	1.8	2.0	2.9	3.0	3.0	Welsch et al., 2006
AT1G02810 !	pectinesterase family protein	1.8	3.7	5.9	6.7	5.6	
AT2G38970	zinc finger (C3HC4-type RING finger) family protein	1.8*	4.7	3.5	3.4	2.4	
AT5G14450 !	GDSL-motif lipase/hydrolase family protein	1.8	2.9	5.7	6.3	6.5	
AT3G50310	protein kinase-related (MAPKKK20)	1.7	2.0	2.6	3.8	4.6	
AT4G15530 !	pyruvateorthophosphate dikinase (PPDK)	1.7	3.0	3.2	4.0	3.6	Parsley et al, 2006
AT5G51550	phosphate-responsive 1 family protein	1.7*	2.7	5.0	6.2	5.0	
AT3G50190	expressed protein	1.7	2.6	1.6	2.1	2.1	
AT4G16563	aspartyl protease family protein	1.7	2.1	3.6	3.2	3.0	
AT2G19590	1-aminocyclopropane-1-carboxylate oxidase, putative	1.7	2.8	4.3	4.7	5.3	
AT1G64170 !	cation/hydrogen exchanger, putative (CHX16)	1.7	3.7	2.9	2.8	3.3	
AT5G48940	leucine-rich repeat transmembrane protein kinase, putative (RCH1)	1.6	2.0	5.2	6.9	10.0	Casamitjana Martinez et al., 2003
AT1G70710	endo-1,4-beta-glucanase (EGASE) / cellulase1 (CEL1)	1.6	2.4	4.1	4.0	4.6	Shani et al., 2003
AT3G57140	patatin-related	1.6	4.5	7.4	7.1	4.9	
AT5G17630 !	glucose-6-phosphate/phosphate translocator, putative	1.6	3.2	2.3	2.7	2.9	
AT4G09760	choline kinase, putative	1.6	2.1	2.5	2.8	1.9	
AT5G64570	glycosyl hydrolase family 3 protein	1.6	4.5	4.7	3.2	2.2	
AT2G01420 !	auxin transport protein, (PIN4)	1.6	3.8	1.5	2.2	3.0	Friml et al., 2002
AT5G25900	ent-kaurene oxidase/ Cytochrome P450 (GA3)	1.6*	2.9	5.3	6.0	6.1	Helliwell et al., 1998
AT2G24280	serine carboxypeptidase S28 family protein	1.6	5.1	3.9	3.9	4.0	
AT1G33790	jacalin lectin family protein	1.6	3.7	5.6	6.4	7.7	
AT1G16070 !	tubby family protein (Tubby-like protein 1)	1.6	12.6	3.0	4.9	3.6	Lai et al., 2004
AT3G15680 !	zinc finger (Ran-binding) family protein	1.5	3.6	2.7	2.9	2.7	
AT2G23050	phototropic-responsive NPH3 family protein	1.5	2.2	2.8	4.0	5.2	
AT3G11690	expressed protein	1.5	2.0	2.1	2.1	2.7	
AT1G66250	glycosyl hydrolase family 17 protein	1.5	2.2	3.2	4.7	4.8	
AT4G28100	expressed protein	1.5	2.3	3.1	2.3	2.0	
AT4G28720	flavin-containing monooxygenase family protein / FMO family protein	1.5	2.4	4.7	6.3	10.5	
AT1G18250	thaumatin, putative	1.5	5.4	2.7	4.1	4.1	
AT5G19520	mechanosensitive ion channel domain-containing protein (MSL9)	1.5	2.4	4.9	6.2	5.9	Haswell et al., 2008
AT5G08260 !	serine carboxypeptidase S10 family protein	1.4	5.2	2.3	2.9	2.3	
AT5G48130	phototropic-responsive NPH3 family protein	1.4	2.5	1.8	3.0	4.5	
AT4G26760	microtubule associated protein (MAP65/ ASE1) family protein	1.4	2.8	1.9	2.2	2.1	
AT3G47960	proton-dependent oligopeptide transport (POT) family protein	1.4	3.8	3.6	3.0	2.2	
AT2G23180	cytochrome P450, putative	1.4	2.5	2.9	2.6	2.4	
AT5G45780 !	leucine-rich repeat transmembrane protein kinase, putative	1.3	3.2	2.1	2.0	1.8	
AT2G40250	GDSL-motif lipase/hydrolase family protein	1.3	2.6	6.5	6.9	7.8	
AT3G07130 !	serine/threonine protein phosphatase family protein	1.3	3.1	2.9	2.4	2.6	

Table 1- continued from previous page

Gene	Description	45 min. Dex/Cyc	4 hrs. Dex/Cyc	2 hrs. Dex	4 hrs. Dex	8 hrs. Dex	Reference
AT3G19380 !	U-box domain-containing protein	1.3	3.2	2.4	2.5	2.2	
AT2G32560	F-box family protein; similar to SKP1 interacting partner 2 (SKIP2)	1.3	2.1	3.1	2.8	2.8	
AT2G21050	amino acid permease, putative, similar to AUX1 (LAX2)	1.3	2.2	3.3	4.2	5.2	
AT1G47840	Hexokinase, putative (HEXOKINASE3)	1.2	2.1	2.2	3.5	3.2	
AT1G51830	leucine-rich repeat protein kinase, putative	1.2	3.0	1.4	2.0	1.4	
AT1G29980	expressed protein	1.2	2.1	2.1	2.0	1.7	
AT1G32190	expressed protein	1.2	2.2	2.5	3.3	3.5	
AT1G22900	similar to disease resistance-responsive family protein	1.2	2.0	1.6	2.0	1.8	
AT4G11310 !	cysteine proteinase, putative	1.2	2.2	1.2	2.4	2.4	
AT3G50410 !	Dof-type zinc finger domain-containing protein (OBP1)	1.1	2.4	2.7	2.7	2.4	Skirycz et al., 2008
AT2G38210	stress-responsive protein	1.1	2.4	2.2	2.2	1.9	
AT5G56580	mitogen-activated protein kinase kinase (MAPKK6)	1.1	2.0	2.3	2.6	2.5	Soyano et al., 2003
AT3G55760	expressed protein	1.1	2.2	2.4	3.7	5.1	
AT1G76790 !	O-methyltransferase family 2 protein	1.1	2.3	2.2	2.6	2.2	
AT4G38410	dehydrin, putative	1.1	3.3	6.9	9.0	6.4	
AT5G62710	leucine-rich repeat family protein / protein kinase family protein	1.1	2.4	2.8	3.3	2.8	
AT4G05390	ferredoxin--NADP(+) reductase, putative	1.0	2.4	2.1	2.4	1.7	
AT3G17840	leucine-rich repeat transmembrane protein kinase (RLK902)	1.0	2.1	2.4	3.0	2.6	Tarutani et al., 2004

Dex/Cyc treatment, when compared to Cyc-treated PLT2:GR seedlings (Table 2). Occasional discrepancies that are observed in the amount of change between microarray and qPCR data are remarkable but have been reported in other studies as well (summarized in Morey et al., 2006). Overall, results from our qRT-PCR analysis are consistent with that revealed by microarray analysis.

PLT2 targets appear redundantly regulated by the PLT gene family

Previous studies have shown that the PLT gene family acts redundantly in root development (Aida et al., 2004; Galinha et al., 2007). This implies that PLT family members regulate a common set of genes, beside their unique protein function. To test the PLT2-specific transcriptional regulation of candidate targets, we compared transcript levels in *plt1-4, plt2-2* roots and *plt1-4* roots of a selection of the previously analyzed set of genes by means of qRT-PCR. Out of the 16 genes tested only three genes show a more than two-fold decrease in expression levels (AT3G62760, AT1G50570, and AT5G60630; Table 2), suggesting a large number of PLT2 target genes identified in our study can be redundantly regulated by other factors. A recent study by Passarinho et al. (2008) reported 139 positively regulated direct targets of the PLT2 homologue BABY BOOM (BBM). When we compared the overlap of direct target genes between the PLT2 candidate gene list and published BBM targets we found an overlap of 25 genes out of the 100 direct PLT2 targets (Table 1). Together, these results suggest extensive redundancy at the level of transcriptional regulation of many target genes by the PLT family members.

Table 2: Validation of microarray data and comparative mutant expression analysis by quantitative RT-PCR. Relative expression levels, as determined by qRT-PCR on three (Dex/Cyc) and two (mutant comparison) independent RNA samples, are represented as the average of the ΔΔCt values ((ΔCt 35SPLT2GR Dex/Cyc - ΔCt 35SPLT2GR Cyc); (ΔCt *plt1-4, plt2-2* - ΔCt *plt1-4*)). Values in bold indicate a consistent 2-fold reduction in expression in *plt1-4, plt2-2* compared to *plt1-4*. The asterisks indicates those genes with a q-value equal to or less than 0.2 within the 45 min. Dex/Cyc microarray experiment. ND, not determined.

Gene	Description	45 min. Dex/Cyc Microarray FC	45 min. Dex/Cyc QRT-PCR FC	*plt1-4,plt2-2* vs. *plt1-4* QRT-PCR FC
AT4G14690	chlorophyll A-B binding family protein / early light-induced protein (ELIP2)	17.5*	2.3 ± 0.0	1.5 ± 0.1
AT3G01840	protein kinase family protein	5.8	1.7 ± 0.2	ND
AT1G56680	glycoside hydrolase family 19 protein	5.7*	2.7 ± 0.6	1.5 ± 0.1
AT3G62760	glutathione S-transferase (GSTF13)	5.5*	4.1 ± 0.7	**-3.4 ± 1.3**
AT5G60630	expressed protein	4.5*	6.2 ± 1.2	**-2.8 ± 0.4**
AT5G56790	protein kinase family protein	4.0*	2.4 ± 0.7	-1.1 ± 0.1
AT5G60890	receptor-like protein kinase (ATR1) (MYB34)	3.7*	3.2 ± 0.3	-1.0 ± 0.0
AT4G28950	Rac-like GTP-binding protein (ARAC7)	3.4*	2.7 ± 0.1	-1.4 ± 1.0
AT1G55760	BTB/POZ domain-containing protein	3.4*	3.2 ± 0.9	2.2 ± 0.4
AT3G15720	glycoside hydrolase family 28 protein	3.2*	2.2 ± 0.4	1.3 ± 0.3
AT4G37430	cytochrome P450 81F1 (CYP81F1) (CYP91A2)	3.2*	2.3 ± 0.4	ND
AT5G44460	calcium-binding protein	3.0*	2.4 ± 0.8	2.1 ± 0.7
AT5G02550	expressed protein	2.8	2.2 ± 0.1	ND
AT1G21090	hydroxyproline-rich glycoprotein family protein	2.7*	2.0 ± 0.2	2.0 ± 0.5
AT4G36830	GNS1/SUR4 membrane family protein	2.6*	2.2 ± 0.6	ND
AT1G50570	C2 domain-containing protein	2.4*	2.0 ± 0.0	**-2.0 ± 0.0**
AT5G47060	senescence-associated protein-related	2.4	2.0 ± 0.2	ND
AT5G13290	protein kinase family protein	2.3	2.0 ± 0.4	ND
AT3G50870	zinc finger (GATA type) family protein MONOPOLE	2.3*	1.9 ± 0.4	-1.2 ± 0.3
AT2G01210	leucine-rich repeat transmembrane protein kinase	2.2	2.3 ± 2.0	1.6 ± 0.2
AT5G14690	expressed protein	2.2	1.7 ± 0.1	ND
AT3G24240	leucine-rich repeat transmembrane protein kinase (RCH2)	2.2	3.8 ± 0.4	ND
AT2G38970	zinc finger (C3HC4-type RING finger) family protein	1.8*	3.2 ± 1.9	1.3 ± 0.4
AT3G14230	encodes a subfamily B-2 of ERF/AP2 transcription factor family (RAP2.2).	1.8	2.1 ± 0.2	ND
AT5G51550	phosphate-responsive 1 family protein	1.7*	3.4 ± 1.0	2.0 ± 0.3

PLT2 directly regulates genes in non-overlapping expression domains in a dose-dependent manner

The *PLT* gene family is suggested to act in a dose-dependent manner; dictating distinct cellular aspects of root development by means of an instructive gradient of PLT protein levels (Galinha et al., 2007). Such a regulatory mechanism implies concentration dependent control over transcription of downstream targets, and consequently, the existence of target expression patterns that overlap only partially with the graded PLT expression domain. Transcriptional targets that depend on high

PLT levels e.g. will be expressed in the stem cell area only whereas targets that require low-levels are expected to be expressed more proximal. To obtain a global overview of the expression patterns of the PLT2 target genes along the longitudinal axis of the root, we investigated their corresponding "digital in situ" expression values from transverse root sections as collected by Brady et al. (2007). The majority of genes (58%) have predicted peak expression values within the domain of highest PLT2 expression. These genes are either solely expressed in the stem cell area or are expressed across the entire meristem yet display decreasing expression values towards the distal meristem boundary. Fewer genes (11%), however are predicted to display different root expression patterns; confined expression in more distal regions of the root or increasing expression values towards the distal end. In order to validate the predicted expression patterns, we constructed GFP promoter fusions for a selection of targets with expression domains that overlap with restricted regions of the PLT2 domain and transformed these into wildtype plants. In most cases (85%), the promoter fusions reflected the predicted expression pattern, suggesting that sufficient upstream regulatory sequence was cloned before the GFP coding sequence. Based on their GFP expression patterns, the targets can generally be categorized

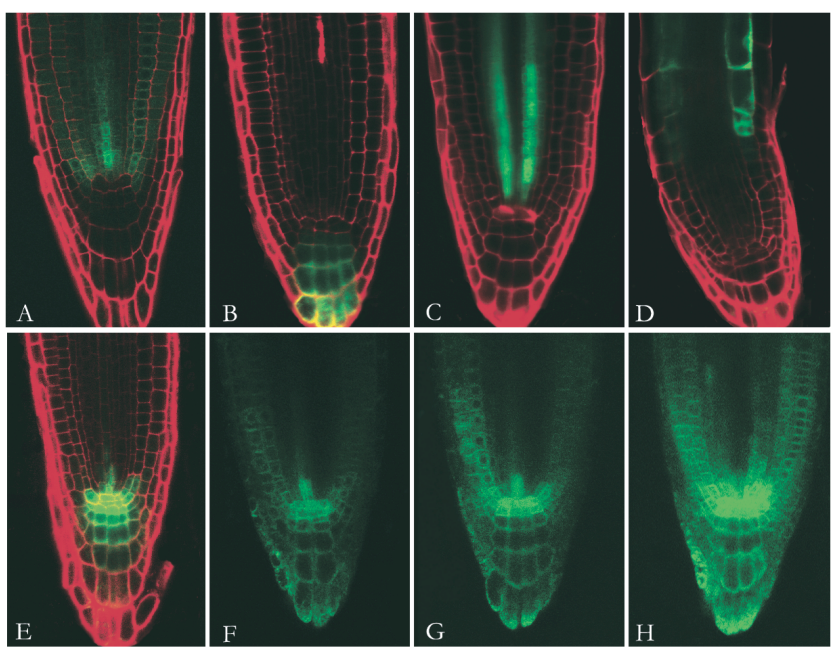

Figure 2: Expression and regulation of direct PLT2 targets. (**A-H**) GFP promoter fusions of five selected direct PLT2 targets in 5 day old wildtype (**A-E**) and 35SPLT2GR (**F-H**) seedlings. (**A**) Graded expression pattern of AT3G15720 (glycoside hydrolase), (**B**) Columella specific expression of AT1G70140 (ATFH8), (**C**) meristem expressed AT3G15680 (Ran-binding zinc finger), (**D**) Elongation zone restricted expression of AT5G02550 (expressed protein), (**E**) stem cell niche specific expression of AT2G03830 (expressed protein). Expression of AT2G03830 in 35SPLT2GR seedlings before Dex induction (**F**), after 4 hrs of induction (**G**), and after 8 hrs of PLT2 activation (H) shows an increase in expression level and expansion of the expression domain.

into three classes corresponding with their domain of expression in relation to that of the threshold levels of the PLT2 gradient that determine cell fate. Out of the 13 targets tested, 7 genes display expression patterns that overlap with intermediate PLT2 levels (three genes shown as example in Fig. 2a-c); 2 genes overlap with the lowest PLT2 levels in the elongation zone (one gene shown as example in Fig. 2d); and 4 genes correlate with the highest PLT2 expression (one gene shown as example in Fig. 2e). These results indicate that direct targets of PLT2 can be expressed in restricted areas within the PLT2 expression domain and might therefore be regulated by PLT2 in a concentration-dependent manner.

To test potential dose-dependent regulation of these targets we crossed the GFP-reporter lines into the 35S::PLT2:GR background. Thus far, we have analyzed the response of the AT2G03830 (expressed protein) regulatory region after 4 and 8 hrs of global PLT2 activation. Initially confined to the stem cell area, promoter activity increases drastically after PLT2 activation and expands into a broader domain (Fig. 2f-h). This observation indicates that AT2G03830 regulation is controlled by PLT2 in a dose-dependent manner and concurrently suggests a mechanism for gene regulation (see discussion).

Functional characterization of PLT2 target genes

To understand the functional output of the PLT2 pathway, we performed a literature search for functional data on the direct PLT2 targets and used Gene Ontology (GO) annotations to search for enriched signatures of biological process or molecular function within the entire set of PLT2 regulated genes. From the 100 direct target genes, our literature search returned 19 genes that have been phenotypically portrayed. Interestingly, the described functions of these direct target genes reflect the many processes that are associated with PLT2 activity (Aida et al., 2004; Blilou et al. 2005; Galinha et al., 2007). Among many putative cell wall modifying enzymes in the target list, endo-1,4-beta-glucanase *CELLULASE1* (*CEL1*) and *BETA-D-XYLOSIDASE 4* (*XYL4*) have been shown to play important roles in cell wall relaxation (Shani et al., 2003; Minic et al., 2004) and root growth (*CEL1* only; Shani et al., 2003). Moreover, with *ACTIN DEPOLYMERIZING FACTOR9* (*ADF9*) and the Rho GTPase *ROP9* we identified a potential connection from PLT2 signaling to dynamic actin reorganization (Dong et al., 2001; Fu et al., 2002), which is associated with meristematic cell identity (Wang et al., 2007). The mitogen-activated protein kinase kinase *atMKK6*, the transcriptional coactivator *ANGUSTIFOLIA* (*AN3*) and the DOF transcription factor *OBF BINDING PROTEIN 1* (*OBP1*) have all been identified as important positive regulators of the cell cycle (Soyano et al., 2003; Horiguchi et al., 2005; Skirycz et al., 2008). An intriguing candidate for mediating PLT2 signaling in control of root pole formation in the developing embryo is the

GATA-type zinc finger *MONOPOLE* (*MNP*). Mutations within this gene result in irregularly arranged cells at the base of the embryo and, eventually, in embryos with no functional root pole (Tal Nawy, personal communication). Together, the nature of these direct targets suggest the existence of a cluster of genes with diverse functions associated with cell specification, proliferation and differentiation/elongation that is under direct control of PLT2.

Our GO term enrichment analysis among the direct targets, revealed a significant overrepresentation of genes annotated to encode proteins involved in phosphorylation processes (molecular function "kinase activity" and biological function "cell surface receptor linked signal transduction "; Table 3). These observations suggest a strong involvement of post-transcriptional protein modifications as important means to directly relay PLT2 information. Although not significantly overrepresented, we found a substantial presence of genes encoding proteins involved in the regulation of transcription (8.1% against a 5.7% background frequency; *p-value* 2.03e-01), suggesting PLT2 function is also mediated through a network of transcription factors (data not shown).

Table 3: Functional characterization of PLT2 targets.

GO Main Category	GO Term	Sample Frequency	Background Frequency	p-value
Direct PLT2 targets				
Molecular Function	kinase activity	16/98 (16.3%)	1274/36621 (3.5%)	1.37e-04
Biological Process	cell surface receptor linked signal transduction	6/98 (6.1%)	150/36621 (0.4%)	1.66e-03
Indirect targets activated by PLT2				
Biological Process	tryptophan biosynthetic process	9/622 (1.4%)	20/36621 (0.1%)	2.16e-08
Molecular Function	Transferase activity	77/622 (12.4%)	2435/36621 (6.6%)	1.59e-04
Molecular Function	DNA binding	64/622 (10.3%)	2085/36621 (5.7%)	5.41e-04
Biological Process	Nucleotide metabolic process	10/622 (1.6%)	78/36621 (0.2%)	1.14e-03
Biological Process	Glucosinolate biosynthetic process	7/622 (1.1%)	36/36621 (0.1%)	2.94e-03
Indirect targets repressed by PLT2				
Molecular Function	Peroxidase activity	24/724 (3.3%)	103/36621 (0.3%)	4.61e-16
Biological process	Response to stimulus	118/724 (16.3%)	3448/36621 (9.4%)	3.42e-06
Molecular Function	Water channel activity	9/724 (1.2%)	37/36621 (0.1%)	4.27e-05
Biological process	Lignan biosynthetic process	6/724 (0.8%)	16/36621 (0.0%)	5.08e-04
Biological process	Cellular carbohydrate biosynthetic process	14/724 (1.9%)	157/36621 (0.4%)	4.09e-03

Functional classification of the activated indirect PLT2 targets revealed a most significant overrepresentation of genes involved in the tryptophan metabolic pathway (Table 3). Apart from supplying tryptophan for protein synthesis, this metabolic pathway is also used to provide precursors for auxin biosynthesis. Furthermore, we found the a clear enrichment for genes involved in nucleotide metabolism, glycoside metabolism, DNA and nucleotide binding and genes with transferase activity (Table 3). Surveying the set of indirectly downregulated genes of PLT2, indicated a strong enrichment for genes encoding proteins with predicted reductase activity (Table 3). Approximately 25% of the genes with suggested reductase activity present in the Arabidopsis genome are downregulated upon PLT2 induction. Moreover, genes that encode proteins involved in cellular metabolic processes, cell wall biosynthesis and response to stimuli are also significantly overrepresented in the set of downregulated genes.

PLT2 directly controls the Tryptophan-dependent auxin biosynthesis pathway

Because of the prominent function of auxin in root development and its role as a candidate plant morphogen, we looked closer into the highly overrepresented class of PLT2 regulated targets associated with the GO terms "tryptophan and glucosinolate biosynthetic processes". Most strikingly, we identified the myb-like transcription factor altered tryptophan (Trp) regulation *ATR1* and the rate-limiting auxin biosynthesis flavin monooxygenase-enzyme *YUCCA8* (Cheng et al., 2006) among the direct targets. ATR1 has been identified as a key homeostatic regulator of Trp metabolism, controlling the expression of several indole-3-acetic acid (IAA) and indolic glucosinolate (IG) biosynthesis genes (Celenza et al., 2005). By investigating cell type–specific transcriptional signatures in the root, Brady et al. (2007) revealed a potential transcriptional module of auxin biosynthesis genes regulated by ATR1 in the inner radial tissues. Since *ATR1* was identified as an immediate target of PLT2, we tested whether the same module of auxin biosynthesis genes could be identified within our list of significantly upregulated genes 2 hrs, 4 hrs, and 8 hrs after PLT2:GR activation. At all three time-points we found positively regulated genes that are induced more than two-fold and belong to the presumptive MYB-domain transcription factor–regulated auxin biosynthesis module (Fig. 3, and Supplementary Table 2; Brady et al., 2007). Apart from the biological implications, this exemplifies how we can benefit from the time series data to reconstruct a PLT2 controlled transcriptional network. In addition to the ATR regulated module, more genes acting in the Trp-dependent auxin and IG biosynthesis pathway were identified as significantly upregulated (Fig. 3, and Supplementary Table 2). Other then *YUCCA8*, which appears to be directly regulated by PLT2, we also identified *YUCCA3* and *YUCCA9* among the indirect targets (Fig. 3). Together, this indicates that PLT2 activity drastically influences the expression of auxin biosynthesis genes,

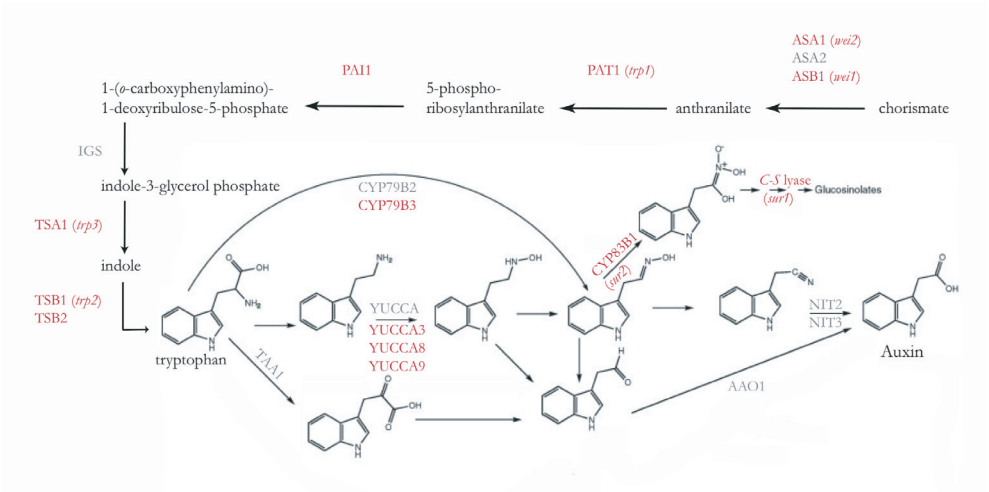

Figure 3: PLT2 controls (rate-limiting) enzymes in the tryptophan-dependent auxin biosynthesis pathways. Chorismate can be converted to tryptophan by a series of enzymatic alterations mediated by different enzymes. Most of these enzymes are under indirect control of PLT2. Trp can be converted via various pathways to IAA and glucosinolates. CYP79B2, CYP79B3 and the YUCCA flavin monooxygenases catalyze rate-limiting steps in a trp-dependent pathway and are all strongly responsive to PLT2 activation. PLT2 responsive genes are depicted in red. Figure adapted from Zhao, 2008.

in part through direct regulation of *ATR1* and *YUCCA8*.

In addition to the investigation into genes that are involved in auxin biosynthesis, we performed a manual search for genes implicated in auxin transport and signaling. This analysis returned auxin transport facilitators among the direct (PIN4 and LAX2) and indirect targets (PIN1), as well as auxin signaling components that can be directly (NPY4 and ARF10) and indirectly (ARF5/MONOPTEROS and IAA30) upregulated by PLT2 (Supplementary Table 2). Thus, the PLT2 pathway appears to directly influence auxin biosynthesis, auxin transport, and auxin signaling during root development, primarily in a positive fashion.

Discussion

Here we present the result of a whole genome microarray approach to identify the direct targets of the PLT2 master regulator of root development. From the multitude of upregulated genes after PLT2 activation we predicted 100 direct targets with high confidence. These genes are consistently upregulated 2 hrs after DEX induced PLT2:GR activation and maintain elevated transcript levels at 4 and 8 hrs of treatment. More importantly, upregulation of these genes was observed in the presence of the protein synthesis inhibitor Cyc both in microarray experiments and by independent qRT-PCR. Recently, the *in vivo* binding of PLT2 to the regulatory sequence of a number of these direct targets was shown (van Dijken, personal communication), demonstrating that PLT2 has transcription factor activity and confirms at least some

direct targets identified by our microarray analysis are directly bound by PLT2 *in vivo*.

The functional basis for the observed redundancy of the PLT gene family in control of root growth and stem cell maintenance was revealed by examining transcript behavior of the direct targets in different mutant backgrounds. We found that in a *plt1* background, removal of PLT2 results in the downregulation of only three direct target genes, out of the 16 genes tested. Moreover, a complementary microarray study identifying direct BBM targets (Passarinho et al., 2008) and preliminary qRT-PCR results from overexpression studies of the close PLT2 homologous AIL5 and AIL7 (data not shown) indicate that other PLT family members can (directly) regulate some of the PLT2 targets as well. This extensive overlap in target regulation between several PLT family members might be due to the result of using constitutive ectopic activation of PLT2 rather than restricting PLT2 action to its endogenous expression domain. Future research should determine which genes are common targets of the PLT gene family and which target genes execute specific PLT protein action.

Experimental data indicates that the PLT gene family collectively defines at least three different cell fates. Specification of discrete cell fates and differentiation states is generally addressed in terms of distinct downstream target expression patterns. Accordingly, the graded signal coming from PLT is thus expected to be converted into more or less binary responses of each target, defining sharp response boundaries. Our expression analysis of a selection of direct PLT2 targets is consistent with these expectations, demonstrating in several cases all-or-none expression in restricted and non-overlapping regions. Moreover, constitutive expression of PLT2 results in a corresponding domain-shift of one of the direct targets revealing its dose-dependent regulation. Interestingly, the GFP expression patterns of the targets tested appear to correlate with specific threshold concentrations in the PLT gradient; targets that are expressed in the stem cell nice, overlapping the region of highest PLT2 expression, (2) targets restricted to the columella and meristematic region, correlating with intermediate PLT2 levels, and (3) target genes active in the differentiation/elongation zone at low PLT2 level presence. Currently we have no phenotypic data on any of the loss-of-function mutants of these genes, but their expression patterns make them interesting candidates for defining region-specific PLT output. Quite the opposite to those target genes with expression patterns that cross multiple PLT2 threshold concentration zones, which are more likely to have general functions in root development. Noteworthy, most target GFP expression patterns not only show region specific expression along the longitudinal axis but also in the radial plane, suggesting additional regulatory factors act in concert with PLT2 to specify their confined expression domains.

Using our target expression data we can now also postulate a molecular mechanism for PLT2 directed transcriptional activation of downstream targets. Transcriptional effectors employ a range of strategies to translate graded signaling into differential gene regulation (reviewed in Ashe and Briscoe, 2005). Though in theory every unique downstream gene expression pattern requires a distinct strategy of transcriptional activation, the known modes of action have been categorized based on general design features. The expression domain of EXPRESSED PROTEIN AT2G03830 (Fig. 2e) suggests it is only activated at peak levels of PLT2, limiting its expression to the stem cell niche. The most parsimonious model would predict that enhancers within the *cis*-regulatory region of this gene have low-affinity PLT2-binding sites that are only occupied at the highest PLT2 concentration. Consequently, if PLT2 protein levels are ectopically elevated this would expand the response of AT2G03830 into a broader domain; which is exactly what is observed in PLT2 overexpression lines. However, binding-site affinity can only account for some PLT2 gradient readouts. For genes activated in the middle and apical part of the gradient, there is a requirement for the integration of combinatorial inputs from PLT2 and other positive and/or negative transcriptional regulators for correct interpretation of the PLT2 gradient. In case of PLT2 overexpression, the expression domain of the target gene that dependent on combinatorial input is not expected to shift accordingly unless activation of the co-regulator is also controlled by PLT2. Impending research focused on identifying PLT2 binding sites within target regulatory regions will increase our understanding of differential gene regulation by graded signaling.

An important function of PLT2 in post-embryonic root development is to facilitate the transgression of cell differentiation along the apical-basal axis; guiding cells with stem cell identity to prolific cell fate to cell differentiation and elongation (Galinha et al., 2007). Functional categorization of our identified direct targets suggests PLT2 relies substantially on transcription factors and posttranscriptional modifications as means to relay this sort of information. In addition, we find that executers of cell behavior can also be directly regulated. The latter group includes those direct target genes that have been implicated to function in processes such as cytokinesis, cell elongation, intracellular trafficking and cell wall composition.

A global survey of the indirect targets suggest that PLT2 function in root development is mediated notably through positive regulation of hormonal pathways and phosphate signaling and negative modulation of cell wall biosynthesis, the phenylpropanoid biosynthetic process and the oxidative stress response. A more in-depth analysis of the nature of these indirect targets will be required to attribute gene-specific contributions to PLT2 downstream function.

With the identification of MNP as a direct target, we have found a good candidate

for mediating PLT information during embryonic root specification. In *monopole* mutants cells at the basal pole of the developing embryo stop dividing at the 8-cell stage, thus before specification of the root progenitor (hypophyseal) cell (Tal Nawy, personal communication). Whether MNP acts as the hypophysis fate determinant, or has a supporting role (e.g. enable basal auxin maximum) is unclear. Similar defects are observed in the *plt1,plt2,plt3* triple mutant embryo, hinting towards a regulatory circuitry in which the PLT gene family redundantly controls *MNP* expression to establish embryonic root pole development. Analysis of *MNP* transcript levels in various *plt* mutant backgrounds will be essential to test this hypothesis. In addition, expression of *MNP* in the developing *plt1,plt2,plt3* embryo under control of the PLT2 promoter might reveal the extent of PLT dependent root pole formation that is mediated through MNP.

In conclusion, the nature of the direct targets provide direct leads to many processes currently associated with PLT2 regulation; from the onset of root specification in the developing embryo to cell proliferation and differentiation in the post-embryonic root.

Previous studies have shown that overexpression of ATR1 and *YUCCA* genes result in elevated IAA levels (Celenza et al., 2005; Zhao et al., 2001; Cheng et al., 2006). However, auxin concentrations in 24 hrs. Dex induced 35S::PLT2:GR plants are only occasionally elevated judging from the DR5 auxin response marker output (Galinha et al., 2007). This suggests the activity of an endogenous mechanism to counterbalance the increased auxin biosynthesis activity through enhanced auxin degradation or through the activation of a precursor diverting pathway. In fact, the key enzymes in glucosinolates biosynthesis, *C-S* lyase/SUR1 or CYP83B1/SUR2, have both been found to be strongly enhanced in response to PLT2 activation. These genes cooperatively convert the intermediate indole-3-acetaldoxime (IOX) for use in the glucosinolate biosynthesis pathway and thus draining the auxin biosynthesis precursor pool (reviewed in Zhao, 2008; Fig. 3). However, our microarray data indicates that induction of PLT2 directly and indirectly interferes with many auxin response related factors and can thus be obstructing the measurement of auxin response (Supplementary Table 2). *In planta* auxin measurements will be required to obtain a more realistic estimate of auxin concentration and distribution.

Earlier studies have uncovered a strong entanglement between PLT activity and the PIN auxin transport facilitators. Restricted *PLT* expression in the root depends on the action of multiple *PIN* genes and, in turn, transcription of *PIN* genes is redundantly maintained by PLT activity (Blilou et al., 2005; Galinha et al., 2007). The combinatorial action of all root expressed PIN genes establishes a robust auxin gradient and basal maximum that is thought to be "translated" by the auxin responsive

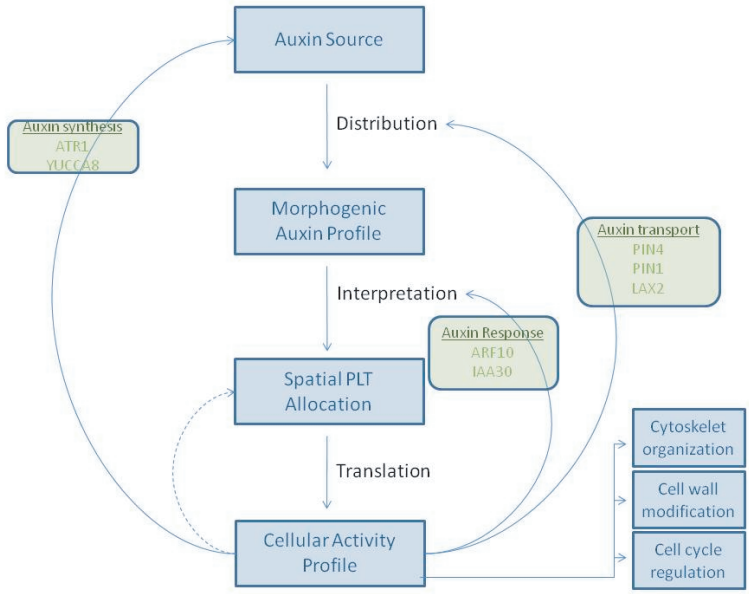

Figure 4: Conceptual framework for auxin/PLT2 mediated positional specification in root development. PIN facilitated transport generates a morphogenic auxin gradient spanning the root meristem. Interpretation of the gradient by auxin signaling pathway components results in graded expression of PLT proteins. In turn, the PLT proteins dictate cellular responses in a concentration dependent manner. The identified PLT2 targets reveal multiple levels of feedback exist between the cellular response (gene activity profile), auxin biosynthesis (morphogen source), auxin distribution (morphogen profile) and potentially also the allocation or activity of PLT proteins. Figure inspired by Jaeger et al., 2008.

PLT genes into distinct cellular responses. In our experiment, we identified *PIN4* as a direct target of PLT2 but none of the other PIN family members. Possibly, since PIN4 is a main contributor to the establishment and maintenance of the basal auxin maximum (Friml et al., 2002), PLT2 exerts direct control over *PIN4* to ensure a stable maximum and thereby its own expression. In addition, *PIN1* is upregulated more than 2-fold as soon as 2 hrs after Dex induction and thus also closely linked with PLT2 activity.

With the identification of two key regulators of local IAA biosynthesis as direct PLT2 targets (*ATR1* and *YUCCA8*), we now have evidence that *PLT* genes not only shape auxin distribution through PIN protein control but may also contribute directly to the auxin concentration profile. Moreover, we identified several factors involved in auxin signaling among the direct (NPY4 and ARF10) and indirect targets suggesting that PLT2 can modulate the cellular response to auxin as well. In other words, cellular activity (e.g. auxin transport and/or biosynthesis) might feedback on positional information encoded by the auxin gradient; coupling the formation and interpretation of the (morphogen) gradient (Fig. 4; see also summarizing discussion).

Methods Summary

Plant materials and steroid treatments

In the experiments described, Arabidopsis thaliana plants of the Columbia (Col-0) and Wassilewskaya (Ws) ecotype were used. The 35S::PLT2:GR conditional overexpressor line and the *plt1-4* and *plt1-4,plt2-2* mutants were described earlier (Aida et al., 2004; Galinha et al., 2007). In general, seedlings were germinated on half-strength Murashige and Skoog medium after 7 days of cold treatment at 4°C and grown under standard lab conditions as described in Willemsen et al., 1998. For Dex treatments, a 10 mM stock was prepared in DMSO and added to the medium at a concentration of 10 μM. For Cyc treatments, a 100 mM stock solution was prepared in ethanol and added to the medium at a concentration of 10 μM. Equal amounts of DMSO and/or ethanol was added to the controls as appropriate.

Microarray data acquisition and statistical analysis

For all microarray experiments, 2 day old seedlings were transferred to conical flasks and grown for 24 hrs in liquid ½ GM medium under continuous light. For the 4 hours and 45 min. direct induction experiment we pre-treated the seedlings for 15 min. with Cyc before adding Dex. After treatment, tissues were flash frozen in liquid nitrogen. We included three biological replicates for each induction experiment and two biological replicates for the complementary mock treatment.

Total RNA was then isolated from the frozen material using the Macherey-Nagel NucleoSpin RNA Plant kit (Bioke, http://www.bioke.com), including the on-column DNAse treatment. Probes for hybridization were generated by Service XS (http://www.servicexs.com) according to standard Affymetrix protocol and hybridized on Affymetrix ATH1 microarray chip.

The microarray raw datasets were normalized using the *affy* package in the statistical environment R (http://www.r-project.org/). The robust multi-array average (RMA) expression measure (Irizarry et al., 2003) was used to convert raw data to log expression values. To assess differential gene expression between samples linear models were applied in the R package Limma (Linear Models for Microarray Data; Smyth, 2004; Smyth, 2005) and p-values derived from a t-test were corrected for multiple testing errors by using the multiple testing correction of Benjamini and Hochberg (1995) with a 5 % false-discovery rate (FDR), corresponding to a Q-value of ≤ 0.05 (Storey and Tibshirani, 2003). Treatments (with Dex or CHX) and time were used as coefficients in the model. Significant differentially expressed genes were subsequently obtained by selecting for genes with more than 2-fold increase or

decrease in expression levels. In the case of 45 min. Dex/cyc treatments however, we arbitrarily raised the q-value cut-off to 0.2 as random variation in gene expression induced by Cyc hampered gene selection at high q-values. Moreover, we applied a 1.5-fold expression value cut-off for target selection to compensate for the short period of PLT2 induction.

Real-time quantitative RT-PCR

Total RNA for reverse transcription was obtained from an independent experiment treating 35S::PLT2:GR plants for 45 min. with Dex and 1 hrs with Cyc, under similar condition as for microarray purposes. RNA was isolated as mentioned. For the *plt1-4* versus *plt1-4, plt2-2* mutant comparison, we dissected root tips at approximately 0.4 cm from the apex and immediately flash froze them separating them into two pools. RNA of each pool was isolated as mentioned.

Each RNA sample (2.5 μg) was reverse transcribed using an oligo-dT primer (Amersham, http://www.amersham.com) and SuperScriptIII (Invitrogen, http://www.invitrogen.com), following the instructions of the manufacturer. Real-time PCR was performed using the ABIPrism7700 Sequence Detector and Cybergreen chemistry (Applied Biosystems, http://www.appliedbiosystems.com). At least two independent amplifications were performed from each cDNA sample. The thermal cycling program started with a step of 2 min at 50 ºC and 10 min at 95 ºC, followed by 40 cycles (15 sec at 95 ºC and 1 min at 60 ºC). Dissociation kinetics analyses of the amplification products confirmed that only the expected products were amplified. Expression levels were calculated relative to ACTIN2 (AT3G18780) using the $2^{-\Delta\Delta C}$ T method, in which mock treated 35S::PLT2:GR seedlings were used as the calibrator (Livak and Schmittgen, 2001). Primers were designed according to the recommendations of Applied Biosystems and are listed in Supplementary Table 3.

Plasmid construction and Plant transformation

Promoter fusions of the selected target genes were generated by insertion of the upstream intergenic region into pGREENII-0229 (http://www.pgreen.ac.uk) with an eGFP-noster fragment. Primers used to amplify the regulatory regions are summarized in Supplementary Table 3. *Arabidopsis* Col-0 plants were then transformed by the floral dip method (Clough and Bent, 1998). In total we analyzed the expression patterns of 13 direct targets in at least 5 independent transformants. Generally (80%), the GFP expression pattern reflected the predicted pattern, suggesting that sufficient upstream regulatory sequence was cloned before the GFP coding sequence.

Gene ontology analysis

nalysis for GO term enrichment was performed using the AMIGO server (www.amigo.geneontology.org/cgi-bin/amigo/go.cgi; Carbon et al., 2009). For the indirect target analysis, only GO terms with more than 5 annotated loci were taken into account. For the direct target analysis, the minimum number of gene products annotated to the term was set to two. Parent categories synonymous with a child category that contained an overlapping gene content of more than two thirds, were omitted to limit redundancy in the same GO category.

Supplemental data to:

Dosage-dependent activation of PLT2 targets establishes a feed-forward network that regulates *Arabidopsis* root development

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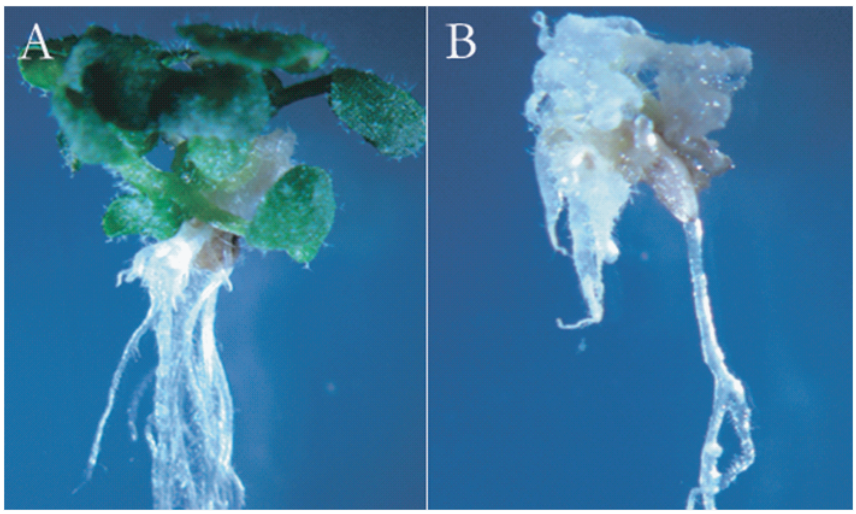

Supplementary Figure 1: **Transient expression of PLT2 induces a meristematic fate switch and promotes ectopic root formation**. (**A** and **B**) Four week old 35S::PLT2:GR plants that have been treated with mock (**A**) or Dex (**B**) continuously three days after germination.

Supplementary Table 1: List of negatively regulated candidate direct target genes of PLT2, ranked according to their negative fold-change at 45 min. of combined Dex/Cyc treatment. Asterisks indicate those genes with a q-value equal to or less than 0.2 within the 45 min. Dex/Cyc experiment; q-value for all genes in other experiments ≤0.05.

Gene	Description	45 min. Dex/Cyc	4 hrs. Dex/Cyc	2 hrs. Dex	4 hrs. Dex	8 hrs. Dex
AT3G14560	expressed protein	-2.2*	-2.1	-2.1	-2.1	-2.3
AT5G08330	TCP family transcription factor	-1.5	-1.0	-2.2	-2.1	-2.0
AT4G37790	homeobox-leucine zipper protein 22 (HAT22)	-1.5	-2.3	-2.9	-2.3	-2.5
AT5G47980	transferase family protein	-1.4	-2.2	-2.2	-2.3	-3.3
AT5G01210	transferase family protein	-1.4	-2.3	-1.8	-2.0	-2.3
AT5G22270	expressed protein	-1.2	-2.1	-1.9	-2.8	-2.2
AT1G67110	cytochrome P450 family protein	-1.1	-2.3	-1.4	-2.0	-1.7
AT1G03850	glutaredoxin family protein	-1.1	-2.3	-2.4	-2.3	-2.9
AT2G35770	serine carboxypeptidase S10 family protein	-1.1	-2.5	-1.4	-2.0	-1.8
AT5G47990	cytochrome P450 family protein	0.9	-2.1	-3.0	-2.0	-2.8

Supplementary Table 2: List of PLT2 direct and indirect targets involved in the auxin biosynthesis, transport and signalling related processes and their respective fold-changes after PLT2 activation.

Gene	Description	45 min. Dex/Cyc	4 hrs. Dex/Cyc	2 hrs. Dex	4 hrs. Dex	8 hrs. Dex
Trypthophan Biosynthesis						
AT5G05730	alpha subunit of anthranilate synthase (ASA1/WEI2); catalyzes the rate-limiting step of tryptophan synthesis	0.9	0.8	2.4	2.0	2.1
AT1G25220	anthranilate synthase beta subunit1; catalyzes the first step of tryptophan biosynthesis (ASB1)	0.9	1.1	1.9	2.0	2.2
AT5G57890	anthranilate synthase beta subunit, putative					
AT5G17990	phosphoribosylanthranilate transferase (TRP1)	0.9	1.0	1.8	1.8	2.3
AT1G07780	phosphoribosylanthranilate isomerase (PAI1); catalyzes the third step in the tryptophan biosynthetic pathway	1.0	0.9	1.2	2.1	2.2
AT5G05590	phosphoribosylanthranilate isomerase PAI2);					
AT1G29410	phosphoribosylanthranilate isomerase (PAI3)					
AT3G54640	tryptophan synthase alpha chain (TSA1); catalyzes the penultimate step in tryptophan biosynthesis	1.0	0.9	2.0	2.0	2.4
AT5G54810	tryptophan synthase beta subunit (TSB1);	0.9	0.8	1.9	2.2	3.2
AT4G27070	Tryptophan synthase beta (TSB2)					
Auxin Biosynthesis						
AT1G04610	flavin-containing monooxygenase (YUCCA3)	0.9	1.2	2.1	6.3	17.3
AT5G11320	flavin-containing monooxygenase (YUCCA8)	1.5	2.4	4.7	6.3	10.5
AT5G43890	flavin-containing monooxygenase (YUCCA9)	1.1	1.1	1.5	1.7	2.1
AT4G39950	cytochrome P450 (CYP79B2)	1.1	1.3	1.7	1.7	1.8
AT2G22330	cytochrome P450 (CYP79B3)	1.0	0.8	1.5	2.4	2.6
Indolic Glucosinolate Biosynthesis						
AT4G31500	cytochrome P450 (CYP83B1/SUR2)	1.1	1.4	4.2	5.0	5.8
AT2G20610	C-S lyase (SUR1)	1.0	0.9	1.4	1.7	2.0
Auxin Conjugation						
AT2G23170	IAA-amido synthase	0.8	0.6	2.0	2.7	2.1
Auxin Transport						
AT1G73590	auxin efflux carrier (PIN1)	1.1	1.2	2.0	2.8	2.9
AT5G57090	auxin efflux carrier (PIN4)	1.6	3.8	1.5	2.2	3.0
AT2G21050	amino acid permease, putative (LAX2)	1.3	2.2	3.3	4.2	5.2
Auxin Signalling						
AT2G23050	NPH3 domain containing protein (NPY4)	1.5	2.2	2.8	4.0	5.2
AT1G19850	auxin response factor 5 (ARF5/MONOPTEROS)	1.0	0.9	1.5	2.1	2.5
AT2G28350	auxin response factor 10 (ARF10)	1.2	3.2	1.7	1.8	2.2
AT3G62100	Aux/IAA protein (IAA30)	1.0	0.9	2.2	3.4	4.4

Supplementary Table 3. Primer sequences used for cloning and QRT-PCR amplicon amplification.

fragment	purpose	fragment size (kb)	Forward primer (5'-3')	Reverse primer (5'- 3')
pAT3G15720	GFP fushion	2.7	CGTTTTTAGTTTCACAATTAGGC	TTGTTGAAGCGAAGGCTTTAC
pAT1G70140	GFP fushion	1.7	CGGTAACTCTTAAGGCCCATT	CAATTCCCAAAAATCTCAGGA
pAT3G15680	GFP fushion	3.9	GGGGTGTTGTGCTTTACGTT	CACGAGGCTTTGAAAGAGTG
pAT5G02550	GFP fushion	1.8	CAACGGCTATGATTTGCTGA	GGATTGGGAAGAGAGAAAAGAGA
pAT2G03830	GFP fushion	3.5	TGCCGTTAATAAAGATTCTTAGCC	TTTTGTGCTATCTTTCTTTTTCTTC
AT4G14690	QRT-PCR	0.2	ATCGCCATGGAGTTATCA	CCCTTTTGACTTTGCCTC
AT3G01840	QRT-PCR	0.2	AATCGTAATGGAGGTTTTGTCTGG	CAAACGCAGAATCAACCGAA
AT1G56680	QRT-PCR	0.2	ACCGAAGTTGGTCCATCACTAAGAC	TATAGATCTTTACCAGGATTCACCC
AT3G62760	QRT-PCR	0.2	AGCCGGAGATACTTACACACTCGC	TTTGTGGTGGGAGCAACG
AT5G60630	QRT-PCR	0.2	GTTCGACTTCATCATTGTAGCTCTT	GGTCTGGTACTATCAGTGTAGTTGA
AT5G56790	QRT-PCR	0.2	CTGGAGCCTCACCGTTTT	AAGCATGAATCAGGTTTACG
AT5G60890	QRT-PCR	0.2	TAAACCGGGACTTACTGACC	CTCGTCAGACAAAGACTCCAA
AT4G28950	QRT-PCR	0.2	TCTTCTTCACCACTCTTCTT	GTAGGAAACTTGTTGCTAGTG
AT1G55760	QRT-PCR	0.2	TCTGATGGAAGCATTGGAG	CCTCTCAAGCACATTCTTTG
AT3G15720	QRT-PCR	0.2	CGTCGGCAGTGGAAGTGAG	GTACAGCAATTGTAGACGCACC
AT4G37430	QRT-PCR	0.2	AGCTATAGGCAAATCAATGG	CATAAGGCCTTTGATGATAACG
AT5G44460	QRT-PCR	0.2	GGTTCGATGACTTTGCTG	GTTCAATCTCTCCTGCTTCAG
AT5G02550	QRT-PCR	0.2	GTCGGAGAGACCAAGCCGTC	ATGGTGATCTTTGCTAACATTGTCG
AT1G21090	QRT-PCR	0.2	GTATCCATCAAGAACAGGCTC	TGAGCTGAATTAGTGGAAGT
AT4G36830	QRT-PCR	0.2	TTCTAGCGATTCTATCGACGACTCT	TAAGCGTTACGTTGGCTATTC
AT1G50570	QRT-PCR	0.2	CTGTTGACGAAGCAGCAC	GATGATATCTGTTGGCTTCT
AT5G47060	QRT-PCR	0.2	GAAGAGAATTTGGCTTCTTC	CAGACACAACAGATCTCGG
AT5G13290	QRT-PCR	0.2	TAACCGGAAGAGACCCGA	CAACTGCAGATGTCTGGATTGGT
AT3G50870	QRT-PCR	0.2	GCATGCGGCATTCGTTTC	GTGAGCACCGTCGGATTCA
AT2G01210	QRT-PCR	0.2	TGTTCTTGATCCTTGTTTGGCT	CCGGCCACGGGTAATCTG
AT5G14690	QRT-PCR	0.2	ATGGCGGGACCCACTGTC	GATATTCCCGCGGCGTTTC
AT3G24240	QRT-PCR	0.2	CTTTACTCATGGCTTCACTCTT	CCGGAGATAGTGAGTTTCTGG
AT2G38970	QRT-PCR	0.2	CAGCAACTCGTGTTTTCTGGG	ATGTCAAGCACCGTGACAAG
AT3G14230	QRT-PCR	0.2	CCAAACTCTGATGAGTCAGACGA	TACAAACTAAGGAACCATCGATCAC
AT5G51550	QRT-PCR	0.2	ATCCGACGGCGGTATTTG	ATTATGCATCGACACAACAAACCC
ACTIN1	QRT-PCR	0.2	GGCGATGAAGCTCAATCCAAA	GGTCACGACCAGCAAGATCAAG

Chapter five

Summarizing discussion

The research described in this thesis focuses on the function of transcription factor family members in control of development, stem cell maintenance, cell differentiation states, and zonation in the *Arabidopsis* root. We show that plants employ conserved genetic factors to regulate stem cell maintenance in root and shoot systems and expanded the notion that the *PLT* gene family (Aida et al., 2004) specifically regulates stem cell properties in the root to a significantly broader role in root development. In addition, we show that members of the PLT gene family can activate transcriptional targets in a dose-dependent fashion to establish a feed-forward network that regulates root growth and development. In this summarizing chapter I will discuss our results in the light of recent insights in stem cell specification and maintenance, morphogen theory and transcriptional networks to give directions to future research.

In chapter **two** we describe the identification of a novel transcription factor that is particularly important for proper fate maintenance of cells residing in the stem cell niche. This homeobox protein, WOX5 (WUSCHEL-RELATED 5), is expressed in the quiescent centre where it dictates minor aspects of QC specification. More importantly, WOX5 appears to regulate columella stem cell maintenance in a non-cell autonomous fashion. Loss of WOX5 function results in differentiated columella stem cells, while conditional overexpression results in the accumulation of cells that are remarkably similar to wildtype distal stem cells. Interestingly, these induced stem cells are now made independent from the organizing activity of the QC. Normally, laser ablation of the quiescent centre results in differentiation of the columella stem cells (van den Berg et al., 1997; Wildwater et al., 2005); in 35S:WOX5 plants the distal stem cell population remained intact after QC ablation. It remains to be resolved whether it is the WOX5 protein that is translocated from the QC to the distal stem cells to instruct stem cell fate or an activated downstream factor. Ectopic expression of WOX5 results in accumulation of induced stem cells only in the distal root area by maintaining stem cell daughter cells in the stem cell state for a prolonged period. These observations suggest the presence of (a) local factor(s) that may act in concert with WOX5 signaling to maintain distal stem cell fate. Potential candidates to act cooperatively with WOX5 in columella stem cell control are the NAC transcription factors FEZ and SMB. These genes control distal stem cell divisions through a self-regulating feedback loop (Willemsen et al., 2009). However, recent data suggest that 35S:WOX5 induced stem cells still accumulate in *fez* and *smb* mutant backgrounds (data not shown), indicating WOX5 acts independent or downstream of FEZ and SMB in stem cell regulation.

In *shr* and *scr* mutants, *wox5* transcripts are either weakly present or totally absent, suggesting WOX5 acts downstream of the SHR/SCR signaling pathway in QC specification and distal stem cell maintenance. However, the proximal meristem of single *scr* and *shr* mutants ultimately differentiates (as opposed to *wox5*)

suggesting additional SHR/SCR controlled factors contribute to niche maintenance. Independently expressing WOX5 in a *scr/shr* background might facilitate molecular identification of these parallel regulators, e.g. through microarray profiling.

It did not escape our notice that the non-autonomous function of WOX5 in distal stem cell signaling in the root is similar to the function of its close homologue WUSCHEL in shoot stem cell maintenance. By reciprocal expression experiments we show that both genes are functionally equivalent in stem cell control, indicating that the organizers of both stem cell systems employ related regulators to provide maintenance signaling. This provides molecular evidence that the stem cell regulatory system originated before root and shoots separated in land plants from which the current higher plants are derived. This hypothesis has recently been substantiated by phylogenetic data, demonstrating that stem cell promoting WUS function was present in a likely common ancestor of gymnosperms and angiosperms (Nardmann et al., 2009). More molecular parallels between shoot and root stem cell niche regulation have been revealed recently, with the identification of CLE40 function in distal stem cell control. In the shoot system, stem cells signal back to the organizing center by secreting the CLAVATA3 (CLV3) dodecapeptide (Kondo et al., 2006), which represses WUS expression in a CLV1/CLV2 receptor-like kinase dependent manner (Brand et al., 2000; Schoof et al., 2000). In the root, differentiating descendants of distal root stem cells express CLE40, a peptide closely related to CLV3, that regulates the domain of WOX5 expression via *ACR4*, a receptor-like kinase of the *CRINKLY4* family (Stahl et al., 2009). Although the molecular factors involved in stem cell homeostasis seem to be similar in both niches, the oligopeptide signaling appear to operate in opposite manner. In contrast to the shoot system, peptide-signaling originating from differentiated cells rather than the stem cells co-regulate the size and position of the root niche. Comparing the expression profile of transcripts from the overexpression alleles of WOX5 and WUS (Leibfried et al., 2005) may result in the identification of more genetic parallels between shoot and root stem cell niche regulation

Chapter **three** describes the functional analysis of four PLT transcription factor family members that are redundantly required for embryonic root development and post-embryonic root growth in a dose-dependent fashion. Sequential deletion of wildtype *PLT* genes result in ever decreasing root and meristem length; culminating in *plt1* $^{-/-}$*plt2* $^{+/-}$ *plt3* $^{-/-}$ *bbm* $^{-/-}$ seedlings that completely lack a root and hypocotyl. Interestingly, the homozygous combination of *plt2* and *bbm* knock-out alleles yields early arrested embryos indicating these genes have additional roles in early embryogenesis. It will be interesting to determine their exact role during the initial embryonic developmental stages through phenotypic analysis and early fate-marker behavior. In addition, the recent uncovering of the transcriptional targets of both

BBM (Passarinho et al., 2009) and PLT2 may facilitate a candidate approach to identify the genetic downstream factor(s) contributing to PLT mediated early embryo development.

Pervasive activation of PLT2:GR in 3 day old seedlings results initially in de novo root development from the shoot meristem region. It is unclear however, which cells are specifically susceptible for the meristematic fate transformation towards root identity. It will be interesting to determine the exact field of morphogenic competence using domain specific promoters to ectopically express PLT2 in distinct regions of the shoot meristem. Moreover, we observed that ectopic root formation is impeded in older seedlings that have already produced several leaves (data not shown). This suggests that apart from spatial limitations there exists also a developmental window of competence to respond to PLT2 with cell fate reversion and ectopic root formation. Chromatin remodeling factors and (cell-specific) differentiation regulators might play a role as competence determinants in this process.

Transcriptional and translational studies of the PLT proteins reveal their graded expression spanning the root meristem with highest expression in the stem cell area, intermediate levels in the transit amplifying zone, and low levels in the differentiation zone. By changing the shape of the PLT expression domain we show that cell fate within the root meristem depends on the amount of PLT cells are subjected to; high PLT levels maintain stem cell fate, intermediate levels control cell division, and low levels are required for cell elongation. These findings demonstrate that fundamental principles of morphogen action can be attributed to a plant transcription factor regulating organ development; meaning, a signaling molecule that acts directly on cells to produce specific responses dependent on the molecule's concentration. Can PLT proteins be therefore considered morphogens? According to the classical definition not, as it requires the morphogen to be synthesized locally in order to spread from the source and form a concentration gradient. Moreover, the classical morphogens are defined not only as regulators of organ growth but also as determinants of morphological organ patterning. In the mature *Drosophila* wing, for example, Decapentaplegic (DPP) morphogen gradient outputs are manifested by the positioning of wing veins along the anteroposterior axis (reviewed in Affolter and Basler, 2007). In contrast, tissue organization in the root remains largely unaltered upon PLT manipulation, excluding PLT proteins as true morphogens by definition.

A more favorable morphogen candidate acting in root development is auxin, although its distribution system differs from that defined for classical morphogens. The auxin gradient spanning the root meristem is instructive in terms of cells specification, tissue patterning and root zonation (Sabatini et al., 1999; Blilou et al., 2005; Grieneisen et al., 2007) and cells react flexibly to changes in auxin levels by modulation of their developmental fate (Sabatini et al., 1999). In this perspective, the auxin responsive PLT proteins rather seem to act as the molecular "translators"

of the instructive auxin gradient. This would require PLT to control expression of direct transcriptional targets in regions corresponding to specific PLT activity levels; a hypothesis investigated in chapter four.

In stem cell control, RETINOBLASTOMA-RELATED (RBR) acts in parallel with PLT; since only the combined modulation of both genes resulted in ectopic stem cell accumulation. Identification of their transcriptional targets may reveal a common stem cell fate controlling pathway where both downstream networks converge on to.

Chapter **four** reports the genome-wide identification of direct target genes of PLT2. In addition, we show that these targets are expressed in non-overlapping expression domains within the root meristem and can be regulated by PLT2 in a concentration-dependent manner. This may provide a molecular explanation for the dosage-dependent regulation of cell differentiation states within the growing root by the PLT protein family reported in chapter three. Currently we have investigated the transcriptional response of just one direct target to ectopic expression of PLT2 and observed rapid expansion of its endogenous expression domain. Conclusive evidence for dosage-dependent activation of PLT2 targets requires the response of more target expression patterns not only in PLT2 activation lines but also in different *plt* mutant backgrounds. In the latter case, however, it is necessary to assess whether the hypothesized decrease of target expression domains is a direct effect of reduced PLT activity or an indirect effect of decreased meristem size (even though this is a result of loss of PLT function). Conditional PLT loss-of-function may circumvent this complication.

The classical theory of positional information by morphogen gradients as postulated by Wolpert (1968) implies that positional specification is a two-step process; (1) diffusion from a local source creates a linear gradient of decreasing morphogen concentration through a field of undifferentiated cells, (2) cells become specified by expressing specific target genes depending exactly on threshold levels in the gradient. As the morphogen gradient is unaffected by the cellular response, the positional values are independent of subsequent processes operating within the developmental field. Our data however, in concert with a growing number of studies (reviewed in Jeager et al., 2008), demonstrate a strong entanglement between positional information and its response system, involving various levels of regulatory feedback of the target tissue into the morphogen gradient. In our case, the most striking example is the direct control of PLT2 over PIN expression. As Grieneisen and collegues (2007) have shown, PIN distribution is the main determinant to generate the auxin gradient across the root meristem. This suggests that the shape of the morphogenic auxin gradient can be modulated through direct feedback in the responsive cells. Whether PLT targets directly regulate PIN polarity remains to be seen, though the GTPase gene family member ROP9 that we identified as a direct PLT2 target is a potential

candidate. At a different level, activation of the tryptophan and auxin biosynthesis pathways upon PLT2 expression suggests that cellular response also feeds back into the morphogen gradient by modulating auxin concentrations. Finally, factors of the auxin response pathway have also been shown to be regulated by PLT2. This in itself creates a whole new regulatory multilevel feedback mechanism. Not only have these Aux/IAA-ARF's have been implicated in regulating *PLT* expression (Aida et al., 2004), they have also been reported to play a role in PIN polarity (Sauer et al., 2006). Together, our findings indicate that root development and maintenance is regulated by a highly tangled hierarchical regulatory system in which recursive procedures (a factor regulating itself) are used to stabilize the morphogenic positional field. Having such extensive regulatory feedback mechanisms between cellular activity and the field of positional information produced by the morphogen allows for dynamic integration of independent cues into the developing system. In principle, the responsive cells determine their relative position in the developmental field based on morphogen concentration, but the form of this field is dynamic and in part determined by feedback from the response system. For example, unfavorable growth conditions may signal independently from the auxin signaling system to slow down cell division in the meristem. Feedback of the root cells into the morphogen gradient is required in order to adjust the auxin gradient according the new conditions; in this, case a shorter root meristem.

A global ontology survey of the indirect targets suggest that PLT2 function in root development is mediated notably through positive regulation of hormonal pathways and phosphate signaling and negative modulation of peroxidase activity, cell wall biosynthesis, the cellular carbohydrate biosynthetic process and general stress responses. Interestingly, the downregulated classes of genes all have been linked to cell-wall extensibility and cell elongation (reviewed in Passardi et al., 2004), suggesting PLT promotes cell proliferation in part through active repression of cell elongation. However, peroxidases have also been associated with reactions that restrict growth (Passardi et al., 2004). A more in-depth analysis of the nature of the individual targets will be required to attribute gene-specific contributions to PLT2 downstream function.

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Samenvatting in het Nederlands

Slechts weinig dieren worden honderd jaar of ouder. Veel planten echter kunnen deze leeftijd eenvoudig bereiken en enkele zijn zelfs in staat meer dan duizend jaar oud te worden. Planten kunnen zo oud worden omdat zij altijd de mogelijkheid behouden nieuwe organen te vormen zoals bladeren, takken en zijwortels. Dit stelt hen in staat te reageren op veranderende omgevingsfactoren en schade aan het systeem te compenseren. Het geheim van de "eeuwige jeugd" ligt verscholen in groepjes van ongespecialiseerde, onvolwassen cellen die zichzelf over zeer lange periodes kunnen handhaven in de volwassen plant. Dit zijn de zogenaamde stamcellen; cellen die zichzelf kunnen vernieuwen en het leven kunnen geven aan een grote verscheidenheid van gespecialiseerde cel-typen.

Stamcellen voelen zich het prettigst in een zogenaamde "niche", een micro-omgeving waar de juiste omstandigheden heersen om hun ongespecialiseerde status te waarborgen. Planten bezitten twee van dit soort stamcel niches. Eén niche bevindt zich in het topje van de plant en is verantwoordelijk voor de vorming van alle zichtbare bovengrondse delen en een andere niche bevindt zich in het tipje van de wortel waar het wortelgroei mogelijk maakt. Hoewel beide stamcel niches op elkaar lijken, was het tot dusver onduidelijk of de moleculaire factoren die ten grondslag liggen aan het behoud van de scheut niche dezelfde zijn als die van de wortel niche.

Stamcellen weten niet uit zichzelf dat ze stamcellen zijn. Daartoe worden ze geïnstrueerd door naburige cellen welke het controlecentrum vormen van de stamcel niche. Van de model plant *Arabidopsis thaliana*, was een belangrijke factor voor stamcel behoud in de scheut al lange tijd bekent. Dit gen, *WUSCHEL* genaamd, komt tot expressie in het controlecentrum en signaleert naar de aangrenzende cellen dat ze stamcellen zijn en moeten blijven. In Hoofdstuk twee van dit proefschrift beschrijven we dat een vergelijkbaar signaleringsmechanisme ook van toepassing is in de stamcel niche van de wortel. Sterker nog, het gen dat zorgt draagt voor de signalering naar de stamcellen blijkt een tweelingbroertje te zijn van *WUSCHEL*, vandaar dat het de naam heeft gekregen *WUSCHEL-RELATED HOMEOBOX 5* (*WOX5*). Verlies van WOX5 functie leidt tot verlies van stamcellen in de wortel, vergelijkbaar met het verlies van WUSCHEL in de scheut. Als ultiem bewijs voor de vergelijkbare functie van beide genen in de verschillende stamcel niches, hebben we beide genen omgewisseld en in elkaars controlecentrum tot expressie gebracht. In beide gevallen kan de expressie van de één compenseren voor het verlies van de ander, wat betekent dat het behoud van stamcellen in zowel de scheut als de wortel afhankelijk is van eenzelfde soort (geconserveerde) genen en signalering.

Vanuit evolutionair perspectief bezien is dit een interessante vinding omdat het zou kunnen betekenen dat de regulatie van stamcellen vanuit een controlecentrum een oeroud mechanisme is, dat dateert uit een tijd waarin wortel en scheut zich nog niet als afzonderlijke stamcel-systemen hadden ontwikkeld. Paleobotanische gegevens suggereren dat landplanten voor het ontstaan van bladeren via splitsende stengels groeiden. Wellicht gaat de overeenkomst in stamcel controle tussen wortel en scheut terug tot dat systeem.

WOX5 is echter niet het enige gen dat een rol speelt bij het behoud van stamcellen in de wortel. Eerder werk binnen onze onderzoeksgroep heeft reeds aangetoond dat twee APETALA2-domein transcriptiefactoren genaamd PLETHORA1 (PLT1) en PLT2 evenzogoed essentieel zijn voor stamcelactiviteit. In Hoofdstuk 3 beschrijven we het onderzoek naar twee nieuwe leden van de *PLT* genfamilie, *PLT3* en *BMM*. Ook deze genen blijken, in combinatie met eerder genoemde PLT's, een aanzienlijke bijdrage te leveren aan het behoud van stamcelidentiteit in de wortel. Belangrijker nog, we tonen aan dat de functie van de *PLT* genen niet beperkt blijft tot de stamcellen in de wortel. De *PLT* genfamilie blijkt onmisbaar tijdens de vroege embryogenese en bij de aanleg van de basale structuren; de wortel en hypocotyl. Ook in de post-embryogene wortel strekt de invloed van *PLT* genen tot buiten de stamcelregio. Namelijk, manipulatie van het PLT expressie domein toont aan dat PLT genen naast stamcelidentiteit ook de zones van celdeling en celstrekking kunnen beïnvloeden.

Een expressiestudie van *PLT* genen laat zien dat PLT eiwitten gradueel gedistribueerd zijn langs de apicale-basale as van het wortelmeristeem; met een maximum accumulatie in de stamcel-regio, gematigde accumulatie in de celdelingzone en lage aanwezigheid in de zone van celstrekking. Dit doet vermoeden dat tijdens de post-embryogene ontwikkeling van de wortel een instructief PLT eiwitgradiënt de verdeling van de wortel in zones reguleert.

In Hoofdstuk 4 rapporteren we onze bevindingen van een genoom-brede studie naar de transcriptionele targets van PLT2. In totaal hebben we 100 directe targets geïdentificeerd, welke ons inzicht verschaffen in de diverse genetische programma's die PLT2 aanwendt teneinde wortelontwikkeling te sturen. Ondermeer vinden we specifieke aanwijzingen naar embryonale wortelontwikkeling, organisatie van het cytoskelet, celwand modificaties, en de cel cyclus. Bovendien laten onze resultaten zien dat PLT2 genen direct kan induceren in niet-overlappende expressiedomeinen op een concentratie afhankelijke manier. Een belangrijke vinding, want het suggereert dat het daadwerkelijk de PLT genen zijn die direct, en op een concentratie-afhankelijke manier, de differentiatie-toestand van de cellen in het wortelmeristeem dicteren middels de regulatie van specifieke genetische programma's.

Een opvallende bevinding anderzijds, is de overrepresentatie van factoren welke door PLT2 gereguleerd worden en betrokken zijn bij hetzij de biosynthese, het transport of de signalering van het plantenhormoon auxine. De PLT genen worden echter weer gereguleerd door ditzelfde auxine; welke zich ook als een informatief gradient manifesteert in de wortel. Dit impliceert een grote mate van verstrengeling tussen de formatie en interpretatie van het instructieve auxin-veld enerzijds en het door PLT2 gemedieerd response-systeem anderzijds.

Hoofdstuk 5, tenslotte, bediscussieert de implicaties van de in dit proefschrift beschreven resultaten en geeft richting aan toekomstig onderzoek naar de rol van WOX5 en PLT transcriptiefactoren in de regulatie van stamcellen en celdifferentiatie tijdens de wortelontwikkeling van *Arabidopsis thaliana.*

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Goed. Nog even wat mensen bedanken en dan is het gedaan. Het is een (onnodig) lang en moeizaam traject geweest,waarvan ik alleen maar kan hopen dat de geëiste investeringen zich in de nabije toekomst terug gaan betalen. Evenzogoed ben ik blij dat het erop zit en tevreden met het resultaat.

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Curriculum Vitae

It was on a warm Friday in the first week of September 1976, that Marijn Luijten was born in Nijmgen; the oldest and, subjectively, most beautiful city of Holland. Not long after his birth he moved to the outskirts of the city, to grow up happy and untroubled in the small town of Wijchen. After receiving his HAVO degree, he picked up a biochemistry study at the International Agricultural Hogeschool Larenstein. There, during a one year internship at the ATO-DLO under supervision of Drs. Gerard Rouwendal, the marvelous world of molecular plant biology unveiled itself to him. Inspired, he started his studies Biology at the University of Nijmegen in 1998, the same year he got his HLO- degree in microbiology/biochemistry from Larenstein. As an undergraduate he performed a first internship in the lab of Prof. Celestina Mariani, where, under supervision of Dr. Ivo Rieu, he worked on identifying the cellular localization of the ethylene receptors. From March 2001 to November 2001, a second internship was performed in the lab of Prof. Hajime Sakai, at DuPont Crop Sciences in Newark, United States, where he worked on the identification of novel genes involved leaf pattern formation and floral phase transition. From September 2002, he has worked in the department of Molecular Genetics at the university of Utrecht, to study the role of transcription factor family members in control of stem cell maintenance and cell differentiation states in the *Arabidopsis* root. Currently, Marijn works at the department of toxicogenetics, LUMC Leiden, in the lab of Dr. M. Tijsterman.