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REVIEW

Building a plant: cell fate specification in the early *Arabidopsis* embryo

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ABSTRACT

Embryogenesis is the beginning of plant development, yet the cell fate decisions and patterning steps that occur during this time are reiterated during development to build the post-embryonic architecture. In *Arabidopsis*, embryogenesis follows a simple and predictable pattern, making it an ideal model with which to understand how cellular and tissue developmental processes are controlled. Here, we review the early stages of *Arabidopsis* embryogenesis, focusing on the globular stage, during which time stem cells are first specified and all major tissues obtain their identities. We discuss four different aspects of development: the formation of outer versus inner layers; the specification of vascular and ground tissues; the determination of shoot and root domains; and the establishment of the first stem cells.

KEY WORDS: *Arabidopsis*, Embryogenesis, Pattern formation, Cell specification, Asymmetric cell division, Cell-cell communication, Auxin

Introduction

Plants can grow for many centuries and new organs, such as leaves, branches, flowers and roots, are continuously added to build elaborate and complex post-embryonic structures. This involves the intricate coordination of asymmetric cell division, cell fate specification and cell-cell communication (positional signalling). Yet, however complex the plant morphology eventually may become, in its essence post-embryonic organogenesis is a reiteration of a basic developmental program: embryogenesis. Starting from a zygote – a single totipotent cell – embryogenesis produces the first tissue precursors as well as the first stem cells, and by the end of embryogenesis the zygote has transformed into a mature embryo that invariably comprises the same basic tissue types of any post-embryonic plant: epidermis, vascular tissues (in vascular plants) and ground tissue (Esau, 1977). During post-embryonic development, these tissues are maintained and differentiate to obtain unique attributes, such as root hair and trichome development for the epidermis (Grierson et al., 2014; Pattanaik et al., 2014), secondary cell wall modification for vascular tissue (Furuta et al., 2014) and Casparian strip formation for ground tissue (Geldner, 2013). Thus, embryogenesis provides an excellent opportunity to study the progression from the very first cell and tissue type specification events to multicellular tissue formation in the absence of tissue homeostasis and cell differentiation.

The embryos of most flowering plants grow by seemingly chaotic, random cell divisions (Pollock and Jensen, 1964; Johri, 1984; Johri et al., 1992), and only a few (e.g. members of the Brassicaceae family, including *Arabidopsis thaliana*) follow a simple, highly regular, predictable pattern (Mansfield and Briarty, 1991; Jürgens and Mayer, 1994). Despite this difference, the relative positioning of identities is highly robust and without exception will give rise to a seedling with a stereotypical organisation, with primary meristems of root and shoot bearing one or two cotyledons at opposite ends, and a radial pattern with concentric tissue layers. Most studies to date have focused on *Arabidopsis* because of its highly regular pattern of cell division; little is known about the molecular and cellular mechanisms underlying patterning in species with less regular divisions.

Much progress has been made in this field over the past decade. In this Review, we describe the current knowledge and recent advances made with regard to understanding the specification and subsequent growth of the different cell types in the *Arabidopsis* embryo. We focus on the globular stage of embryogenesis, as this is the phase during which all the basic tissues are initiated; we consider this developmental stage as a model that is well suited to study how plants regulate asymmetric cell division, cell-cell communication and identity specification. In particular, we focus on major events such as the separation of inner and outer cell fate, ground and vascular tissue formation, shoot and root specification, and the initiation of stem cells, discussing the key players involved in each of these important developmental processes. Finally, we propose future directions that should help to generate a better understanding of cell fate specification in the early embryo.

Cell division patterns in the early *Arabidopsis* embryo

In *Arabidopsis*, the embryo undergoes a highly ordered sequence of cell divisions, during which the emerging tissues are specified and patterned (Fig. 1). Thus far, our knowledge of embryonic cell division patterns and shape has come mostly from studies using (optical) two-dimensional (2D) sections (Mansfield and Briarty, 1991; Jürgens and Mayer, 1994; Scheres et al., 1994). As development occurs in three spatial dimensions, many questions regarding cell shape and division plane control have thus remained unanswered. However, a recent description of cellular patterns and volumes in 3D during embryogenesis has greatly improved our understanding of early *Arabidopsis* embryogenesis (Yoshida et al., 2014).

Arabidopsis embryogenesis begins with fertilisation of the egg cell by one of the two sperm cells that are delivered by the pollen tube (Dumas and Rogowsky, 2008). After fertilisation, the zygote quickly elongates along the future apical-basal axis before undergoing its first division (Fig. 1). This division is asymmetric and produces a smaller apical and a larger basal cell; the apical cell generates the entire embryo except for its very basal end, whereas the basal cell undergoes a series of transverse divisions that will ultimately generate a file of seven to nine cells, of which all but the uppermost one will form the extra-embryonic suspensor (Fig. 1). After the first asymmetric division, the apical cell undergoes a series of rapid cleavage divisions that partition the original cell volume (Yoshida et al.,

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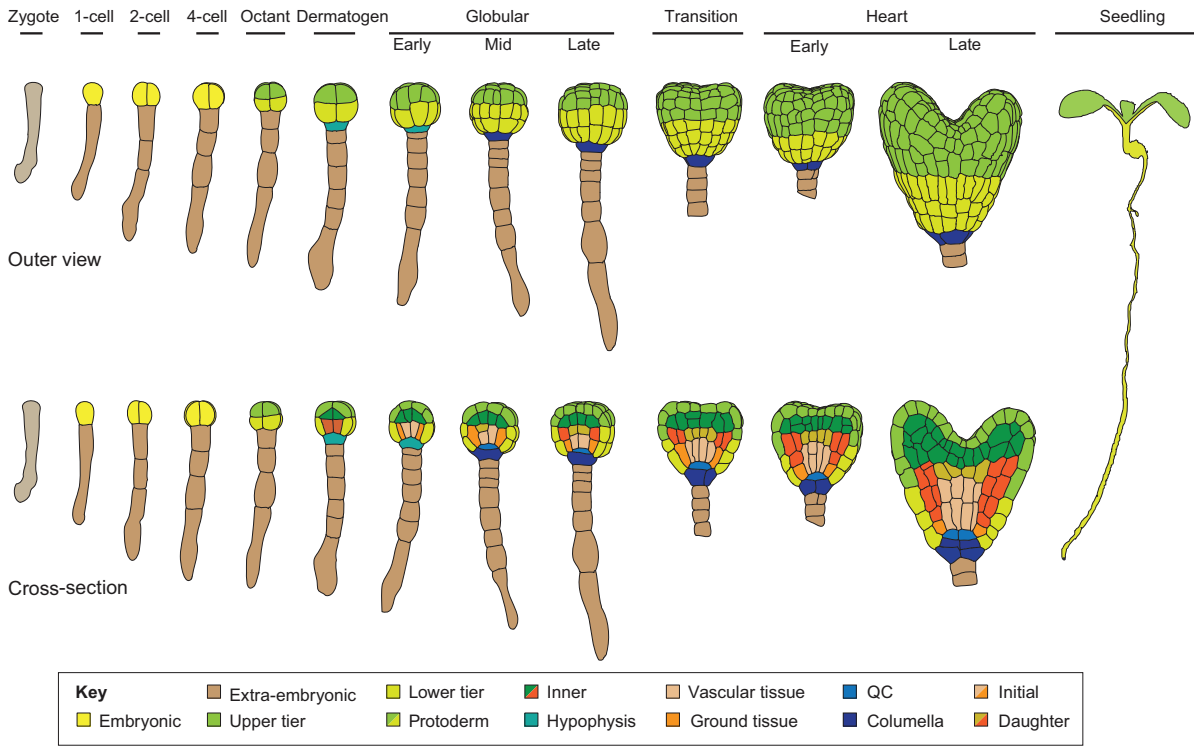

Fig. 1. *Arabidopsis* embryo development. Surface view and longitudinal cross-sections of a developing *Arabidopsis* embryo. Cells are coloured according to their lineage, as indicated in the key. Based on data from Yoshida et al. (2014).

2014). It first performs two rounds of longitudinal divisions at right angles to one another to produce four cells of equal size. This is followed by a transverse division that separates the two tiers, generating the octant stage embryo (Fig. 1). At this stage, the upper tier is slightly, but significantly, smaller than the lower tier (Yoshida et al., 2014). All cells in both tiers then undergo a tangential division, giving rise to eight inner cells and eight outer cells (dermatogen stage). This division separates the protoderm (the precursor of the epidermis) from the inner tissues (the precursors of the ground and vascular tissues) (Fig. 1). Recent work shows that the outer cells of the dermatogen stage embryo are more than twice the volume of the inner cells, a feature that had been impossible to detect in 2D sections (Yoshida et al., 2014). In the following stages, both the orientation of cell division and volumetric asymmetry are very regular in the lower half of the embryo, whereas they are less constrained in the upper half.

The next round of divisions that forms the early globular stage is a central formative event. The outer, protodermal cells divide anticlinally, only to extend the outer layer. By contrast, the inner cells divide longitudinally. Here, the four basal cells form larger, outer ground tissue precursors and smaller, inner vascular precursors. At about the same time, the uppermost cell of the suspensor is specified as the hypophysis and divides asymmetrically to form a smaller lens-shaped cell that is the precursor of the quiescent centre (QC) and a larger basal cell that is the precursor of the distal stem cells of the root meristem (Fig. 1).

The specification of different cell identities during embryogenesis is tightly controlled by specific molecular pathways and is often marked by the onset of specific gene expression patterns. In the following sections, we discuss our current understanding of the cellular and molecular events that take place during the formation of outer (protoderm) versus inner layers, the specification of vascular and ground tissues, the determination of shoot and root domains, and the establishment of the first stem cells.

The separation of inner and outer fates

At the octant stage, all cells of the embryo proper divide along a tangential plane, aligned along the apical-basal axis, giving rise to two cell populations with very different identities: the protoderm and the inner cells (Fig. 1). What dictates this tangential division plane is not clear. The WUSCHEL-RELATED HOMEOBOX (WOX) transcription factors, together with auxin signalling (Box 1), have been implicated in this process. Tangential division is perturbed in *wox2* mutants (Haecker et al., 2004, Breuninger

Box 1. Auxin: a versatile patterning molecule

Auxin is a versatile plant signalling molecule that plays a central role in nearly all aspects of growth and development (Zhao, 2010). Auxin is interpreted through a short nuclear signalling pathway. When auxin levels are low, AUXIN RESPONSE FACTORs (ARFs), which are transcription factors, are bound and inhibited by unstable, nuclear AUX/IAA proteins (Reed, 2001; Guilfoyle and Hagen, 2007). When levels increase, auxin binds to the $SCF^{TIR1/AFB}$ ubiquitin ligase complex and increases the affinity of this enzyme for its substrates, the AUX/IAAs (Gray et al., 2001; Dharmasiri et al., 2005; Kepinski and Leyser, 2005). The ubiquitylation and subsequent degradation of AUX/IAAs by the 26S proteasome releases ARFs from inhibition, thereby allowing them to modulate the expression of their target genes, which in turn mediate auxin-dependent growth and development.

The active form of auxin is indole-3-acetic acid (IAA), a tryptophan-like molecule. An obvious question is how such a structurally simple molecule can elicit such a wide variety of cellular responses. Part of the answer probably lies in the large size of the TIR1/AFB receptor, AUX/IAA and ARF families (Remington et al., 2004; Dharmasiri et al., 2005); the mixing and matching of different members within these families provides each cell with a unique response machinery, thus enabling a tailored auxin response. In the embryo, for example, ARF expression patterns are dynamic and divergent, forming a prepattern that enables specific auxin responses in each cell type (Rademacher et al., 2011).

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et al., 2008), and this phenotype is further enhanced in *wox2 wox8* double-mutant or *wox1 wox2 wox3* triple-mutant combinations, or when *wox2* single or *wox2 wox8* double mutants are combined with mutations in *MONOPTEROS* [*MP*; also known as *AUXIN RESPONSE FACTOR 5* (*ARF5*)] (Breuninger et al., 2008). Additionally, auxin response inhibition perturbs tangential division, suggesting that there might be a direct link between auxin response and protoderm formation (Yoshida et al., 2014). However, embryos that ectopically express a nondegradable version of the AUXIN/INDOLE ACETIC ACID (AUX/IAA) protein BODENLOS (BDL), which non-specifically inhibits AUXIN RESPONSE FACTORs (ARFs), do eventually form a separate outer layer, indicating that auxin response is not strictly required for epidermis formation (Rademacher et al., 2012).

The anatomical delineation of the outer layer and inner cells coincides with a change in the gene expression pattern of *ARABIDOPSIS THALIANA MERISTEM LAYER 1* (*ATML1*), which belongs to a family of homeodomain leucine zipper class IV (HD-ZIP IV) transcription factor genes (Mukherjee et al., 2009). *ATML1* expression is detected as early as the one-cell stage embryo, but becomes restricted to the protodermal layer at its inception at the transition from the octant to dermatogen stage (Fig. 2) (Lu et al., 1996; Sessions et al., 1999; Takada and Jürgens, 2007). The upstream regulators that provide this positional information are unknown but appear to be auxin- and microRNA-independent (Takada and Jürgens, 2007; Nodine and Bartel, 2010). A more fundamental question is the degree to which the process of protoderm specification requires specific regulatory input. The outermost cells are by definition in contact with the external environment. Hence, one might expect a role for the environment in epidermis cell specification. Endosperm tissues (the second product of double fertilisation) surround the embryo and provide it with nutrients as it develops. Studies have shown that the endosperm indeed plays a role in epidermal cell differentiation during embryogenesis (Tanaka et al., 2001; Yang et al., 2008). However, embryos grown in culture are also able to form a proper epidermis (Custers et al., 1997), suggesting that directional signalling from the endosperm is not crucial for epidermal specification. Alternative mechanisms could involve the integration of genetic programs with the physical properties of the outside cells that will be distinct from those of cells that are surrounded by other cells.

The closest homologue of *ATML1*, *PROTODERMAL FACTOR 2* (*PDF2*), is ubiquitously expressed in 4-cell stage embryos, but is confined to the outermost layer by the early globular stage (Fig. 2) (Abe et al., 2003). Strong double-mutant combinations of *atml1* and *pdf2* show severe phenotypes associated with defects in epidermal cell specification that lead to embryo lethality, whereas weak double-mutant combinations produce only a few leaves that lack the epidermis and exhibit mesophyll cells on their surface instead (Abe et al., 2003; San-Bento et al., 2014). ATML1 and PDF2 have been shown to bind *in vitro* to a *cis*-regulatory element called the L1 box (Abe et al., 2001, 2003; Nakamura et al., 2006). This motif is present in the promoters of many epidermis-specific genes, including *ATML1* and *PDF2*. It has thus been suggested that *ATML1* and *PDF2* regulate their own activity to reinforce protodermal identity (Abe et al., 2003; Takada and Jürgens, 2007). However, the presence of an L1 box is only an absolute requirement for the expression of *PDF2* and not for *ATML1*, indicating that other factors are likely to be involved in the regulation of *ATML1* expression (Abe et al., 2001; Takada and Jürgens, 2007). It was recently demonstrated that ATML1 is not only necessary but also sufficient for protodermal identity (Peterson et al., 2013; Takada et al., 2013). *ATML1* overexpression leads to ectopic *ATML1* promoter activity in the inner tissues of post-embryonic seedlings as well as the ectopic expression of other epidermis-specific genes, and induces epidermis-specific traits in non-epidermal tissues. ATML1 can therefore be considered as a master transcriptional regulator for epidermal cell specification in the shoot.

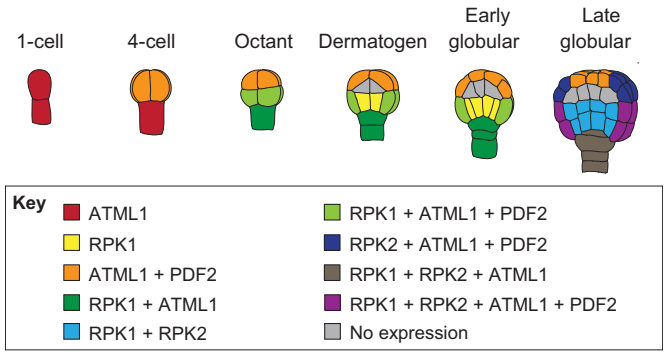

Fig. 2. Radial patterning during early embryogenesis. Expression patterns of genes involved the separation of outer and inner cell fates. Based on data from Takada and Jürgens (2007), Abe et al. (2003) and Nodine et al. (2007).

As the outer cells start to express epidermis-specific genes at the transition from the octant to the dermatogen stage, the inner cells are marked by the expression of *MP* (Hardtke and Berleth, 1998), the auxin efflux carrier PIN-FORMED 1 (PIN1) (Friml et al., 2003) and *ARGONAUTE 10* [*AGO10*; also known as *ZWILLE* (*ZLL*) or *PINHEAD* (*PNH*)] (Moussian et al., 1998), which is a member of the ELONGATION INITIATION FACTOR 2C (EIF2C)/AGO class of proteins. However, to date, no genes have been identified that are sufficient to establish these inner cells. This could be because the upper and lower inner halves of the embryo are specified through independent mechanisms. This is conceivable, as division patterns in the upper half are very different from those in the lower half from this stage onwards (Yoshida et al., 2014). Moreover, the upper and lower inner tiers start to express different gene sets. For instance, the expression of *WUSCHEL* (*WUS*), a marker for the shoot apical meristem (SAM), initiates in the upper tier inner cells (Mayer et al., 1998), whereas the expression of the root apical meristem (RAM) markers *TARGET OF MONOPTEROS 5* (*TMO5*) and *TMO7* initiates in the lower tier inner cells around this stage (Schlereth et al., 2010).

In the globular stage embryo, two closely related leucine-rich repeat (LRR) receptor-like kinases, RECEPTOR-LIKE PROTEIN KINASE 1 (RPK1) and RPK2 [also known as TOADSTOOL 2 (TOAD2)], are redundantly required for correct outer and inner cell fate separation (Nodine et al., 2007). RPK1 and RPK2 are first expressed at the octant and globular stages, respectively (Fig. 2). In *rpk1 rpk2* double mutants, an embryo-lethal phenotype at the late globular stage is observed, with excessive cell division defects and aberrant protodermal cell morphologies, especially in the basal half of the embryo, where RPK1 and RPK2 expression overlaps. Furthermore, *ATML1* is initially expressed in cells at the position of the protoderm, but fails to be maintained at the globular stage in these mutants. Similarly, the vascular primordium markers *SHORT ROOT* (*SHR*) and *AGO10* are initially correctly expressed but, by the globular stage, all cells of the basal tier of *rpk1 rpk2* double-mutant proembryos ectopically express *SHR* and *AGO10*. This suggests that RPK1 and RPK2 act by restricting inner cell fate to the central domain, rather than by positively regulating inner identity.

So far, RPK1 and RPK2 have not been linked to any of the known embryonic pathways. Similarly, the ligands that bind these kinases

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during embryo development as well as their downstream signalling components are unknown. The types of ligands thought to interact with LRR receptor-like kinases are varied (Diévart and Clark, 2004; Fiers et al., 2007). Interestingly, RPK2 has been shown to act downstream of CLAVATA3 (CLV3) in the regulation of meristem maintenance (Kinoshita et al., 2010). Candidate RPK2 binding partners during embryogenesis could therefore be members of the CLV3/EMBRYO SURROUNDING REGION (CLE) family. So far, signalling pathways comprising CLE polypeptides have been demonstrated to regulate various aspects of shoot, root and vascular meristem function. However, CLE8 was recently found to regulate basal embryo division patterns, hinting at the possibility of a thus far unexplored role for this family of small secreted peptides in embryonic development (Fiume and Fletcher, 2012).

Ground tissue pattern formation

At the early globular stage, the longitudinal division of the inner cells leads to the formation of vascular and ground tissue precursor cells (Fig. 1). Whether the vascular and ground tissues are specified simultaneously or whether one is specified before the other and/or controls its formation is still an open question. Furthermore, although much is known about the later aspects of ground tissue formation (reviewed by Pauluzzi et al., 2012), genetic approaches have not yet revealed the crucial components of embryonic ground tissue specification; globular stage defects have not been reported in mutants for well-known players of ground tissue pattern formation. Thus, ground tissue specification might use factors that are entirely different from those used later during tissue maintenance. Alternatively, it is possible that early roles for the late-acting factors are being obscured by redundancy in function.

Current knowledge of ground tissue specification in the embryonic root is centred around the GRAS family transcription factor SHR (Helariutta et al., 2000; Nakajima et al., 2001). SHR and its target SCARECROW (SCR) (Di Laurenzio et al., 1996; Levesque et al., 2006), which is also a GRAS family member, are indispensable for the periclinal division of the ground tissue daughter cell that generates separate endodermis and cortex layers (collectively called ground tissue). Although *SHR* and *SCR* are already expressed at the globular stage (Fig. 3), embryos homozygous for the *shr* or the *scr* mutation display defects at the early heart stage, when the periclinal division that doubles the ground tissue is absent (Scheres et al., 1995; Helariutta et al., 2000; Wysocka-Diller et al., 2000). As a result, both *shr* and *scr* mutants contain only one ground tissue layer upon germination. In *scr* mutants, this ground tissue layer displays both cortex and endodermis characteristics. By contrast, *shr* mutants exhibit a loss of endodermal identity, while ectopic SHR overexpression induces endodermal fate (Helariutta et al., 2000; Nakajima et al., 2001). Thus, SHR is required for endodermal identity, and both SHR and SCR are required for the asymmetric periclinal division of ground tissue daughter cells. Furthermore, SHR is expressed in the stele in both the nucleus and the cytoplasm, but moves outward via plasmodesmata (PD) (see Box 2) into the adjacent endodermis and QC, where it is exclusively present in the nucleus (Vatén et al., 2011). It has been shown that this movement of SHR is necessary for its function; nonmobile forms of SHR fail to rescue radial or vascular patterning defects (Gallagher et al., 2004; Gallagher and Benfey, 2009; Carlsbecker et al., 2010; Vatén et al., 2011).

Another factor known to play an early role in ground tissue patterning is SCHIZORIZA (SCZ) (Mylona et al., 2002; Pernas et al., 2010; ten Hove et al., 2010). The *scz* mutant was independently isolated in screens for genes involved in root epidermis development and for genes involved in QC specification and stem cell maintenance (Mylona et al., 2002; ten Hove et al., 2010). Cloning of *SCZ* revealed

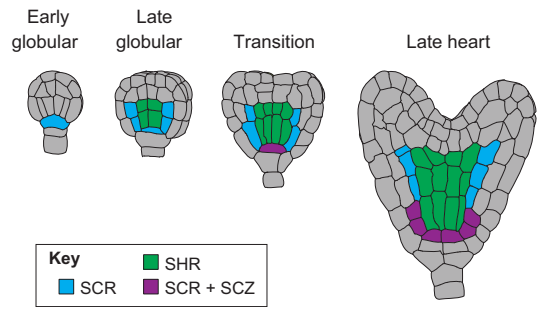
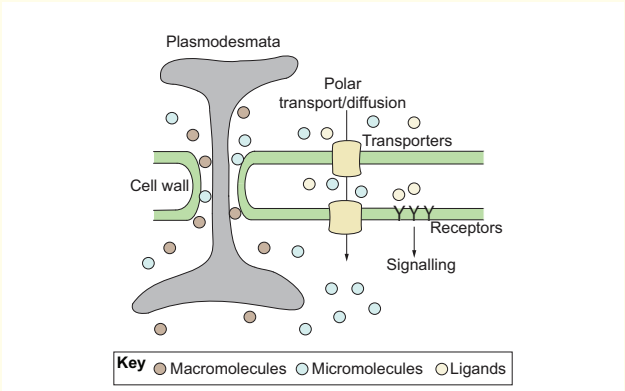


Fig. 3. Expression patterns of genes involved in ground tissue pattern formation. Expression patterns are based on data from Helariutta et al. (2000), Wysocka-Diller et al. (2000) and ten Hove et al. (2010). Earlier expression of *SHR*, *SCR* and *SCZ* is not well documented.

Box 2. Plasmodesmata and intercellular communication mechanisms



Intercellular communication is essential to orchestrate development (Van Norman et al., 2011; Wendrich and Weijers, 2013). The rigid cell walls of plants provide physical support but hamper information exchange. Plants have thus evolved several mechanisms to overcome this barrier, including the secretion of signals from one cell to receptors on another cell and the polar transport of plant hormones. In addition, plants use endoplasmic reticulum-containing channels called plasmodesmata (PD; see figure) to form cytoplasmic continuities between cells in order to exchange molecules (Robert and Friml, 2009). With limited pore sizes, PD allow small molecules to passively pass between cells (Ding et al., 1992). Macromolecules, such as RNAs and proteins (especially transcription factors), have also been shown to move across PD to regulate plant growth (Brosnan and Voinnet, 2011; Wu and Gallagher, 2012; Spiegelman et al., 2013). The permeability of PD is regulated by the deposition of callose and also by organelle-nucleus-PD signalling and reactive oxygen species (Benitez-Alfonso et al., 2009; Zavaliev et al., 2011; Burch-Smith and Zambryski, 2012). Many efforts have been made to identify elements that are responsible for the movement of non-cell-autonomous proteins (NCAPs). However, a universal sequence has not been detected, suggesting that a multi-pathway mechanism or another, yet to be identified, mechanism controls NCAP transport (Lucas et al., 2009). The restricted movement of TMO7 indicates that a regulatory mechanism exists, in this case at the early globular stage (Schlereth et al., 2010). A detailed investigation of PD development and callose accumulation during embryogenesis might provide more insight into how plants build up their communication network.

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that it is synonymous with *HEAT SHOCK TRANSCRIPTION FACTOR B4* (*HSFB4*), a gene with similarity to heat shock transcription factors (Pernas et al., 2010; ten Hove et al., 2010). In *scz* mutants, cells at multiple positions in the root meristem are affected in cell type specification and show a mixed cell type identity (Mylona et al., 2002; ten Hove et al., 2010). *SCZ* mRNA is expressed in the QC, ground and vascular tissues, with the highest levels of expression occurring in the QC, ground tissue stem cells and their immediate daughters, although SCZ acts mainly from the cortex cell layer (ten Hove et al., 2010). There, it promotes cortex fate and suppresses endodermis identity, after the asymmetric division of the ground tissue stem cell daughter, possibly through downregulation of *SHR* and *SCR*. Additionally, SCZ controls the separation of cell fate in the surrounding layers.

So far, it is unclear whether SCZ directly modulates cell fate in the cell layers surrounding the cortex by moving out of its transcriptional domain (similar to SHR) or whether it acts through an unknown intermediate factor. Initial ground tissue patterning defects in *scz* mutants occur in heart stage embryos, with ground tissue carrying out an aberrant periclinal division, resulting in an ectopic ground tissue layer observed at the torpedo stage. The inner ground tissue daughter cells continue to perform one or more periclinal divisions, generating two or more ectopic layers (Mylona et al., 2002; Pernas et al., 2010; ten Hove et al., 2010). Combining *scz* and strong *scr* mutations leads to a striking defect in ground tissue development, in which it appears that neither the cortex nor the endodermis develops (Pernas et al., 2010). However, as no globular defects have been reported for the *scz scr* double mutant, it remains unclear whether ground tissue is not specified or whether it is just not maintained during embryogenesis. Two observations suggest that the latter scenario is more plausible. First, *SCZ* expression cannot be detected at the globular stage when ground tissue is likely to be specified, but is only found in ground tissue cells from the heart stage onwards (Fig. 3). Second, a double-mutant combination of *scz* with a weaker *scr* allele (as well as a weak *scz shr* double mutant) was previously shown have one or two ground tissue layers (Mylona et al., 2002). More in-depth marker analyses in the early embryo should help to distinguish between these possibilities.

In summary, despite having a detailed description of the genetic networks involved in ground tissue development, our understanding of the initial events that set up this tissue is limited, and dedicated approaches will have to be devised in order to dissect how ground tissue is formed.

Formative vascular divisions in the early embryo

The same division that gives rise to the ground tissue generates the first vascular precursor cells (Fig. 1). Hence, all root vascular tissues are derived from four provascular initial cells in the early globular stage embryo that undergo several rounds of oriented, periclinal divisions to create a vascular bundle of up to 40 cells by the end of embryogenesis, showing a diarch pattern with a central xylem axis and two phloem poles (Scheres et al., 1994; reviewed by De Rybel et al., 2014a). Although virtually nothing is known about the actual specification events that generate these cells, much progress has recently been made in understanding the formation of vascular tissue during embryogenesis (Fig. 4). These studies have focused mainly on the auxin-dependent transcription factor MP. During embryogenesis *mp* mutants display defects in the characteristic divisions that generate the vascular tissues and altogether fail to establish an embryonic root (Berleth and Jürgens, 1993; Hardtke and Berleth, 1998; Hamann et al., 1999, 2002). Transcript profiling studies have identified several direct MP target genes encoding transcription

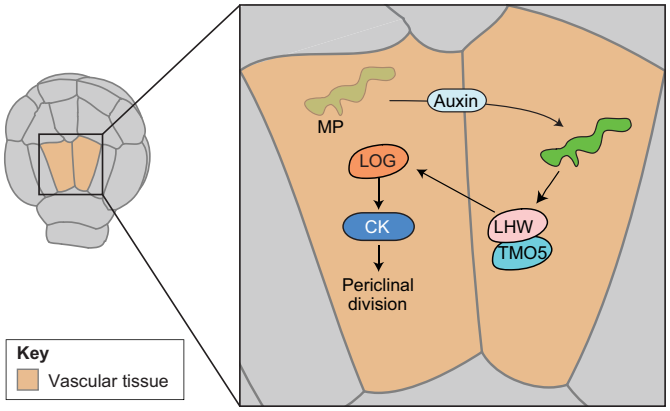

Fig. 4. Vascular tissue formation in the early globular stage embryo. In future vascular and ground tissue stem cells, auxin activates the expression of MP, which in turn triggers the expression of its target genes, including *TMO5*. TMO5 and its dimer partner LHW promote cytokinin (CK) signalling by activating the expression of *LOG4*, which encodes an enzyme involved in the final step of CK biosynthesis. The resulting auxin-CK interaction promotes periclinal cell division.

factors that act downstream of MP in root initiation, indicating that MP function in root initiation is mediated by successive transcriptional steps (Schlereth et al., 2010). One of these factors, the basic helix-loop-helix (bHLH) transcription factor TMO5, marks the provascular initial cells in the early globular stage embryo, suggesting that it mediates MP functions in the proembryo in a cell-autonomous fashion. Whereas *tmo5* single mutants do not display any phenotype, *tmo5 tmo5-like1* (*t5l1*) double mutants show a reduced vascular bundle with monarch symmetry, having a single phloem pole and a xylem pole (Schlereth et al., 2010; De Rybel et al., 2013). These defects are almost identical to those seen in a previously described bHLH mutant, *lonesome highway* (*lhw*; also known as *bhlh146*) (Ohashi-Ito and Bergmann, 2007). The additional removal of close TMO5 homologues (*t5l1*, *t5l2* and *t5l3*) causes a further reduction in vascular size due to an increasing loss of periclinal divisions in vascular precursor cells (De Rybel et al., 2013). Similarly, a double-mutant combination of *lhw* and its close homologue *lonesome highway-like 3* (*lhl3*) displays more severe phenotypic defects in vascular tissue formation (Ohashi-Ito et al., 2013). Interaction studies *in vitro* and *in vivo* revealed heterodimeric complexes between the TMO5 and LHW bHLH clades (Ohashi-Ito and Bergmann, 2007; De Rybel et al., 2013). Interestingly, at the globular stage, the expression patterns of TMO5 and LHW overlap only in provascular cells, suggesting that only these cells accumulate a dimeric complex. Later in development, this overlap becomes restricted to the young xylem cells. Ectopic co-expression of TMO5 and LHW specifically triggers periclinal divisions in all cell types, suggesting that the TMO5-LHW dimer is both necessary and sufficient both to control the periclinal divisions involved in the establishment of the embryonic vascular tissue (Fig. 4) and for the maintenance of the vasculature in the post-embryonic root (De Rybel et al., 2013). However, given that xylem cells do not themselves divide periclinally and that TMO5/LHW misexpression induces periclinal divisions both cell-autonomously and non-cell-autonomously, it is likely that these genes control periclinal divisions through a diffusible factor.

Recently, LONELY GUY 4 (LOG4), an enzyme involved in the final step in the biosynthesis of the plant hormone cytokinin (CK), was identified as a direct target of the TMO5-LHW transcription factor complex (De Rybel et al., 2014b). CK is well known for its important

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role in later aspects of root vascular patterning (reviewed by Miyashima et al., 2013a). *log4* mutants do not show any phenotype, but embryonic vascular tissue development and patterning are severely affected in *log1 log2 log3 log4 log5 log7 log8* septuple mutants, indicating a collective requirement for LOG function during CK formation in the vascular tissue (De Rybel et al., 2014b). Furthermore, the treatment of *tmo5 t5l1* or *lhw* mutants with CK was sufficient to increase the number of periclinal cell divisions, causing reversal to diarch patterns and even wild-type cell numbers in *lhw*, indicating that LOG-derived CK is a major contributor to the vascular function of TMO5-LHW (Fig. 4). An independent study that focused on the LHW-T5L1 complex identified *LOG3*, *LOG4* and *APH6* as its direct targets (Ohashi-Ito et al., 2014). Here, *TMO5* promoter-driven LOG3 expression was able to rescue the vascular pattern defect of the *log3 log4 log7* triple mutant, confirming that local LHW-induced and TMO5/T5L1-induced active CK production is vital for the activation of vascular cell division and, hence, the establishment of vascular patterning. Previously, a mutual inhibitory interaction between auxin and CK was shown to direct patterning of the vascular tissue in distinct domains (Mähönen et al., 2006; Bishopp et al., 2011a,b). These new findings now show that interactions between the same two hormones control cell division. Simulations using a mathematical model showed that a core network of auxin-CK interactions can explain how these two distinct processes are coordinated (De Rybel et al., 2014b). Clearly, many questions still remain, but this work successfully connects several layers of regulation in embryonic tissue formation.

The determination of shoot and root domains

After the first two rounds of cell division, the octant stage embryo can be anatomically separated into two developmental domains, comprising the upper and lower tier cells, which contribute to the formation of aerial tissues and the hypocotyl and root tissues, respectively (Fig. 1). After this separation, these two domains are fundamentally different and cell division patterns are more regular in the lower than in the upper domain (Yoshida et al., 2014). The differential expression of *WOX2*, *WOX8* and *WOX9* also marks these distinct domains (Fig. 5) (Haecker et al., 2004); *WOX2* is expressed in the apical domain, whereas *WOX8* is expressed in the suspensor, including the future hypophysis, and *WOX9* is activated in the basal domain (Haecker et al., 2004). The expression of *WOX8/9* in the suspensor is regulated by a WRKY-type transcription factor, WRKY2 (Fig. 5) (Ueda et al., 2011). Loss of *WOX8* and *WOX9* leads to misregulated cell division in the lower tier cells, suggesting a role for *WOX8/9* in basal cell identity (Breuninger et al., 2008).

At what stage are these domains explicitly specified as 'shoot' and 'root', and what mechanisms instruct this decision? Although only a few factors are known to influence this step, important insight has come from the analysis of two families of transcription factors: the class III HD-ZIP family (Emery et al., 2003) and the AP2-domain PLETHORA (PLT) family (Fig. 5) (Aida et al., 2004; Galinha et al., 2007). HD-ZIP III genes are known to regulate formation of the SAM, the radial (adaxial/abaxial) pattern in the embryo, the boundary between the SAM and the cotyledons, and the dorsal/ventral pattern of leaves during post-embryonic development (Prigge et al., 2005). The HD-ZIP III genes *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*), *ARABIDOPSIS THALIANA HOMEOBOX 8* (*ATHB8*) and *ATHB15* are expressed in the upper tier cells at the globular stage of embryogenesis and are redundantly required for post-embryonic shoot maintenance (Emery et al., 2003). The expression of HD-ZIP III genes is controlled by microRNA 165/166 (miR165/166) family members (Mallory et al., 2004), which are expressed in the abaxial region of the embryo (Fig. 5) (Williams et al., 2005; Miyashima et al., 2013b). The *PLT1*, *PLT2*, *PLT3* [also known as *AINTEGUMENTA-LIKE 6* (*AIL6*)] and *BABYBOOM* (*BBM*; also known as *PLT4*) genes, by contrast, occupy a domain that is roughly complementary to the HD-ZIP III domain and are expressed in lower tier cells at the early globular stage (Fig. 5) (Aida et al., 2004; Galinha et al., 2007). Ectopic expression of *PLT1* or *PLT2* using constitutive promoters induced all organ identities that originate from the basal region of the embryo, i.e. hypocotyl, root and root stem cell niche, demonstrating a dominant role for PLT1 and PLT2 in basal cell fate determination.

Studies on the *TOPLESS* (*TPL*) gene, which encodes a transcriptional co-repressor (Long et al., 2006), linked the functions of HD-ZIP III and PLT genes (Fig. 5) (Smith and Long, 2010). In the *tpl* mutant, *PLT1* and *PLT2* are ectopically expressed at both ends of the embryo, leading to a double-root seedling. TPL directly binds the PLT promoters and, indeed, these genes are required for the double-root phenotype (Smith and Long, 2010). The double-root phenotype can be suppressed by a mutation in the HD-ZIP III gene *PHB* that renders the mRNA resistant to microRNA regulation. Thus, HD-ZIP III genes can antagonise PLT function in promoting an apical root. Finally, ectopic expression of HD-ZIP III genes using the *PLT2* promoter transforms basal cells into a second SAM and creates a double-headed seedling. This study revealed antagonistic roles for the PLT and HD-ZIP III genes in determining apical and basal cell fate (Fig. 5). Whether the PLT and HD-ZIP III genes directly regulate each other's expression is not clear, and how exactly they interact to maintain the boundary between apical and basal cells requires further investigation.

Establishing the first stem cells

One of the most extraordinary features of plants is that they continuously grow and generate new organs during their life cycle. This growth and regeneration originates from stem cells that reside

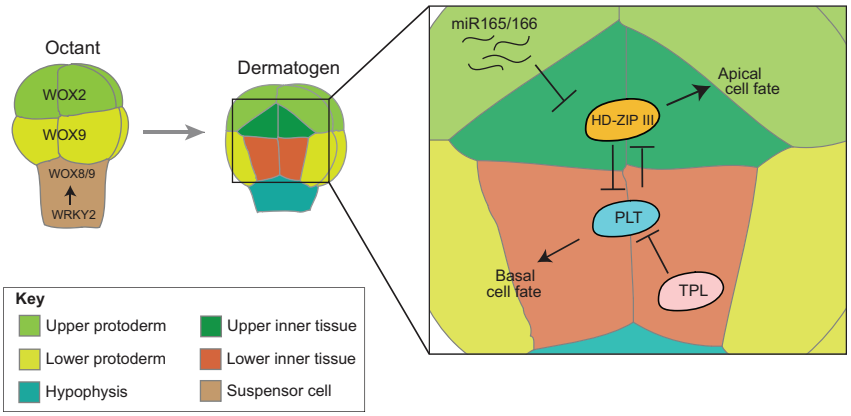

Fig. 5. Apical-basal fate separation in the early embryo. At the octant stage, the differential expression of *WOX2* and *WOX8/9* marks the apical-basal domain. *WOX8/9* expression in the suspensor is regulated by WRKY2. At the dermatogen stage, the transcriptional co-repressor TPL inhibits transcription of its targets, including the basally expressed PLT genes, whereas the expression of HD-ZIP III genes in the apical part is controlled by miR165/166 family members. PLT and HD-ZIP III genes antagonistically determine apical and basal embryo polarity.

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in the SAM, the RAM and the cambium. Within the SAM and RAM, a small population of cells, called the organising centre, divides slowly to provide cell supplies for the surrounding stem cells (Weigel and Jürgens, 2002). The organising centres are also important for maintaining the stem cell identity of adjacent cells (Scheres, 2007). However, regardless of their type, all stem cells originate during embryogenesis and therefore their determination is of vital importance for post-embryonic development.

Currently, *WUS* and *WOX5* are recognised as markers for the shoot and root organising centres, respectively (Sarkar et al., 2007), and these genes are activated at the globular stage of embryogenesis (Mayer et al., 1998; Haecker et al., 2004). True markers for the stem cells themselves, however, have not yet surfaced, making it difficult to study their origin. Whether such universal stem cell markers exist in plants is also unclear. The development and maintenance of the SAM and RAM are thoroughly discussed in several excellent reviews (Aichinger et al., 2012; Lau et al., 2012; Perales and Reddy, 2012; Perilli et al., 2012; Wendrich and Weijers, 2013). Here, we focus on the first SAM and RAM determination steps in the developing embryo.

Several molecular pathways have been identified that regulate the organisation of the SAM. Many of the genes involved are expressed during early embryogenesis, which makes these pathways candidates for controlling stem cell establishment. In addition to well-known factors such as the KNOTTED-1 homeodomain protein homologue SHOOT MERISTEMLESS (STM) (Long et al., 1996) and class III HD-ZIP factors (Prigge et al., 2005), further genes were recently shown to regulate *WUS* expression and SAM formation (Fig. 6). For example, the class II HD-ZIP genes *ATHB2*, *HOMEOBOX ARABIDOPSIS THALIANA 1* (*HAT1*), *HAT2*, *HAT3* and *ATHB4*, which are known to be involved in the shade avoidance response, carpel margin development and leaf polarity (Bou-Torrent et al., 2012; Reymond et al., 2012; Ruberti et al., 2012), were recently shown to be expressed in the early embryo (Turchi et al., 2013). In *hat3-3 athb4-1* double-mutant seedlings, SAM activity, as indicated by the expression of *WUS* and *CLV3* (Fletcher et al., 1999; Schoof et al., 2000), is reduced. Several class II HD-ZIP genes, including *HAT2*, *HAT3* and *ATHB4*, are directly regulated by the class III HD-ZIP gene *REV*, suggesting that the HD-ZIP III genes might regulate development through activation of the HD-ZIP II genes (Fig. 6) (Brandt et al., 2012; Reinhart et al., 2013).

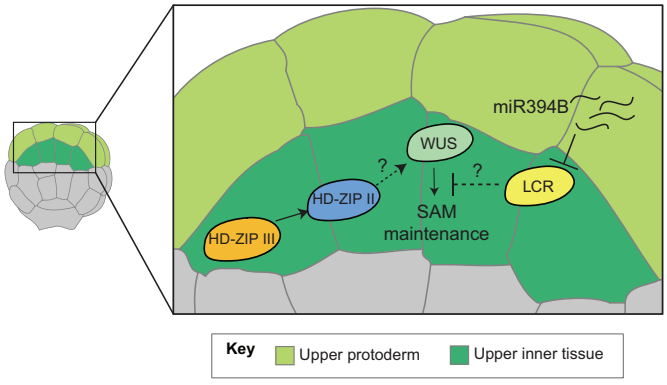

Fig. 6. SAM formation in the mid-globular stage embryo. In the upper inner tissue, HD-ZIP III genes regulate *WUS* expression, possibly through activation of HD-ZIP II genes, and hence SAM formation. The non-cell-autonomous action of miR394B from the protodermal layer represses LCR activity in the subtending cells, enabling the expression of *WUS*, which in turn regulates SAM maintenance.

Recently, the importance of miR394B, acting non-cell-autonomously from within the protodermal layer, during SAM formation has been uncovered. In a screen for enhancers of the weak *ago10-1* mutant that induces SAM defects at low frequency, the *mir394b* mutant was found to dramatically enhance the SAM defect (Knauer et al., 2013). *miR394B* is ubiquitously expressed at the early globular stage, and during SAM establishment its expression is restricted to the L1 layer (Fig. 6). However, *miR394B* transcripts can be detected in the subjacent three layers, suggesting a non-cell-autonomous function of *miR394B* in SAM formation. The direct target of *miR394B* is *LEAF CURLING RESPONSIVENESS* (*LCR*), which contains a SKP2-like F-box domain, suggesting that it functions by targeting proteins for degradation by the 26S proteasome (Bai et al., 1996; Jones-Rhoades and Bartel, 2004; Knauer et al., 2013). In the *mir394b* mutant, which harbours a single nucleotide mutation that disables the degradation of *LCR* mRNA, *CLV3* and *WUS* expression initiate correctly but fail to be maintained afterwards, indicating that the removal of *LCR* is crucial for SAM maintenance but not for its initiation (Knauer et al., 2013). Thus, although regulators of SAM function have been identified (Fig. 6), there is clearly a gap in our knowledge with regards to SAM establishment during embryogenesis.

The ontogeny of the RAM is very different from that of the SAM, as it involves the specification of the uppermost suspensor cell, the hypophysis. This cell further divides asymmetrically to form a smaller lens-shaped cell and a larger basal cell (Fig. 1). Both the hypophysis and its lens-shaped descendant express the *WOX5* marker (Sarkar et al., 2007), and the specification of the hypophysis can thus be regarded as the initiation of the root meristem. Hypophysis specification is regulated by the plant hormone auxin (Fig. 7), and mutations in components of auxin biosynthesis, transport, perception or response all cause defects in hypophysis division and RAM formation (reviewed by Möller and Weijers, 2009). An auxin response maximum, detected through the use of the synthetic auxin response reporter DR5 (Friml et al., 2003), can be observed in the uppermost suspensor cell, i.e. the hypophysis, upon its specification. This auxin response maximum is the result of polar PIN1 localisation (Friml et al., 2003) and, hence, polar auxin transport (PAT; see Box 3). Up until this stage, PIN1 is expressed without polarity, but it then begins to be localised to the basal cell membrane in the provascular cells next to the hypophysis and actively transports auxin into the hypophysis, which triggers gene expression changes and thereby contributes to hypophysis specification (Fig. 7) (Lokerse and Weijers, 2009; Weijers and Friml, 2009). MP, in addition to regulating vascular tissue formation (as discussed above), also controls hypophysis specification (Fig. 7), and *mp* mutant embryos show aberrant hypophysis division (Hardtke and Berleth, 1998). Mutation of *bdl* (*iaa12*), which stabilises the BDL protein, also leads to an *mp*-like phenotype (Hamann et al., 1999, 2002). Notably, *MP* and *BDL* are active in cells adjacent to the hypophysis, suggesting that a non-cell-autonomous factor is needed to regulate hypophysis determination. Auxin transport from MP-expressing cells to the hypophysis also depends on MP (Weijers et al., 2006); hence, auxin itself represents such a non-cell-autonomous signal (Fig. 7). However, since auxin response reporters mark more suspensor cells than just the hypophysis (Friml et al., 2003), it is likely that additional signals are required for the specification of the hypophysis. Among the direct MP targets, the small bHLH transcription factor TMO7 was shown to behave as such a second signal. *TMO7* is expressed in cells adjacent to the hypophysis, but the TMO7 protein moves into the

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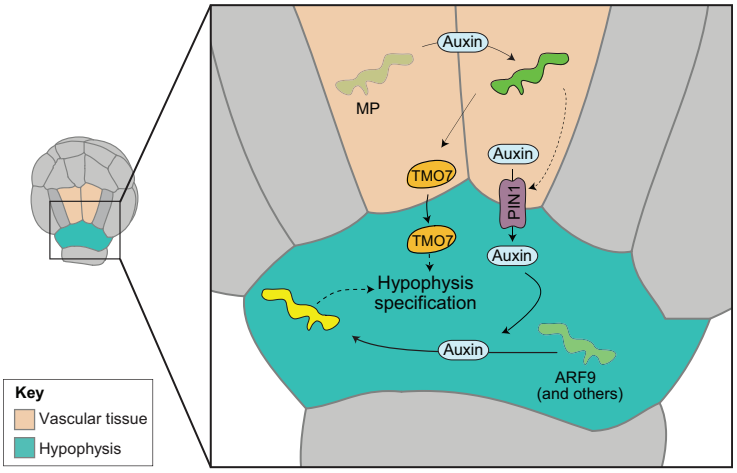

Fig. 7. Hypophysis specification in the early globular stage embryo. In future vascular and ground tissue cells, MP activates its downstream targets, including *TMO7*. TMO7 moves from the provascular cells to the uppermost suspensor cell. Additionally, MP promotes PIN1-dependent auxin transport to the uppermost suspensor cell. Here, both auxin response (through ARF9 and other ARFs) and TMO7 are required to specify the hypophysis.

hypophysis (Fig. 7) (Schlereth et al., 2010). Suppression of *TMO7* by RNA interference or an artificial microRNA leads to abnormal hypophysis division and rootless seedlings, similar to *mp* mutants, demonstrating the biological significance of *TMO7* in hypophysis determination.

RAM initiation thus requires inductive signalling from adjacent cells, although the downstream response that induces hypophysis identity is still under investigation. By systematically analysing the expression patterns of AUX/IAA and ARF families, the IAA10 and ARF9 proteins were identified as likely candidates for mediating the auxin response in the hypophysis (Fig. 7) (Rademacher et al., 2011; Rademacher et al., 2012). The *MP*/*BDL-TMO7* and *ARF9-IAA10* pathways might cooperatively specify the hypophysis by converging upon the same targets or, alternatively, they might act independently.

Box 3. Polar auxin transport

Auxin is unique among plant hormones in that it has a dedicated transport system. This so-called polar auxin transport (PAT) is an important mechanism in plants that channels auxin into specific tissues in order to trigger or maintain certain developmental responses. PAT is facilitated by auxin influx and efflux carriers, whose polar subcellular localisations determine the directionality of auxin flow (reviewed by Zažímalová et al., 2010). Auxin flow directionality mainly depends on the polarised subcellular localisation of the PIN-FORMED (PIN) auxin transporters (Petrásek et al., 2006; Wiśniewska et al., 2006). Conversely, auxin regulates its own transport by controlling the expression of PIN genes, PIN protein redistribution and degradation (Vieten et al., 2005; Paciorek et al., 2005), thereby contributing to a complex pattern of feedback regulation. PAT and the establishment of auxin maxima control embryo development. Accordingly, auxin distribution changes dynamically at key steps of embryo development (Friml et al., 2003). After the first division of the zygote, apical cell development requires PAT (Friml et al., 2003). At this stage, auxin is locally produced in the cells of the suspensor (Robert et al., 2013). In these cells, PIN7 is polarly localised toward the proembryo (Friml et al., 2003) and mediates directional auxin flow to the proembryo, where the resulting auxin response maximum contributes to its specification (Lokerse and Weijers, 2009; Weijers and Friml, 2009). At the early globular stage, the onset of localised auxin biosynthesis in the proembryo is required for PIN1 polarisation in the inner proembryonic cells, resulting in a basal auxin response maximum and specification of the future root pole (Friml et al., 2003). Thus, PAT and distinct local auxin sources orient apical-basal axis formation plant during embryogenesis (Robert et al., 2013; Wabnik et al., 2013).

Another output of the auxin response that connects to stem cell properties is represented by the PLT genes. The expression of *PLT1* and *PLT2* initiates in the lower tier cells at the octant stage and later in the lens-shaped cell after the asymmetric division of the hypophysis, whereas the *PLT3* and *BBM* genes initiate expression from the heart stage onwards, with highest expression occurring in the provascular cells and the lens-shaped cell (Aida et al., 2004; Galinha et al., 2007). The *plt1 plt2 plt3 bbm* quadruple mutant significantly impairs RAM formation and produces rootless seedlings (Galinha et al., 2007) and, importantly, this can be correlated with defects in hypophysis division. It is not clear whether these division defects are a consequence of impaired lower tier development, or whether PLT proteins act in the hypophysis cell itself. *PLT1* and *PLT2* expression depends on MP activity (Aida et al., 2004), although they are not immediate targets of MP (Schlereth et al., 2010). This is in line with the recent finding that PLT genes are auxin regulated, but that this control is very slow (Mähönen et al., 2014).

Thus, at least three molecular components appear to converge downstream of auxin to regulate the specification of the hypophysis. In order to better understand hypophysis determination, the next challenge is to identify the direct targets of *TMO7*, *ARF9* and the PLT genes. This should provide insights into the molecular pathways by which plants can specify a cell as the stem cell progenitor, and trigger its appropriate asymmetric cell division.

Conclusions

Most studies on pattern formation are conducted in post-embryonic tissues. However, in plants, the initial specification and establishment of tissues occurs in the early embryo, and the patterns that are established during embryogenesis are maintained post-embryonically during growth. In this Review, we have focused on a crucial stage in embryo development that condenses many key morphogenetic processes: the globular stage. At this developmental stage, all tissues obtain their identities and, at the same time, the stem cells are first specified. Although many components of the genetic pathways that are active during these major patterning events have been identified, many questions still remain. For example, most of the identified genes are involved in cell fate maintenance and not initiation. True cell fate determinants thus remain to be identified. This could be due to difficulties in recognising mutants that are defective in early patterning events; mutations that compromise tissue specification are likely to cause dramatic growth defects that may inflict early lethality, thus hampering the identification of such genes through forward-

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genetic approaches. The development of novel techniques for gene identification by tissue-specific transcriptomics might help to gain more insight into the important mechanisms underlying embryo development.

Plant embryos are deeply embedded in seed and fruit and this, together with their small size, poses challenges in imaging. The recent 3D reconstruction of early embryogenesis has greatly improved our view of the formative events that take place during these early developmental stages (Yoshida et al., 2014). With this novel tool, many old and new developmental questions can now be addressed, but it inevitably cannot answer all questions. The next challenge will be the development of new techniques for the 3D live imaging of growing plant embryos, something that has contributed so much to the understanding of (a)symmetric cell division events, corresponding growth and patterning during development in animal model systems (Clarke, 2009; Toya et al., 2010; Truong and Supatto, 2011).

As described in Box 2, cell-cell communication via PD is important for pattern formation in plants, but how does cell-cell communication contribute to the establishment of a pattern during plant embryogenesis? It is known that overaccumulation of callose decreases the permeability of PD and therefore obstructs cell-cell communication, and in the dominant *CALLOSE SYNTHASE 3* mutant *cals3-2d* many developmental defects are observed during embryogenesis (Vatén et al., 2011), confirming the importance of PD communication in embryo development. It is therefore important to understand how plants establish and regulate cell-cell communication during embryogenesis. The establishment of a 3D PD transportation map should provide more information as to whether cell-cell communication is involved in the determination of domains and/or the specification of specific cell types.

Most of our knowledge on plant embryogenesis is derived from *Arabidopsis*. This is because of its invariant embryonic cell division pattern, which allows for lineage analysis, as well as its small genome size, rapid life cycle and transformation ability. But how does patterning occur in other plant species and how have the patterning mechanisms that operate during embryogenesis evolved? Several signalling processes involved in setting up the embryo pattern are evolutionarily ancient, as is the structure of the embryo itself, as an even more ancient innovation than seeds (Radoeva and Weijers, 2014). Would it be possible to trace back key events in plant embryo patterning? Genome sequences are readily available for various lower land plant model systems (Bowman et al., 2012), and the continuing efforts of The 1000 Plants Initiative (OneKP or 1KP) are generating large-scale gene sequencing data for over 1000 plant species. Phylogenetic analyses using such data should help to clarify the evolutionary trajectory of known embryo patterning genes during land plant evolution, as well as their origin and ancestral function. We thus anticipate that, within the coming years, several of the major questions in this field will be answered, providing us with a detailed view of the important cell fate decisions that build a plant.

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Competing interests

The authors declare no competing or financial interests.

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