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ARABIDOPSIS: A RICH HARVEST 10 YEARS AFTER COMPLETION OF THE GENOME SEQUENCE

Embryogenesis – the humble beginnings of plant life

Ive De Smet[1,2,†], Steffen Lau[2,†], Ulrike Mayer[1] and Gerd Jürgens[1,2,*]

[1]*Center for Plant Molecular Biology, University of Tübingen, Auf der Morgenstelle 3, D-72076 Tübingen, Germany, and*
[2]*Department of Cell Biology, Max Planck Institute for Developmental Biology, Spemannstraße 35, D-72076 Tübingen, Germany*

Received 16 November 2009; revised 23 December 2009; accepted 4 January 2010.
*For correspondence (fax +49 7071295797; e-mail gerd.juergens@zmbp.uni-tuebingen.de).
†These authors contributed equally to this work.
Dedicated to the memory of Andreas Müller (1935–1992), pioneer of genetic research on Arabidopsis embryogenesis.

SUMMARY

Each plant starts life from the zygote formed by the fusion of an egg and a sperm cell. The zygote gives rise to a multicellular embryo that displays a basic plant body organization and is surrounded by nutritive endosperm and maternal tissue. How the body organization is generated had already been studied before the genome sequence of *Arabidopsis thaliana* was completed 10 years ago, but several regulatory mechanisms of embryo development have since been discovered or analysed in more detail. Although this progress did not strictly depend on the availability of the genome sequence itself, several advances were considerably facilitated. In this review, we mainly address early embryo development, highlighting general mechanisms and crucial regulators, including phytohormones, that are involved in patterning the embryo and were mainly analysed in the post-genome decade. We also highlight some unsolved problems, provide a brief outlook on the future of Arabidopsis embryo research, and discuss how the knowledge gained from Arabidopsis could be translated to crop species.

Keywords: apical-basal axis, radial pattern, root initiation, auxin, primary meristem, cotyledon initiation.

INTRODUCTION

Regardless of whether the adult plant is an ephemeral weed or a long-lived sequoia tree, its developmental origin is essentially the same: a seed containing a simple mature embryo that displays an apical–basal axis of polarity and a radial pattern of concentric tissue layers perpendicular to the apical–basal axis. The axis of polarity has, at its top end, the primary shoot meristem, which is flanked by one or two cotyledons, and the primary root meristem at its bottom end. How this basic body organization is established during embryogenesis is the focus of this review.

Embryogenesis starts with fusion of an egg cell with a sperm cell to form the zygote. In parallel, another female gamete within the same ovule, the diploid central cell, fuses with the other sperm cell delivered by the same pollen tube to give rise to the endosperm, which nourishes and protects the developing embryo (Dumas and Rogowsky, 2008). In addition, the developing embryo and the endosperm are surrounded by the maternal tissue of the ovule, which is gradually transformed into the seed coat. Thus, the activities of six potentially different genomes may influence plant embryogenesis: diploid zygote, triploid endosperm, diploid maternal tissue, haploid egg cell, diploid central cell and haploid sperm cells. But although the endosperm plays quite an important role during late embryogenesis (Tanaka *et al*., 2001; Garcia *et al*., 2003; Kondou *et al*., 2008), it is not required *per se* for proper development during the early pre-globular stages (Weijers *et al*., 2003). This conclusion is supported by the occurrence of somatic embryogenesis, which implies that extrinsic signals from the endosperm or the maternal tissues are not critical for embryo development and patterning.

During embryo development, the body axes and the basic body plan of the plant are laid down. This starts with establishment of the apical–basal axis, followed by establishment of the radial axis, and finally establishment of bilateral symmetry. Importantly, the root and shoot stem cell pools, which are essential for the quasi indefinite post-embryonic growth, are also specified during this early phase of pattern formation and morphogenesis (Figure 1a) (De Smet and Jürgens, 2007; Scheres, 2007). During the subsequent maturation phase, storage reserves accumulate and ultimately the embryo prepares for developmental arrest.

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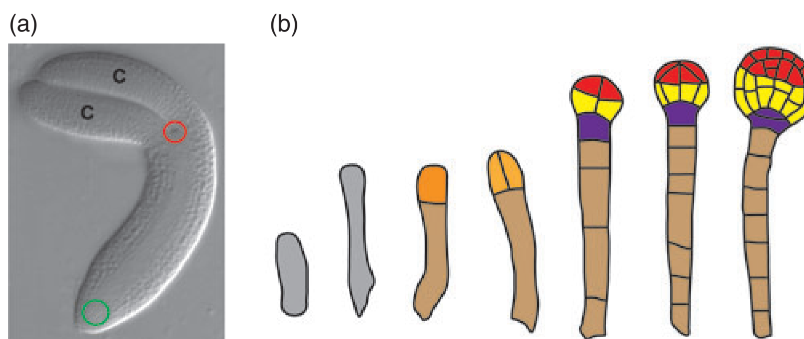

Figure 1. Embryogenesis in *Arabidopsis thaliana*.
(a) A bent-cotyledon stage embryo showing the two cotyledons (C) and both the root (green circle) and shoot (red circle) stem cell niches.
(b) The elongated zygote (grey) divides asymmetrically to give rise to an apical (orange) and a basal cell (brown). The apical cell undergoes a series of cell divisions resulting in the upper tier (red) and lower tier (yellow) of the embryo proper. The basal cell undergoes transverse cell divisions to form the suspensor. After a transverse asymmetric cell division, the uppermost suspensor cell, also called the hypophysis (purple), contributes to root pole formation.

Arabidopsis embryogenesis is by no means representative of all flowering plants. A prominent special feature of Arabidopsis is the nearly invariant cell division pattern during early embryogenesis, when pattern formation occurs, and this may be related to the small size of the Arabidopsis embryo (Figure 1b). This regularity has been successfully used to identify the origin of developmental defects in patterning mutants (Jürgens *et al.*, 1991). However, the molecular and cellular mechanisms that govern this coordinated development, as well as the concomitant establishment of cell identities, are still rather poorly understood.

EARLY EMBRYOGENESIS AND THE ESTABLISHMENT OF AN APICAL–BASAL AXIS OF POLARITY

The Arabidopsis zygote elongates about threefold along the future apical–basal axis, followed by an asymmetric division that generates a small embryonic apical daughter cell and a larger extra-embryonic basal daughter cell (Mansfield and Briarty, 1991; Jürgens and Mayer, 1994). The basal daughter cell and its derivatives repeatedly divide horizontally, thus forming a filamentous suspensor 6–9 cells long. In contrast, the apical daughter cell and its derivatives undergo a series of highly coordinated cell divisions with changing division planes to form a globular embryo proper (Figure 1b) (Mansfield and Briarty, 1991; Jürgens and Mayer, 1994). These two cell lineages differ not only in division plane orientation but also in gene expression and prospective cell fate.

Several proteins such as GNOM, YODA (YDA), MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3), MPK6 and SHORT SUSPENSOR (SSP) are involved in the very early events of zygote development. In the respective mutants and in the phenotypically related *grounded* (*grd*) mutants, the zygote fails to elongate properly and instead divides almost equally (Mayer *et al.*, 1993; Lukowitz *et al.*, 2004; Wang *et al.*, 2007; Bayer *et al.*, 2009). However, despite their similar early phenotype, no straightforward connection appears to exist between the ADP-ribosylation factor guanine–nucleotide exchange factor (ARF–GEF) GNOM on the one hand and members of a mitogen-activated protein kinase cascade, such as YDA (a MAPKK kinase), MPK3 and MPK6, and the interleukin-1 receptor-associated kinase (IRAK)/Pelle-like kinase SSP on the other hand. Although the transcription factor targets of this kinase cascade in early embryogenesis are currently unknown, GNOM is one of the better-studied proteins of Arabidopsis. GNOM is involved in the polar recycling of PIN-FORMED (PIN) auxin efflux carriers to the basal plasma membrane, and the main defects in *gnom* mutant embryos are attributed to the strongly perturbed directional auxin transport (Steinmann *et al.*, 1999; Geldner *et al.*, 2003). But what exactly leads to the zygotic defect in *gnom* is not known, especially as no zygotic phenotype was reported for multiple *pin1,3,4,7* knockout mutants (Friml *et al.*, 2003), suggesting that auxin transport is not necessarily involved in zygote elongation. However, there is evidence for a function of auxin in establishing the cell fate of the apical daughter cell of the zygote. Its proper specification not only involves a PIN7-mediated auxin response maximum (Friml *et al.*, 2003) but also auxin signalling that is dependent on the auxin response factor MONOPTEROS (MP)/AUXIN RESPONSE FACTOR 5 (ARF5) and its AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) inhibitor BODENLOS (BDL)/IAA12 (Hamann *et al.*, 1999; Lau *et al.*, 2008).

Another group of genes that has been implicated in the earliest events of embryogenesis is the *WUSCHEL RELATED HOMEOBOX* (*WOX*) gene family, and this not only because of the striking expression pattern of some of its family members. *WOX2* and *WOX8* are expressed in the zygote, and, after its division, *WOX2* is expressed in the apical cell and *WOX8* in the basal cell (Haecker *et al.*, 2004). *WOX9* does not appear to be expressed in the zygote but is expressed in the basal cell and possibly also in the apical cell (Haecker *et al.*, 2004; Wu *et al.*, 2007). Although defects in the *wox2* mutant can already be observed in the apical

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daughter cell of the zygote, developmental abnormalities mainly arise at later stages, for example during protoderm formation (Haecker *et al.*, 2004; Breuninger *et al.*, 2008). Interestingly, *wox8 wox9* double mutant and strong *wox9* single mutant embryos display more pronounced defects than *wox2* embryos do: development of the apical and basal cell lineages is strongly impaired, and growth arrest is observed as early as the zygote stage (Wu *et al.*, 2007; Breuninger *et al.*, 2008).

PROTODERM FORMATION AND THE ESTABLISHMENT OF A RADIAL AXIS

Protoderm formation is one of the earliest patterning events during Arabidopsis embryogenesis, taking place at the transition from the octant to the dermatogen stage, when the eight cells of the embryo proper divide tangentially (Figure 2a). The outer cell layer thus formed is designated the protoderm and eventually differentiates into the epidermis. The protoderm cells divide anticlinally, thus maintaining the integrity of the outer layer. Disregarding general cell-division mutants, the *WOX2* knockout mutant appears to rather specifically affect the tangential cell divisions at the octant stage and the anticlinal cell divisions in the protoderm, a phenotype that is enhanced by knockout of *WOX1* and *WOX3* or *WOX8*, and also by knockout of *MP* (Haecker *et al.*, 2004; Breuninger *et al.*, 2008). The precise role of these *WOX* genes during protoderm formation remains to be determined. Nonetheless, protoderm formation is accompanied by the establishment of complementary expression domains, being confined to either the protoderm or the inner cells, of previously co-expressed genes. For example, *ZWILLE* (*ZLL*) expression becomes restricted to the inner cells, whereas expression of *ARABIDOPSIS THALIANA MERISTEM LAYER 1* (*ATML1*) and *PROTODERMAL FACTOR 2* (*PDF2*) is confined to the protoderm (Figure 2a) (Lu *et al.*, 1996; Lynn *et al.*, 1999; Abe *et al.*, 2003; Takada and Jürgens, 2007; Tucker *et al.*, 2008). How such expression patterns are established and maintained has not been determined. Interestingly, double mutant embryos lacking the receptor-like kinases RECEPTOR-LIKE PROTEIN KINASE 1 (RPK1) and TOADSTOOL 2 (TOAD2) have outer cells that abnormally express markers for inner cell fates (Nodine *et al.*, 2007). In addition, however, vascular markers are expressed in ground tissue, suggesting more complex perturbation of embryogenesis (Nodine *et al.*, 2007).

How the provascular cells are specified is still unknown. In contrast, specification of the endodermis and cortex cells by asymmetric division of the ground tissue and the role played by the transcription factors SHORTROOT (SHR) and SCARECROW (SCR) in this process have been studied in detail (reviewed by Jenik *et al.*, 2007).

HYPOPHYSIS SPECIFICATION AND PRIMARY ROOT MERISTEM FORMATION

Hypophysis specification initiates formation of the primary root meristem. The hypophysis divides asymmetrically into

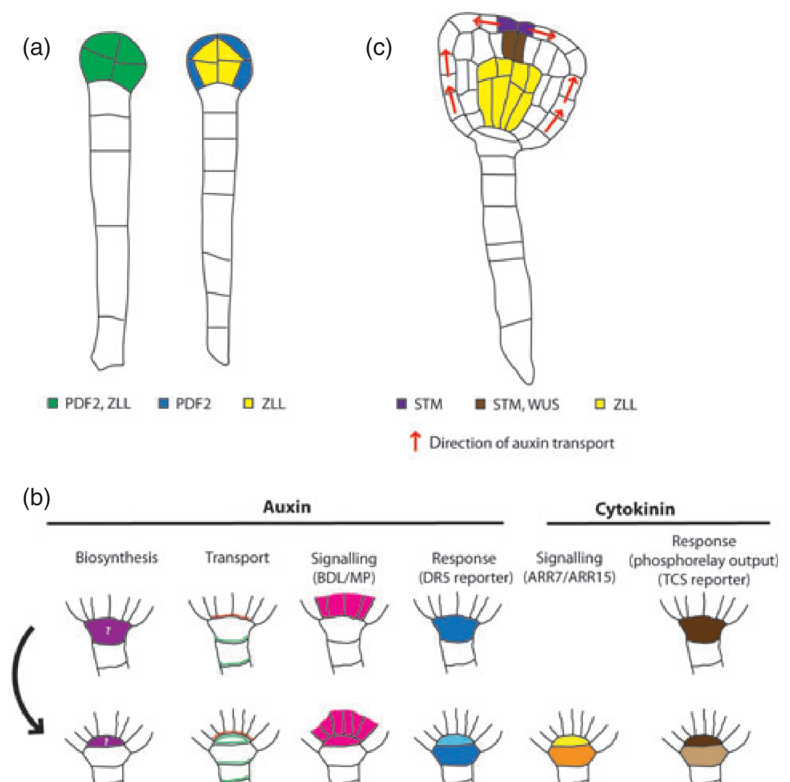

Figure 2. Cell fate and cotyledon specification in early Arabidopsis embryogenesis.
(a) Protoderm formation at transition from the octant (left) to the dermatogen stage (right) is accompanied by the differential restriction of apically expressed genes such as *PDF2* and *ZLL*.
(b) Specification of the hypophysis depends on a BDL/MP-dependent signal from the superhypophyseal cells (pink), which elicits an auxin response in the hypophysis, and on a cytokinin response in the hypophysis (upper row). Subsequently, a complex overlay of auxin and cytokinin signalling specifies the upper lens-shaped cell and the lower cell (lower row). Whether auxin biosynthesis (purple) occurs in the hypophysis and the lens-shaped cell is unclear, as indicated by a question mark. Basal localization of PIN1 (red) and PIN7 (green) results in a downward auxin flow. Different colours indicate low (light blue, yellow, light brown), versus high (dark blue, orange, dark brown, pink) response or signalling activity, respectively.
(c) Shoot meristem establishment depends on the expression of STM, WUS and ZLL, and cotyledon formation depends on directional auxin transport in the protoderm towards incipient cotyledons.

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an upper lens-shaped cell that gives rise to the four cells of the quiescent centre of the root meristem, and a larger basal cell that generates the lower tier of stem cells for the columella (Figures 1b and 2b). In addition, adjacent cells from the apical cell lineage are presumably recruited by signalling from the quiescent centre to become the upper tier of stem cells for root tissues (van den Berg *et al.*, 1997). If the hypophysis is not specified properly, its division plane is mis-oriented, and a root meristem is not formed, which eventually results in rootless seedlings.

Hypophysis specification is linked to auxin signalling. Mutations affecting auxin biosynthesis [*typtophan aminotransferase of arabidopsis 1 (taa1) tryptophan aminotransferase related 1 (tar1) tar2*, Stepanova *et al.*, 2008; *yucca 1 (yuc1) yuc4 yuc10 yuc11*, Cheng *et al.*, 2007), directional auxin transport (*pin1 pin3 pin4 pin7*, Friml *et al.*, *2003*) or auxin response (*mp*, Berleth and Jürgens, 1993; Hardtke and Berleth, 1998; *bdl*, Hamann *et al.*, 1999, 2002; *auxin resistant 6 (axr6)*, Hobbie *et al.*, 2000; Hellmann *et al.*, 2003; *transport inhibitor response 1 (tir1) auxin signaling F-box protein 1 (afb1) axr1-like (axl) afb2 afb3*, Dharmasiri *et al.*, 2005; *axl axr1*, Dharmasiri *et al.*, 2003, 2007] interfere with the specification of the hypophysis, resulting in rootless seedlings.

The auxin response mediated by MP and BDL acts non-cell-autonomously on hypophysis specification, being confined to the provasculature of the central region immediately adjacent to the hypophysis (Weijers *et al.*, 2006). Here, MP promotes PIN1-mediated auxin transport to the hypophysis and the induction of one (or more) predicted mobile signal(s) that also move(s) into the future hypophysis (Weijers *et al.*, 2006). How these two signals together specify the fate of the target cell is still unknown. However, one specific response is MP-dependent activation of the *WOX5* gene in the hypophysis (Sarkar *et al.*, 2007). Downstream of auxin biosynthesis and transport, auxin flux or cellular auxin concentration might mediate the translocation of BREVIS RADIX (BRX) from the plasma membrane to the nucleus, which modulates auxin-dependent processes.

As MP and BDL act from the provascular cells, the presence of these cells is critical for specification of the hypophysis and the subsequent establishment of a functional root meristem. Therefore, at present, it is not clear whether mutants that fail to form the provascular cells as well as a root affect primary root meristem formation directly or only indirectly (Gagne and Clark, 2007; Nodine *et al.*, 2007; Gagne *et al.*, 2008; Nodine and Tax, 2008; Song *et al.*, 2008). For example, the phosphatases POLTERGEIST (POL) and POL-LIKE 1 (PLL1) such as hypophysis specification (Scacchi *et al.*, 2009) were proposed to redundantly control asymmetric cell divisions in the primary shoot and root, and to specifically affect asymmetric cell division of the hypophysis (Song *et al.*, 2008).

Apart from auxin, the phytohormone cytokinin also seems to play a prominent role in establishment of the primary root stem cell niche, i.e. quiescent centre and surrounding stem cells. The locally and temporally defined interaction between auxin and cytokinin controls a differential phospho-relay output that is transiently required for hypophysis-derived daughter cells to generate a functional root stem cell system (Müller and Sheen, 2008). Auxin antagonizes cytokinin signalling in the basal daughter cell of the hypophysis by inducing the expression of ARABIDOPSIS RESPONSE REGULATOR 7 (ARR7) and *ARR15*, which are feedback repressors of cytokinin signalling (Figure 2b) (Müller and Sheen, 2008).

Furthermore, proper hypophysis specification is also impaired in the *bim1* mutant, which was originally shown to be affected in brassinosteroid signalling (Yin *et al.*, 2005). BES INTERACTING MYC-LIKE PROTEIN 1 (BIM1) interacts with DORNRÖSCHEN (DRN) and DRN-LIKE (DRNL), which are involved in auxin signalling, providing a possible point of cross-talk between auxin and brassinosteroid signalling (Chandler *et al.*, 2009). This is further supported by similar defects in the *brx* mutant (Scacchi *et al.*, 2009), which shows impaired auxin responsiveness, most likely as a consequence of altered brassinosteroid biosynthesis (Mouchel *et al.*, 2006).

PRIMARY SHOOT MERISTEM FORMATION AND COTYLEDON INITIATION

Shoot meristem and cotyledon specification occur concomitantly in Arabidopsis embryogenesis, with the primary shoot meristem forming between the two out-growing cotyledon primordia from the globular stage onwards (Figure 2c). Although the processes underlying the two different specification events are linked, they are not dependent on each other, as evidenced by mutants, such as *shoot meristemless* (*stm*) or *wuschel* (*wus*), that lack a primary shoot meristem but not the cotyledons (Barton and Poethig, 1993; Laux *et al.*, 1996). Conversely, mutants such as *laterne* or *pin1 pinoid* (*pid*) lack cotyledons but not the primary shoot meristem (Jürgens *et al.*, 1991; Furutani *et al.*, 2004; Treml *et al.*, 2005).

The processes that establish the primary meristems of shoot and root share related features. The two meristems form at opposite ends of the provasculature, the root meristem at its basal end and the shoot meristem at its apical end. Furthermore, the functional organization of both meristems depends on functionally interchangeable members of the WOX family of homeodomain transcription factors, WOX5 in the root meristem and WUSCHEL (WUS) in the shoot meristem (Laux *et al.*, 1996; Mayer *et al.*, 1998; Sarkar *et al.*, 2007). *WUS* expression is first detected in the four inner cells of the upper tier of the dermatogen-stage embryo, continues in their daughter cells above the provasculature, and is eventually confined to the cells of the organizing centre of the shoot meristem (Mayer *et al.*, 1998). How *WUS* expression is initiated and subsequently regulated during embryogenesis is not known. In addition to observations that the SNF2-class ATPase SPLAYED (SYD)

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targets *WUS* and that a short regulatory region in the *WUS* promoter is sufficient for typical *WUS* expression, *WUS* expression in the shoot apical meristem is known to be positively regulated by cytokinin (Bäurle and Laux, 2005; Kwon *et al.*, 2005; Gordon *et al.*, 2009). It is especially interesting in the latter context that WUS apparently maintains meristem function by repressing the transcription of several Arabidopsis response regulators that are involved in the negative feedback loop of cytokinin signalling (Leibfried *et al.*, 2005), thus providing a basis for self-stimulatory *WUS* expression (Gordon *et al.*, 2009).

In addition to *WUS*, the *KNOTTED1*-like homeobox (*KNOX*) gene *STM* is also indispensable for initiation of the primary shoot meristem (Barton and Poethig, 1993; Long *et al.*, 1996; Lenhard *et al.*, 2002). The nuclear localization of STM depends on BEL1-like homeodomain transcription factors, and the triple knockout of *ARABIDOPSIS THALIANA HOMEOBOX1* (*ATH1*), *PENNYWISE* (*PNY*) and *POUND-FOOLISH* (*PNF*) phenocopies the *stm* mutant (Cole *et al.*, 2006; Rutjens *et al.*, 2009). *STM* is expressed in cells that give rise to the primary shoot meristem, and its expression persists in all shoot apical meristems (Long *et al.*, 1996).

Expression of *STM* depends on the partially redundant putative transcription factors *CUP-SHAPED COTYLEDON 1–3* (*CUC1–3*), which are initially expressed in a central stripe across the apical half of the embryo (Aida *et al.*, 1997, 1999; Takada *et al.*, 2001; Vroemen *et al.*, 2003; Hibara *et al.*, 2006). In *cuc1 cuc2* double mutants, the cotyledons are not separated and look cup-shaped, *STM* is not expressed, and no primary shoot meristem is formed; expression of *CUC1* under the control of the 35S promoter leads to enhanced *STM* expression and the formation of ectopic meristems (Aida *et al.*, 1997, 1999; Takada *et al.*, 2001). The mode of action of *KNOX* genes has mainly been linked to cytokinin- and gibberellin (GA)-dependent signalling (reviewed in Hay *et al.*, 2004), and STM has actually been shown to induce the expression of cytokinin biosynthesis genes and genes encoding GA-deactivating enzymes (Jasinski *et al.*, 2005; Yanai *et al.*, 2005).

Class III homeodomain-leucine zipper (HD-ZipIII) proteins are also required for establishment of the shoot meristem. Several mutant combinations of *REVOLUTA* (*REV*), *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *CORONA* (*CNA*) and *ATHB8* lack a shoot meristem, in addition to displaying other developmental defects (Emery *et al.*, 2003; Prigge *et al.*, 2005). *ZWILLE* (*ZLL*), a member of the *ARGONAUTE* (*AGO*) family, appears to act in the same pathway. *ZLL*, which is apparently only weakly expressed in the primary shoot meristem during early embryogenesis, but rather strongly in the adjacent vascular primordium cells, controls stem cell maintenance non-cell-autonomously (Moussian *et al.*, 1998; Lynn *et al.*, 1999; Tucker *et al.*, 2008). *ZLL* negatively regulates the accumulation of miR165/166 in the primary shoot meristem, which themselves negatively regulate HD-ZipIII genes (Mallory and Vaucheret, 2006; Liu *et al.*, 2009). *ZLL* is therefore required to keep class III HD-Zip levels sufficiently high in the primary shoot meristem. Interestingly, accumulation of PHB and PHV transcripts has to be prevented in the lower part of the embryo proper at the heart stage via a SERRATE–miRNA165/166-dependent pathway in order for primary root development to occur (Grigg *et al.*, 2009).

Although normal *CUC1* and *CUC2* expression has been shown to be linked to auxin signalling (Aida *et al.*, 2002; Furutani *et al.*, 2004), and *WUS* expression during somatic embryogenesis appears to be auxin-dependent (Su *et al.*, 2009), primary shoot meristem initiation and maintenance are not developmental processes that depend on auxin signalling. In fact, auxin has been implicated in the repression of meristematic features, especially in the context of cotyledon or leaf primordium formation (Furutani *et al.*, 2004; Hay *et al.*, 2004, 2006). Cotyledon formation, on the other hand, strongly depends on auxin signalling, and several mutants affected in auxin biosynthesis, auxin transport or auxin response are also affected with respect to cotyledon initiation or formation (reviewed in Chandler, 2008).

At the late-globular stage of embryogenesis, auxin is transported in an apical to basal direction in inner cells, but towards the incipient cotyledon primordia in protodermal cells, i.e. in a basal to apical direction in the lateral protoderm (Steinmann *et al.*, 1999; Benková *et al.*, 2003). The apical localization of PIN1 is generally regulated by the serine/threonine kinase PID that phosphorylates PIN1, and a quadruple knockout of *PID* and its three closest homologues – *PID2*, *WAG1* and *WAG2* – abolishes cotyledon formation (Christensen *et al.*, 2000; Friml *et al.*, 2004; Cheng *et al.*, 2008). The *pid pin1* and *pid enhancer of pinoid* (*enp*) (*laterne*) double mutants also lack cotyledons (Jürgens *et al.*, 1991; Furutani *et al.*, 2004, 2007; Treml *et al.*, 2005). In these two mutant combinations, the expression domains of *STM* and *CUC* genes extend into regions where the cotyledons would normally form, and additional knockout of *STM* or *CUC* genes restores cotyledon formation (Furutani *et al.*, 2004; Treml *et al.*, 2005). This indicates that high auxin concentrations induce cotyledon formation by preventing *CUC* and *STM* expression and/or activity in cotyledon primordia (Furutani *et al.*, 2004).

In contrast to the *CUC* genes, *ASYMMETRIC LEAVES 1* (*AS1*), a MYB transcription factor, and *ASYMMETRIC LEAVES 2* (*AS2*), a LOB domain transcription factor, repress meristematic features and are expressed in cotyledon primordia (Byrne *et al.*, 2000, 2002; Ori *et al.*, 2000; Iwakawa *et al.*, 2002). The main function of *STM* appears to be negative regulation of the *AS1*/*AS2* pathway, as *AS1* or *AS2* knockout makes *STM* dispensable for primary shoot meristem initiation and maintenance (Byrne *et al.*, 2000, 2002). The *AS1*/*AS2* pathway appears to act in parallel to auxin signalling as indicated by data regarding leaf initiation (Hay *et al.*, 2006).

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EMBRYOGENESIS AND AUXIN

Among the known phytohormones, it is predominantly auxin that has been implicated in embryo development and patterning (Santner and Estelle, 2009; Santner *et al.*, 2009; Wolters and Jürgens, 2009). Nonetheless, it might be premature to assume that auxin is the only phytohormone involved in early embryogenesis. There is at least the possibility that the large number of auxin-related reports on embryogenesis simply reflects the research interest in this field, and not necessarily the dominant role of auxin during embryogenesis.

Unlike for other phytohormones, directional transport of auxin has been demonstrated extensively. Active PIN-dependent asymmetric auxin efflux is required for axis formation, patterning and organ initiation at all developmental stages, even during early embryogenesis (Figure 3a) (Steinmann *et al.*, 1999; Friml *et al.*, 2002, 2003; Blilou *et al.*, 2005), and forms a strong buffer to maintain normal auxin gradients within the embryo (Weijers *et al.*, 2005). The correct spatial and temporal auxin distribution in the embryo by vectorial transport predominantly driven by PINs is connected to non-polar auxin efflux mediated by ATP-binding cassette (ABC) proteins of the B sub-family, such as ABCB1 and ABCB19 (Mravec *et al.*, 2008).

The proper (polar) localization of PIN proteins – which depends on trafficking, recycling, degradation and reversible phosphorylation – plays a crucial role in establishing the necessary auxin gradients. This is indicated by the mutant phenotypes of factors involved in these processes, such as the ARF–GEF GNOM (Steinmann *et al.*, 1999), the Rab5-related GTPase ARA7/RAB-F2B (Dhonukshe *et al.*, 2008), the Rab–GEF VACUOLAR PROTEIN SORTING 9A (VPS9A) (Goh *et al.*, 2007; Dhonukshe *et al.*, 2008), the retromer complex member VPS29 (Jaillais *et al.*, 2007), the SERINE/THREONINE PROTEIN PHOSPHATASE 2A (PP2A) (Michniewicz *et al.*, 2007), the protein kinase PID (Friml *et al.*, 2004) and the endosomal sorting complex required for transport (ESCRT)-related proteins CHARGED MULTIVESICULAR BODY PROTEIN/CHROMATIN MODIFYING PROTEIN1A (CHMP1A) and CHMP1B (Spitzer *et al.*, 2009). Similar defects can be mimicked by auxin transport inhibitors (Hadfi *et al.*, 1998; Larsson *et al.*, 2008; Hakman *et al.*, 2009), and embryonic defects can be explained by the mis-localization of auxin efflux carriers (Peer *et al.*, 2009).

Proteolysis mediated by the ubiquitin/26S proteasome-dependent pathway plays a crucial role in plant growth and development, including embryogenesis. Ubiquitination involves the transfer of ubiquitin to substrate protein, bound by E3 ligating enzymes (Pickart, 2001). SCF E3 complexes comprise Skp1, Cullin, an F-box protein and the small RING protein Rbx1 (Pickart, 2001). One of the best-characterized SCF ubiquitin ligases is the SCF^{TIR1} complex, which mediates auxin responses by the auxin-dependent degradation of AUX/IAAs (Lau *et al.*, 2008) (Figure 3b). The disruption of components of this degradation pathway often results in embryonic defects, which in most mutants leads to rootless seedlings. For example, mutations in MP

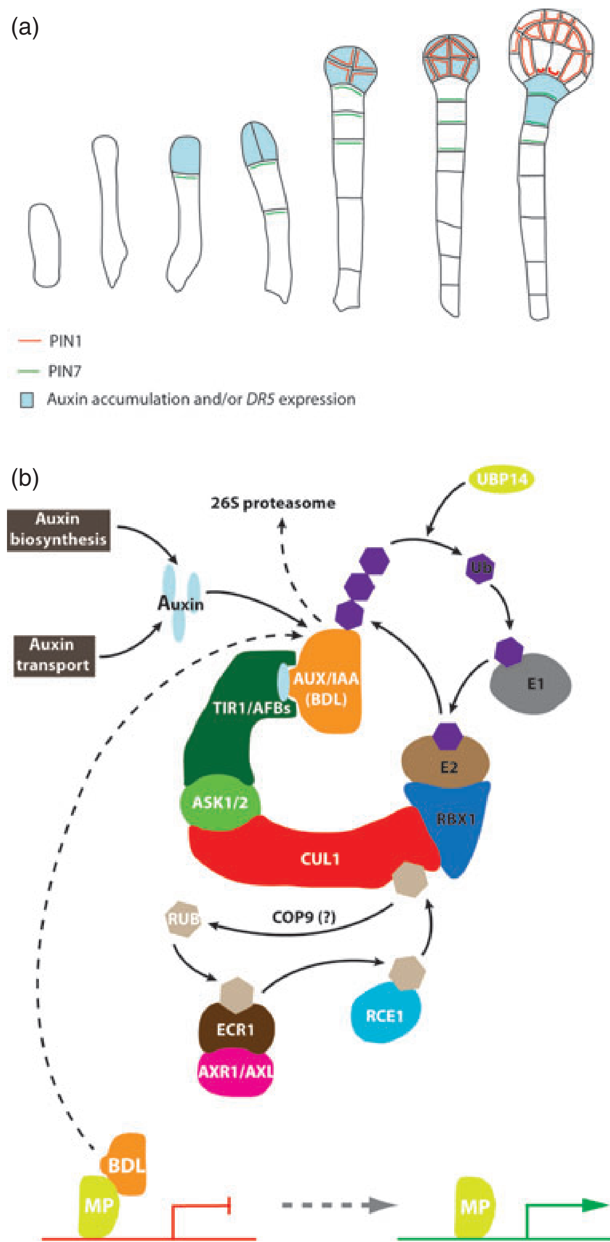

Figure 3. Auxin transport and response in early Arabidopsis embryogenesis.
(a) Initially auxin is transported from the basal to the apical cell(s), resulting in an auxin response in the apical cell(s). After reversion of the direction of auxin transport, an auxin response is observed in the hypophysis and the subhypophyseal cell.
(b) Protein degradation, namely the degradation of AUX/IAAs (such as BDL) to release activating ARFs (such as MP) from inhibition plays a crucial role in the auxin response. Mutations in the factors indicated using white text give rise to similar early embryonic defects and/or rootless seedlings.

or BDL (Berleth and Jürgens, 1993; Hardtke and Berleth, 1998; Hamann *et al.*, 1999, 2002; Weijers *et al.*, 2006), TIR1 and AFBs (Dharmasiri *et al.*, 2005), AXR6/CULLIN 1 (CUL1) (Hobbie *et al.*, 2000; Shen *et al.*, 2002; Hellmann *et al.*, 2003; Quint *et al.*, 2005), RELATED-TO-UBIQUITIN (RUB) proteins (Bostick *et al.*, 2004), subunits of a heterodimeric RUB-activating enzyme, namely AXR1, AXL and E1 C-TERMINAL RELATED 1 (ECR1) (Dharmasiri *et al.*, 2003, 2007), ARABIDOPSIS SKP1 HOMOLOGUE 1 (ASK1)/ASK2 (Liu *et al.*, 2004) or the ubiquitin-specific protease UBIQUITIN-SPECIFIC PROTEASE 14 (UBP14) (Doelling *et al.*, 2001) all result in such defects (Figure 3b). However, depending on the associated F-box protein, these E3 ubiquitin ligases act as key regulators of numerous plant signal transduction pathways (Schwechheimer and Calderon-Villalobos, 2004; Lechner *et al.*, 2006; Ho *et al.*, 2008). Is it conceivable that some of the early defects in the respective mutants are not caused by interference with auxin signalling? For instance, *cul1-1* (Shen *et al.*, 2002) and *axr1 axl* (Dharmasiri *et al.*, 2007) have very early embryonic defects, namely at the first division of the zygote, although these defects were not observed in *tir1 afb1 afb2 afb3* mutants (Dharmasiri *et al.*, 2005). Alternatively, there might be a higher degree of redundancy in the TIR1–AFB protein family than revealed so far. Moreover, does the *axr1* mutation enhance the apical–basal defects of *bdl* mutants (Hamann *et al.*, 1999) because of its stabilizing effect on additional AUX/IAAs or because other signal transduction pathways are affected? It is at least tempting to speculate that auxin is not the only regulator of (early) embryogenesis.

At present, there is very little evidence for a clear role of other phytohormones in embryo patterning, although a role of cytokinin in primary root stem cell specification has recently been demonstrated (Müller and Sheen, 2008) (see above), and other data suggest a role for brassinosteroids in embryo development (Chandler *et al.*, 2009; Scacchi *et al.*, 2009). GA and abscisic acid (ABA), on the other hand, have mainly been implicated in later stages of embryo development, controlling seed dormancy and germination (Holdsworth *et al.*, 2008).

A system that operates via plasma membrane-associated receptor-like kinases, which have been implicated in several aspects of Arabidopsis development (De Smet *et al.*, 2009), could be a means of conveying positional information independently of phytohormones during embryogenesis. However, although a few receptor-like kinases have been shown to be involved in tissue specification and differentiation in the embryo (Tanaka *et al.*, 2002, 2007; Nodine *et al.*, 2007; Nodine and Tax, 2008; Tsuwamoto *et al.*, 2008), very little is known about the upstream extracellular signals and downstream targets of these kinases. An alternative system for local interactions between cells involves transcription factor movement. However, this has not been conclusively demonstrated in embryogenesis, although evidence for SHR movement in the seedling root suggests that the same mechanism could also operate in embryos (reviewed by Jenik *et al.*, 2007).

THE *ARABIDOPSIS THALIANA* GENOME SEQUENCE AND BEYOND

How did embryogenesis research benefit from knowledge of the genome sequence?

Decades ago, genetic control mechanisms of Arabidopsis embryogenesis attracted the attention of several researchers (Müller, 1963; Meinke and Sussex, 1979; Jürgens *et al.*, 1991; Mayer *et al.*, 1991). A large number of mutants with specific or more general defects were identified, and the cloning of EMBRYO DEFECTIVE 30 (EMB30)/*GNOM* pioneered the molecular identification of a diverse set of genes involved in embryogenesis (Shevell *et al.*, 1994; Busch *et al.*, 1996).

At present, 1–2% of the annotated Arabidopsis genes have been shown to be important for embryogenesis (Tzafrir *et al.*, 2003, 2004; Meinke *et al.*, 2008, 2009), but many more are very likely to be involved in it. With the release of the Arabidopsis genome sequence in 2000 (Arabidopsis Genome Initiative, 2000), the mapping of mutants became easier, and reverse genetic approaches such as TILLING, gene silencing and insertional disruption of genes became possible (McCallum *et al.*, 2000a,b; Budziszewski *et al.*, 2001; McElver *et al.*, 2001; Till *et al.*, 2003; Henikoff *et al.*, 2004; Ossowski *et al.*, 2008). Although not all of these technical advances have been extensively exploited in embryo research so far, they supported major novel findings. Unfortunately, a major feature of many embryo patterning mutants appears to be low phenotypic penetrance, suggesting redundancy within gene families or between genetic pathways. The genome sequence is an invaluable tool with regard to genetic redundancy, as closely related genes are easily identified (Dharmasiri *et al.*, 2005, 2007; Hust and Gutensohn, 2006; Long *et al.*, 2006; Muralla *et al.*, 2007; Nodine *et al.*, 2007; Sitaraman *et al.*, 2008).

On a genome-wide level, few studies of gene expression during embryogenesis have been undertaken, mainly due to technical difficulties in accessing the small developing embryo. This is in contrast to post-embryonic growth and development, for which transcriptional changes in diverse processes and cell types have been described in detail (Birnbaum *et al.*, 2003; Zimmermann *et al.*, 2004; Schmid *et al.*, 2005; Brady *et al.*, 2007; De Smet *et al.*, 2008; Goda *et al.*, 2008). However, similar studies might soon also be possible for embryos using improved techniques of fluorescence-activated cell sorting or laser-capture microdissection in combination with micro-array analyses or deep sequencing. Transcript profiling of older embryos has already been tackled using laser-capture micro-dissection,

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showing that approximately 65% of all genes are expressed in the developing embryo from the late-globular stage onwards, increasing to 77% at the torpedo stage (Casson *et al.*, 2005; Spencer *et al.*, 2007). Similarly, while detailed (often genome-wide or evolutionary-scale) studies of 5′ regulatory regions and putative transcription factor targets have been performed for a wide variety of genes and developmental processes (Levesque *et al.*, 2006; Nemhauser *et al.*, 2006; Uchida *et al.*, 2007), this has only been performed to a limited extent for genes regulating specific embryonic processes (Takada and Jürgens, 2007; Cole *et al.*, 2009; Kawashima *et al.*, 2009). By extrapolation from data on *Phaseolus coccineus* (scarlet runner bean), one study identified *cis*-regulatory sequences that activate transcription in the suspensor (Kawashima *et al.*, 2009). In summary, although knowledge of the Arabidopsis genome sequence has clearly eased the problems related to genetic redundancies and of identifying genes involved in embryo development, several of the major regulators known today had already been described previously.

How can the new insights be translated to (model) crop species?

Upon fertilization, a very regular cell division and gene expression pattern results in the formation of tissues and organs that make up the Arabidopsis embryo. However, this regularity is not observed in many other plant species (Johri *et al.*, 1992), making it a future challenge to determine whether research on this model system yields insights that can be translated to economically important plant species. Currently, insights gained from the Arabidopsis model system are being investigated in crop species such as maize and rice, mainly based on genomic information (Zimmermann and Werr, 2005, 2007; Nardmann and Werr, 2006; Nardmann *et al.*, 2007; Chandler *et al.*, 2008). Of course, research in these crop species is by no means fully dependent on Arabidopsis research. Critical components of embryogenesis had been identified in those species independently of Arabidopsis (Sentoku *et al.*, 1999; Itoh *et al.*, 2005), and the rice genome sequence is also available (Yu *et al.*, 2002). Differences in embryogenesis programs could be revealed by comparing the Arabidopsis embryo transcriptome with the embryo transcriptome of other plant species such as the conifer *Pinus taeda* (Cairney and Pullman, 2007).

How did signalling mechanisms evolve? Comparisons between the genomes of the moss *Physcomitrella patens*, the lycopod *Selaginella moellendorffii* and flowering plants such as Arabidopsis showed that, for example, the auxin signalling machinery – which plays an essential role in Arabidopsis embryo patterning – was possibly in place when plants colonized the land (Rensing *et al.*, 2008; Paponov *et al.*, 2009). However, *P. patens* and *S. moellendorffii* appear to have a supplementary mechanism of nuclear auxin signalling that is absent in flowering plants (Paponov *et al.*, 2009). Further comparisons between land plants and unicellular algal lineages revealed that the well characterized AUX/IAA–ARF–TIR1/AFB-dependent auxin signalling pathway is absent in the latter (Lau *et al.*, 2009). Thus it will be interesting to determine the evolutionary time point at which auxin and other signalling mechanisms arose. Such genome comparisons can provide insights into the origin of signalling pathways and how these pathways evolved to eventually give rise to the specific developmental programs that govern angiosperm development in general and Arabidopsis embryogenesis in particular.

PERSPECTIVES

The Arabidopsis genome sequence allowed us to make a leap forward with respect to elucidation of the basic regulatory processes that control embryogenesis in this model plant species. There are still many aspects to be investigated before we have a full picture of the developmental and physiological mechanisms that guide embryogenesis, but we are confident that the missing links will be revealed in the coming years.

Importantly, minimal sets of non-housekeeping genes need to be identified for fundamental developmental processes such as establishment of polarity, determination of the organismal axes, control of orientation of cell division planes, and specification and maintenance of cell identities. For instance, it is not clear at present how apical and basal cell identities are established after zygote division. Is this due to a partitioning of transcripts during zygote development? Or is it due to differential transcription after the asymmetric zygote division? In addition, there is the long-standing hypothesis of suppression of embryo fate in the suspensor by the embryo proper (Vernon and Meinke, 1994; Zhang and Somerville, 1997; Baroux *et al.*, 2001; Vernon *et al.*, 2001). Although developmental arrest of the embryo proper, either by mutation or through toxin-mediated cell ablation, results in unusual suspensor divisions and occasionally in secondary embryo development (Vernon and Meinke, 1994; Zhang and Somerville, 1997; Baroux *et al.*, 2001; Vernon *et al.*, 2001; Weijers *et al.*, 2003), it is not clear how this would actually work. A research field that has largely been neglected is the presence of signals in the cell wall. For tobacco embryogenesis, it has been shown *in vitro* that the presence of the original zygote cell wall is required for the maintenance of cell polarity and the apical–basal axis, as well as for typical suspensor formation (He *et al.*, 2007). In this respect, it is interesting to note that a cell wall-associated arabinogalactan protein epitope has been localized to the embryo proper of Arabidopsis (Hu *et al.*, 2006). And finally, the role of the various phytohormones in embryogenesis and their intricate cross-talk needs to be clarified in more detail.

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