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Early plant embryogenesis — dark ages or dark matter?

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In nearly all flowering plants, the basic body plan is laid down during embryogenesis. In Arabidopsis, the crucial cell types are established extremely early as reflected in the stereotypic sequence of oriented cell divisions in the developing young embryo. Research into early embryogenesis was especially focused on the role of the infamous tryptophan derivative auxin in establishing embryo polarity and generating the main body axis. However, it is becoming obvious that the mere link to auxin does not provide any mechanistic understanding of early embryo patterning. Taking recent research into account, we discuss mechanisms underlying early embryonic patterning from an evolutionary perspective.

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Introduction

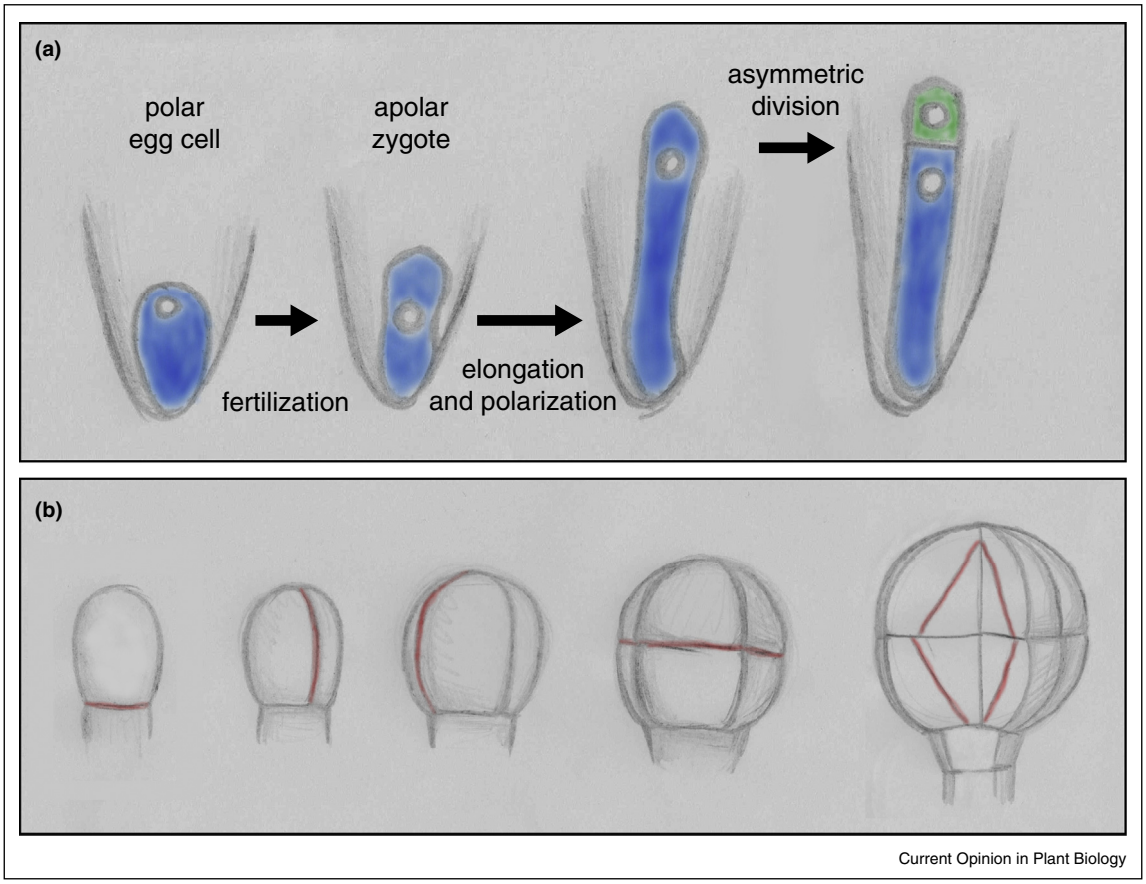
Like all multicellular organisms, flowering plants usually start their development as a single, fertilized cell — the zygote. In plants, cells are surrounded by a rigid cell wall and can therefore not move. In order to generate a complex morphology, cells have to implement different programs of oriented cell division and cell elongation. Although a large number of factors involved in the establishment of the plant body plan [1,2] have been identified, little is known about the underlying molecular mechanisms that lead to the different cell identities in the early embryo. In *Arabidopsis thaliana*, the division of the zygote is asymmetric, giving rise to a smaller apical and a larger basal cell (Figure 1a). The latter undergoes a limited number of transverse cell divisions to form the suspensor — a filamentous structure that plays an important role in supporting the developing embryo [3,4]. Rather than dividing repeatedly in transverse orientation, the apical daughter cell divides twice longitudinally to form the 4-cell proembryo. Transverse divisions of all four cells create an 8-cell proembryo with an upper and a lower tier each consisting of four cells. Asymmetric tangential (aka periclinal) divisions of all 8 cells then form an outer protoderm layer — the precursor of the epidermis — and a mass of inner cells [5, Figure 1b]. These asymmetric cell divisions initiate radial pattering of the embryo. Stem cell niches for the future shoot and root are subsequently established in the upper tier and at the boundary between proembryo and suspensor, respectively, connected by precursor cells of the vascular strand (stele). With the embryonic leaf primordia growing out of the upper tier, all major organ primordia of the seedling have been established. This stereotypic development is characteristic of *Brassicaceae* family members. Although very different cell division patterns may occur in embryogenesis of other plant species, they still yield seedlings of essentially the same body organization [6].

In this review, we will focus on the initial steps of embryogenesis including embryo initiation, establishment of the apical–basal axis of the embryo and the asymmetric cell divisions generating the main cell types. By including data from algae and lower land plants (dark ages), we discuss possibly conserved modules that drive early embryogenesis (illuminate the dark matter), with an emphasis on recent experimental advances.

Setting the stage: egg–zygote transition

Fertilization transforms the egg cell into a zygote, initiating a regulated cell division program in a formerly quiescent cell. The fertilization event itself could trigger embryogenesis if egg and sperm cell provide factors that combine to work like a two-component system. How this might happen in higher plants is at present poorly understood. Transient calcium peaks are associated with the fusion of the gametes, but the molecular identity of the signaling output is unclear [7,8]. Some insight into the activation of a zygotic genetic program might be gained from the unicellular alga *Chlamydomonas reinhardtii*. This haploid alga forms gametes of *plus* and *minus* mating type under nitrogen-starvation [9]. The *plus* and *minus* gametes express homeodomain proteins of the TALE superclass with the BELL-related Gamete-specific *plus*1 (Gsp1) expressed in *plus* gametes and the KNOX-related Gsm1 expressed in *minus* gametes. Ectopic expression of Gsp1 in *minus* gametes leads to Gsp1/Gsm1 heterodimerization and is sufficient to activate a zygotic transcriptional program [10,11]. Could this hetero-dimerization of BELL and KNOX homeodomain transcription factors be conserved for activation of a zygotic transcrip-

Figure 1



Early Arabidopsis embryogenesis. **(a)** Polarization of the zygote followed by asymmetric division. The apical cell (green) gives rise to the proembryo. **(b)** Patterning in 3D. Schematic illustration of the first embryonic cell divisions up to the 16-cell stage. The latest division plane is depicted in red.

tional program in higher plants? Knockout mutations in one of the four BELL-like transcription factors (BELL1) in *Physcomitrella patens* lead to normal gametophytic development but egg cells are morphologically changed and cannot form embryos. Overexpression of PpBELL1 leads to ectopic formation of sporophytes on gametophytic caulonema cells, possibly by BELL1-MKN2 (KNOX) hetero-dimerization [12••]. With the sporophyte being the dominant generation in higher plants, KNOX genes likely evolved beyond regulating a zygotic transcriptional program to allow for the appearance of more complex sporophytes [13,14]. Nonetheless, a fundamental role for heterodimerizing transcription factors in activating a sporophytic genetic program in the zygote may still be retained in flowering plants.

Breaking the stage: polarization of the zygote — Episode I

In Arabidopsis, the mature egg cell is a polarized cell and aligned with the polarized ovule [15,16]. After fertilization, however, the egg cell is reorganized such that for a transient period the nucleus is positioned centrally and the large egg cell vacuole is partitioned into smaller, evenly distributed vacuoles [17,18] (Figure 1a). These morphological changes suggest that the zygote goes through a transient non-polar phase. Subsequently, the zygote repolarizes, elongates in its future apical–basal axis, and its nucleus moves towards the cell apex followed by asymmetric cell division. In addition, the large vacuole is reformed at the basal pole [19]. How is symmetry broken in the zygote?

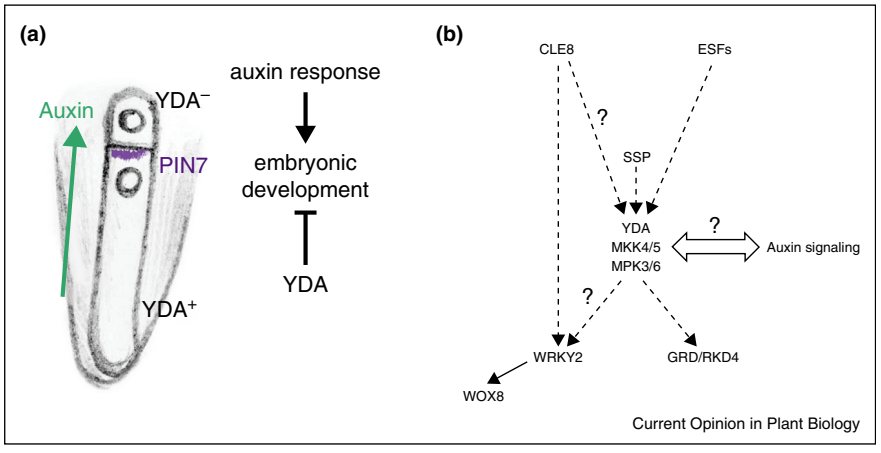
Mutations in the mitogen-activated protein (MAP) kinase kinase kinase gene *YODA (YDA)* imply the involvement of a MAP kinase pathway in breaking zygote symmetry, in addition to its well-known role in stomata formation during leaf development [20]. Loss-of-function mutants of *yda* and double mutants of the downstream MAP kinases *mpk3 mpk6* show reduced zygote elongation and equal-sized daughter cells after mitosis [20,21]. Instead of repeatedly dividing transversely to form the filamentous suspensor, the basal daughter cell and its

derivatives often display longitudinal cell divisions. These cells are incorporated into the proembryo, resulting in shorter suspensors or embryos lacking a recognizable suspensor. Conversely, constitutively active variants of YDA (YDA-CA) lead to over-proliferation of the suspensor, often resulting in filamentous structures that apparently consist only of suspensor cells. This suggests that the embryonic YDA pathway is differentially regulated between the daughter cells of the zygote and promotes suspensor identity (or represses embryo identity) (Figure 2a) [20,22]. How YDA activity is controlled is at present unclear, but a few pieces of the puzzle have emerged: The membrane-associated pseudokinase SHORT SUSPENSOR (SSP) seems to play a role in YDA activation right after fertilization by an unusual parent-of-origin effect [23]. Accumulating specifically in sperm cells of pollen, *SSP* transcripts are translated after fertilization, that is, in the zygote [23]. The functional role of this translational regulation is not clear at present but it is interesting to note that *SSP* is a fairly recent, *Brassicaceae*-specific paralog of *BSK1* [24]. BSKs are abundant signaling partners in various receptor kinase signaling pathways in development and innate immunity [25]. Ectopic expression of *SSP* in leaves leads to strong gain-of-function phenotypes that mimic those of *YDA-CA* variants [23]. Thus, the presence of the SSP protein might be sufficient to cause unrestricted activation of the YDA signaling pathway. In analogy, the transient presence of SSP in the zygote and its basal daughter cell might be sufficient to activate YDA. The *ssp* phenotype, however, is much weaker than *yda* loss-of-function phenotypes, indicating another signaling input for YDA activation (Figure 2b). A family of cysteine-rich peptides, named EMBRYO SURROUNDING FACTORs (ESFs) have been proposed to work upstream of YDA since they can influence suspensor development and genetically interact with the YDA pathway [26]. They are supposedly secreted by the endosperm to influence apical–basal polarity and suspensor length; however, analysis of recent transcriptome profiles of early embryos reveals that ESF transcripts might also be present in the proembryo [27–29].

Polarization of the zygote — Episode II

In *wrky dna binding protein 2 (wrky2)* mutants, the zygote elongates normally but divides in a morphologically symmetric fashion with a centrally located division plane and large vacuoles in the apical as well as the basal daughter cell [30]. In later development of the embryo, the boundary between proembryo and suspensor appears less defined with frequent aberrant cell divisions within the suspensor lineage. Thus, some aspects of polarization of the zygote as well as cell fate acquisition of the daughter cells seem impaired in *wrky2*. The equal size of the daughter cells by itself is most likely not a direct cause for this abnormality because the size ratio of the daughter cells of the zygote is variable between flowering plant species whereas the cell fates are distinct [6,31]. Transcription factor WRKY2 activates *WOX8* expression in the zygote and in the suspensor [30]. Single *wox8* mutants do not display any obvious embryonic defects but *wox9* and *wox8 wox9* double mutants show irregular cell proliferation in the suspensor [32,33]. *WRKY2* is preferentially expressed in the basal daughter cell and the suspensor. How is *WRKY2* expression and activity spatially regulated? The secreted peptide CLAVATA3/ESR-RELATED 8 (CLE8) might play a role in this context since mutations in *cle8* cause loss of *WOX8* expression in suspensor cells and similar early-embryonic phenotypes as in *wox8 wox9* double mutants [34]

Figure 2



Signaling events breaking zygote symmetry. **(a)** The embryonic YDA pathway is differentially regulated in the daughter cells of the zygote, actively repressing embryonic development in the basal cell. PIN7 localization at the apical membrane of the basal cell leads to auxin accumulation in the proembryo. **(b)** Signaling cascades involved in apical–basal patterning. How YDA signaling and auxin response antagonistically interact is at present unknown. Dashed arrows indicate positive interaction, the solid arrow direct activation. Question marks indicate speculative connections.

(Figure 2b). The regulation of transcription factors of the WOX family by CLE-sensing receptor kinase pathways seems to be a recurring theme [35–37]. It is therefore tempting to speculate that a CLE-receptor pathway might also regulate WRKY2 activity in the early embryo. Recently, it was shown that WRKY2 and WRKY34 are targets of MPK6 in pollen [38]. It is thus tempting to ask if WRKY2 acted downstream of the YDA-MPK3/6 MAP kinase cascade and how CLE8 might fit in.

Polarization of the zygote — Episode III

The RWP-RK protein GROUNDED (GRD/RKD4), which might act as a transcriptional regulator, appears to be a necessary factor for the response to the YDA MAP kinase signaling cascade, although it does not appear to be a direct target [39•,40]. Loss-of-function phenotypes morphologically resemble those of *yda* and *ssp*, indicating that its activity is important for embryonic patterning at earliest stages, promoting basal cell fate. Interestingly, induced and prolonged overexpression of *GRD* in postembryonic tissues induces embryo-like structures resembling somatic embryos [39•,40]. In *Marchantia polymorpha*, deletion of an Arabidopsis RWP-RK homolog causes defects in germ cell formation, and overexpression causes cells to adopt egg-like transcriptomes [41••,42••]. Down-regulation by artificial microRNA, however, causes proliferation of the egg cell without fertilization, indicating that RKDs might keep egg cells quiescent and could be involved in egg-to-zygote transition. Compared to the Arabidopsis embryo, the simple morphology of the liverwort sporophyte might mask the function of RKDs after fertilization. Alternatively, GRD/RKD4 might not directly regulate zygote polarity but could be responsible for an early sporophytic or embryonic transcriptional program in higher plants.

Stage differences: differentiation in 3D

Multicellular organisms often benefit from the division of labor between their various, differentiated cell types. Cell types can be generated in different ways. For example, an external signal can instruct adjacent cells differentially. Another way would be to distribute molecules differentially during the division of a polarized cell. In Arabidopsis, many tissue-initializing cell divisions are asymmetric and often accompanied by a change in division orientation compared to previous division planes [43]. Extending earlier microscopic observations [5], recent work generating 3D embryonic images provided evidence for additional asymmetric divisions during early Arabidopsis embryogenesis [44•]. Symmetric cell divisions have been described to follow the 'shortest wall' rule [45,46]. Inhibition of transcriptional auxin response alters the plane of division from periclinal to anticlinal (asymmetric to symmetric) at the transition from 8-cell to 16-cell stage [44•]. This implies that asymmetric division is genetically controlled and auxin seems to be part of it. However, care has to be taken with generalizing division rules, since cell division also depends on stochasticity, tissue-type and tension [47–49].

In early embryogenesis the following asymmetric cell divisions appear to play fundamental roles in patterning: (a) the division of the zygote (asymmetric), (b) the following two cell divisions in the proembryo — longitudinal as opposed to the repeated transverse divisions in the suspensor (change of orientation), (c) the periclinal divisions laying down the protoderm during the transition from 8-cell to 16-cell stage (change of orientation), (d) the cell divisions in the lower tier of the 16-cell and 32-cell embryo establishing ground tissue and vascular tissue initials (asymmetric and change of orientation) and (e) the division of the uppermost suspensor cell known as hypophysis (asymmetric). In essence these divisions ultimately partition the proembryo into a 3-dimensional structure by giving rise to apical–basal and radial polarity axes (Figure 1b).

In contrast to flowering plant embryos, filamentous green algae like *Klebsormidium flaccidum* show neither asymmetric divisions nor changes in division orientation, lack most of the auxin response machinery components [50] and morphologically resemble a suspensor. Microspore-derived embryos from *Brassica napus* following the zygote-like pathway stay filamentous until stress induces differentiation of a terminal cell to mimic zygotic embryogenesis [51]. Embryos generated this way express auxin efflux carriers PIN1 and PIN7, and terminal/apical differentiation can be blocked by inhibition of polar auxin transport [52]. If the aforementioned YDA pathway represses embryonic development and thus formally counteracts auxin response, it is not clear what relieves the apical cell of the embryo from this repression. The recurrent theme of the terminal cell(s) of a filament initiating differentiation is recapitulated with a set of different experiments eliminating the embryo (initial cell) by toxin, microdissection or laser ablation [53,54,55•]. In all these cases, suspensor cells adjacent or close to the site of experimental interference abnormally express markers of auxin response and subsequently form secondary embryos [55•,56]. This abnormal auxin response resembles the normal auxin response in the apical daughter cell of the zygote that coincides with the initiation of proembryo development.

Both computer modeling [56] and recent experimental work [57,58] couple auxin transport to auxin production in orienting the apical–basis axis of the embryo. According to these results, an auxin source as visualized by YUCCA expression exists in the cells of the early suspensor from where auxin is then transported via the suspensor-expressed auxin efflux facilitator PIN7 to the apical/terminal cell where it accumulates (Figure 2a). At later stages, a second auxin source is established in the upper proembryo potentially via TAA1 and YUCCA action

which is, according to the model, important for the reversal of auxin flow and differentiation of the root pole [57,59–61]. Although the idea of auxin being involved in embryo initiation is appealing, there are still a number of loose ends. For example, if PIN7 plays a major role for auxin transport into the apical/terminal cell, how is PIN7 expression set up only in suspensor cells? Apart from the PIN auxin efflux facilitators, auxin influx has also been shown to play a role in early patterning. LAX genes seem to be preferentially active in the apical or basal domains at early embryo stages and mutant embryos have a blurred boundary between proembryo and suspensor [62].

Perspectives

In this review we have emphasized an evolutionary perspective for interpreting experimental findings that were largely obtained in Arabidopsis zygotic embryogenesis or Brassica microspore embryogenesis. One might thus ask how general is the mechanism of embryo initiation inferred from studies in these two systems. Is it confined to the *Brassicaceae* or dicots or does it apply to flowering plants at large? Another important question concerns the origin of the apical–basal axis of the embryo. Is it just the consequence of establishing the embryo fate of the apical cell, which then interacts with the basal cell to generate regions along the proembryo-suspensor axis? A case in point is the local interaction of proembryo cells through the mobile transcription factor TMO7 with the suspensor to specify the fate of the hypophysis as founder of the root meristem [63]. Yet another basic feature of embryo patterning is the occurrence of asymmetric cell divisions generating daughter cells of different fates, and some of these asymmetric divisions require transcriptional auxin response. Is this a general phenomenon and how is this process regulated molecularly? The role of auxin in developmental processes is not clear at all. Does it elicit different cellular responses in a concentration-dependent manner? Or does it rather trigger preexisting cellular programs in a switch-like manner [64,65]? In the basal land plant *Marchantia polymorpha*, diverse cellular responses including the 3-dimensional organization of the plant body can be generated with only a minimal number of auxin response components [66•,67•]. It might thus be wise to consider other signaling molecules and pathways as relevant players in early embryo patterning as outlined in the section on breaking the symmetry of the zygote.

Conflict of interest

The authors declare no conflict of interest.

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