Title:

New cues for body axis formation in plant embryos

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Abstract

Plant embryogenesis initiates with the fusion of sperm and egg cell, and completes the generation of the basic outline of the future plant. Here, we summarize the recent findings about the signaling cascade triggering the zygotic transcription, and the intracellular events and regulatory factors involved in the formation of the two major body axes. We highlight the lack of systematic *de novo* transcriptional activation in the zygote, and emphasize the importance of cytoskeletal reorganization to polarize the zygote and control the first asymmetric division that establishes the apical–basal axis. Finally, the limited knowledge of mechanisms that control the cell divisions separating the inner and outer cell layers is summarized and we propose approaches to enhance our understanding of basic principles of plant embryogenesis.

Introduction

In both animals and plants, sexual reproduction produces male and female gametes, which are fused to generate the zygote. In plants, zygotic development marks the transition from the gametophytic haploid life phase to the sporophytic diploid life phase. Therefore, the gametophytic state is reprogrammed to a new sporophytic state that initiates the series of transcriptional events directing embryogenesis. Thereafter, the zygote and the embryo generate the body axes as the basis of subsequent pattern formation. In *Arabidopsis thaliana*, the apical–basal axis is defined by the asymmetric division of the zygote, which generates a small apical daughter cell and a large vacuolated basal daughter cell (Figure 1) [1]. The apical daughter cell gives rise to the spherical proembryo by divisions in different orientations. In contrast, the basal daughter cell continues to divide horizontally to produce a filamentous suspensor that connects the proembryo to maternal tissues [1,2]. Each cell in the 8-cell stage proembryo performs an asymmetric (periclinal) division that separates an outer protoderm layer from a mass of inner cells. These divisions generate the radial axis in the 16-cell embryo (Figure 1). The protoderm differentiates as the epidermis, and the

stem cell niche of the shoot meristem arises from the inner lineage [3-5], showing that the basic body plan is already generated at such an early stage although patterning of organs takes place post-embryonically.

Onset of zygotic/embryonic transcriptional program

In animals, early embryogenesis depends on maternal RNAs deposited in the unfertilized egg cell, and the transcription of the zygotic genome is activated only after several cycles of embryonic cell division [6,7]. In plants, *de novo* transcription takes place in the zygote and the contribution of parental genomes have been analyzed using transcriptomes from hybrids between different accessions and mutants [8-10]. Several contradictory results were reported, possibly due to differing degrees of contaminating maternal tissues [11,12]. A consensus emerges that in angiosperms, there is no concerted maternal to zygotic transition, and the zygotic transcriptional machinery is active after completion of fertilization. This was shown for genes that are transcribed *de novo* immediately after fertilization [11]. Yet other genes are gradually activated transcriptionally or silenced during early stages of embryogenesis [13]. This progressive activation of zygotic genes was recently confirmed by the RNA sequencing of isolated

zygotes in rice and maize [14*,15]. By comparing the transcripts in sequential stages of zygote development, it was shown that the maternal transcripts are abundant at early stages, but *de novo* transcripts become gradually predominant during the first zygotic cell cycle.

Recent work identified a signaling pathway that activates transcription of specific genes after fertilization. *SHORT SUSPENSOR* (*SSP*) encodes a membrane-associated pseudokinase, and its mRNA is inherited from sperm [16]. In the zygote, *SSP* mRNA is translated to activate the mitogen-activated protein (MAP) KK kinase YODA (YDA) (Figure 2a) [16,17]. The downstream MAP kinases MPK3/6 phosphorylates the transcription factor WRKY2, which in turn activates the transcription of *WUSCHEL HOMEOBOX8* (*WOX8*), by directly binding to its promoter with maternally-derived factors HOMEODOMAIN GLABROUS11/12 (HDG11/12) [18**,19]. WOX8 and its closest homolog, WOX9, redundantly regulate both zygote polarization and embryo patterning, although their direct targets remain unknown [20]. *WOX* genes appear to be plausible targets of zygotic *de novo* expression in Arabidopsis, and also in rice [21]. *WOX8* and *WOX9* are derived from an ancestral clade of *WOX* genes represented by

WOX13 in Arabidopsis [22]. This clade contains one gene in the liverwort *Marchantia polymorpha* [23], and three genes in the moss *Physcomitrella patens*, two of these being expressed and required for the first cell division of the zygote [24]. In spite of the deep evolutionary conservation of the role of *WOX* genes in zygotic development, *SSP* is *Brassicaceae*-specific variant of *BSK* gene family [25] and thus it is not likely that the parental cooperation that regulates the activation of the *WOX* cascade in Arabidopsis is widely conserved amongst plant species.

Apical–basal axis formation: zygote polarization

Although the cell division patterns during embryogenesis are quite diverse amongst plant species [26], the zygotic transverse division that produces the apical and basal cells is common to most species of land plants, including Arabidopsis, rice, mosses and liverworts [24,27-29], implying the fundamental role of zygote polarization during apical–basal axis formation. In Arabidopsis, the unfertilized egg cell shows polar organization, which is marked by the apical position of the nucleus (Figure 1) [30]. At fertilization however, the nucleus becomes positioned centrally [31]. After the zygote

elongation, the nucleus again positions close to the apical apex, and thus the first zygotic division is asymmetric, producing a small apical cell [1]. The living dynamics of the intracellular repolarization were visualized by live-cell imaging of developing Arabidopsis zygotes [32,33**]. The preexisting alignment of microtubules (MTs) and actin filaments (F-actins) in the egg cell is disorganized after fertilization, and then MTs organize into a subapical transverse ring to restrict the direction of zygote elongation, whereas F-actin forms an apical cap and longitudinal arrays to move the nucleus to the apical tip (Figure 2b). Similar associations of cytoskeleton features and nuclear migration are observed in tip-growing cells, such as fern protonema and Arabidopsis root hair [34,35], implying that the zygote might utilize the tip growth machinery to polarize. Although it is still possible that maternal polarity determinants are inherited from the egg cell, the dramatic intracellular changes in the elongating zygote imply that egg cell polarity is lost after fertilization, and subsequently the zygote establishes its polarity *de novo* (Figure 2b). Fertilization-triggered loss of polarization might be a general feature of plant zygotes, since dramatic cell shrinkage is reported in various

species [33**,36-38], and F-actin organization is remodeled during the fusion of gamete nuclei (karyogamy) in rice [39].

The molecular determinants of the apical–basal axis are still unknown. As in most animals, the plant sperm cell could provide a cue to orient the zygote polarity [40,41]. This remains unclear in angiosperms, because of anatomical constraints on the site of sperm fusion with the egg cell *in vivo* and *in vitro* fertilization [42,43]. Some signals might originate from the embryo-surrounding tissue, endosperm, because the cysteine-rich peptide encoded by *EMBRYO SURROUNDING FACTOR1* (*ESF1*) is produced in endosperm and regulates YDA cascade during embryo patterning [44].

In addition, it is still unclear which genes regulate the diverse processes that lead to the zygote polarization. As described above, the directional zygote elongation and the polar nuclear migration depend on MTs and F-actin, respectively [33**], but these steps are genetically inseparable because, for example, the defects of zygote length and the division asymmetry are enhanced in a correlative manner as more mutations of *SSP*, *WRKY2* and *HDG11/12* are combined [18**]. Until now, several candidates have been identified as the zygote polarity regulators. MicroRNAs likely control some factors

involved in early step of embryogenesis including the first division of the zygote [45,46*]. DNA methylation may also participate in zygote polarization, because asymmetry of the zygote division is impaired at low penetrance in the *DNA METHYLTRANSFERASE1* (*MET1*) mutant *met1-6* [47]. The defect might be related to increased expression of *YDA* in *met1-6* compared with wild type. YDA activation might also rely on fluctuation of genomic DNA methylation that has been reported between the egg cell and the 1-cell embryo [48]. In the near future, the zygotic transcriptome analyses will identify the target genes of these pathways, as well as the downstream factors of SSP-WOX8 cascade. Combination of these findings with the zygote live-imaging analysis of various spatiotemporal events, such as organelles dynamics and epigenetic changes, will identify the molecular mechanism responsible for the asymmetric cell division of the zygote.

Radial axis formation: separation of inner and outer cells

After the formation of the apical–basal axis, the proembryo generates the second body axis, the radial axis, by the asymmetric anticlinal cell division separating an outer

protoderm from inner cell mass (Figure 1). Several genes show asymmetric expression in relation with formation of the radial axis after the transition from the 8- to the 16-cell stages (Figure 2c). WUSCHEL (WUS) expression is initiated in the inner four cells [4], while miR165/166 microRNA is strongly expressed in the outer lower tier [49] (Figure 2c). Recently, various intracellular reporters were generated to investigate radial polarity in 8-cell embryos [50**]. The anticlinal asymmetric cell division that defines the outer layer of cells does not involve nuclear migration and cell elongation as in the case of the asymmetric division of the zygote. Similarly, the cortical MT seems to lack a prominent orientation before the formation of mitotic structures, such as preprophase bands (PPBs) and spindles (Figure 2c) [50**], implying that the cell polarization mechanism involved is distinct from the zygote polarization. Interestingly, the boron transporter BOR1 localizes to the inner membranes when expressed under the control of embryo-specific *WOX2* promoter (Figure 2c) [50**]. Therefore, the localization of BOR1 can be utilized as a radial polarity marker, although its native roles in embryos are presently unclear.

Although there is still very little known about the molecular mechanism regulating the protoderm formation, the plant hormone auxin and the transcription factor WOX2 were identified to contribute to the asymmetric periclinal cell division [20,51]. The 3D-reconstruction analysis of cell division patterns showed that the auxin response is important to shift from the default anticlinal cell division to the periclinal division pattern [51]. However, cell division pattern defects are common in auxin mutants and thus it is unclear how auxin regulates the early divisions of the embryo and whether the effects observed at this stage are directly related to auxin signaling [52].

WOX2, together with its redundant paralogs WOX1, WOX3, and WOX5, regulates the periclinal cell division, and subsequently represses the auxin pathway and promotes a cytokinin signal important for the definition of the shoot meristem niche among the inner cell mass [20,53**]. WOX proteins repress the expression of *MIR166B*, one of the miR165/166 genes that target HOMEODOMAIN-LEUCINE ZIPPER CLASS III (HD-ZIP III) transcription factor genes including *PHAVOLUTA* (*PHV*) [54]. Overexpression of a miR165/166-resistant version of *PHV* (*rPHV*) restores the shoot phenotype of *wox1235* quadruple mutant, but does not rescue the periclinal division defect [53**].

Therefore, WOX proteins would have another yet unidentified targets that act in radial axis formation. Recent transcriptome profiles of 8/16-cell, early and late globular embryos have been obtained [55**]. This gene expression atlas was generated by combining RNA sequencing and a cell purification system based on a two-component transgenic strategy. Based on the comprehensive comparison of the spatial and temporal expression profiles, it was reported that the inner lower cells in the 16-cell embryo already initiate the gene expression to differentiate into vasculature, which becomes recognizable only after the 32-cell stage. Such a stage-specific database will be ideal to identify novel regulators on radial division and protoderm establishment.

Conclusions and perspectives

As discussed in this review, recent studies utilizing novel techniques have identified various genes and intracellular events regulating embryogenesis. Using species other than Arabidopsis has clarified certain aspects. But it is shocking that phylogenetic studies of the origin of embryo axes has not been undertaken, despite the fact that the zygote polarization is common in diverse plant species. Various major questions have

remained unanswered. How are gametes genome reprogrammed transcriptionally if there is little to support that zygotic transcriptional activation takes place? What are the determinants and the molecular mechanisms that establish the apical–basal and the radial axes? As shown in animals embryos, single cell technologies [56,57] in combination with dynamic imaging and phylogenetic perspectives will help to answer these old but basic questions in the near future.

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Figure legends

Figure 1. Schematic diagram of the Arabidopsis embryogenesis. Egg cell polarity is disrupted by fertilization. Later asymmetry of the zygote is established and determines the apical–basal axis. The radial axis is formed when the inner and outer cells are separated at 8-16 cell stage embryo. Solid and dashed arrows indicate sequential and nonsequential steps, respectively.

Figure 2. Key factors and events during early embryogenesis. (a) WRKY2 originates from both parents. The paternally-derived SSP triggers the YDA MAP kinase cascade, which in turn phosphorylates WRKY2, which becomes activated. The activated WRKY2 and the maternally inherited HDG11/12 directly bind to the promoter of *WOX8* and initiate its transcription *de novo* in the zygote. (b) Several intracellular events and putative regulators of the zygote polarization. (c) Molecular markers and regulators of radial patterning. The cortical microtubules (MT) become organized to form the preprophase band (PPB) at the future cell division plane. BOR1 localizes on

the inner cell membranes when it is expressed under the control of *WOX2* promoter (WOX2pBOR1), but the endogenous expression of BOR1 is unknown.

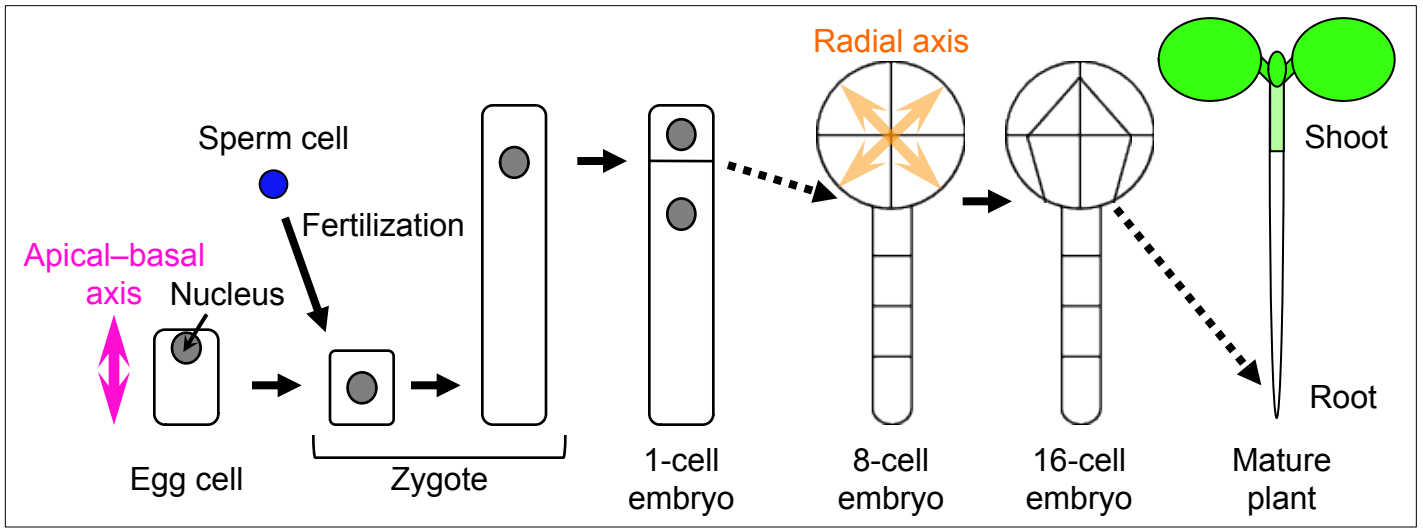
Radial axis
Sperm cell
Fertilization
Apical–basal
axis
Nucleus
Shoot
Root
Egg cell
Zygote
1-cell
embryo
8-cell
embryo
16-cell
embryo
Mature
plant

Figure 1

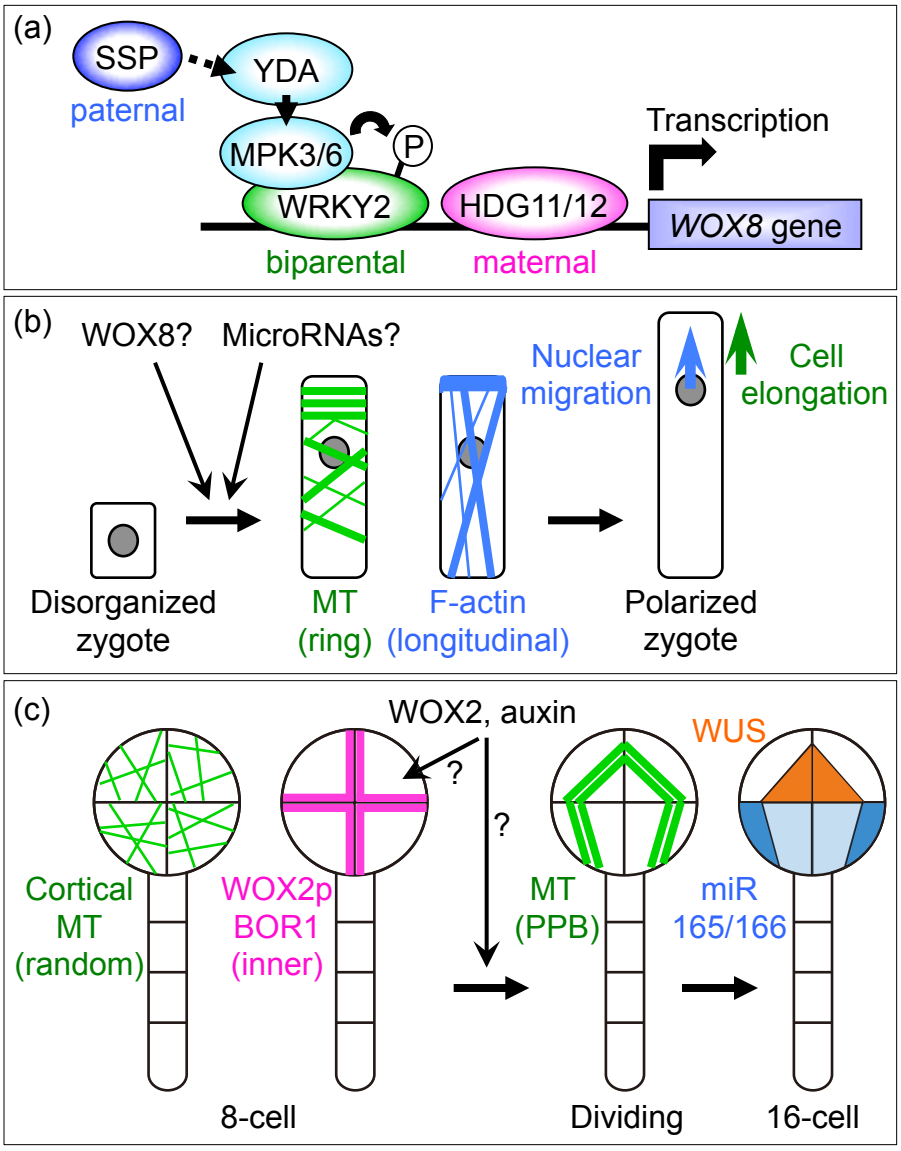
(a)
SSP
paternal
YDA
MPK3/6
P
WRKY2
HDG11/12
biparental
maternal
Transcription
WOX8 gene
(b)
WOX8?
MicroRNAs?
Nuclear migration
Cell elongation
Disorganized zygote
MT (ring)
F-actin (longitudinal)
Polarized zygote
(c)
WOX2, auxin
?
?
WUS
Cortical MT (random)
WOX2p BOR1 (inner)
MT (PPB)
miR 165/166
8-cell
Dividing
16-cell

Figure 2