Cell fate specification and maintenance in the female gametophyte and transcriptional profiling of early embryos in *Arabidopsis thaliana*

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1. Summary

The life cycle of flowering plants alternates between a diploid sporophytic and a haploid gametophytic phase. Successful reproduction depends on fate specification of the gametic cells within the embryo sac and on double fertilization. This double fertilization event is initiated by fusion of the central cell with one of the two sperm cells as well as fusion of the egg cell with the other sperm cell, which gives rise to the endosperm and the embryo, respectively. However, the molecular mechanisms of cell fate specification during female gametophyte development remain elusive and gene expression programs in different cell types of early embryos are largely unknown. Here, we show that *ALTERED MERISTEM PROGRAM 1* (*AMP1*) is required to prevent cell fate substitution within the embryo sac in Arabidopsis. Loss-of-function mutations in *AMP1* result in supernumerary egg cells at the expense of synergid cells allowing for twin embryo formation. However, generation of twin embryos usually precludes endosperm formation, which eventually leads to ovule abortion. Remarkably, in such a case, ovule abortion can be overcome by delivering functional supernumerary sperm cells in *tetraspore (tes)* pollen, thereby enabling the formation of twin plants. *AMP1* expression in sporophytic tissue is sufficient to suppress the formation of supernumerary egg cells and the twin-embryo phenotype, indicating that one or more mobile factors are involved in synergid fate specification and that the surrounding tissue can contribute to the patterning process of the female gametophyte. This work highlights the importance of specifying two synergids and only one egg cell within the female gametophyte to ensure successful reproduction.

The second part of this thesis includes a technique that integrates nuclear sorting by flow cytometry and subsequent microarray analysis to generate transcriptome datasets of different cell types in the Arabidopsis early embryo. The results presented indicate that the majority of genes are similarly expressed in the pro-embryo and the suspensor but that a number of genes with supposedly important functions during embryogenesis showed different expression patterns. This technique and the resource presented will assist further studies of early embryogenesis. In addition, this technique can also be applicable to other inaccessible tissues with a limited number of cells.

2. Zusammenfassung

Der Lebenszyklus von Blütenpflanzen lässt sich in die diploide Sporophyten- sowie haploide Gametophytengeneration unterteilen. Die erfolgreiche Fortpflanzung ist dabei abhängig von der Spezifizierung der Gameten innerhalb des Embryosackes und von der sogenannten doppelten Befruchtung. Die doppelte Befruchtung wird durch die Fusionsprodukte der Zentralzelle sowie der Eizelle mit jeweils einem Spermium eingeleitet, woraus im späteren Verlauf der Entwicklung jeweils das Endosperm und auch der Embryo enstehen. Die molekularen Mechanismen welche zur Zellspezifizierung während der Entwicklung des weiblichen Gametophyten führen, sind jedoch weitestgehend unbekannt - genauso wie die Transkriptome der verschiedenen Zelltypen des frühen Embryos. In der vorliegenden Arbeit wurde zum einen gezeigt, dass *ALTERED MERISTEM PROGRAM 1 (AMP1)* unabdingbar ist, um einen Wechsel vom einen in den anderen Zelltyp im Arabidopsis Embryosack zu verhindern. *Loss-of-function* Mutationen in *AMP1* führen zu einer erhöhten Anzahl an Eizellen auf Kosten von Synergiden was die Ausbildung von Zwillingsembryonen erlaubt. Allerdings entsteht in Samenanlagen mit Zwillingsembryonen im Normalfall kein Endosperm woraufhin diese letzendlich absterben. Bemerkenswerterweise kann das vorzeitige Absterben der Samenanlagen verhindert werden, indem über *tetraspore (tes)* Pollen überschüssige und gleichzeitig funktionsfähige Spermien zur Verfügung gestellt werden. Dadurch kann es auch zur Bildung von lebensfähigen Zwillingspflanzen kommen. Expression von *AMP1* nur im maternalen Gewebe ist hinreichend, um nicht nur die Ausbildung von überschüssigen Eizellen sondern auch die von Zwillingsembryonen zu unterbinden. Dies deutet wiederum darauf hin, dass sowohl einer oder mehrere mobile Faktoren als auch das maternale Gewebe an der Synergidenspezifizierung beteiligt sein können. Dieser Teil der Arbeit hebt die Bedeutung der Spezifizierung von zwei Synergiden und nur einer Eizelle im weiblichen Gametophyten hervor, um die Fortpflanzung sicherzustellen.

Im zweiten Teil der Arbeit wird eine Technik beschrieben, welche die Aufreinigung von Zellkernen mittels Durchflusszytometrie mit anschliessender Expressionsanalyse über Microarrays verbindet und damit die Erstellung von gewebespezifischen Transkriptomen des Arabidopsis Embryos ermöglicht. Diese Methode zusammen mit der bereitgestellten Resource an Expressionsdaten wird für künftige Untersuchungen von Nutzen sein. Zusätzlich kann diese Technik auch für andere, unzugängliche Gewebe mit geringer Zellzahl angewendet werden.

3. Introduction

3.1 Cell fate specification and maintenance in the female gametophyte of Arabidopsis

The plant life cycle undergoes alternation between the sporophytic and the gametophytic phase. In flowering plants, the formation of the male and the female gametophytes is integral to double fertilization. In Arabidopsis, the functional megaspore undergoes three rounds of sequential nuclear divisions and following cellularization generates four cell types with seven cells during female gametophyte development (Kagi and Gross-Hardt, 2007). These seven cells are: two accessory cells called synergids, which are required for pollen tube attraction; one egg cell, which fuses with one of the two sperm cells giving rise to the embryo; the homo-diploid central cell, which fuses with the other sperm cell giving rise to the nourishing tissue called endosperm; and three antipodal cells, of which the function remains unclear (Sundaresan and Alandete-Saez, 2010) (**Fig. 1**). Successful double fertilization requires appropriate cell fate specification and maintenance of the cells within the female gametophyte as well as male-female communication (Dresselhaus, 2006). Different genetic screens have been performed in order to identify genes that are involved in cell fate determination during female gametophyte development (Yang et al., 2010). However, many of the mutations identified are in genes involved in basic processes, such as pre-mRNA splicing, cell cycle regulation, *etc* (Yang et al., 2010). For instance, loss-of-function mutations in several spliceosome factor genes, e.g. *LACHESIS (LIS), CLOTHO (CLO)/GAMETOPHYTIC FACTOR 1 (GFA1)*, *ATROPOS (ATO)* as well as in *WYRD* (*WYR*) that encodes a putative plant ortholog of the inner centromere protein (INCENP) leads to ectopic expression of an egg cell marker in the synergid cells (Gross-Hardt et al., 2007b; Moll et al., 2008; Kirioukhova et al., 2011). It is worth noting that *LIS* is strongly expressed in gametic cells and reduction of *LIS* expression specifically in the egg cell impairs the development of the synergid(s) (Volz et al., 2012). Therefore, this inhibition signal from the egg cell to some extent regulates synergid development indicating the importance of cell-cell communication within the female gametophyte (Volz et al., 2012). Nevertheless, the synergids expressing egg cell markers do not seem to turn into functional egg cells. On the other hand, it has been shown that the synergids can turn into egg cell once the egg cell is ablated (Lawit et al., 2013). Interestingly, the non-cell autonomous regulation in the female

gametophyte does not only occur in Arabidopsis, but was also reported for maize (Krohn et al., 2012). The secreted peptide Zea Mays Egg Apparatus1 (ZMEA1)-LIKE (ZmEAL1) from the egg cell is required for prevention of antipodals from adopting central cell fate (Krohn et al., 2012).
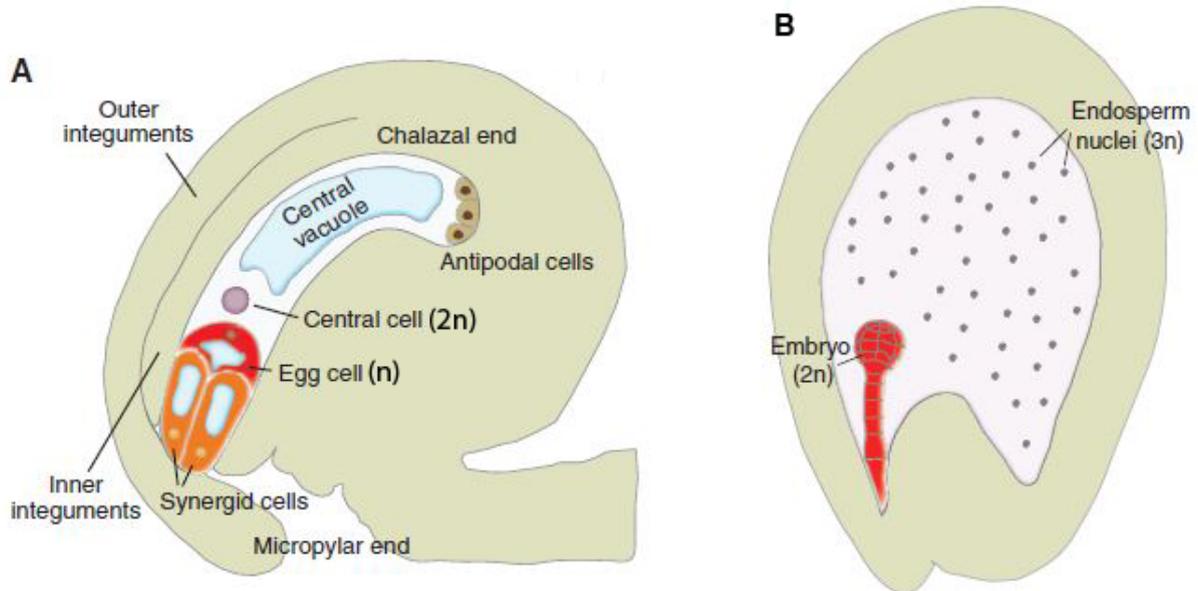
Adapted from (Sundaresan and Alandete-Saez, 2010)

Figure 1. Female gametophyte and double fertilization products in Arabidopsis

(A) Female gametophyte; (B) Double fertilization products

3.2 Apical-basal patterning in Arabidopsis early embryogenesis

In flowering plants, embryogenesis starts with the fertilization product of egg and sperm cell called zygote. In Arabidopsis, the asymmetric division of the zygote generates a smaller apical and a larger basal cell (Jürgens, 2001). The smaller apical cell eventually gives rise to most parts of the embryo whereas the larger basal cell forms a file of 6-9 cells called suspensor that undergoes programmed cell death during late stages of embryogenesis (Jürgens, 2001). Evidently, the two daughter cells originating from the zygote adopt distinct developmental fates and often differ in morphology (Jürgens, 2001). Even though an asymmetric division of the zygote occurs in most of the flowering plant species, there are also exceptions where the zygote undergoes a morphologically symmetric division or an asymmetric division with the apical daughter cell being larger than the basal daughter cell (Lau et al., 2012). Furthermore, the ensuing divisions in the apical cell and the basal cell also vary between species (Johri,

1992). In Arabidopsis, as in other plant species, the mechanism of the establishment of the apical-basal axis and the maintenance of the cell fates are not well understood. One of the differences between the apical cell and the basal cell is delineated by the expression of a group of *WUSCHEL HOMEOBOX (WOX)* family transcription factors (Haecker et al., 2004; Breuninger et al., 2008; Ueda et al., 2011) (**Fig. 2**). The establishment of zygote polarity requires the transcription factor *WRKY2* which activates expression of *WOX8*, and possibly also *WOX9* (Ueda et al., 2011). The expression of *WOX2* and *WOX8* could already be detected in the zygote, but the expression separates precisely after the asymmetric division of the zygote (Haecker et al., 2004) (**Fig. 2**), thereby indicating the establishment of different cell identities. Besides *WOX* family genes, apical-basal axis formation also depends on the YODA (YDA) signaling pathway. Mutations in genes such as *YDA, GROUNDED (GRD), SHORT SUSPENSOR (SSP)* or *MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3)/MPK6*, which are supposed to be involved in the YDA signaling pathway result in cell elongation defects in the zygote. As such, this leads to a small basal cell, which affects suspensor development or causes partial loss of suspensor identity (Lukowitz et al., 2004; Wang et al., 2007; Bayer et al., 2009; Jeong et al., 2011; Waki et al., 2011; Musielak and Bayer, 2014). Conversely, hyperactive YDA produces unusually long suspensors and pro-embryo development is often inhibited (Lukowitz et al., 2004). Even though *GRD* was recently shown to be a downstream component in YDA signaling (Jeong et al., 2011; Waki et al., 2011)*,* how exactly YDA downstream signaling transduction works is unclear.
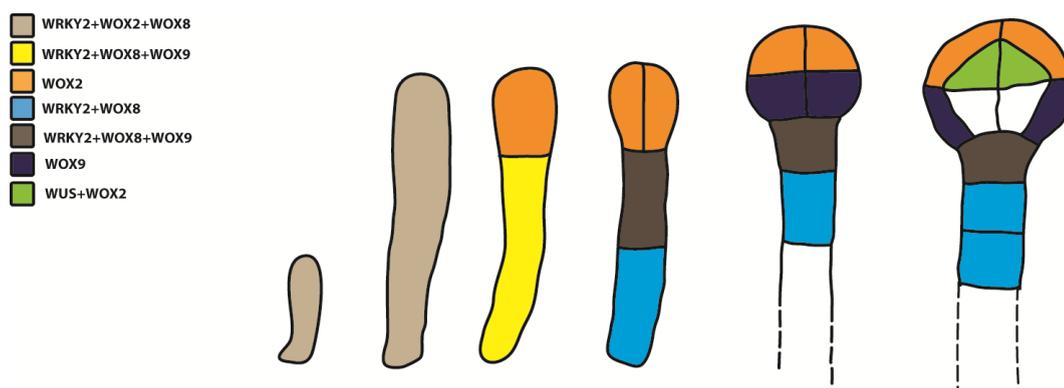

Figure 2. Expression pattern of *WRKY2* and *WOX* family genes [Modified, based on (Lau et al., 2012)]

Auxin is involved in most plant developmental processes. In addition to the two pathways mentioned above, the auxin signaling pathway also contributes to the

establishment of the apical-basal axis and is notable after the division of the zygote (Friml et al., 2003). It was reported that the apical auxin maximum requires transport of auxin by the auxin efflux carrier PIN-FORMED 7 (PIN7), and the accumulation of auxin in the apical cell possibly together with other factors is required for the specification of the apical cell identity (Friml et al., 2003) (**Fig. 3**). Transverse, instead of vertical, division of the apical cell could be observed in several auxin signaling pathway mutants [e.g. *pin7*, *bodenlos* (*bdl)*] indicating that apical cell identity is impaired (Hamann et al., 1999; Friml et al., 2003). Interestingly, auxin is not only required for the specification of apical embryonic cell identity, but also important for preventing the suspensor cells to acquire embryonic fate, as evidenced by embryo formation from the suspensor cells if auxin response is suppressed in the suspensor (Rademacher et al., 2012). Likewise, excessive divisions in the suspensor adopting embryo identity have been reported in other mutants, for instance, *twin2, suspensor 1 (sus1)* and *amp1* (Schwartz et al., 1994; Zhang and Somerville, 1997; Vernon et al., 2001; Vidaurre et al., 2007)*.* The mechanism of such a transformation, however, still needs to be determined.
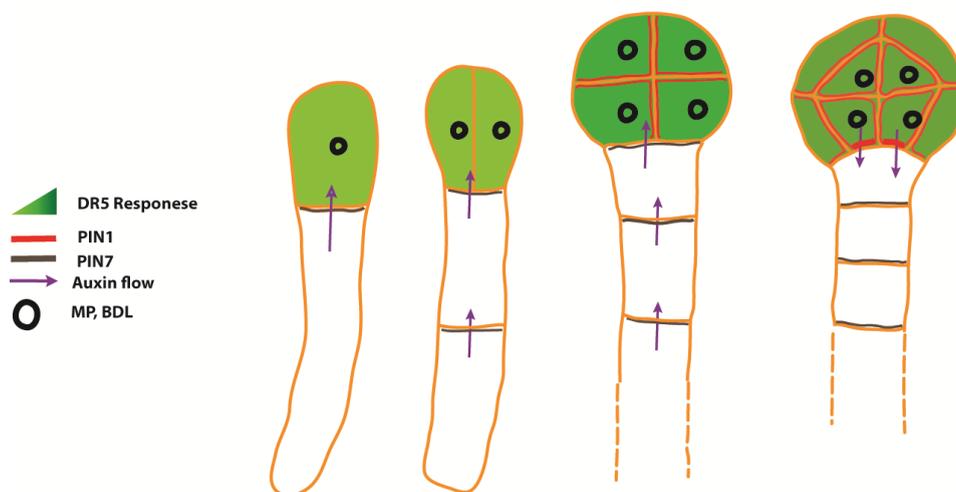
Figure 3. Auxin signaling in early embryogenesis [Modified, based on (Lau et al., 2012)]

3.3 Transcriptomics in Arabidopsis early embryos

Gene expression studies revealed that different transcriptional programs are initiated in different cell types from early stages of Arabidopsis embryogenesis and early embryonic patterning is marked by setting up diverse transcriptional domains (Le et al., 2010; Lau et al., 2012; Belmonte et al., 2013). Evidently, the distinct expression

programs in different cell types contribute to the apical-basal axis formation (Friml et al., 2003; Haecker et al., 2004). Furthermore, it was suggested that some factors in the pro-embryo inhibit embryo formation in the suspensor, as inferred from the onset of embryogenesis in the suspensor after chemical ablation of the pro-embryo (Weijers et al., 2003). The transcriptional profiling of different domains in Arabidopsis embryos or whole embryos from different stages has been reported from several studies by using different methods (Le et al., 2010; Xiang et al., 2011; Belmonte et al., 2013). Nevertheless, the analysis of transcriptional programs in the apical and basal cell lineages at earlier stages of embryogenesis, which will be instrumental for a better understanding of early embryo patterning, is still lacking. Several methods have been applied for various tissues and different organisms, e.g. Laser capture micro dissection (LCM) (Kerk et al., 2003; Nakamura et al., 2004; Espina et al., 2006; Paulsen et al., 2009), Fluorescence-activated cell sorting (FACS) (Birnbaum et al., 2003; Jayasinghe et al., 2006; Shigenobu et al., 2006), Translating ribosome affinity purification (TRAP) (Zanetti et al., 2005; Mustroph et al., 2009) and Isolation of nuclei tagged in specific cell types (INTACT) (Deal and Henikoff, 2010, 2011). However, TRAP and INTACT are currently under optimization for special tissues such as plant embryos. The advantage of LCM is the capability of isolating specific tissues without the need of generating transgenic plants and it has been shown that LCM can be used for sectioning of different parts of Arabidopsis ovules and embryos (Kerk et al., 2003; Espina et al., 2006; Paulsen et al., 2009; Le et al., 2010; Wuest et al., 2010; Belmonte et al., 2013). Nonetheless, LCM requires high precision in sectioning target cells in order to avoid contaminations from neighbouring tissue. Consequently, precise isolation of certain cell types by LCM, such as cells in *WUS* expression domain in early embryos, which are deeply embedded within an early embryo could be considerably challenging. The integration of Fluorescence-activated cell sorting (FACS) with gene expression analysis has been reported in many studies, such as purification of Drosophila embryonic cell populations and isolation of different cell types in Arabidopsis roots (Birnbaum et al., 2003; Shigenobu et al., 2006). Yet, most of the studies in plants, if not all, were based on generation of wall-free protoplasts from certain tissues (Birnbaum et al., 2003; Nawy et al., 2005). However, such a method is very difficult to apply to Arabidopsis early embryos, in particular in large amount. Interestingly, it was reported that fluorescently labelled nuclei from the companion cells of the phloem could be isolated using flow cytometry by so called fluorescence-activated nuclear sorting (FANS) for transcriptome

analysis (Zhang et al., 2008). Therefore, the application of FANS to early embryos will pave the way for transcriptional profiling of special, not easily accessible, cell types.

4. Aim of this work

Plant sexual reproduction is dependent on the formation of both male and female gametophytes. In Arabidopsis, failure of proper specification of distinct cell types within the embryo sac often leads to ovule abortion without fertilization events or with only a single fertilization event. However, the underlying molecular basis of cell specification in the female gametophyte is not well understood. In particular, the reason for specifying only one egg cell and two synergids, as well as the consequences of the generation of functional supernumerary egg cells are elusive. Therefore, one of the aims is to investigate how the different cell types are specified within the Arabidopsis female gametophyte and its impact on plant reproduction.

The asymmetric division of the zygote generates a smaller apical cell and a larger basal cell. The morphologically distinct apical and basal cells adopt different cell fates and develop into most part of the embryo and the suspensor, respectively. So far only a handful of genes were shown to be differentially expressed in the apical and basal cell lineages, which were described previously to exert biological function in apical-basal axis establishment. In addition, the approach to manipulate early embryos is technically challenging. Our aim is to develop a method that can be employed for transcriptional profiling of distinct cell types in early embryos and possibly other inaccessible tissues.

5. Results and discussion

5.1 Cell-fate specification in the female gametophyte of Arabidopsis and twin-embryo formation in *amp1*

In Arabidopsis, the female gametes and accessory cells are specified during female gametophyte development (Yang et al., 2010). Although some hints on cell fate specification and maintenance of different cell types within the embryo sac have been reported (Kagi and Gross-Hardt, 2007), little is known about the molecular cues of cell fate specification in the embryo sac. Here, it is shown that the cell fate acquisition or maintenance of the synergids requires *AMP1*. In *amp1* mutants, twin embryos were observed in fertilized ovules and this phenotype can only be observed when *amp1* is used as the homozygous mother plant in reciprocal crosses. It appears that *amp1* cells destined to become synergids adopt egg cell fate and thereby supernumerary egg cells are generated. Likewise, ovules with supernumerary functional egg cells were reported in the maize *indeterminate gametophte1 (ig1)* and the Arabidopsis *eostre* mutants (Kermicle, 1971; Guo et al., 2004; Pagnussat et al., 2007). Although the presence of an auxin gradient in the female gametophyte has recently been challenged (Lituiev et al., 2013), positional information still seems to play a role in cell fate determination in the embryo sac. In the *eostre* mutant, nuclear migration defects could be observed due to misexpression of *BEL-LIKE HOMEODOMAIN 1 (BLH1)* in the embryo sac. As a consequence, an additional functional egg cell is generated at the expense of a synergid, thereby allowing the production of twin-zygote like structures (Pagnussat et al., 2007). However, whether the cell fate switch happens prior to nuclear migration or afterwards still needs to be determined. Furthermore, supernumerary egg cells observed in the maize *indeterminate gametophyte 1 (ig1)* mutant is due to a prolonged free nuclear division phase (Guo et al., 2004; Evans, 2007). Similar to *eostre*, an extra egg cell *in ig1* can be fertilized but in this case the zygotes could progress further to late stage embryos (Kermicle, 1971; Guo et al., 2004; Pagnussat et al., 2007). In contrast to the previously reported mutants, the *amp1* embryo sac contains nuclei expressing an egg cell marker located at the synergid nucleus position at earlier stages. The additional egg cells ultimately translocate to the primary egg cell position instead of the synergid position, which indicates that egg cell fate could be specified at the synergid cell position and the misspecification or cell fate substitution in *amp1* appears to happen prior to nuclear migration. However, the underlying mechanism of nuclear

migration is currently unclear. Abnormal female gametophyte development has been reported in mutants involved in microtubule-related components (Tanaka et al., 2004; Pastuglia et al., 2006). It is possible that the microtubule arrangement is altered in *amp1*, which affects with the nuclear movement.

AMP1 has been isolated in several genetic screens (Helliwell et al., 2001; Saibo et al., 2007; Vidaurre et al., 2007; Liu et al., 2013), but the molecular function of *AMP1* has not fully been resolved yet. In this study, the expression of *AMP1* in the surrounding sporophytic tissue is sufficient to suppress the supernumerary egg cell(s) as well as twin-embryo phenotypes, suggesting that the process of synergid specification also involves the surrounding maternal tissue of the embryo sac. A recent report showed that ER-localized AMP1 is involved in miRNA-mediated translational inhibition. Since some of the miRNA pathway components and a few targets are expressed in the female gametophyte and the surrounding sporophytic tissue (Wuest et al., 2010; Schmidt et al., 2011; Sanchez-Leon et al., 2012; Schmid et al., 2012; Li et al., 2013), it is conceivable that AMP1 might inhibit the translation of unknown miRNA targets, thereby maintaining synergid cell fate. Alternatively, since *AMP1* encodes a putative glutamate carboxypeptidase it might act as a peptidase that generates small peptides (Helliwell et al., 2001). Nonetheless, what exactly *AMP1* does to specify or maintain synergid cell fate in the female gametophyte needs to be further examined. It has been shown that mutations in *AMP1* only change the expression level of a handful of genes (Helliwell et al., 2001), but future proteomics studies might facilitate unraveling the molecular function of *AMP1*.

In contrast to the previously reported gametophytic mutants that rest on the ectopic egg cell marker expression in the synergids (Gross-Hardt et al., 2007a; Moll et al., 2008), the *amp1* embryo sac showed supernumerary functional egg cells. Notably, in the case of two egg cells and one synergid in the *amp1* embryo sac, we were able show that sperm cells can freely choose any of the female gametes. Given the conflicting results from previous studies at large based on manipulating sperm cells about the favor of fusion processes between the sperm cells and the female gametes (Nowack et al., 2006; Chen et al., 2008; Frank and Johnson, 2009; Aw et al., 2010; Hamamura et al., 2011), this work, therefore, also laid the current doubts on the choices of sperm cells with regard to fertilizing the egg cell or the central cell.

Furthermore, in *amp1* ovules one functional synergid is sufficient to attract a pollen tube and fulfill fertilization which is consistent with previous studies (Higashiyama et al., 2001). It is worth noting that the number of synergids also varies between species, e.g. in *peperomia*-type embryo sacs only one synergid can be found and in *plumbagella*- and *plumbago*-types there are no synergids observed in the embryo sac, whereas in *Amborella* three synergids are specified (Johri, 1992; Friedman, 2006). This raises questions regarding the number of synergids needed for successful fertilization and the reason to eliminate the other likely redundant synergids after fertilization in Arabidopsis. The analysis of *amp1* ovules also suggests that the specification of two synergids might not only be important for pollen tube attraction but also for the prevention of alternative fertilization events that as a consequence leads to twin-embryo formation, thereby reducing reproduction. Taken together, this work provides a different view on the specification of synergid cells and sets the stage for the profound understanding of the fundamental mechanism of double fertilization.

5.2 Development of a method for generating transcriptional profiles of different cell types of the early Arabidopsis embryo

Embryo development depends on tightly controlled spatial and temporal gene expression programs. Thus, generation of comprehensive transcriptional profiles of different cell types in the embryo would contribute to a better understanding of how distinct cell types are established and maintained during early embryogenesis. However, the isolation of specific cell types from the deeply embedded embryo is one of the daunting tasks. Here, we have developed a method for isolating nuclei from specific cell types of the Arabidopsis early embryo by FANS in combination with transcriptome analysis. With this method, we were able to isolate target nuclei from as early as one-cell stage embryos and possibly also zygotes that are deeply embedded in the ovules. Compared to LCM, this method largely avoids contamination from surrounding tissues and in addition it allows collection of *de novo* synthesized mRNA from the nuclei. The quality and quantity of RNA has been regarded as the critical factor for downstream applications. With our method, low amounts of RNA with RIN (RNA Integrity Number) numbers of approximately six could be achieved and this RNA was used for RNA amplification and microarray analysis. By comparing the transcriptome

from nuclei of whole early embryos with the transcriptome from whole cells of whole early embryos, it could be shown that the data generated with these methods are comparable. Collectively, this method can be widely used for characterizing gene expression of deeply embedded cell types with limited number of cells, such as stem cell population in the embryos.

The comparison of transcriptome datasets between the pro-embryo and the suspensor showed that most transcripts are similarly expressed in both pro-embryo and suspensor of early embryos, but for certain transcripts there were differences. Several genes found to be apically expressed in the data set were previously characterized to be functionally important during early embryogenesis. For example, *MONOPOLE (MNP)/HANABA TANARU (HAN)* was shown to be expressed in the apical cells of the embryo and to be involved in the boundary set-up between pro-embryo and suspensor (Nawy et al., 2010). *PIN1*, known as an auxin efflux carrier, is expressed in the pro-embryo cells mediating auxin flow from apical cells to the hypophysis, which is critical for embryonic root initiation (Friml et al., 2003; Weijers et al., 2006). Moreover, the apically expressed *WOX2* plays a fundamental role in the establishment of the apical domain (Haecker et al., 2004). Additionally, we have validated the expression pattern of additional candidate genes that showed differential expression according to our data set by promoter fusion analysis and *in situ* hybridization. Moreover, we have examined the mutants for several candidate genes and most of them, however, do not show embryonic abnormal phenotypes which might be due to functional redundancy or the lack of null mutant alleles. Taken together, our data set along with previously reported ones provides insights into the gene regulatory networks in embryogenesis and our method can be applied for analysis of dynamic transcriptional programs in various domains during progression of embryo development in the future. Furthermore, the application of new tools such as CRISPR/Cas might overcome the limitations of characterizing the function of embryo-expressed genes and unveil the contribution of distinct gene expression programs to the early embryo patterning.

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7. Publications and Manuscripts

7.1   Twin plants from supernumerary egg cells in Arabidopsis

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Running title: Dizygotic twins in Arabidopsis

Summary

Sexual reproduction of flowering plants is distinguished by double fertilization – the two sperm cells delivered by a pollen tube fuse with the two gametic cells of the female gametophyte – the egg and the central cell – inside the ovule to give rise to the embryo and the nutritive endosperm, respectively [1]. The pollen tube is attracted by non-gametic synergid cells, and how these two cells of the female gametophyte are specified is currently unclear. Here, we show that *ALTERED MERISTEM PROGRAM 1 (AMP1)*, encoding a protein associated with the endoplasmic reticulum [2], is required for synergid cell fate during Arabidopsis female gametophyte development. Loss of *AMP1* function leads to supernumerary egg cells at the expense of synergids, enabling the generation of dizygotic twins. However, if twin embryos are formed, endosperm formation is prevented, eventually resulting in ovule abortion. The latter can be overcome by the delivery of supernumerary sperm cells in *tetraspore (tes)* pollen [3], enabling the formation of twin plants. Thus, both primary and supernumerary egg cells are fully functional in *amp1* mutant plants. Sporophytic *AMP1* expression is sufficient to prevent cell-fate change of synergids, indicating that one or more AMP1-dependent mobile signals from outside the female gametophyte can contribute to its patterning, in addition to the previously reported lateral inhibition between gametophytic cells [4-6]. Our results provide insight into the mechanism of synergid fate specification and emphasize the importance of specifying only one egg cell within the female gametophyte to ensure central cell fertilization by the second sperm cell.

Highlights

- Egg cells remain fully functional in *amp1* mutants.

- Synergids become fully functional supernumerary egg cells, giving rise to embryos.

- Twin-embryo phenotype is suppressed by *AMP1* expression in somatic tissue of the ovule.

- Supernumerary sperm cells rescue developmental arrest of twin embryos.

Results and discussion

Several female-gametophytic defect mutants have been isolated from different genetic screens [7]. Most of these mutants, if not all, hardly proceed to successful double fertilization and are unable to produce viable fertilization products. Recent reports showed that loss-of-function mutations in several spliceosome factor genes, e.g. *LACHESIS (LIS)* or *CLOTHO/GAMETOPHYTE FACTOR 1 (CLO/GFA1)*, as well as in *WYRD (WYR)*, which encodes a putative plant ortholog of the inner centromere protein (INCENP), lead to the ectopic expression of an egg cell marker in synergid cells [4, 5, 8]. It has been proposed that egg cell expression of *LIS* is required for synergid development [6]. However, the presumed additional egg cells in *lis*, *clo* or *wyr* appear not to be functional. Synergids can also trans-differentiate to egg cell-like cells when the egg cell is ablated, and supernumerary egg cells have been proposed to be present in the *eostre* mutant of Arabidopsis likely due to the trans-differentiation of synergids [9, 10]. In the *eostre* mutant, in which *BEL1-LIKE HOMEODOMAIN 1 (BLH1)* is mis-expressed in the embryo sac, zygote-like structures were observed after pollination, but these structures did not give rise to embryos [10]. Several sporophytic defects have been reported for *amp1* mutants, including an enlarged shoot apical meristem, the early onset of flowering and the over-proliferation of suspensor cells, which occasionally leads to the formation of secondary embryos in later development [11-13]. To elucidate the details of secondary embryo formation in *amp1*, we examined ovules from *amp1-10* mutant plants from very early stages onwards. Surprisingly, instead of suspensor-derived secondary embryos, which would be arranged in tandem as reported previously, we observed young twin embryos

that were arranged side-by-side and thus appeared not to be suspensor-derived (Figures 1A-B). To corroborate that loss of *AMP1* function is causal for this early twin-embryo phenotype, we analyzed two more *amp1* alleles: *amp1-13*, another T-DNA allele, and the EMS-induced allele *amp1-1*, carrying a premature stop codon. Like *amp1-10*, *amp1-13* appears to be a null mutant (unpubl. data; [13]). Indeed, the other two alleles showed the same twin-embryo phenotype in fertilized ovules (Figures1C-D), although at somewhat different frequencies (Figure 1E). Since the early twin-embryo phenotype of *amp1-1* was rescued by two genomic *AMP1* constructs, *gAMP1* (0 twin-embryo pairs in 303 ovules) and *gAMP1:3xGFP* (0 twin-embryo pairs in 704 ovules), we concluded that lack of *AMP1* was causative for the early twin-embryo phenotype. However, ovules containing twin embryos aborted at early stages such that twin embryos did not develop beyond the early-globular stage of embryogenesis (Figures S1A-C). This was likely linked to the fact that 95% (n=111) of ovules containing twin embryos clearly lacked endosperm, which indicated that the supernumerary embryo was formed at the expense of central cell fertilization. However, 19 of 265 fertilized ovules containing twin embryos showed autonomous central cell divisions (Figures S1D-F). To discern possible parental effects for the early twin-embryo phenotype, we performed reciprocal crosses between wild-type and *amp1-10* mutant plants. While pollination of homozygous *amp1-10* plants with wild-type pollen resulted in twin embryos at a similar frequency as in the case of self-pollinated homozygous *amp1-10* mutant plants, no twin embryos were observed when wild-type plants were pollinated with *amp1-10* pollen (Figure1E). To trace back this maternal defect, ovules of emasculated *amp1-10* flowers were analyzed. Often two or three cells with the nucleus at the egg cell position were observed, instead of only one as in wild-type embryo sacs (Figures 1 F-H). And in line with this result, the egg cell markers *pEC1.1::HTA6:3xGFP* and *gAT2G21740 (EC1.2):3xGFP* [14] were often expressed in two or even three cells in *amp1-10* mutant embryo sacs whereas no supernumerary putative egg cells were observed in wild-type (Figures 2A-E and Figures S2A-D). Since the total number of cells at the micropylar end was not changed in *amp1* female gametophytes as compared to wild-type (Figures S1G-H''), the additional putative egg cell(s) must have been generated at the expense of some other cell(s). As the synergids usually flank the single egg cell they were prime candidates for

such a fate substitution. Indeed, this idea was supported by the expression of the synergid marker *pNTA>>nTdtomato* (based on [15]). While in wild-type almost always two cells expressed this marker and the fluorescently labeled nuclei were positioned at the micropylar end of the cells, five different categories were distinguished in *amp1-10*: (i) embryo sacs showed wild- type-like synergid marker expression; (ii) two cells expressed the synergid marker, but in one cell the nucleus was shifted to an egg cell nucleus-like (ECL) position; (iii) two cells expressed the synergid marker and in both cells the nucleus was shifted to an ECL position; (iv) only one cell expressed the synergid marker; (v) no cell expressed the synergid marker (Figures 2F-K and Figure S2E). These varied effects on synergid marker expression and nucleus position suggested that gametophytic cells destined to be synergids can adopt egg cell fate. To experimentally examine this idea, we analysed *amp1-10* mutant ovules for expression of both the egg cell marker *pEC1.1::HTA6:3xGFP* and the synergid cell marker *pNTA>>nTdtomato* (Figure 2L-M). The vast majority of wild-type ovules contain two nuclei expressing only the synergid marker at the micropylar end, in addition to one nucleus expressing the egg cell marker. In contrast, *amp1-10* mutant ovules displayed eight different categories of expression patterns and nuclear positions, with approximately 40% of these ovules harboring one or two nuclei towards the micropylar end that expressed both the egg cell and the synergid marker (categories II, III, IV and VI; Figure 2L,M). Quantitative analysis of the single marker line *pEC1.1::HTA6:3xGFP* revealed that in 26% of the ovules that expressed the egg cell marker, there was at least one nucleus at the micropylar end expressing that marker (Figures S2B-C). These results demonstrate that indeed the supernumerary putative egg cell(s) derive from transformed or mis-specified synergids that might still retain the characteristic position of the nucleus at the micropylar end. Taken together, the above results indicated that *AMP1* is required to prevent synergids from taking on egg cell fate. The persistent synergid marker *pNTA>>nTdtomato* was occasionally detected not only in nuclei at the egg cell position but – due to the stability of the fluorescent protein – also in one of the twin embryos (9.9%, n = 378 fertilized ovules) (Figures 3A-C) in contrast to wild-type embryos (not shown), clearly demonstrating that converted synergids when fertilized gave rise to embryos and were therefore fully functional egg cells. That supernumerary putative egg cells did not

autonomously undergo embryo development without fertilization was inferred from the observation that both embryos of the same twin pair in all GFP-expressing ovules (n = 36 twin pairs) expressed the paternally introduced early embryo marker *pS4::nGFP* (Figure S3A and Figures 3D-G). To discern which *amp1* mutant ovules were preferentially fertilized we analyzed ovules with GFP expression of the egg cell marker in wild-type and *amp1-10* (Figure S2D). Before fertilization, ovules displayed one, two or three putative egg cells in roughly equal numbers. This distribution was changed after fertilization because the vast majority of *amp1-10* ovules containing three putative egg cells remained unfertilized whereas the other categories of *amp1* ovules were preferentially fertilized (Figure S2D, compare left with right). These results were supported by the observation that only 50% of the *amp1-10* ovules were fertilized and of those only about 20% (n=623) contained twin embryos (Figure S2F). Thus, at least one cell with synergid properties appears to be required for successful fertilization.

Even though the above mentioned lack of endosperm as well as the lack of central cell fertilization in the case of early twin embryos (Figures 3H-K and Figure S3B) already suggested that sperm cells from only a single pollen tube fused with female gametes in *amp1*, we performed a mixed-pollination experiment to distinguish sperm from different pollen tubes. A mixture of pollen carrying one or the other of the two embryo markers *pATML1::n3xGFP* and *pARF13>>nTdtomato* (Figure S3C) was used for pollinating *amp1-10* mutant plants. All the twin-embryo pairs examined (n = 22) expressed only one or the other of the two fluorescent markers (not shown), which indicated that each twin-embryo pair originated from the pair of sperm delivered by a single pollen tube. Thus, embryo pairs in *amp1* mutants were genetically identical dizygotic twins.

Abortion of ovules with twin embryos should be overcome by delivering more than two sperm cells with a single pollen tube to achieve triple fertilization. This idea was based on the following observations: (i) central-cell marker expression was not changed in *amp1* (Figures S3D-G); (ii) a supernumerary putative egg cell persisted in *amp1* fertilized ovules containing an embryo and endosperm (Figures 3L-N: 16 of 125 ovules; Figure S3H-I: 12 of 130 ovules), which indicated no principal problem with central cell fertilization in *amp1* embryo sacs

containing two egg cells. Pollen of the *tetraspore (tes)* mutant often contain more than two

sperm cells [3]. In contrast to self-pollinated *amp1* or pollination of *amp1* with wild-type pollen,

pollination of *amp1* with *tes* pollen strongly decreased the percentage of endosperm absence

in ovules containing twin embryos (Figure 4I). Moreover, pollination of *amp1* with *tes* pollen

produced twin torpedo and bent-cotyledon stage embryos, which germinated as twin seedlings

to give rise to twin adult plants (Figure 4). Cell-cell communication has been proposed to play

a central role for cell fate specification in the Arabidopsis female gametophyte [4, 6]. We

therefore investigated whether *AMP1* acts cell-autonomously or rather non-cell autonomously

during synergid specification. The genomic *AMP1:3xGFP* fusion, which fully rescued the *amp1*

supernumerary egg cell and twin-embryo phenotypes, was strongly expressed in the

sporophytic tissue and the synergids, and weaker expression was sometimes detected in the

egg cell (Figure 1I). *AMP1* expression at earlier stages of ovule development was only

detected in the sporophytic tissue (Figure 1J). Given the strong *AMP1* expression in

sporophytic tissue, we explored whether that expression contributed to proper synergid fate

specification. Interestingly, *amp1* heterozygous plants only very rarely produced twin embryos

(1 case in 469 ovules) and supernumerary egg cells (1 case in 121 ovules), indicating that

sporophytic *AMP1* expression was principally able to mediate synergid fate specification. This

finding was corroborated by the rescue of both mutant phenotypes in 10 transgenic lines

expressing *AMP1* from the *35S* promoter which is active in the surrounding sporophytic tissue

but not in the female gametophyte itself ([16, 17]; Figures S3J-K; 0 twin-embryo pairs in 358

ovules). Both phenotypes were also rescued in 19 transgenic lines expressing *AMP1*

specifically in the synergids, using the *NTA* promoter ([15]; 0 twin-embryo pairs in 132 ovules).

Intriguingly, both mutant phenotypes could also be rescued by expressing *AMP1* in the

neighboring central cell (24 transgenic lines, 0 twin-embryo pairs in 351 ovules) and in the egg

cell (25 transgenic lines, 0 twin-embryo pairs in 426 ovules). Thus, synergid specification

requires an AMP1-dependent signal that is likely mobile and can be provided by neighboring

cells including the sporophytic tissue of the ovule. There are distinct features that set *amp1*

mutants apart from previously reported mutants with compromised synergid identity such as *lis*

on which the lateral inhibition model for gametophytic cell fate identity was based [4]. Unlike

lis, *amp1* embryo sacs contained fully functional primary egg cell and supernumerary putative egg cell that gave rise to twin plants if supernumerary sperm were provided. Furthermore, *lis* is a gametophytic mutant, *LIS* was strongly expressed in egg cell and central cell, and egg cell expression was essential for synergid development [4,6]. In contrast, the dizygotic twin and supernumerary egg cell phenotypes of *amp1* mutant can be rescued by sporophytic contribution of *AMP1* expression, which suggests that the AMP1-dependent signal for promoting or maintaining synergid cell fate can be provided by the gametophyte-surrounding maternal tissue. Thus, synergid fate might not simply be the result of preventing egg cell fate by lateral inhibition among the gametophytic cells at the micropylar end, but the outcome of a distinct process also involving input from the surrounding sporophytic tissue. How *AMP1* might contribute to the production of a synergid-promoting signal is not clear at present. *AMP1* has been discussed to function as a glutamate carboxypeptidase, possibly influencing cytokinin levels or modulating levels of signaling molecules [18-20]. However, the expression of the sensitive synthetic cytokinin sensor *TCSn::GFP* [21] was not altered in *amp1* compared to wild-type ovules (Figures S3L- M). More recently, *AMP1* has been localized to the ER and implicated in miRNA-mediated translational inhibition [2]. Whatever its exact molecular function, where in the ovule *AMP1* is expressed appears not to be critical, suggesting that *AMP1* mRNA or protein might move between cells or be required for the production of a likely mobile signal for synergid identity.

Our analysis of the twin-embryo phenotype of *amp1* mutants also sheds light on the boundary conditions for double fertilization in plant reproduction, which involves two sperm cells and the four cells at the micropylar end of the female gametophyte: two synergids, one egg cell and one central cell. Ovules with twin embryos but no endosperm as well as ovules with one developing embryo and endosperm plus one persisting unfertilized egg cell strongly suggest that the two sperm cells of a fertilizing pollen tube are free to choose their mating partners. This settles the controversial issue of potential mating preferences, which has largely been addressed by manipulating sperm cells [22-26] and a mutant in which specifically the central cell is not fertilized [27]. The occurrence of twin embryos without endosperm in *amp1* ovules also has implications regarding the number of synergids, which are required for pollen tube

attraction [28]. Their number varies between species [29]. One synergid is sufficient for pollen tube attraction such that any other synergid in the same ovule needs to be eliminated actively in order to prevent fertilization by another pollen tube [28,30]. Our study now suggests that this rather cumbersome procedure might nonetheless have been selected for in evolution because the alternative – two egg cells and one synergid at the micropylar end – reduces the probability of successful reproduction.

Supplemental information

Supplemental Information includes three figures, Figure legends, Supplemental Experimental Procedures and Supplemental References, and can be found with this article online at XXX.

Author contributions

J.K. conducted the experiments, all three authors designed the study, analysed the data and wrote the manuscript.

Acknowledgments

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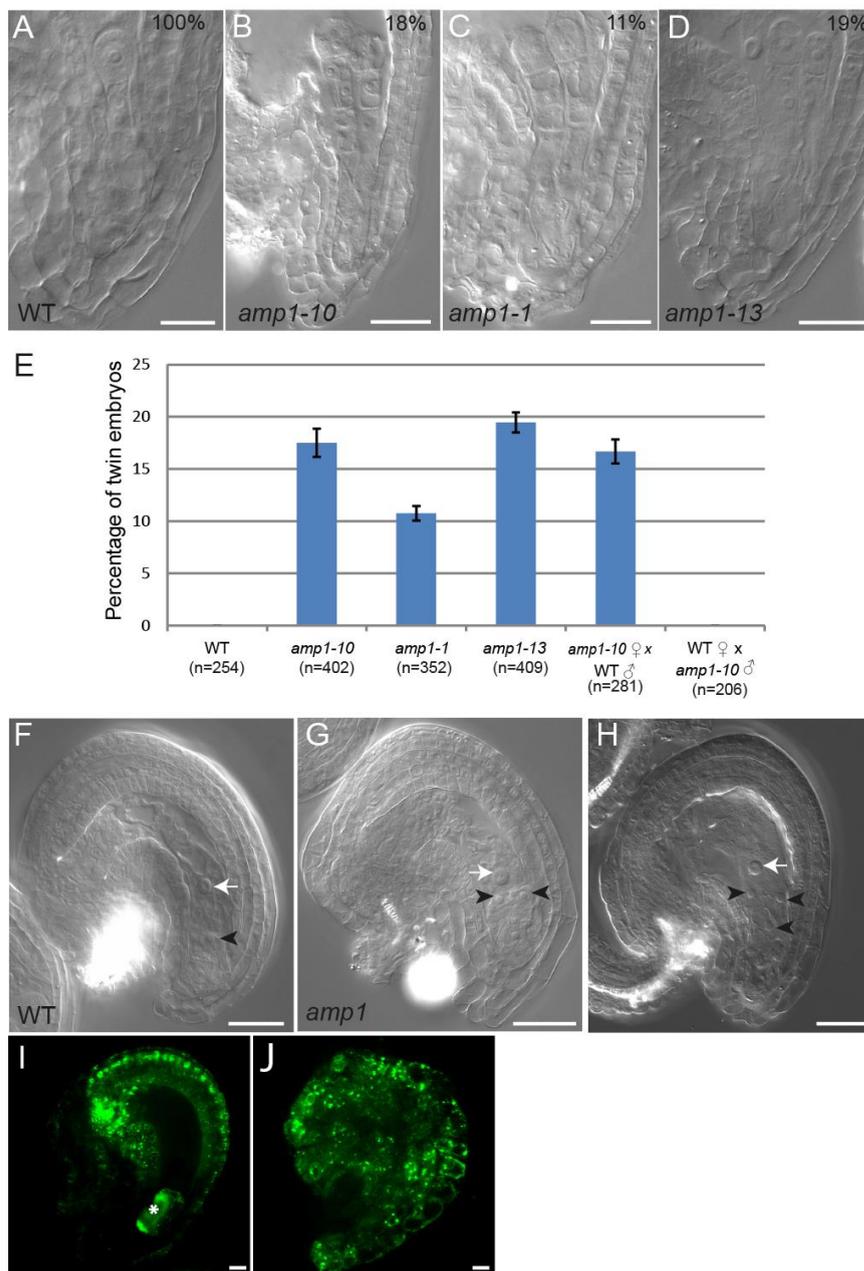
Figure 1

Figure 1. Twin embryos and supernumerary putative egg cells in *amp1* mutants

(A-E) Developing embryos. Single embryo in a wild-type (WT) ovule (A), twin embryos in *amp1-10* (B), *amp1-1* (C) and *amp1-13* (D) mutant ovules. (E) Frequency of twin embryos (expressed as percentage of fertilized ovules) in WT, *amp1* and reciprocal crosses (mean ± SD). (F-H) Unfertilised ovules. One egg cell in WT (F), two or three putative egg cells in *amp1-10* (G and H). Black arrowhead, egg cell-like nucleus; white arrow, central cell nucleus. (I, J) *gAMP1:3GFP* expression in mature (I) and developing (J) ovule; asterisk, synergid. Scale bars, 25 µm (A-H), 10 µm (I, J). See also Figure S1.
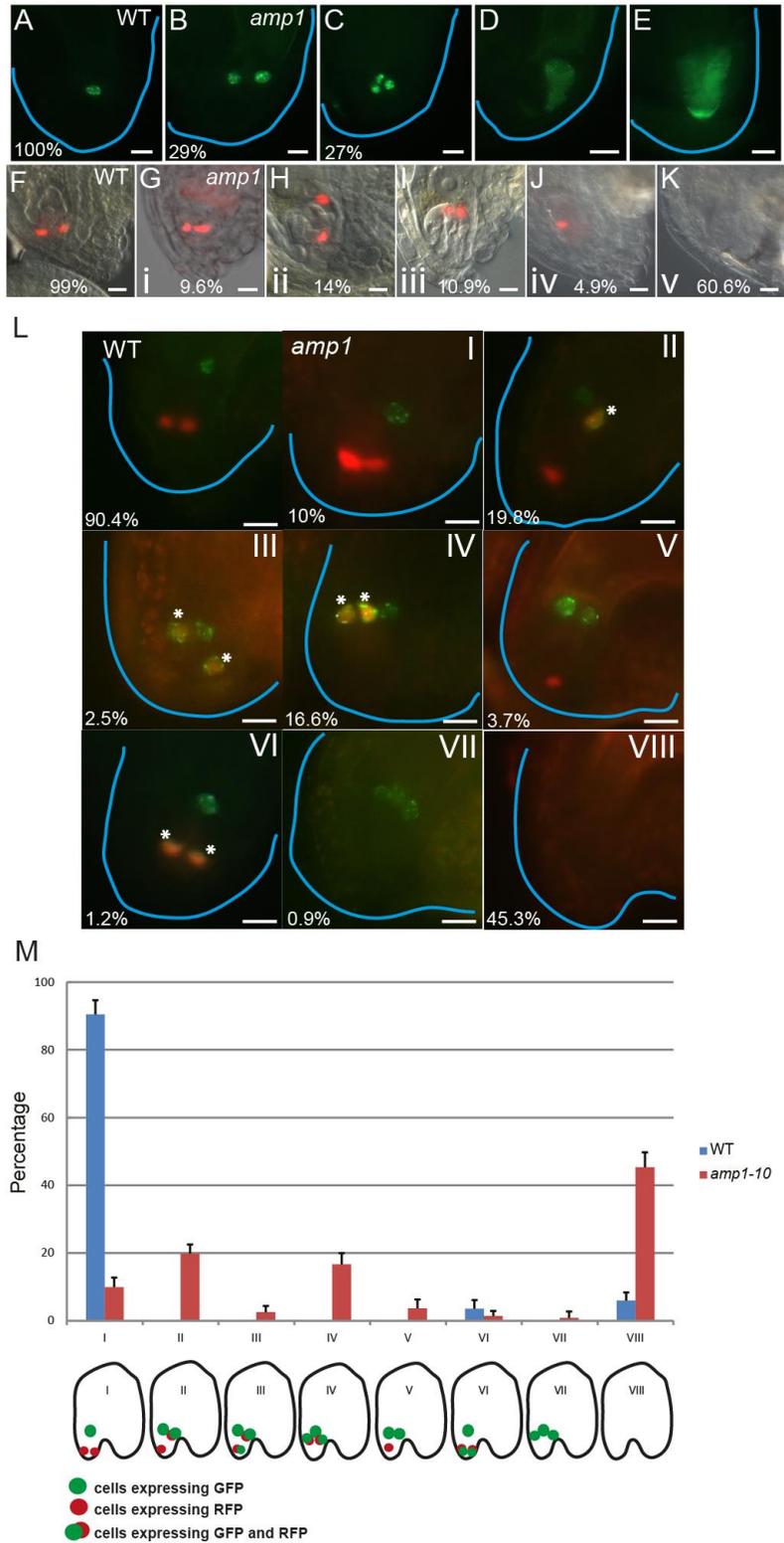
Figure 2

Figure 2. Synergids expressing egg cell marker in *amp1* mutant ovules

(A-E) Egg cell marker expression in wild-type (WT) and *amp1-10* (ovules outlined in blue).

(A-C) *pEC1.1::HTA6:3GFP* in WT (A) and *amp1-10* (B-C); (D-E) *gAt2g21740(EC1.2):3GFP*

in WT (D) and *amp1-10* (E). (F-K) Synergid marker (*pNTA>>nTdtomato*) expression and

nuclear position in WT (F) and *amp1-10* (G-K). (L) Expression of *pEC1.1::HTA6:3GFP* and *pNTA>>nTdtomato* in WT and *amp1-10* (ovules outlined in blue). Asterisks, co-expression of both markers. (M) Quantitative analysis of co-expression of egg cell marker and synergid marker (mean±SD; n=445 for WT and n=480 for *amp1-10*); categories are the same as in (L). Scale bars, 10 µm. See also Figure S2.
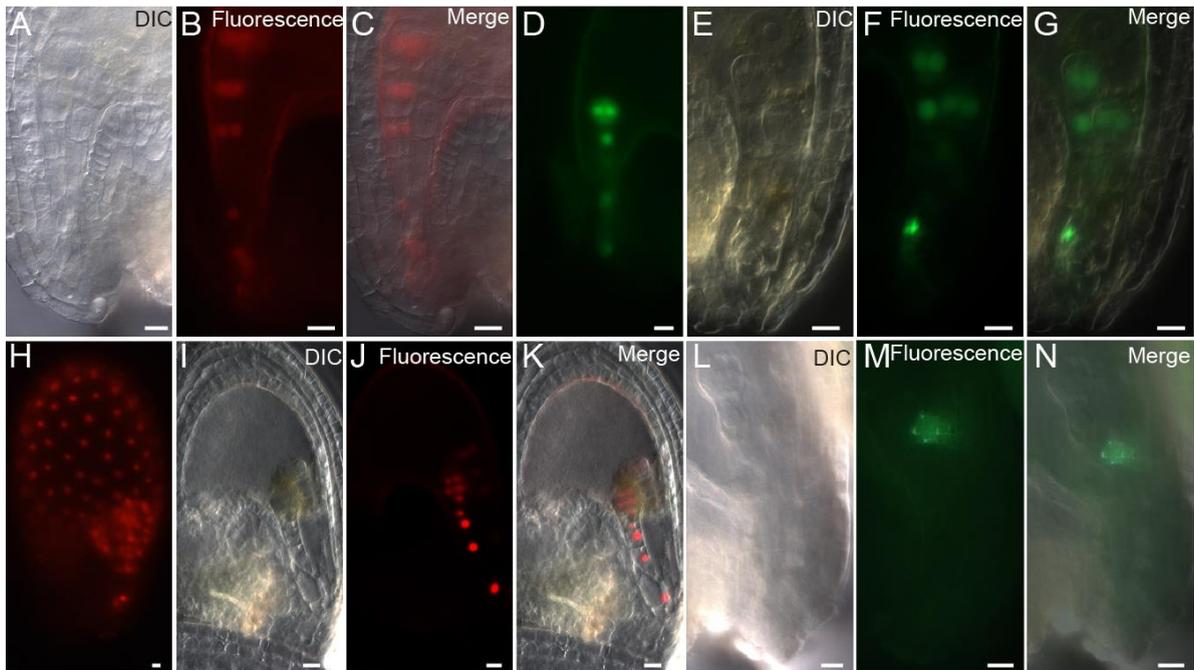

Figure 3

Figure 3. Supernumerary putative egg cells can be fertilized to give rise to embryos

(A-C) Persistence of the synergid marker *pNTA>>nTdtomato* in one of the twin embryos in *amp1-10* ovule. (D-G) Twin embryos are fertilization products. (D) Wild-type (WT) ♀ x *pS4::nGFP* ♂; (E-G) *amp1-10* ♀ x *pS4::nGFP* ♂. (H-K) No endosperm formation in ovule containing twin embryos. (H) WT ♀ x *pRPS5A>>nTdtomato* ♂, (I-K) *amp1-10* ♀ x *pRPS5A>>nTdtomato* ♂. (L-N) Egg cell marker *pEC1::HTA6:3GFP* in unfertilized egg cell next to non-fluorescent developing embryo in *amp1-10* ovule. Scale bars, 10 µm. See also Figure S3.
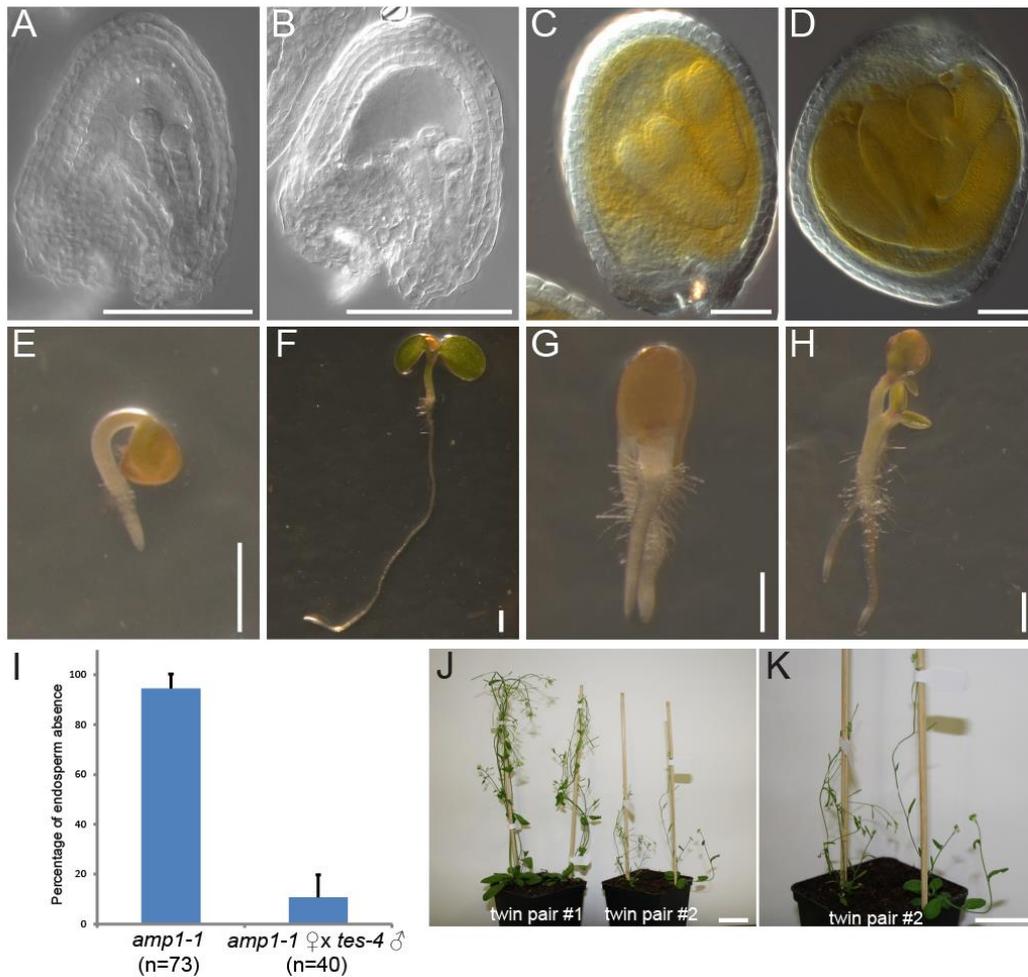
Figure 4

Figure 4. Twin seedling and plant formation after pollination of *amp1* ovules with *tes-4* pollen

(A-D) Cleared ovules of selfed *amp1* (A), *amp1-1* ♀ x Ws ♂ (B) and *amp1-1*♀ x *tes-4* ♂(C, D). Scale bars, 0.1 mm. (E-H) Germinated F1 seedlings from (E,F) *amp1-1* ♀ x Ws ♂. (G,H) *amp1-1* ♀ x *tes-4* ♂. Ws, Wassilewskija wild-type. (I) Reduced frequency of endosperm absence by fertilization with supernumerary sperm. (n, total number of ovules containing twin embryos; mean ± SD). (J-K) Adult twin plants. (J) Two independent twin pairs. (K) One twin

of pair #2 appears weaker than the other. Scale bars, 0.05 cm (E-H), 2 cm (J, K).
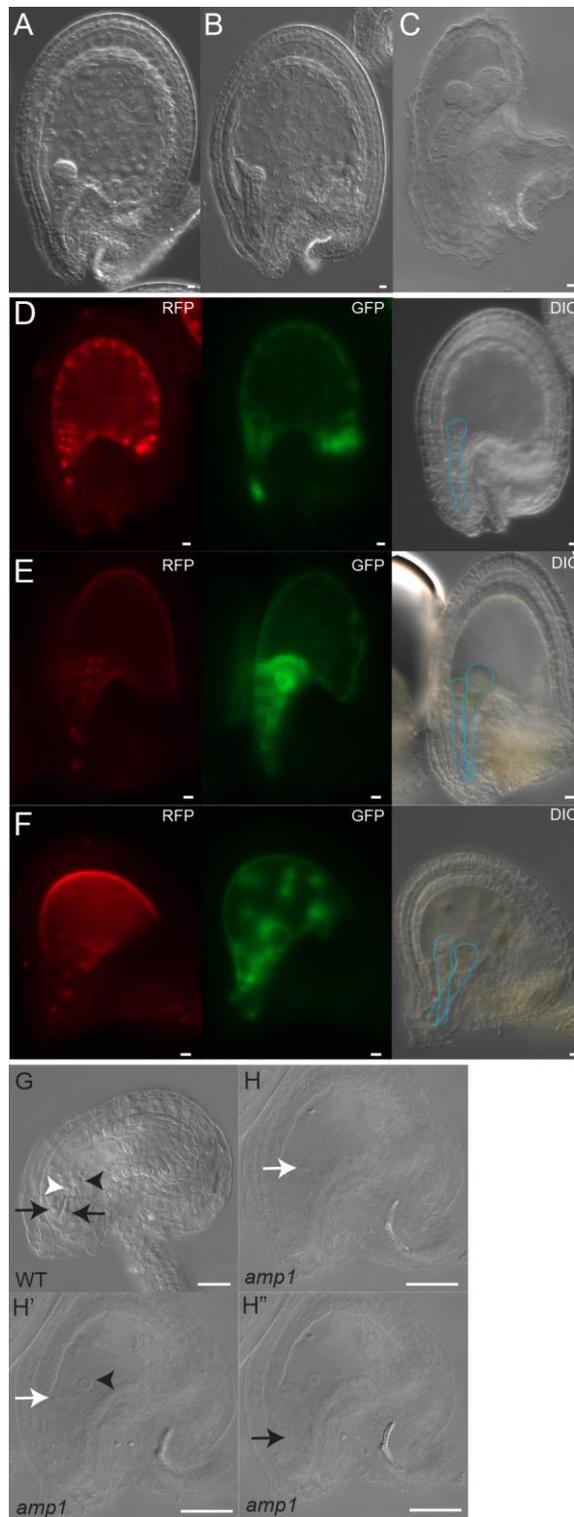
Figure S1

Figure S1 related to Figure 1. Developmental arrest of twin embryos in absence of endosperm development and the total number of synergids and egg cells in *amp1* ovules.

(A-C) Post-fertilization development. Wild-type (WT) embryo at early-globular stage (A), *amp1-10* ovule containing single embryo (B) and developmental arrest of *amp1* ovule containing twin

embryos (C). (D-F) Endosperm and embryo development. (D) Single embryo with endosperm development in WT; (E,F) twin embryos in *amp1-1* ovules with (E) persisting central cell and (F) central cell autonomous divisions. *pMEA::3xGFP amp1-1* ♀ **×** *pRPS5A>>nTdtomato* ♂. Green, central cell marker *pMEA::3xGFP*; red, endosperm and embryo marker *pRPS5A>>nTdtomato*; blue, embryo outline. (G-H'') WT embryo sac (G) and *amp1-10* embryo sac at different focal planes (H-H''). Black arrowhead, central cell nucleus; white arrowhead, egg cell nucleus; white arrow, egg cell-like nucleus; black arrow, synergid nucleus. Scale bars, 10 µm.
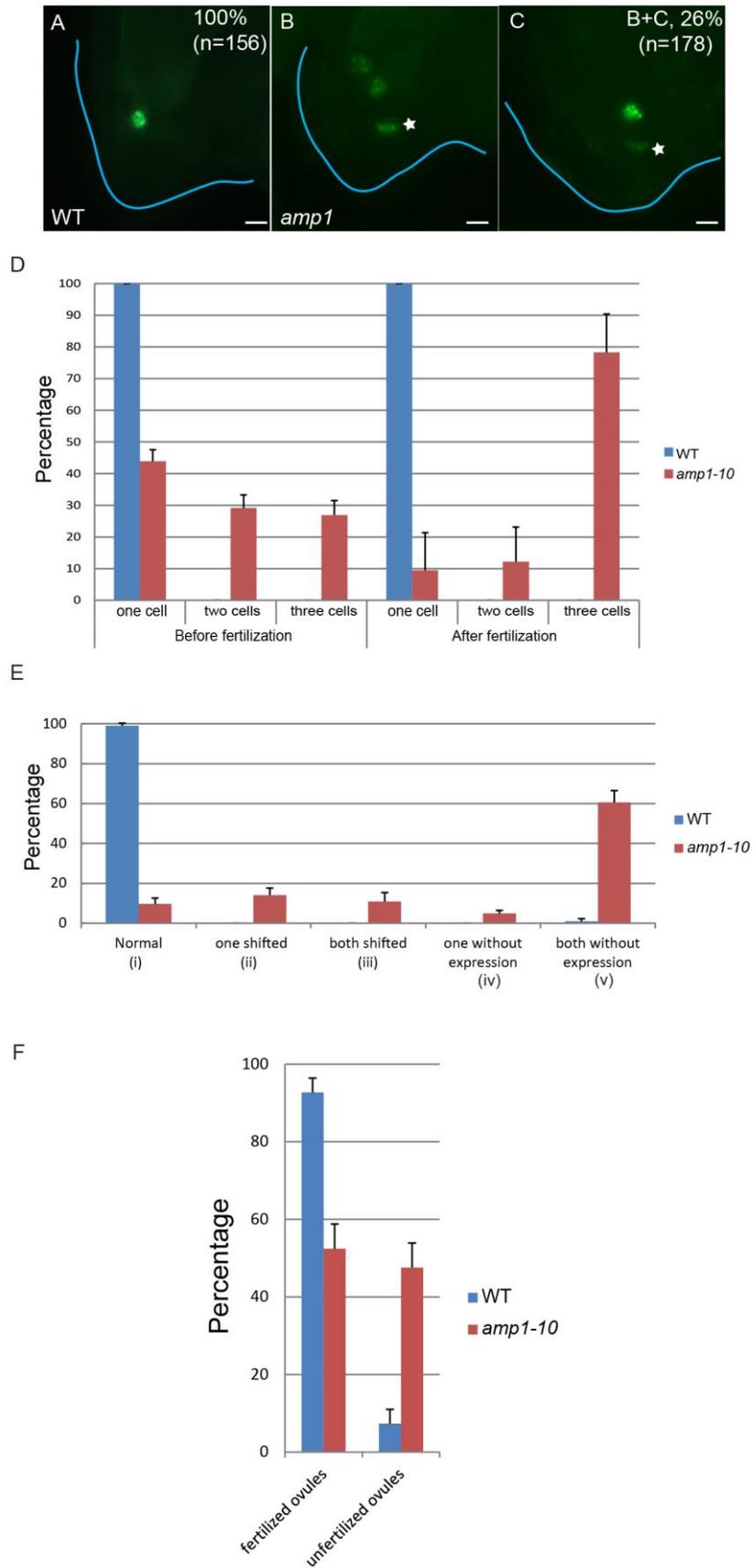
Figure S2

Figure S2 related to Figure 2. Ovule abortion correlates with synergids expressing egg cell marker.

(A-D) Expression of egg cell marker *pEC1.1::HTA6:3xGFP*. (A) Wild-type (WT), (B,C) *amp1-10* (star, putative egg cell nucleus at the synergid nucleus position; ovules outlined in blue); (D) quantification of unfertilized ovules expressing egg cell marker before and after fertilization (mean ± SD; before fertilization: n=193 for WT and n=88 for *amp1-10*; after fertilization: n=41 for WT and n=122 for *amp1-10*). (E) Quantitative analysis of synergid marker expression shown in Figure 2F-K (mean ± SD; n=294 for WT and n=956 for *amp1-10*). (F) Quantitative analysis of ovule abortion in WT and *amp1-10* (mean ± SD; n=474 for WT and n=1123 for *amp1-10*). Scale bars, 10 µm.
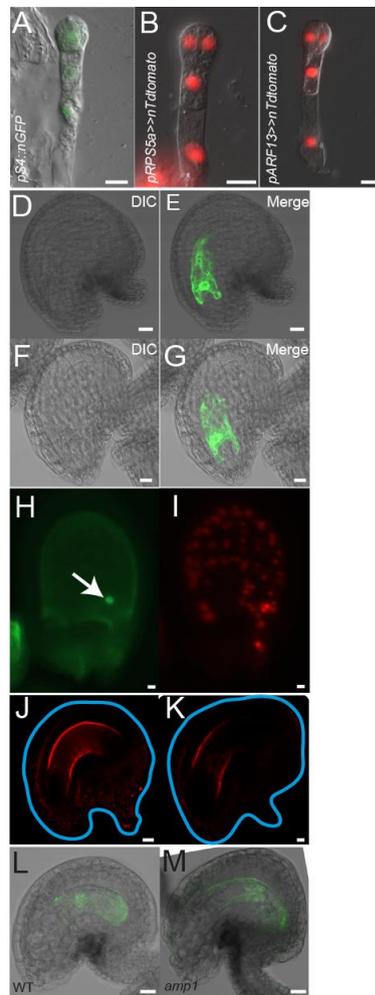
Figure S3

Figure S3 related to Figure 3. Fluorescent-reporter expression in embryos and embryo sacs of *amp1* ovules.

(A-C) Embryonic markers expressed in WT: (A) *pS4::nGFP*, (B) *pRPS5a>>nTdtomato*, (C) *pARF13>>nTdtomato*. (D-G) Central-cell marker *pMEA::3xGFP* expressed in wild-type (WT) (D, E) and *amp1-1* (F, G) ovules. (H, I) Persisting egg cell (H; white arrow, GFP signal) in ovule with embryo and endosperm (I; RFP signals) (9.5%, n=130). *pEC1.1::HTA6:3xGFP* (egg cell marker) *amp1-10* ♀× *pRPS5A>>nTdtomato* (embryo and endosperm marker) ♂. (J,K) *p35S>>nTdtomato* expression in ovule at maturity (J); non-transgenic WT ovule at maturity as control (K) (ovules outline in blue). (L, M) cytokinin sensor *TCSn::GFP* expression in WT (L) and *amp1-10* (M) ovules. Scale bars,10 µm.

Supplemental experimental procedures

Plant material and growth conditions

All the Arabidopsis (*Arabidopsis thaliana*) lines used in the experiments were of the Col-0 ecotype except for Ws and *tes-4*, which is in the Ws background. The *amp1* mutant alleles used were *amp1-1*, *amp1-10* (SALK_021406) and *amp1-13* (SALK_022988). *tes-4* (N9353) was obtained from Nottingham Arabidopsis Stock Centre (NASC). The marker lines *pNTA>>nTdtomato*, *pARF13>>nTdtomato* and *pRPS5A>>nTdtomato* were kindly made available by Martin Bayer(MPI for Developmental Biology, Tübingen). *pATML1::n3xGFP* was described previously [1]. Plants were grown at 22°C to 24°C in a growth chamber under a 16-h/8-h light/dark cycle. For growth under sterile conditions, seeds were surface-sterilized and grown on half-strength Murashige and Skoog (MS) agar plates containing 10g l^{-1} sucrose.

Molecular cloning and plant transformation

For complementation, a 4525 bp genomic fragment including promoter and 3'UTR of *AMP1* was PCR-amplified using primers *gAMP1s* and *gAMP1as* and sub-cloned into the *pGEM-T* vector (Promega). The fragment was finally introduced into *pGIIKantNos* [2] to yield *gAMP1*. To generate the *gAMP1:3xGFP* fusion construct, a 4396bp genomic fragment without the stop codon of *AMP1* was PCR-amplified using *gAMP1s* and *gAMP1as2* primers and sub-cloned into *pGEM-T*, then the fragment was introduced into *pGIIKan3xGFP* [3].

For the rescue constructs, the *AMP1* CDS was PCR-amplified using *AMP1*(cds) s and *AMP1* (cds) as primers and sub-cloned into *pGEM-T*. Then the *AMP1* CDS was once more PCR- amplified using *AMP1* (cds) s and M13F primers. Eventually, the fragment was introduced into *pGIIKantNos* (26). *pNTA* was PCR-amplified using primers *pNTAs* and *pNTAas* and sub-cloned into *pGEM-T*. The *CaMV 35S* promoter was kindly provided by Martin Bayer. Finally, the two promoters were introduced into *pGII KanAMP1CDS*.

For the *pEC1::HTA6:3xGFP* construct, the *HTA6* CDS without stop codon was PCR-amplified using primers *HTA6s* and *HTA6as* and introduced into *pGIIKan3xGFP* [3] to result in *pGIIKanHTA6:3xGFP* vector, and *pEC1* was PCR-amplified using primers *pEC1s* and *pEC1as* and sub-cloned into *pGEM-T*. Then *pEC1* was cut out of *pGEM-T-pEC1* and introduced into *pGIIKanHTA6:3xGFP*.

To generate *gAt2g21740:3xGFP*, a 1378 bp genomic fragment without stop codon was PCR- amplified using *gAt2g21740s* and *gAt2g21740as* and introduced into *pGIIKan3xGFP* [3].

For the *pS4::nGFP* construct, an *At3g10100* promoter fragment was PCR-amplified using primers *pS4s* and *pS4as* and sub-cloned into *pGEM-T*. Then *pS4* was cut out from *pGEM-T* and introduced into *pGIIKann3xGFP* [3] what resulted in *pS4::nGFP*.

To generate *pMEA::3xGFP*, *pMEA* was PCR-amplified using primers *pMEAs* and *pMEAas* and sub-cloned to *pGEM-T*. Then *pMEA* was cut out from *pGEM-T* and introduced into *pGIIKan3xGFP* [3].

Plant transformations were performed by floral dip as described previously [4]. Transformants were screened on half-strength MS agar containing either 15mg l^{-1} phosphinothricin or 62.5mg l^{-1} kanamycin.

For each rescue experiment, three independent homozygous plant lines were analyzed.

Microscopy

For differential interference contrast (DIC) microscopy, ovules from approximately 5 days after fertilization were mounted in clearing solution containing chloral hydrate, water, and glycerol (ratio w/v/v: 8:3:1) or Hoyer's solution for scoring twin-embryo phenotype and analyzing marker expression in the twin embryos. For the analysis of female gametophytes, flowers were emasculated and 24 hours later, ovules were mounted in Hoyer's solution.

For fluorescence analysis, ovules were mounted in 10% glycerol (v/v) and embryos were dissected from the ovules. Confocal microscopy analysis was carried out by an Olympus IX81 confocal laser scanning microscope (image acquisition software: FV10-ASW; objectives: UPlanSApo x40). Images were further processed using Adobe Photoshop and Illustrator software. Zeiss Axio Imager (image acquisition software: AxioVision; camera: AxioCam HRc; objectives: Plan-APOCHROMAT 20x, 40x and 63x oil were used for wide-field and DIC images). Seedlings were observed using SteREO Zeiss Discovery V.20 (image acquisition software: AxioVision; camera: AxioCam Rev 3).

Genotyping

dCAPS genotyping primers were designed by dCAPS finder 2.0
(http://helix.wustl.edu/dcaps/) and primers for T-DNA insertion lines were
designed by http://signal.salk.edu/tdnaprimers.2.html.
Primer sequences used in this study are as follows:

Oligo name	Sequence (5'–3')
gAMP1s	GGTACCAGAAGAAAGGAAGAGGAGGGA
gAMP1as	CTCGAGGTCTTGAATATGATTAGATTAT
gAMP1as2	CTCGAGTGTGAAACCTCCTTTAAGAGCT
AMP1(cds) s	CTGCAGATGTCACAACCTCTCACCACCA
AMP1(cds) as	GGATCCTCATGTGAAACCTCCTTTAAGA
M13F	CGCCAGGGTTTTCCCAGTCAC
pNTAs	CGGGGTACCGCCAGGTACTACTAAGACGAG
pNTAas	CCCAAGCTTGAGTGAAGGAAATGAGAGGTG
HTA6s	GAATTCATGGAATCCACCGGAAAAGTG
HTA6as	GGATCCAGCTTTCTTTGGAGACTTGACTG
pEC1s	AAAACTGCAGTGCCTTATGATTTCTTCGGTTT
pEC1as	CGGGGTACCTTCTCAACAGATTGATAAGGTCGAAA
gAt2g21740s	AAACTCGAGTAAATGTTCCTCGCTGACG
gAt2g21740as	AAAGGATCCAAGTTTCACAGAGGAAGGC
p35s (s)	CGGGGTACCGCCCCAGATTAGCCTTTTCAAT
p35s (as)	CCCAAGCTTTCCCCCGTGTTCTCTCCAA
pS4s	AAACTGCAGGGTTTCGTGAGAGAGGACT
pS4as	AAAGGATCCTGCCGGAAAAATCGATTAAG
amp1–1 dCAP s	TTTCTATATTTATCAGTGGCTGGAACTCG
amp1–1 dCAP as	CTTTCCCTTCTAAGAGCTTGCTCAG
pMEAs	CGGGGTACCAATAGGTCGAGAAAATGCTGT
pMEAas	ACGCGTCGACTAACCACTCGCCTCTTCT
LBb1.3 Left border primer for Salk lines	ATTTTGCCGATTTCGGAAC
amp1–10 genotyping primer sense	CGCTGGGAGTGCTAATATACG
amp1–10 genotyping primer antisense	GTCTATAGACGGTATCCCGGC

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7.2 Cell type-specific transcriptome analysis in the early Arabidopsis thaliana embryo

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Running title: Arabidopsis early embryo profiling

ABSTRACT

In multicellular organisms, cellular differences in gene activity are a prerequisite for differentiation and establishment of cell types. In order to study transcriptome profiles, specific cell types have to be isolated from a given tissue or even the whole organism. However, whole-transcriptome analysis of early embryos in flowering plants has been hampered by their size and inaccessibility. Here we describe the purification of nuclear RNA from early stage *Arabidopsis thaliana* embryos using fluorescence-activated nuclear sorting (FANS) to generate expression profiles of early stages of the whole embryo, the proembryo, and the suspensor. We validated our datasets of differentially expressed candidate genes by promoter-reporter gene fusions and *in situ* hybridization. Our study revealed that different classes of genes with respect to biological processes and molecular functions are preferentially expressed either in the proembryo or in the suspensor. This method especially can be used for tissues with a limited cell population and inaccessible tissue types. Furthermore, we provide a valuable resource for research on Arabidopsis early embryogenesis.

KEY WORDS: Fluorescence-activated nuclear sorting, Proembryo, Suspensor, Transcriptome analysis

Abbreviation	Full name
FACS	Fluorescence-activated cell sorting
FANS	Fluorescence-activated nuclear sorting
LCM	Laser capture microdissection
TRAP	Translating ribosome affinity purification
INTACT	Isolation of nuclei tagged in specific cell types
PE	Proembryo
SUS	Suspensor
EMB	Whole embryo
nEMB	Nuclei from whole embryo
nPE	Nuclei from proembryo
nSUS	Nuclei from suspensor
cgPE	Cellular globular-stage proembryo
cgSUS	Cellular globular-stage suspensor
cgSEED	Cellular globular-stage entire seed excluding embryo
cEMB	Cellular whole embryo
cKAN1	Cellular KANADI 1 expression domain adult shoot

INTRODUCTION

Multicellular organisms are made up of various cell and tissue types consisting of differentiated cells which all derive from pluripotent, undifferentiated progenitor cells. Since these cell and tissue types fulfill a plethora of different functions during the life cycle, progenitor cells have to undergo coordinated changes in spatial and temporal gene expression programs during differentiation. Comprehensive characterization of transcriptional profiles is therefore of great importance to understand the establishment and maintenance of specific cell types. In the case of embryogenesis in flowering plants with the embryos often being deeply embedded in the maternal seed tissue, however, the isolation of cells from specific cell types is already a very challenging task. In general, several existing methods have been employed to overcome such difficulties for different tissues and organisms, such as laser capture microdissection (LCM), fluorescence-activated cell sorting (FACS), translating ribosome affinity purification (TRAP), and isolation of nuclei tagged in specific cell types (INTACT) (Bonner et al., 1972; Emmert-Buck et al., 1996; Heiman et al., 2008; Deal and Henikoff, 2010). At present TRAP and INTACT are still under optimization in order to be widely used for special tissues such as those in plant embryos (Palovaara et al., 2013). LCM has been used in different studies to isolate tissues from sectioned material without the need of generating transgenic plants (Kerk et al., 2003). Recently, parts of different tissues inside the *Arabidopsis thaliana* seed including the embryo were isolated by LCM and the different expression profiles were analyzed (Spencer et al., 2007; Le et al., 2010). Nonetheless, LCM requires high precision during tissue excision in order to avoid contamination from adjoining cells. Additionally, since the used material originates from tissue sections, only parts of the cell can be effectively collected. Consequently, precise isolation of certain cell types, such as shoot apical meristem cells, which are deeply embedded within the embryo, is a considerable challenge. Evidently, FACS in combination with gene expression analysis has been broadly employed for many studies, such as purification of *Drosophila melanogaster* embryonic cell populations (Cumberledge and Krasnow, 1994; Shigenobu et al., 2006), clinical applications (Jayasinghe et al., 2006; Jaye et al., 2012), and isolation of different cell types in *Arabidopsis* root and shoot tissue (Birnbaum et al., 2003; De Smet et al., 2008; Yadav et al., 2014). Most of the FACS

studies in plants were based on the generation of protoplasts from easily accessible tissues and therefore this method is very difficult to apply to *Arabidopsis* embryos, in particular in large amount. In contrast, fluorescently labeled nuclei from the companion cells of phloem root tissue were isolated by fluorescence-activated nuclear sorting (FANS) for further transcriptome analysis (Zhang et al., 2008). Importantly, even though there are obvious differences between nuclear and cytoplasmic RNA composition, several reports showed that the diversity of nuclear and total cellular RNA is overall comparable (Steiner et al., 2012; Palovaara et al., 2013).

In light of specific advantages and disadvantages of the different techniques mentioned above, we combined fluorescent-activated sorting of nuclei (FANS) with linear RNA amplification and microarray analysis to characterize the transcriptomes of two cell types – the proembryo (PE) and suspensor (SUS) – in the early *Arabidopsis* embryo originating from a single cell – the zygote – as well as the whole embryo (EMB). Our strategy was to label nuclei with nuclear localized GFP (nGFP) driven by cell-type specific promoters only active either in the cells of the proembryo or the suspensor, or uniformly active in the whole embryo. GFP-positive nuclei were sorted by flow cytometry and afterwards standard ATH1 microarray chips were used for transcriptome analysis. Our analysis demonstrated that specific transcripts are differentially expressed between the proembryo and suspensor at early stages of embryogenesis, including genes that were previously reported to be differentially expressed *in vivo* (Lau et al., 2012). The datasets were further validated by promoter-reporter fusion analysis and *in situ* hybridization for a subset of genes that were preferentially expressed in one or the other cell type. Additionally, we also compared our nuclear whole embryo transcriptional profile with that of manually isolated, early-stage whole embryos as well as with publicly available data. In summary, we developed a robust method in order to generate comprehensive expression profiles of specific cell types in *Arabidopsis* early embryos. In particular, this method can be widely used for characterizing gene expression of deeply embedded cell types with a limited number of cells. In addition, we provided a comprehensive resource for the earliest stages and tissues of Arabidopsis development.

RESULTS
Identification of embryo-specific marker lines

In order to obtain marker lines that show specific expression during the early stages of *Arabidopsis* embryogenesis in the proembryo, suspensor, or whole embryo, we first screened the GAL4-GFP enhancer trap collection from the Haseloff lab (Haseloff, 1999). Tracing back expression from microscopic analysis of seedling roots, one of the Haseloff lines (N9322) showed specific suspensor expression and the insertion locus was identified by TAIL-PCR to position 610bp upstream of the *AT5G42203* coding sequence (supplementary material Fig. S1). We then cloned about 2kb upstream region including 5' untranslated region (5' UTR) sequences for both the neighboring *AT5G42200* and *AT5G42203* genes fused to n3xGFP in order to check whether one or the other of the two promoters could recapitulate the expression pattern of the enhancer trap line. Regarding the expression pattern of the different transgenic lines, the promoter containing the upstream region of the *AT5G42200* gene showed specific expression only in the suspensor from the embryonic 2-cell stage onward (Fig. 1A,B).

Second, according to published data, the *DORNROESCHEN (DRN)* gene (*AT1G12980*) was shown to be expressed exclusively in the proembryo until early globular stage (Chandler et al., 2007; Cole et al., 2009). Therefore, we cloned the upstream region of *DRN* together with its 3'UTR as was described before (Chandler et al., 2007). Indeed, the expression pattern for this construct in transgenic embryos fit the published data for *DRN* (Fig. 1C,D).

Finally, initial experiments to test our method (data not shown) indicated embryonic expression of the *AT3G10010* locus. Indeed, when we checked the expression pattern of the cloned promoter-reporter construct for *AT3G10010* it fit the expected embryonic expression (Fig. 1E,F).

FANS analysis and microarray results

The individual fluorescent marker lines showing specific expression in proembryo, suspensor, or whole embryo nuclei were subsequently used to generate cell type-specific nuclear transcription profiles of the early *Arabidopsis* embryo. Since we were not able to recover protoplasts from early embryonic stages due to the embryonic cell wall and cuticle being recalcitrant to enzymatic digestion, we developed a workflow that enabled us to efficiently extract nuclei from ovule tissue with embryonic stages ranging from 1- to 16-cell stages. We isolated ovules from young siliques and fixed

them with 4% paraformaldehyde in order to maintain nuclear integrity. Additionally, by fixing the cellular contents we made sure that the transcriptional status of the nuclei did not change during the subsequent extraction and separation steps. After nuclear extraction, approximately 1000 GFP-positive nuclei from ovules of about 100 siliques were purified for the different marker lines on average by flow cytometry (supplementary material Fig. S2). Pools of approximately 3000 GFP-positive nuclei were used for RNA extraction representing one biological replicate.

After RNA amplification and biotinylation, the transcriptome analyses were carried out in biological triplicates with a standard Affymetrix ATH1 genome array, which covers roughly 71% of the to date presumed 33602 total *Arabidopsis* genes (Lamesch et al., 2012). The MAS5 normalized probe set signals (supplementary material Table S1) were gcRMA (gene chip robust multi-array average) normalized and log2 transformed (supplementary material Table S2). When we compared microarray probe sets only detected as present (P) in the MAS5 normalization algorithm for raw values across all three replicates, they showed a chip coverage of 34, 32, and 25% for nEMB (nuclei from whole embryo), nPE (nuclei from proembryo), and nSUS (nuclei from suspensor), respectively. The lower coverage for the nSUS is due to the lower concordance in present (P), marginal (M), and absent (A) MAS5 calls between all three nSUS replicates compared to nPE or nEMB values (supplementary material Fig. S3). Nevertheless, there is substantial overlap of expressed genes designated as three times present (3xP) in the MAS5 calls between the three samples (supplementary material Fig. S4). We used the gcRMA values for correlation analysis of the biological replicates for nuclear transcriptomes from the whole embryo, proembryo, and suspensor, which showed high correlation with PCCs (Pearson correlation coefficient) ranging from 0.976 to 0.987 (supplementary material Table S3). Taken together, we detected a substantial number of genes that are active in the proembryo and/or the suspensor, as well as the whole embryo during *Arabidopsis* early embryogenesis.

Analysis of global expression profiles

To gain an initial overview of the general expression patterns of nPE and nSUS genes active during early *Arabidopsis* embryogenesis, we grouped the aforementioned MAS5 3xP array elements according to their gene ontology (GO) terms "biological

process" and "molecular function" retrieved from the Arabidopsis information resource website (TAIR, www.arabidopsis.org). Disregarding the term 'other cellular processes', the percent-wise highest enriched terms in nPE regarding the GO category "biological processes" were 'protein metabolism', 'developmental processes', and 'cell organization and biogenesis' (Fig. 2A,B). Together with the highest nPE-enriched terms in the category "molecular function" being 'DNA or RNA binding', 'protein binding', and 'nucleotide binding', these results can be explained by the importance for the proembryo of faster development and growth compared to the filamentous suspensor. On the contrary, the most prominent terms enriched for nSUS in the category "biological process" were 'response to stress', 'response to abiotic or biotic stimulus', and 'transport' (Fig. 2C). In the category "molecular function" we found enrichment for 'other enzyme activity', 'hydrolase activity', and 'transporter activity' (Fig. 2D). The suspensor undergoes programmed cell death (PCD) during maturation stages of embryogenesis, however the underlying mechanism still remains elusive (Bozhkov et al., 2005). Our analysis showed that the major categories of suspensor-expressed genes are 'response to stress' and 'response to abiotic or biotic stimulus'. Moreover, developmental PCD can be induced by stress or environmental stimulus (Bozhkov et al., 2005; van Doorn and Woltering, 2005), implying that suspensor PCD during embryogenesis might share similar signaling components with that of stress or environmental stimulus-induced PCD. Interestingly, the suspensor cell walls have to render the rapid stretching of the suspensor cells possible by continuous loosening for normal embryo development to take place (Babu et al., 2013), which is brought about for example by glycoside hydrolases and a plethora of cell wall remodeling factors (Cosgrove, 2001; Minic and Jouanin, 2006). Evidently, the suspensor has also been implicated in providing the proembryo with nutrients and plant hormones to be delivered by transporter proteins (Kawashima and Goldberg, 2010).

Differentially expressed candidate genes

In order to find significantly differentially expressed candidate genes between the nPE and nSUS samples, a rank product analysis was conducted with a percentage of false positives smaller than 0.1 and a change of greater than two-fold. A total of 307 and 180 array elements corresponding to 335 and 181 locus identifiers were enriched for

nPE and nSUS, respectively (supplementary material Tables S4 and S5). Among those we found genes that were previously shown to be differentially expressed and important for patterning and specification processes during embryogenesis e.g. *PIN-FORMED 1* (*PIN1, AT1G73590*), *WUSCHEL-RELATED HOMEOBOX 2* (*WOX2, AT5G59340*), *HANABU TARANU* (*HAN, AT3G50870*), *OBF BINDING PROTEIN 1* (*OBP1, AT3G50410*), or *FUSCA3* (*FUS3, AT3G26790*) (Aida et al., 2002; Friml et al., 2003; Kroj et al., 2003; Haecker et al., 2004; Skirycz et al., 2008; Nawy et al., 2010). Several other genes previously reported as being preferentially expressed in the suspensor (Friml et al., 2003; Haecker et al., 2004; Breuninger et al., 2008) did either not pass the stringent statistical analysis (*WOX9/AT2G33880*), or were not detectable with the microarray (*PIN7/AT1G23080, WOX8/AT5G45980*). However, even though, for example, PIN7 was under the microarray detection limit, we were able to detect its mRNA in all three nSUS samples by qRT-PCR (data not shown), which indicates the existence of false negatives in our data set due to sensitivity thresholds. In addition to the already mentioned auxin efflux carrier PIN1 and in light of the fact that auxin was indirectly shown to accumulate in the cells of the proembryo at early embryonic stages (Friml et al., 2003), we were also able to detect auxin biosynthesis genes *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1, AT1G70560)* and *YUCCA4* (*YUC4, AT5G11320*) as well as *MYB77* (*AT3G50060*), which is involved in auxin signal transduction (Cheng et al., 2007; Shin et al., 2007; Stepanova et al., 2008). This is in accordance with the evidence demonstrating the importance of auxin signaling in cell division and differentiation during early embryogenesis (Lau et al., 2012).

GO analysis of the suspensor-enriched transcript list showed only one significant class, namely lipid localization (supplementary material Fig. S6). On the contrary, analysis of the proembryo-enriched transcripts revealed many classes (supplementary material Fig. S7), most prominent being the terms 'pattern specification process', 'chromatin organization', 'cell cycle', 'ribosome biogenesis', and 'translation'. In contrast to the suspensor, the acquisition of the multidimensional structure in the proembryo requires the strict regulation of cell division and cell differentiation (Jürgens, 2001). The high cell-division rate is usually coordinated by cell cycles and the transition from cell division to cell differentiation is concomitant

with chromatin remodeling, as well as the change of gene expression and protein synthesis rate (Kristensen et al., 2013).

Collectively, our results revealed that except for the morphological differences, the proembryo and the suspensor also appear distinct in gene expression profile during early embryogenesis. Furthermore, our GO analysis indicated an increased complexity of cellular activities in the proembryo compared to the suspensor.

Microarray data validation by promoter expression analysis and *in situ* hybridization

To further validate the microarray results, we randomly selected twelve genes statistically enriched for the proembryo and nine for the suspensor for global expression analysis. Promoters including 5'UTR regions each approximately 2kb in length were constructed to drive expression of n3xGFP or n3xRFP. In most cases, the expression patterns of the promoter fusions were in concordance with the microarray results (Table 1, Fig. 3). In the one- or two-cell stage embryo, there was no exclusive expression in either the suspensor or the proembryo detectable but rather a broad expression in all cells of the whole embryo with differences in expression strength between proembryo and suspensor was visible. Interestingly, some genes showed specific expression in one cell type but not the other (Table 1, Fig. 3A). Others showed global expression in the whole embryo at earliest stages but were later restricted to only one cell type (Fig. 3B). Moreover, reporter expression for several constructs remained universal in the whole embryo, which only later appeared stronger in one cell lineage and weaker in the other (Fig. 3C). Three promoter-fusion constructs did not confer any visible GFP expression in the embryo, which might be due to missing elements important for proper expression or a false-positive signal from the microarray (not shown). Taken together, the expression patterns of the promoter fusions are overall in concordance with the differences found in the statistical analysis of the microarray data. Minor discrepancies between the promoter fusion and the microarray data can most likely be attributed to the stability and low turnover rate of GFP protein inside the plant cell. In total, we tested 21 promoters fused to n3xGFP or n3xRFP of which only 3 were not embryo-expressed. Of the 18

embryo-expressed genes, 16 recapitulated the microarray results of differentially expressed transcripts (Table 1).

As promoter fusion constructs in some cases may not fully recapitulate true gene expression due to the possible lack of critical regulatory elements, we performed *in situ* hybridization for some of the proembryo- and suspensor-enriched transcripts. Overall, the *in situ* hybridization results for the selected, differentially expressed candidate genes (*AT1G04645*, *AT5G46230*, *AT5G61030*, *AT1G28300*, and *AT3G44750*) were consistent with the microarray analysis (Fig. 4A-C). However, we could not detect any signal in the early embryo for *AT2G46690* (data not shown). Since also the n3xGFP fusion did not give any signal, this is probably a false-positive signal on the microarray. Moreover, the promoter-fusion analysis for two proembryo-enriched genes (*AT5G61030* and *AT3G44750*) did not correlate with our microarray analysis since the corresponding reporter-gene constructs indicated ubiquitous expression in all cells of the embryo (supplementary material Fig. S5). The *in situ* hybridization for these two genes, however, showed stronger signals in the proembryo at early stages of embryogenesis (Fig. 4B,C), indicating a possible lack of certain regulatory elements in the respective promoter regions cloned or post-transcriptional regulation of the endogenous gene. Furthermore, the validation of the differentially expressed genes by *in situ* hybridization was not only additive to, but also complementary with the promoter fusion analysis. In summary, the promoter-fusion studies and *in situ* hybridization results for in total 23 genes strongly correlated with the results of the microarray analysis which emphasizes the high quality of the whole dataset.

Comparison with publicly available transcriptome data

Recently, LCM was used in combination with microarrays to generate a very elaborate expression atlas of various seed compartments including the embryo at different developmental stages of the ovule (Le et al., 2010; Belmonte et al., 2013). Among other tissue types, cellular expression profiles were created for the proembryo and suspensor at the globular-embryo stage which we here term cellular globular proembryo (cgPE) and cellular globular suspensor (cgSUS), respectively (Le et al., 2010). We MAS5-normalized and log2-transformed the raw values from the cgPE and cgSUS replicates as described above (supplementary material Tables S1 and S2). To

see if the corresponding nuclear and cellular datasets were comparable, we first checked the overlap of MAS5 calls 3xP. However, unlike the higher overlap between nuclear samples (nPE/nSUS 64.5%, supplementary material Fig. S4), there are substantially fewer array elements shared between nPE/cgPE (47.2%) and nSUS/cgSUS (38.7%, Fig. 5A,B) and furthermore, these percentages are very similar for nPE/cgSUS or nSUS/cgPE (data not shown).

To exclude that the observed expression differences mainly originate from the different subcellular mRNA pools tested and/or being due to different fixation methods, we manually isolated intact whole embryos at 16-32 cell stages and directly extracted RNA without prior fixation. After amplification and microarray hybridization, samples were analyzed as mentioned above (cEMB, supplementary material Tables S1 and S2). Interestingly, we observed a much stronger overlap of MAS5 calls 3xP between nEMB and cEMB of about 70% in contrast to the comparison between our nuclear and the published LCM data (Fig. 5C). Additionally, after testing the normalized and transformed values of all replicates for comparability by box plot analysis (supplementary material Fig. S8A), we performed hierarchical cluster analysis to group the different expression profiles. In summary, all replicates of one specific experiment group together and there are two main clusters consisting of 1) all nuclear (nPE, nSUS, nEMB) plus the cellular embryo sample (cEMB) and 2) the cellular globular-embryo samples (cgPE, cgSUS). In cluster 1) there are subgroups of nuclear samples and the cellular embryo sample (supplementary material Fig. S8B). These differences are further corroborated in a principal component analysis (PCA) plot where cgPE clusters with cgSUS, nPE with nSUS, and nEMB with cEMB. However, the cgPE and cgSUS cluster is farther away from the two other clusters. We can conclude that since the influence of fixation, nuclear RNA, and age of embryos on the observed expression profiles seems to be subtle, the main factor for these discrepancies between the LCM-derived and our datasets must be the different extraction techniques and RNA amplification protocols.

To compare the nuclear and cellular datasets beyond *in silico*, we compared the expression values of the LCM data for genes we tested with the promoter-fusion constructs and *in situ* hybridization (Table 1). For three constructs not showing expression in the embryo, the LCM values were consistent with our microarray results indicating the same false-positive results (*AT1G31400*, *AT2G46690*, *AT1G48470*).

The LCM array element values for seventeen genes showing expression in the embryo were consistent with our results (*AT1G77580*, *AT5G05940*, *AT2G35605*, *AT5G61030*, *AT1G64220*, *AT1G28300*, *AT5G22650*, *AT5G66940*, *AT3G44750*, *AT3G55660*, *AT3G62480*, *AT1G04645*, *AT1G54160*, *AT3G52780*, *AT5G07440*, *AT1G74190*, *AT2G32100*). Four genes appeared as false negatives as the expression values were very low and often the MAS5 call was absent for both the proembryo and the suspensor replicates (*AT5G26270*, *AT3G17290*, *AT5G43510*, *AT5G46230*).

Recently, a report described the expression patterns of multiple auxin response factors (ARF) during early *Arabidopsis* embryogenesis (Rademacher et al., 2011). Four of the tested ARF promoters there were designated as only being expressed in the endosperm but not the embryo itself (*ARF12/AT1G34310*, *ARF17/AT1G77850*, *ARF21/AT1G34410*, *ARF23/AT1G43950*) and except for *ARF12*, these genes were in essence not expressed in our dataset (supplementary material Table S2). In the LCM dataset however, all four genes were at least weakly expressed within the suspensor. This suggests contamination of at least the suspensor samples with surrounding endosperm in the LCM dataset. Since it is essentially impossible to accurately dissect tissue with LCM in the third dimension, the list of apparent suspensor genes is likely contaminated by endosperm-expressed genes. On the contrary, with our methodology, we could minimize contamination with cellular or nuclear material of embryo-surrounding cells.

DISCUSSION

In this study we described and validated a nuclear extraction and purification protocol for expression analysis of inaccessible cell types in the *Arabidopsis thaliana* seed. Given that the unequal distribution of some transcripts in the early embryo leads to distinguishable cell types and likewise the unequal distribution of specific transcripts was reported in the apical and basal cells of tobacco embryos (Breuninger et al., 2008; Lau et al., 2010; Ueda et al., 2011), we reasoned that these cell types might be a well suited test field for our method and that the generation of expression profiles for the proembryo and suspensor of early Arabidopsis embryos will provide insights into better understanding of early embryo development. Several of the statistically-enriched candidate genes for the proembryo were previously described to have important functions during early embryogenesis, some of which were shown as

proembryo-enriched expressed genes in our dataset. For example, *HANABA TANARU* (*HAN*) was shown to be expressed in the apical cell of the embryo and plays a role in setting up the boundary between proembryo and suspensor (Nawy et al., 2010). *PIN-FORMED 1* (*PIN1*), known as an auxin efflux facilitator, is expressed in the proembryo cells, mediating auxin flow from apical cells to the hypophysis (Friml et al., 2003), which is in turn critical for root initiation. Another apically expressed gene is the homeobox transcription factor *WUSCHEL RELATED HOMEOBOX 2* (*WOX2*) plays a fundamental role in the establishment of the apical domain (Haecker et al., 2004). Moreover, the suspensor-expressed gene *FUSCA3* (*FUS3*) lacks the apical expression due to the repression by *DICER-LIKE1* (*DCL1*), and early matured embryos in the *dcl1* mutant show ectopic expression of *FUS3* in the proembryo (Willmann et al., 2011). All these examples initially substantiated our results as these genes were not only present in one or the other dataset but were among the statistically most significant ones.

The *in vivo* expression analyses using promoter-GFP fusion constructs as well as *in situ* hybridization strongly correlated with the microarray results for the candidate genes tested. This demonstrated the validity of the microarray results after stringent statistical analysis from expression data generated for specific tissues in the *Arabidopsis* embryo at the earliest developmental stages.

Comparison with published expression data generated from respective cellular embryonic tissues by laser capture microdissection (Le et al., 2010) apart from certain similarities revealed major differences in types of genes expressed in the given tissues. Potentially there are many factors influencing the final transcriptomic data. These include 1) the plant accession used, 2) the developmental stage of the tissue studied, 3) the RNA composition (cellular, cytoplasmic, nuclear), 4) different fixation approaches 5), RNA extraction, and 6) RNA amplification method. By comparison of our nuclear RNA transcriptome results with those from cellular RNA of non-fixed embryos – assuming the influence of accession and RNA extraction method as marginal – we conclude that the RNA amplification or probably tissue isolation approaches has the greatest impact. This notion seems reasonable because we used a commercial kit and a polyT primer whereas the LCM RNA was amplified with a polyT/random primer mixture (Le et al., 2010).

Crosschecking *in vivo* expression results, we did not see any disadvantages of our transcriptomic data except a certain proportion of false-negatives in detection of low-expressed genes. On the contrary, we propose that our approach has certain advantages, most importantly one being the possibility to study any tissue of interest and the other being a decreased risk of contamination with embryo-surrounding cells compare to LCM. Even though there is the necessity for transgenics in order to use our approach, it is nevertheless applicable to any other transformable plant or animal tissue to generate expression data from a given cell type. Importantly, the method described here not only enables expression studies to be performed but also has the potential to study DNA and histone modifications.

MATERIALS AND METHODS

Plant materials and growth conditions

All *Arabidopsis thaliana* lines used are *Col-0*. The GAL4-GFP enhancer-trap lines generated by the Haseloff lab were obtained from the Nottingham Arabidopsis Stock Centre (NASC). For growth under sterile conditions, seeds were surface-sterilized with 25% bleach, washed three times, and grown on half-strength Murashige and Skoog (MS) containing 0.8% agar plates containing 10 g/l sucrose. Seedlings were transferred to soil and grown at 22°C to 24°C in a growth chamber under a 16 h/8 h light/dark cycle.

Molecular cloning and genotyping

TAIL-PCR was performed as previously described (Liu and Chen, 2007). All genomic fragments (899bp-2000bp upstream of ATG) for the promoter-GFP fusions except for *pDRN* were PCR-amplified and sub-cloned into *pGEM-T* vector (Promega). The n3xRFP was assembled from PCR-amplified monomers in *pGII Kan* vector. All fragments were finally introduced into *pGII Kan:n3×GFP* (Takada and Jurgens, 2007) or *pGII Kan:n3×RFP*. A *pAT3G10100* fragment was introduced into *pGII Kan:n3xGFP* which resulted in *pAT3G10100::nGFP*.

The n2xGFP was amplified from *pGII Kan:n3xGFP* and introduced into *pGII Kan*. For generating *pDRN:n2×GFP:DRN 3'UTR*, a 1378bp *DRN 3'UTR* fragment was PCR-amplified and sub-cloned into *pGEM-T*. Then the *DRN* 3'UTR was

introduced into *pGII Kan:n2×GFP* generating *pGII Kan:n2×GFP:DRN 3'UTR*. A 4.2kb DRN promoter upstream of the start codon was PCR-amplified and sub-cloned into *pGEM-T*. *pDRN* was finally introduced into *pGII Kan:n2×GFP:DRN 3'UTR* generating *pDRN:n2×GFP:DRN 3'UTR*.

Nuclear isolation

Fresh Arabidopsis thaliana ovules were collected in RNAlater buffer (QIAGEN) and kept in fixation buffer (0.1% Paraformaldehyde in RNAlater) for 5-10 min and ground thoroughly using the pestle in the 1.5ml tube. The CelLytic™ PN kit (Sigma) was used for the following procedures.

FANS

Fluorescently labeled nuclei were identified by plotting peak GFP fluorescence (513/17) against autofluorescence (575/25) using a MoFlo Legacy (Beckman Coulter) FACS fitted with a 488 nm laser (100 mW) triggering off the FSC (forward scatter channel). Tests with co-staining with propidium iodide to label free nuclei identified the same GFP population therefore staining was deemed unnecessary. Flow cytometric analyses were carried out as follows: 1x PBS pH 7.0, 70 µM stream, ~60.5 / ~60.0 psi, ~95 kHz, 1-2 single drop envelope.

Manual isolation of embryos

Isolation was performed essentially as previously described (Nodine and Bartel, 2012). In brief, early-globular stage embryos were squeezed out from the ovules on a microscope slide and washed three times in water and subsequently collected in RNAlater. 40-50 embryos were pooled per biological replicate.

RNA extraction and amplification

The sorted positive nuclei were collected in RNA extraction buffer [10 mM Tris-HCl pH 7.9, 50 mM EDTA pH 7.9, 0.2 M NaCl, 0.5% SDS, 0.5 mg/ml RNase inhibitor (Fermentas), 600 µg/ml proteinase K] (Khodosevich et al., 2007). The buffer containing the GFP-positive nuclei was incubated at 55°C with vigorous shaking for 10-15 min. The total volume was adjusted to 600 µl RNase-free water and an equal volume of phenol pH 4.2 was added. The solution was vortexed thoroughly and kept

on ice for 5 min and afterwards centrifuged at 14,000 for 10 min at 4°C. The aqueous phase was transferred into a new tube and an equal volume of phenol:chloroform (1:1) was added. The solution was mixed thoroughly and kept on ice for 5 min and centrifuged at 14,000 g for 10 min at 4°C. The aqueous phase was transferred into a new tube and equal volumes of isopropanol and 20 µg glycogen were added. Then the solution was mixed thoroughly and kept at -20°C overnight and centrifuged at 16,100 g for 45 min at 4°C. Following the centrifugation, the resulting pellet was washed with 70% cold ethanol and dried at room temperature. The pellet was eventually dissolved in RNase-free water. For DNase treatment, a commercial kit (DNase I, Fermentas) was used and afterwards the RNeasy Micro Kit (QIAGEN) was used for RNA cleanup.

One to three ng of total RNA was used for cDNA synthesis and amplification (Arcturus® RiboAmp® HS PLUS RNA Amplification Kit) and the resulting cDNA was fragmented and labeled using the ENZO BioArray™ Single-round RNA amplification and biotin labeling system. 12.5 µg of fragmented cDNA was hybridized on Affymetrix GeneChip ATH1 Arabidopsis Genome Array.

Microarray data analysis

Microarray data analyses were performed using diverse packages implemented in "R" (v2.14.2; http://www.r-project.org). Log2-based expression estimates were obtained from .CEL files using "gcRMA" (v2.26.0) (Wu et al., 2004). Differentially expressed genes were identified by "RankProducts" (v2.26.0) using 100 permutations and a percentage false positive (pfp) cut-off of 0.05 (Breitling et al., 2004). Present, marginal, and absent calls were calculated using MAS5 as implemented in the "affy" package (v1.32.1). Gene ontology (GO) grouping of genes (nuclei, MAS5 3x present) was created with the GO annotations online tool available on the TAIR website (https://www.arabidopsis.org/tools/bulk/go/index.jsp). Singular enrichment analysis (SEA) was carried out with AgriGO (Fisher test method, Yekutieli adjustment, p<0.01; http://bioinfo.cau.edu.cn/agriGO/analysis.php). All Venn diagrams were generated with a combination of BioVenn (http://www.cmbi.ru.nl/cdd/biovenn/), Venn diagram plotter (http://omics.pnl.gov/software/venn-diagram-plotter), and Adobe Illustrator. Quality control analyses (Box plot, hierarchical clustering of

samples, principal component analysis) of all biological replicates were performed with CLC Main Workbench software (version 6.6.2).

RNA *in situ* hybridization

The primers for probe synthesis are listed in supplementary material Table S6. The fragments for the sense and antisense probes were PCR-amplified and inserted into *pBSK⁻* or *pGEM-T* vectors. *In vitro* transcription was performed with T7 or SP6 primers and with Fermentas in vitro transcription kit. Both ends of young siliques were cut off and the middle part was fixed in cold fixation solution (4% Paraformaldehyde in DEPC-treated water, 0.1% Tween-20). A conventional plastic syringe was used for vacuum infiltration and the samples were kept overnight in the fixation solution at 4°C. Following 1×PBS incubation for 2x30 min, the samples were dehydrated through a graded ethanol series (30%, 40%, 50%, 60%, 80%, 90%, 95%) for 1 hour each and finally embedded in paraffin. Paraffin-embedded samples were microtome-sectioned to 6 μm thickness. The procedures of hybridization and staining were performed as described (Schlereth et al., 2010).

Microscopy

For differential interference contrast (DIC) microscopy and fluorescence analysis, ovules were mounted on slides containing clearing solution [chloral hydrate, water, and glycerol (ratio w/v/v: 8:3:1)]. For fluorescence analysis, embryos were gently squeezed out from ovules and mounted in 10% glycerol (v/v). An Olympus IX81 confocal laser scanning microscope (image acquisition software: FV10-ASW; objectives: UPlanSApo *x*40) was used for confocal microscopy analysis. Images were further processed using Adobe Photoshop software. Zeiss Axio Imager (image acquisition software: AxioVision; camera: AxioCam HRc; objectives: Plan-APOCHROMAT ×20 and ×40) was used for wide-field and DIC images and images were further processed with AxioVision SE64 Rel. 4.9.1 software.

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Competing interests

The authors declare no competing financial interests.

Author contributions

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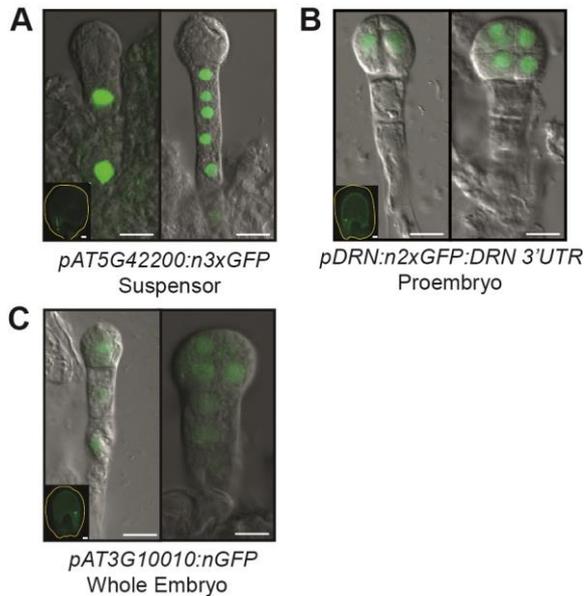
Figure 1. Specific marker lines used for FANS. (A,B) Proembryo marker line at 2-cell (A) and early globular stage (B). (C,D) Suspensor marker line at 2-cell (C) and 8-cell stage (D). (E,F), Whole embryo marker line at 1-cell (E) and 4-cell stage (F).
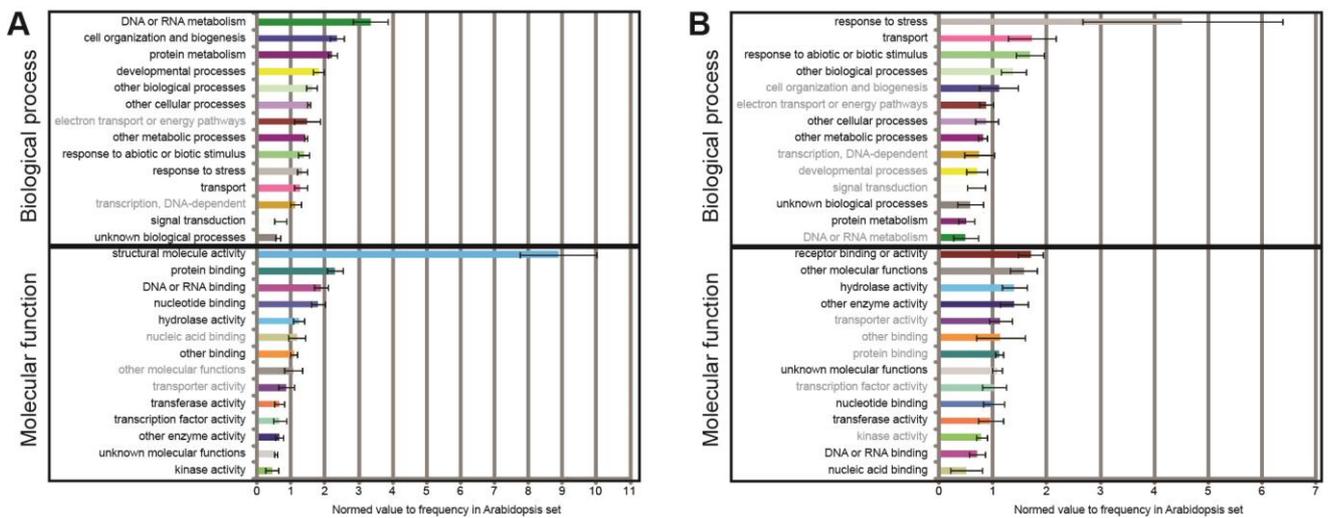

Figure 2. Gene ontology (GO) analysis of genes expressed in nuclei of proembryo and suspensor. (A) Proembryo "biological processes".(B) Suspensor "biological processes". (C) Proembryo "molecular function". (D) Suspensor "molecular function".
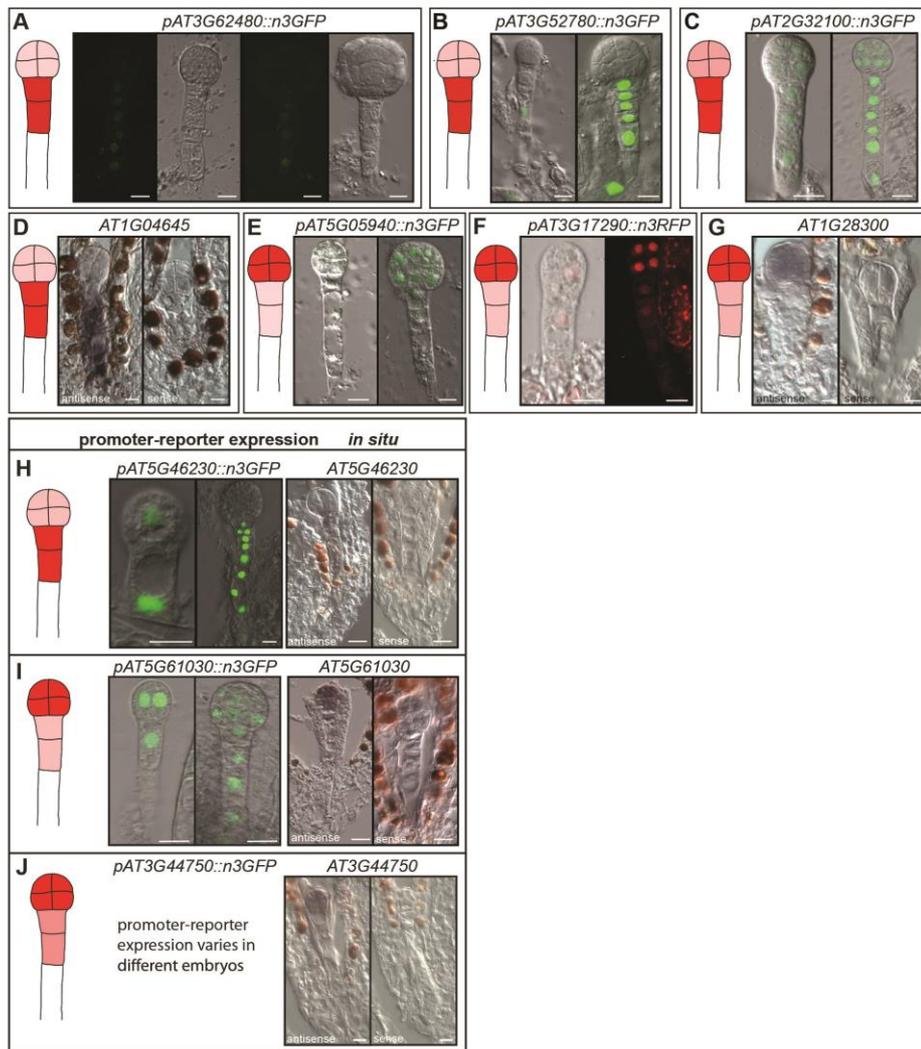
Figure 3. Promoter fusion analysis and *in situ* hybridizations for selected differentially expressed candidate genes in early embryos. (A-G) Temporal promoter-reporter expression and *in situ* hybridization of suspensor-enriched genes (A-D) and proembryo-enriched genes (E-G) during early embryogenesis. (H-J) Comparison of promoter-reporter expression and *in situ* hybridization for the same genes enriched in suspensor (H) and proembryo (I and J). Color shading in the schematic representation of Arabidopsis embryo indicates the expression levels according to the microarray dataset (dark red: stronger expression; light red: weaker expression). Scale bars, 10 μm.
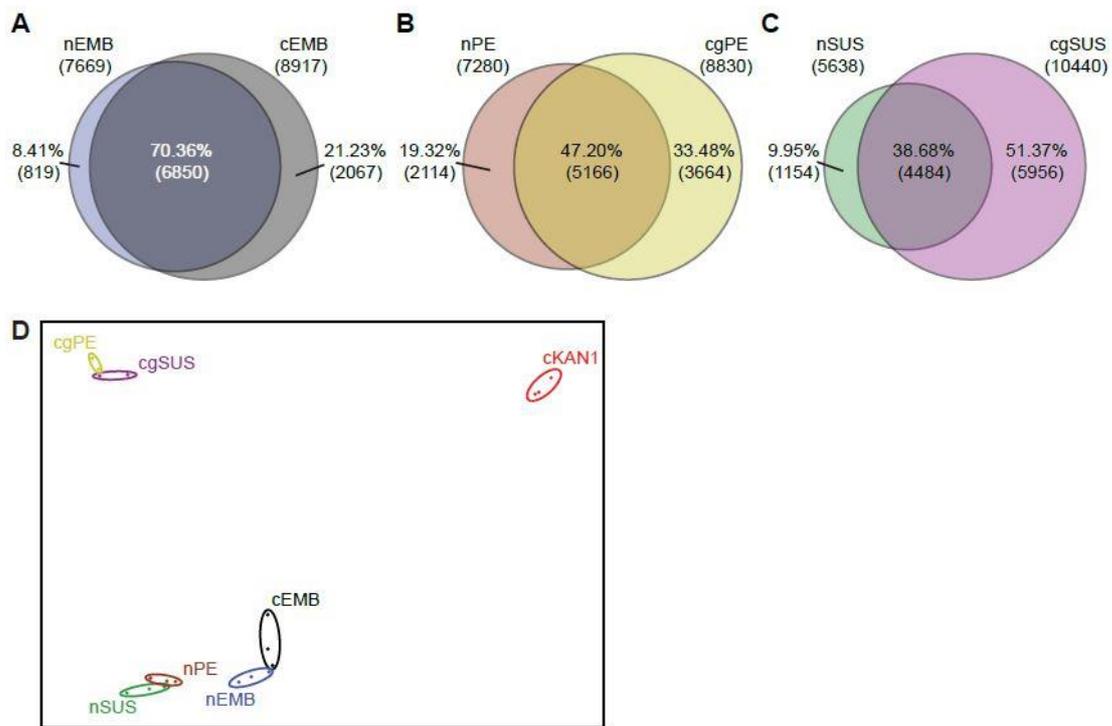
Figure 4. Comparison of nuclear and cellular transcriptome data from different tissue types. (A-C) Venn diagrams showing overlap of MAS5 3x present calls between nEMB and cEMB (A), nPE and cgPE (B), and nSUS and cgSUS (C). (D) Principal component analysis of biological replicates from the different nuclear and cellular tissue types. nPE = nuclei from proembryo, nSUS = nuclei from suspensor, nEMB = nuclei from whole embryo, cEMB = cells from whole embryo, cgPE = cellular globular-stage proembryo, cgSUS = cellular globular-stage suspensor, cKAN1 = cellular KANADI 1 expression domain in the shoot.
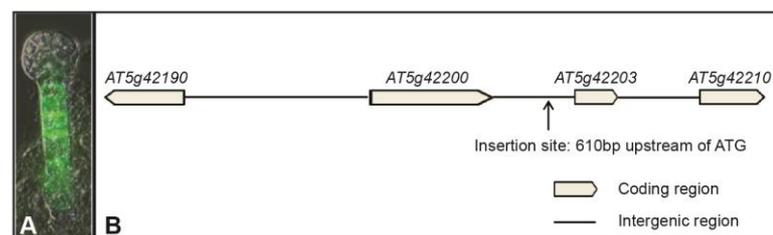

Figure S1. Enhancer-trap line N9322 and identification of genomic insertion site. (A) Suspensor and hypophysis expression at globular stage. (B) Insertion site of T-DNA determined by TAIL-PCR.
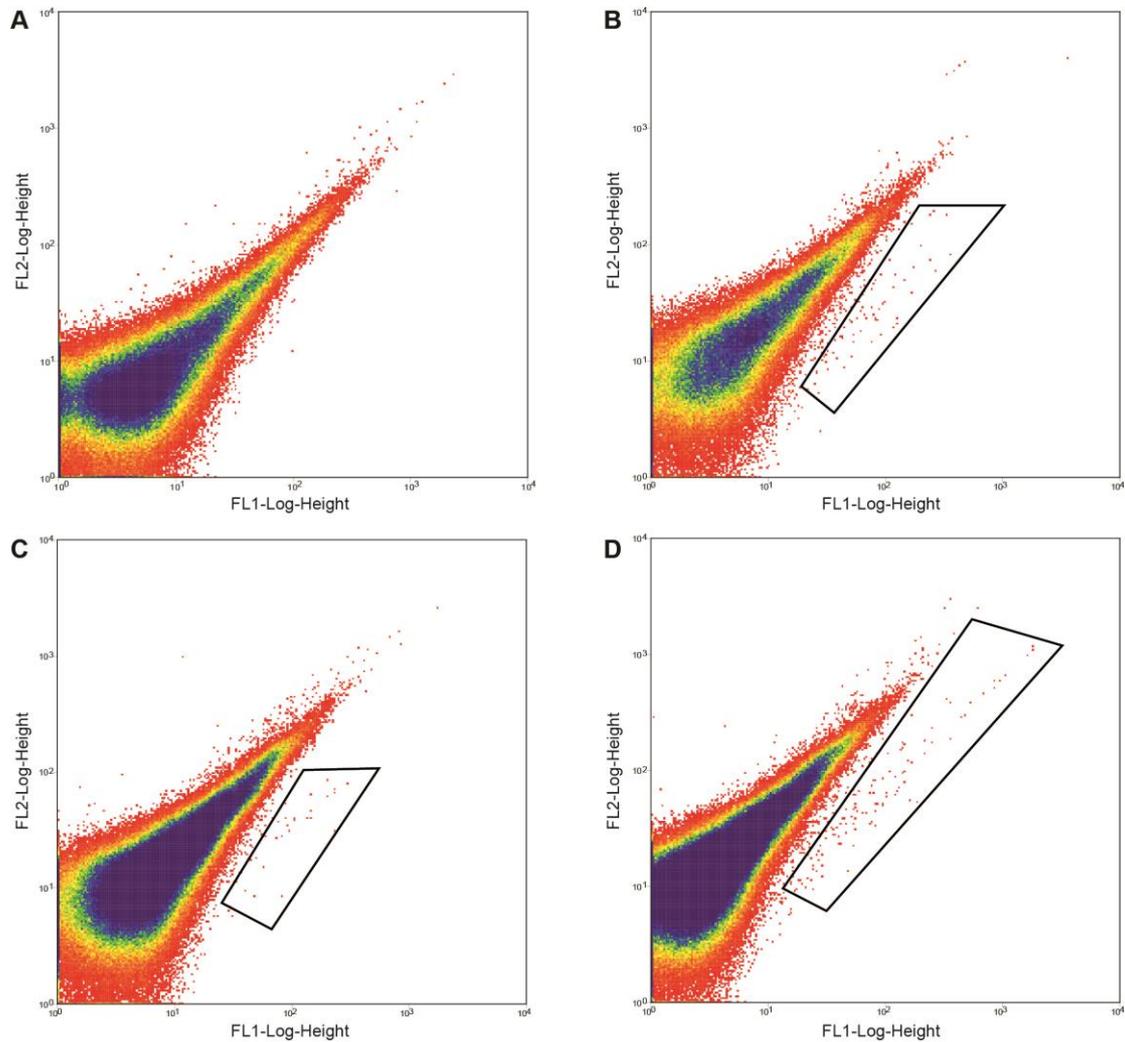
Figure S2. Scatter plots of FANS for GFP-tagged nuclear samples. (A) Mock sample. (B) Suspensor marker line *pAT5G42200:n3xGFP*. (C) Proembryo marker line *pDRN:n2xGFP:DRN 3'UTR*. (D) Whole embryo marker line *pAT3G10010:nGFP*. Fluorescent nuclei were detected by plotting the GFP channel (FL1, log, 513/17, x-axis) against auto-fluorescence (FL2, log, 575/25, y-axis) and drawing a gate around the GFP-positive events.
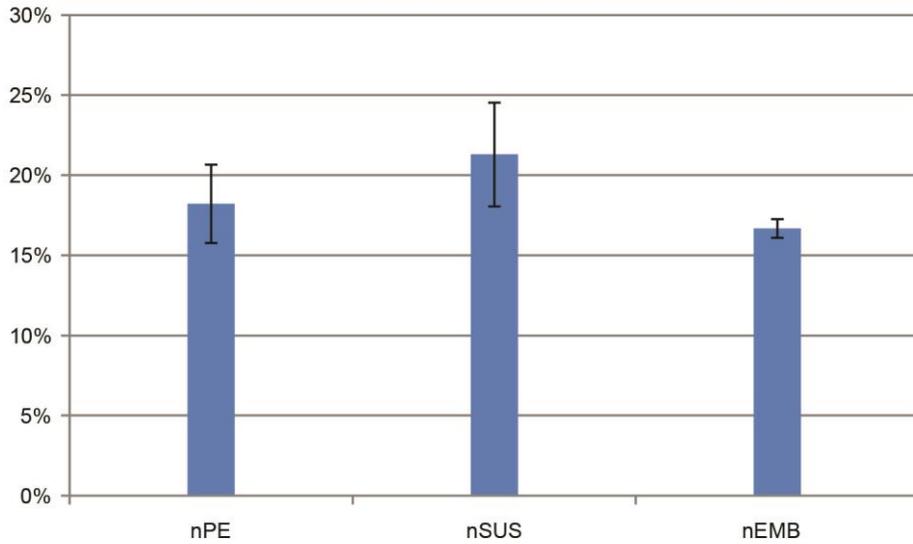
Figure S3. Percentage and standard deviation of MAS5 calls not correlating across three biological replicates. Replicates were compared to each other and the average percentage of calls not matching was calculated.
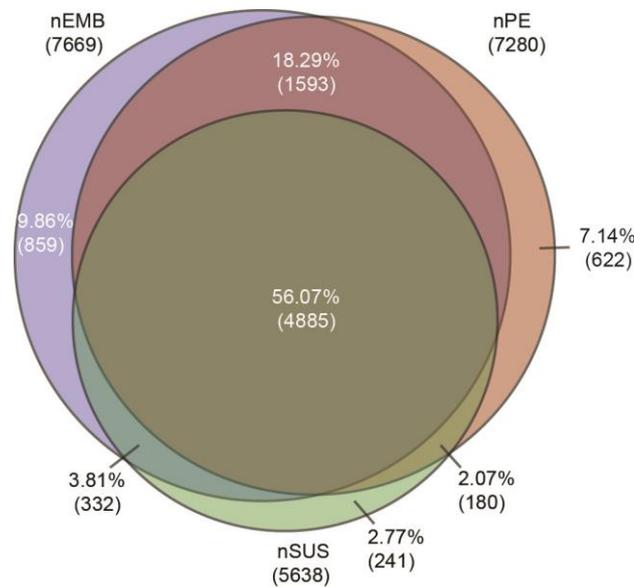

Figure S4. Venn diagram showing overlap of genes expressed in nuclei of the proembryo, suspensor, and whole embryo. For the analysis, only array elements with calls of 3x present (P) across all three biological replicates were used.

DRN (AT1G12980)

A

PIN7 (AT1G23080)

B
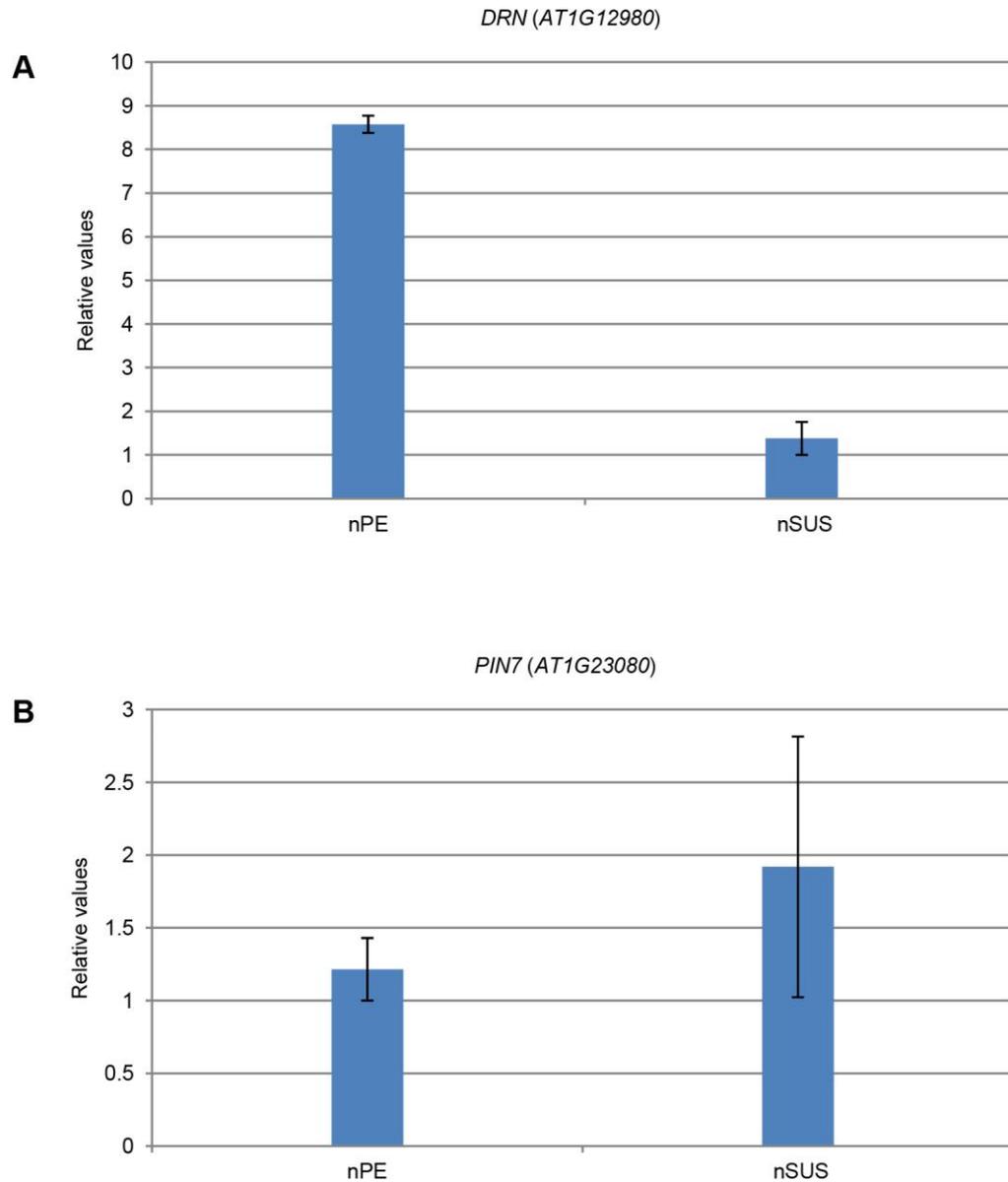
Figure S5. Relative mRNA levels detected by qRT-PCR analysis. (A) *PIN7* relative transcript levels are more abundant in nSUS compared to nPE. (B) *DRN* relative transcript levels are more abundant in nPE compared to nSUS. Average values and standard error are given for two biological replicates for nuclear RNA from both proembryo (nPE) and suspensor (nSUS). ***** *P<0.01* (Student's *t*-test).

Figure S6. Qualitiy analysis of biological replicates from different nuclear and cellular tissue types. (A) Box-plot analysis. (B) Hierarchical clustering analysis (Pearson correlation, complete linkage).

Table 1. Overview of differentially expressed candidate genes used for *in vivo* validation of microarray results.

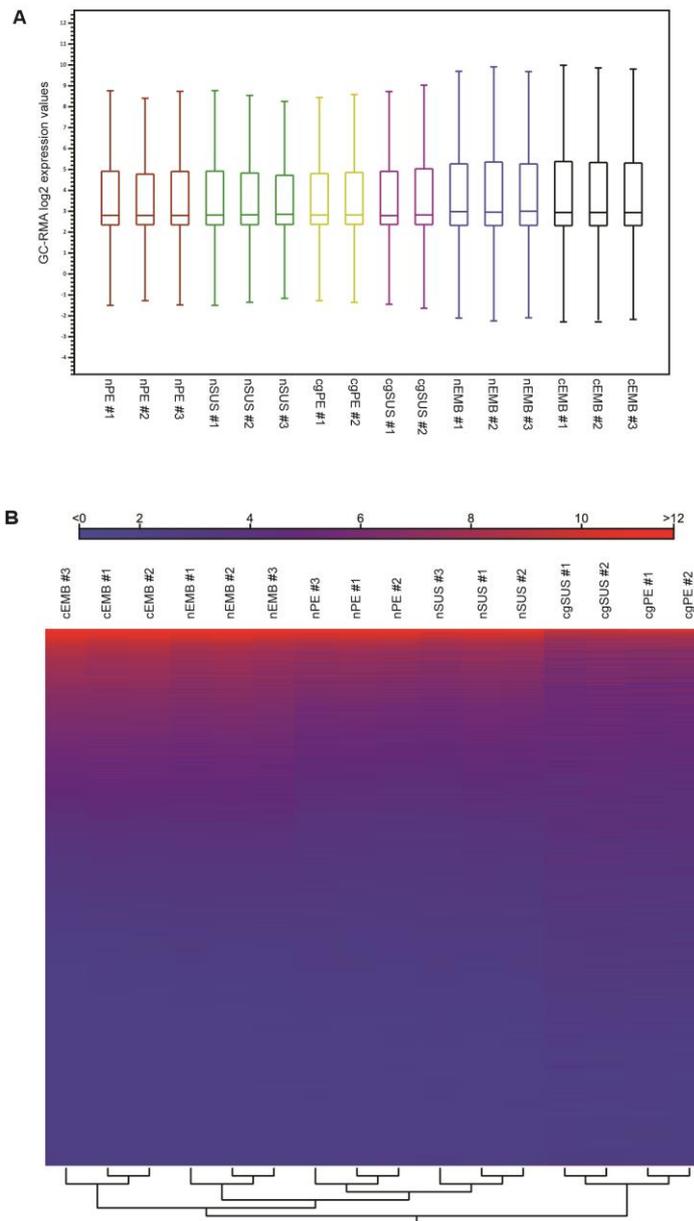For all constructs a short description of the expression patterns in transgenic embryos is given. Gene expression tested by *in situ* hybridizations is indicated with an X. Results of the RankProduct analysis for fold change (FC) are indicated. Additionally, average MAS5 expression values of the three replicates are given for nPE and nSUS samples (decreasing values from red to blue) and the genes overlapping with the embryo-specific analysis results are designated with asterisks. av., average; cl, class; EMB, Whole embryo; FC, fold change; PE, Proembryo; SUS, Suspensor.

Proembryo enriched transcripts tested

Locus	Probe set ID	Promoter expression analysis *in vivo*	*In situ*	FC:(cl1/cl2)	av. MAS5 nPE	av. MAS5 nSUS	embryo-specific
AT5G26270	246888_at	EMB, stronger PE		35.21	5141.74	155.85	*
AT1G77580	259760_at	globular stage PE		7.07	315.51	85.40	*
AT5G05940	250756_at	EMB, at late globular/early heart stage stronger PE		6.50	326.50	65.65	*
AT3G17290	258459_at	EMB, at 8/16-cell stage stronger PE	X	4.44	324.18	90.10	*
AT2G35605	266641_at	EMB, stronger PE		4.41	2616.68	850.30	
AT5G61030	247575_at	EMB, stronger PE	X	3.92	1181.90	365.75	
AT1G31400	262555_at	no expression		3.70	464.74	202.79	*
AT1G64220	262336_at	globular stage PE		3.44	555.46	235.96	
AT1G28300	245669_at	not available	X	3.41	448.10	244.37	*
AT5G22650	249901_at	inconsistant expression		3.41	5074.09	2203.15	
AT5G43510	249157_at	PE early heart stage		3.15	362.94	159.05	*
AT3G44750	252625_at	inconsistant expression	X	2.31	1970.12	885.23	
AT3G55660	251778_at	late globular stage hypophysis and lower tier		2.22	584.78	366.01	*

Suspensor enriched transcripts tested

Locus	Probe set ID	Promoter expression analysis *in vivo*	*In situ*	FC:(cl2/cl1)	av. MAS5 nPE	av. MAS5 nSUS	
AT2G46690	266322_at	no expression	X	8.60	119.51	618.06	
AT3G62480	251212_at	SUS		5.98	100.65	683.83	
AT1G48470	261305_at	no expression		5.67	139.09	603.22	*
AT1G04645	264610_at	not available	X	4.92	2343.15	10066.28	
AT1G54160	263158_at	EMB, stronger SUS		4.39	210.41	588.35	
AT3G52780	252004_at	SUS		4.17	366.63	1419.88	
AT5G46230	248889_at	EMB, stronger SUS	X	3.87	166.79	413.70	
AT5G07440	250580_at	EMB, stronger SUS		3.51	451.98	1162.80	
AT1G74190	260253_at	SUS		3.30	27.33	166.46	*
AT2G32100	265724_at	EMB, stronger SUS		2.84	252.65	613.39	*

Supplemental tables S1-14 can be downloaded online

7.3 Early Embryogenesis in Flowering Plants: Setting Up the Basic Body Pattern

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Early Embryogenesis in Flowering Plants: Setting Up the Basic Body Pattern

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Keywords

zygote, apical-basal axis, root pole initiation, radial pattern, shoot meristem initiation, cotyledon initiation

Abstract

Early embryogenesis is the critical developmental phase during which the basic features of the plant body are established: the apical-basal axis of polarity, different tissue layers, and both the root pole and the shoot pole. Polarization of the zygote correlates with the generation of apical and basal (embryonic and extraembryonic) cell fates. Whereas mechanisms of zygote polarization are still largely unknown, distinct expression domains of WOX family transcription factors as well as directional auxin transport and local auxin response are known to be involved in early apical-basal patterning. Radial patterning of tissue layers appears to be mediated by cell-cell communication involving both peptide signaling and transcription factor movement. Although the initiation of the shoot pole is still unclear, the apical organization of the embryo depends on both the proper establishment of transcription factor expression domains and, for cotyledon initiation, upward auxin flow in the protoderm. Here we focus on the essential patterning processes, drawing mainly on data from *Arabidopsis thaliana* and also including relevant data from other species if available.

Contents

INTRODUCTION

The basic body pattern of a multicellular organism is established from the zygote—the fertilized egg cell—during embryogenesis. In flowering plants, embryogenesis lays down the basis for a stereotyped seedling displaying a simple body organization of two superimposed patterns. Along the main apical-basal axis of polarity, the apically located shoot meristem, which is usually flanked by one or two cotyledons, is linked with the basally located root meristem via the hypocotyl and seedling root. The perpendicular radial pattern comprises a series of concentrically arranged tissue layers, from the outermost epidermal tissue via the ground tissue to the centrally located vascular tissue. Although the body organization of the seedling looks similar in different flowering plant species, its developmental origin can vary between species. For example, members of the Brassicaceae family (such as *Arabidopsis thaliana*) display distinct, nearly stereotypic cell-division patterns in early embryogenesis, whereas embryos of other flowering plant species grow by seemingly random cell divisions (62, 63, 66, 94). In the former group of species, the origin of seedling tissues and organs can thus be easily traced back to specific cells or groups of cells in the early embryo (**Figure 1**). Although this correlation might suggest a causal link between the spatial regulation of cell divisions and pattern formation in the early embryo, *A. thaliana* mutants such as *fass* (*fs*) displaying altered cell-division planes nonetheless generate a normal body organization, whereas morphogenesis is compromised (147). Thus, the stereotypic cell-division pattern seen in *A. thaliana* embryos expresses, but is not instrumental to, developmental decisions and might facilitate such decisions in the early embryo comprising very few cells.

This review covers recent studies that address molecular mechanisms underlying the origin of the apical-basal axis of polarity, the initiation of both the root meristem and the shoot meristem as well as the cotyledons, and radial patterning. It also discusses the parental contributions to gene activity in early embryogenesis in regard to their potential role in early patterning events. For ease of reference, **Table 1** lists the gene abbreviations and full names referred to in this review.

Zygote: fertilization product of egg and sperm cell

ZYGOTE POLARITY AND ELONGATION

Zygote Polarity

In flowering plants, the zygote is formed by the fusion of the egg cell with one of the two sperm cells delivered by the pollen tube (reviewed in 25). Like the egg cell, the zygote is usually polarized with respect to the relative position of nucleus and vacuole. However, egg cell polarity and zygote polarity are different in some species, suggesting that the latter might be established independently of the former.

In many species, the egg cell has its nucleus located toward the chalazal end of the ovule (i.e., apically) and usually has a large vacuole located toward the micropylar end (i.e., basally). This is, for example, the case in *A. thaliana*, *Capsella bursa-pastoris*, and *Nicotiana tabacum* (tobacco), in all of which zygote organization resembles egg cell organization (94, 95, 103, 131, 170); polarity—as inferred from nucleus and vacuole position—appears thus to be maintained after fertilization. However, this was shown not to be the case in *A. thaliana* and probably *N. tabacum*. A transient symmetric stage, in which the nucleus is located centrally and smaller vacuoles are distributed rather evenly within the cell, developmentally separates the polarized egg cell from the similarly polarized zygote (29, 103, 151, 170). In *A. thaliana*, the transcription factor WRKY DNA-BINDING PROTEIN 2 (WRKY2) is involved in the polarization of the zygote by transcriptionally activating *WUSCHEL RELATED HOMEOBOX 8* (*WOX8*) and possibly *WOX9* (151). *WRKY2* is dispensable for the establishment or maintenance of egg cell polarity, which corroborates the notion that egg cell and zygote polarity are not intimately linked (151). Even stronger effects of fertilization on zygote polarity are, for example, observed in *Oryza sativa* (rice), *Zea mays* (maize), and *Papaver nudicaule*, in all of which egg cell polarity is reversed after fertilization. Whereas the nucleus localizes to the micropylar/basal end of the egg cell and the large vacuole to the chalazal/apical end, the opposite is the case in the zygote (25, 114, 123).
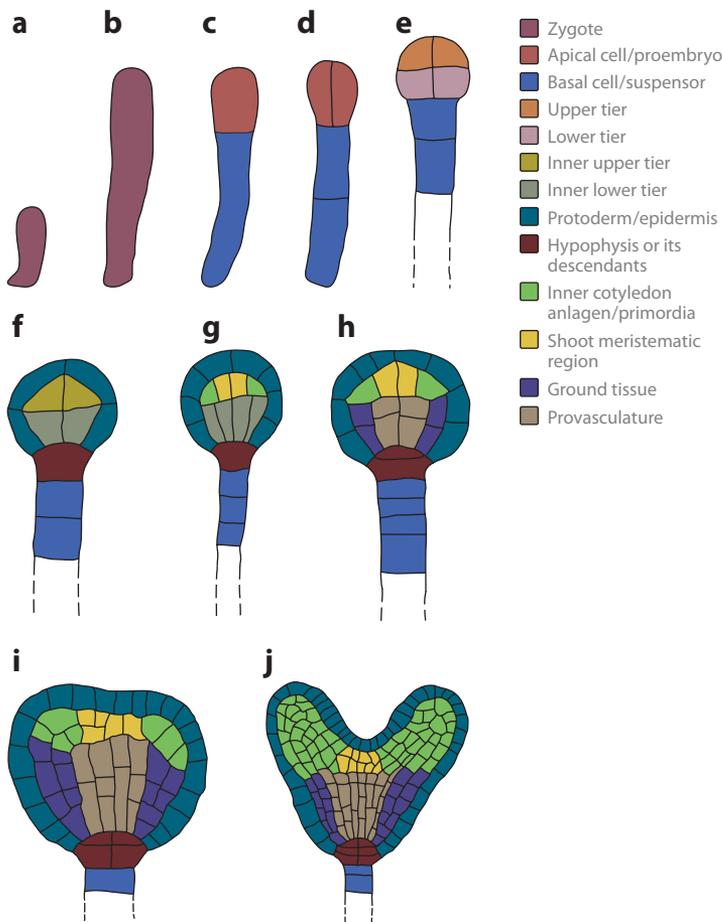
Figure 1

Early embryogenesis in *Arabidopsis thaliana*. Panels show longitudinal sections of embryos during consecutive developmental stages: (*a*) zygote, (*b*) elongated zygote, (*c*) one-cell stage, (*d*) two- or four-cell stage, (*e*) octant stage, (*f*) dermatogen stage, (*g*) early-globular stage, (*h*) mid-globular stage, (*i*) transition stage, and (*j*) heart stage. Groups of developmentally related cells are color-coded. Embryos not drawn to scale.

Zygote Elongation

The *A. thaliana* zygote not only becomes polarized but also elongates approximately threefold along its apical-basal axis before it divides. This elongation depends on the GDP/GTP exchange factor for small G proteins of the ARF class (ARF-GEF) GNOM (GN). If *GN* is knocked out, elongation and asymmetric division are compromised, but GN targets in the zygote are not known (98, 132). Zygote elongation or its asymmetric division also depends

Shoot meristem: group of self-replenishing cells at the shoot apex that sustain shoot growth and the formation of lateral organs such as leaves and flowers

Table 1 Gene abbreviations and full names used in this review

Abbreviation	Full name
ACR4	ARABIDOPSIS CRINKLY 4
AGO1	ARGONAUTE 1
ALE1/2	ABNORMAL LEAF-SHAPE 1/2
ARR7/15	ARABIDOPSIS RESPONSE REGULATOR 7/15
AS1/2	ASYMMETRIC LEAVES 1/2
ATDEK1	ARABIDOPSIS THALIANA DEFECTIVE KERNEL 1
ATH1	ARABIDOPSIS THALIANA HOMEOBOX 1
ATHB8/15	ARABIDOPSIS THALIANA HOMEOBOX 8/15
ATML1	ARABIDOPSIS THALIANA MERISTEM LAYER 1
BBM/PLT4	BABY BOOM/PLETHORA 4
BDL/IAA12	BODENLOS/INDOLE-3-ACETIC-ACID 12
BIM1	BES INTERACTING MYC-LIKE PROTEIN 1
BOP1/2	BLADE-ON-PETIOLE 1/2
CLE40	CLV3/ESR-RELATED 40
CLV3	CLAVATA 3
CUC1/2/3	CUP-SHAPED COTYLEDON 1/2/3
CUP	CUPULIFORMIS
DCL1	DICER-LIKE 1
DRN	DORNRÖSCHEN
DRNL	DORNRÖSCHEN-LIKE
ENP/MAB4	ENHANCER OF PINOID/MACCHI-BOU 4
FDH	FIDDLEHEAD
FS	FASS
GN	GNOM
GRN/RKD4	GROUNDED/RWP-RK DOMAIN 4
HAN	HANABA TARANU
KAN1	KANADI 1
KN1	KNOTTED 1
KNAT1/BP	KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 1/BREVIPEDICELLUS
LOG	LONELY GUY
LTP1	LIPID TRANSFER PROTEIN 1
MKK4/5	MITOGEN-ACTIVATED PROTEIN KINASE KINASE 4/5
MP/ARF5	MONOPTEROS/AUXIN RESPONSE FACTOR 5
MPK3/6	MITOGEN-ACTIVATED PROTEIN KINASE 3/6
NAM	NO APICAL MERISTEM
NPH4/ARF7	NONPHOTOTROPIC HYPOCOTYL 4/AUXIN RESPONSE FACTOR 7
OSH1	Oryza sativa homeobox 1
OSTF1	Oryza sativa transcription factor 1
PDF1/2	PROTODERMAL FACTOR 1/2
PHB	PHABULOSA
PHV	PHAVOLUTA

(Continued)

Table 1 (*Continued*)

Abbreviation	Full name
PID	PINOID
PID2	PINOID 2
PIN1/3/4/7	PIN-FORMED 1/3/4/7
PLT1/2/3	PLETHORA 1/2/3
PNF	POUND-FOOLISH
PNY	PENNYWISE
QHB	quiescent-center-specific homeobox
REV	REVOLUTA
RPK1	RECEPTOR-LIKE PROTEIN KINASE 1
SCR	SCARECROW
SHR	SHORT-ROOT
SSP	SHORT SUSPENSOR
STM	SHOOT MERISTEMLESS
TAA1	TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1
TAR1/2	TRYPTOPHAN AMINOTRANSFERASE RELATED 1/2
TMO7	TARGET OF MONOPTEROS 7
TOAD2	TOADSTOOL 2
TPL	TOPLESS
WAG1/2	WAG 1/2
WOX1/2/3/5/8/9	WUSCHEL RELATED HOMEOBOX 1/2/3/5/8/9
WRKY2/33	WRKY DNA-BINDING PROTEIN 2/33
WUS	WUSCHEL
YDA	YODA
YUC1/4/10/11	YUCCA 1/4/10/11
ZLL/AGO10	ZWILLE/ARGONAUTE 10
ZMCUC3	Zea mays CUP-SHAPED COTYLEDON 3
ZMNAM1/2	Zea mays NO APICAL MERISTEM 1/2

on the interleukin-1 receptor-associated kinase (IRAK)/Pelle-like kinase SHORT SUSPEN-SOR (SSP), the MAPKK kinase YODA (YDA), MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3), MPK6, and the RWP-RK family protein GROUNDED (GRN)/RWP-RK domain 4 (RKD4), which functions as a transcriptional regulator (7, 58, 89, 154, 155). There is evidence that *SSP*, *YDA*, *MPK3*, and *MPK6* as well as MITOGEN-ACTIVATED PROTEIN KINASE KINASE 4 (MKK4) and MKK5 act in the same pathway (7, 155), but the direct targets of this hypothetical kinase pathway in the zygote remain unknown. However, it might be meaningful that a close homolog of WRKY2, WRKY33, is phosphorylated by MPK3 and MPK6 (96, 162).

ZYGOTIC GENOME ACTIVATION

Zygotic genome activation already occurs in the zygote in flowering plants. For *N. tabacum*, evidence has been presented that deposited maternal transcripts are not sufficient for zygote elongation and division, but that this process requires zygotic de novo transcription (170). In *Z. mays* and *N. tabacum*, transcripts not present in egg and sperm cells accumulate in the zygote, which indicates that these transcripts

Cotyledon: leaf formed in the developing embryo

Root meristem: group of self-replenishing cells at the root tip that sustain root growth

Ground tissue: primordium that will give rise to two tissue layers, endodermis and cortex

are made de novo in the zygote (110, 125, 170). Comparable experiments have not been done in *A. thaliana*. However, in both *A. thaliana* and *Z. mays*, genes whose expression has not been detected in pollen are expressed in the zygote from the paternal allele (130, 151), implying zygotic genome activation at the zygote stage in these species.

This de novo expression of paternal genes in the zygote also indicates that the paternal genome is not generally silenced in the zygote or early embryo. This idea has received support from other studies (120, 156, 165), although in these cases it cannot be clearly distinguished between transcripts delivered by the pollen and de novo transcription from the paternal alleles in the zygote. However, whereas *Z. mays* displays an equivalent parental contribution in the zygote and during early embryo development (101), in *A. thaliana* maternal transcripts appear to predominate during early embryogenesis (5). This maternal predominance is thought to result from the downregulation of the paternal alleles by the maternal chromatin small interfering RNA (siRNA) pathway, whereas the activation of the paternal alleles during the course of embryogenesis is thought to be mediated by maternal histone chaperone complex CAF1 (5). However, it cannot be excluded that the maternal predominance during early *A. thaliana* embryogenesis is mainly or also due to transcript carryover from the egg cell rather than specific downregulation of the paternal alleles. Hence, the two aforementioned mechanisms (the chromatin siRNA pathway and activity of the CAF1 complex) could generally be involved in zygotic genome activation. In conjunction with a supposed stronger transcript contribution of the egg cell as compared with the sperm cell, mechanisms delaying the zygotic genome activation would prolong the predominance of transcripts derived from the maternal alleles.

Some observations argue against general differences between paternal and maternal alleles in *A. thaliana*. For example, both paternal and maternal histone H3 variants are replaced by de novo synthesized H3 variants in the zygote (50, 51). And although imprinting is quite common in the angiosperm endosperm, only a few genes imprinted in the embryo have been reported so far (56, 90, 118). The maternal-to-zygotic transition thus appears to already commence in the zygote. In contrast to animals, however, because there is pronounced postmeiotic gene expression in both female and male gametophytes followed by postfertilization gene expression, the maternal-to-zygotic transition might more appropriately be called the gametophytic-to-sporophytic transition. This transition might be completed sooner or later, presumably depending mainly on species-specific velocities of development during early embryogenesis. In this view, the longer it takes for the zygote and its progeny to divide, the earlier in developmental time the gametophytic-to-sporophytic transition might occur.

ZYGOTE DIVISION AND SEPARATION OF APICAL AND BASAL CELL FATE

In the vast majority of flowering plant species, the zygote divides transversely, generating an apical daughter cell and a basal daughter cell, whereas in some species oblique or longitudinal divisions occur (62, 133). When the zygote divides transversely, the two daughter cells may be quite different in size, depending on the position of the plane of cell division. In *Ricinus communis* and *Triticum aestivum* (wheat), for example, the zygote divides "symmetrically," generating two daughter cells of equal size (74, 133). In other species, zygotes divide asymmetrically. Whereas in *Coriaria nepalensis* and *Anethum graveolens*, for example, the apical daughter cell is larger than the basal one, in *A. thaliana* the apical daughter cell of the zygote is smaller than the basal one (94, 133). There seems to be no general rule regarding the size ratio of the apical daughter cell and the basal daughter cell of angiosperm zygotes (133).

Nonetheless, the division of the zygote might still—directly or indirectly—separate apical and basal cell fate and hence might also consolidate or establish the apical-basal axis of

polarity, which is then maintained throughout plant life. Some evidence supports this view. In both *Z. mays* and *N. tabacum*, the apical daughter cell of the zygote exhibits a transcriptional profile distinct from the basal counterpart (48, 113). In *A. thaliana*, two developmental pathways, in addition to the YDA pathway mentioned above, have been linked to apical-basal axis establishment after zygote division: One involves the transcription factors WOX8, WOX9, and WOX2, whereas the other is auxin dependent, involving the auxin efflux regulator PIN-FORMED 7 (PIN7) as well as the transcriptional regulators MONOPTEROS (MP)/AUXIN RESPONSE FACTOR 5 (ARF5) and BODENLOS (BDL)/INDOLE-3-ACETIC-ACID 12 (IAA12) (see below) (**Figure 2**).

Besides *WOX8*, whose expression in the zygote is induced by WRKY2, *WOX2* is also expressed in the zygote (40, 151). After zygote division, though, these two genes are not coexpressed anymore; *WOX2* is expressed in the apical daughter cell of the zygote, and *WOX8* (together with *WOX9*) is expressed in the basal (40). *WOX9*, which is assumed to be
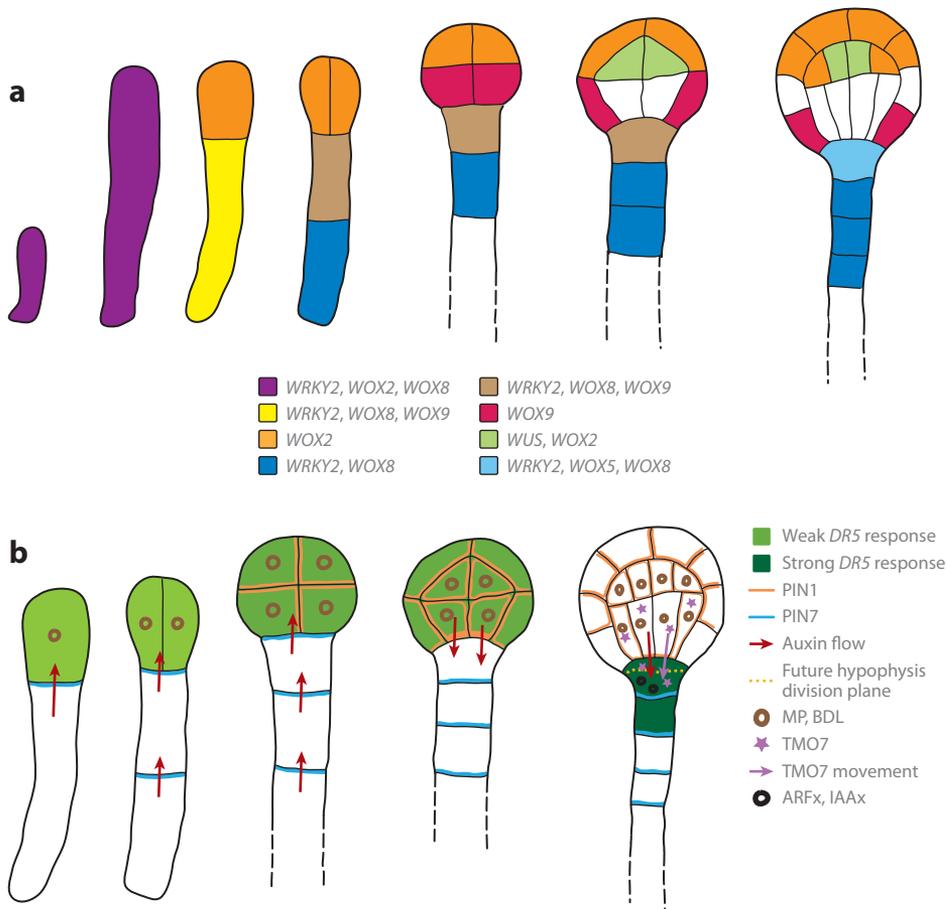

Figure 2

Apical-basal patterning and hypophysis specification in early embryogenesis of *Arabidopsis thaliana*.
(*a*) Expression patterns of *WRKY2* and early-expressed *WOX* genes. (*b*) Auxin signaling and hypophysis specification. Embryos not drawn to scale.

a target of WRKY2 as well, might already be expressed in the zygote and possibly also in the apical daughter cell of the zygote (40, 151, 163). WOX8 and WOX9 are supposed to signal from the basal to the apical daughter cell for proper *WOX2* expression to occur (10). However, because there are stronger defects in *wox8 wox9* or *wox9* alone than there are in *wox2* mutant embryos, WOX8 and WOX9 appear to have additional, WOX2-independent functions in early development (10, 40, 163). *WRKY2* is coexpressed with *WOX8* and partially with *WOX9* during the earliest stages of embryogenesis (40, 151) (**Figure 2***a*), which could account for the early expression of these two *WOX* genes in the basal lineage. The problem of the separation of apical and basal cell fate, however, would not be solved with this extension of the WOX pathway; instead, the problem would be shifted from understanding *WOX2*, *WOX8*, and *WOX9* transcript distribution to understanding *WRKY2* transcript distribution.

The auxin-dependent pathway implicated in apical-basal axis establishment during *A. thaliana* embryogenesis becomes relevant immediately after zygote division, when auxin is transported from the basal to the apical daughter cell via PIN7 (30) (**Figure 2***b*). The auxin response in the apical descendant of the zygote triggered by this directional auxin transport might be important for its proper specification, as evidenced by its transverse instead of longitudinal division in *bdl*, *mp*, *mp bdl*, and *pin7* mutant embryos (30, 42). *MP* encodes an ARF, *BDL* encodes an AUXIN (AUX)/IAA inhibitor, and both are expressed in the apical cell lineage (41, 43); *MP* and *BDL* form a system of two interconnected feedback loops that can be modulated by auxin via the degradation of BDL protein (76). The initial transport of auxin to the apical cell(s) might thus be sufficient to establish expression of these two important developmental regulators. But, comparable to the WOX/WRKY case, the next step on the hierarchy ladder has to be taken now, and how PIN7-mediated basal-to-apical auxin transport is set up must be determined.

HYPOPHYSIS SPECIFICATION AND ROOT POLE FORMATION

Importance of Auxin in Hypophysis Specification and Root Pole Formation

The root pole is the basal end of the angiosperm embryo. In *A. thaliana*, the specification of the founder cell of the root meristem is not the result of a (spatially) isolated developmental program, but the consequence of developmental events that take place in the apically adjoining cells (157).

One of these events is the overall reversal of the above-mentioned basal-to-apical auxin flow from the dermatogen stage onward. The PIN1 auxin efflux regulator formerly nonpolarly distributed in the cells of the embryo proper starts to become localized predominantly to the basal side of the lower inner cells, and the formerly apically localized PIN7 becomes localized to the basal side of the suspensor cells. In consequence, auxin accumulates in the hypophysis and the subhypophyseal cell as indicated by the auxin response reporter *DR5* (30) (**Figure 2***b*).

This accumulation of auxin in the hypophysis appears to be crucial for its specification and subsequent root pole formation, as suggested by the fact that impairment of auxin biosynthesis and transport as well as auxin signaling interfere with these processes. The auxin-biosynthesis multiple mutants *yucca 1* (*yuc1*) *yuc4 yuc10 yuc11* and *tryptophan aminotransferase of arabidopsis 1* (*taa1*) *tryptophan aminotransferase related 1* (*tar1*) *tar2* as well as the auxin transport quadruple mutant *pin1 pin3 pin4 pin7* are rootless, just like seedlings in which the phosphorylation status-dependent polar PIN1 localization is reversed from the basal to the apical side in the inner cells of the embryo proper by the misexpression of the PIN1-phosphorylating serine/threonine kinase PINOID (PID) (19, 30, 31, 102, 139). Moreover, the regulation of *PIN1* expression involves MP and its inhibitor BDL (157). This might explain why the knockout of *MP*, or mutations causing the stabilization of BDL, lead to the non- or misspecification of the hypophysis and subsequent failure to form

a root (157). Thus, MP-BDL-dependent auxin signaling in the cells of the embryo proper indirectly ensures the accumulation of auxin in the hypophysis, where signaling through another ARF-AUX/IAA pair presumably mediates the actual specification process (157) (**Figure 2b**). Recently, detailed expression analysis revealed several *ARF* candidates expressed in the hypophysis (117).

Additional Factors Involved in Hypophysis Specification and Root Pole Formation

In addition to auxin, other molecules likewise serve as mobile signaling cues for hypophysis specification. TARGET OF MONOPTEROS 7 (TMO7), a small transcriptional regulator whose expression is regulated by MP and BDL, also moves from the provascular cells into the hypophysis and contributes to its specification (128) (**Figure 2b**). SHORT-ROOT (SHR) might also move there, as inferred from the expression of *SCARECROW* (*SCR*) in the hypophysis (106, 164). Although SCR does not appear to be necessary for hypophysis specification itself—as indicated by the apparently normal hypophysis division in the *scr* mutant—SCR is subsequently required for proper root pole formation (164). Similar considerations apply to the *PLETHORA* (*PLT*) genes *PLT1*, *PLT2*, *PLT3*, and *BABY BOOM* (*BBM*)/*PLT4* and to *WOX5*. The expression of some of them depends on *MP* and its close homolog *NONPHOTOTROPIC HYPOCOTYL 4* (*NPH4*)/*ARF7* or is initiated in the hypophysis in an *MP-BDL*-dependent fashion, but at least *WOX5* is mainly required for root organization of later developmental stages and root stem cell maintenance (3, 34, 40, 122).

Although auxin signaling is of central importance for root pole initiation, it is not the only plant hormone signaling pathway involved. The brassinosteroid signaling component *BES INTERACTING MYC-LIKE PROTEIN 1* (*BIM1*) and the AP2 transcription factors DORNRÖSCHEN (DRN) and DORNRÖSCHEN-LIKE (DRNL), which interact with BIM1, are required for proper hypophysis division and root formation, suggesting that auxin-brassinosteroid crosstalk is involved in root pole initiation (16, 17, 169). In addition, the requirement of two feedback repressors of cytokinin signaling, *ARABIDOPSIS RESPONSE REGULATOR 7* (*ARR7*) and *ARR15*, for the same process indicates the necessity to dampen cytokinin signaling (105). This dampening happens specifically in the lower derivative of the hypophysis via *ARR7* and *ARR15*, whose expression depends on auxin (105) and hence possibly also indirectly on MP-BDL-dependent signaling.

Positional Information During Root Initiation

The fate of the hypophysis thus appears to be determined by its position at the basal end of the early embryo rather than its descent from the basal daughter cell of the zygote. Indeed, the clonal origin of the hypophysis might not be relevant for root pole initiation. In the *hanaba taranu* (*han*) mutant, expression domains of genes are shifted apically so that genes normally expressed only in the suspensor replace "apical" genes in the lower half of the embryo proper. As a consequence, it is not the histologically still-discernable hypophysis that becomes the founder cell of the future root pole, but rather cell(s) from the lower-tier descendants (108). As in the wild type, the cell(s) to be recruited for root pole formation appear to be those closest to cells with an apical cell fate.

In an even more extraordinary case of atypical embryonic root initiation, which occurs in the *topless-1* (*tpl-1*) mutant, a root is initiated not only basally but also apically and, interestingly, like in *han*, in an MP-independent fashion (87, 108). TPL, a cosuppressor that binds to BDL and probably other AUX/IAAs as well as indirectly to jasmonate ZIM-domain (JAZ) repressor proteins and directly to WUSCHEL (WUS), might recruit histone deacetylases to repress gene expression (70, 86, 115, 141; reviewed in 73). The *tpl-1* mutation is a dominant negative mutation relieving the repression

Provasculature: cells that will give rise to the vasculature (the conductive tissue)

of TPL targets; especially derepression of the TPL targets *PLT1* and *PLT2* leads to the formation of a secondary root pole (135).

Many angiosperm species—including various monocots and, e.g., *Pisum sativum* (pea)—do not exhibit a cell that clearly corresponds to the *A. thaliana* hypophysis, i.e., a single uppermost derivative of the basal daughter cell of the zygote that invariably divides into a smaller upper lens-shaped and a larger lower cell to give rise to the quiescent center and the columella of the root meristem, respectively (reviewed in 59). Nevertheless, these species of course also form a root, and they may do so by employing signaling pathways similar to those in *A. thaliana*, which specify the hypophysis in a position-dependent manner. In *O. sativa*, the *WUS*-type homeobox gene *quiescent-center-specific homeobox* (*QHB*) is—similar to *WOX5* in *A. thaliana*—expressed in a few cells at the basal pole of the embryo; in *Z. mays* and *O. sativa*, an *SCR* homolog might play a role in root patterning (40, 67, 68, 82, 83). The developmental significance of the singular hypophysis in *A. thaliana* might thus mainly relate to the minimal number of cells that constitute the embryo at the very early stage when the root pole is initiated.

RADIAL PATTERNING AND PROTODERM SPECIFICATION

Separation of Inner and Outer Fate in the Early Proembryo

In *A. thaliana*, the beginning of radial patterning is marked by the tangential divisions of the cells of the embryo proper in the octant-stage embryo. The eight outer cells thus formed are the founder cells of the protoderm, and during embryogenesis the eight inner cells will give rise to, e.g., the provasculature and the ground tissue (66, 94, 126) (**Figure 1**). Like apical-basal axis establishment, these tangential divisions have been linked to the action of *WOX* genes and *MP*. In *wox2* and, with a higher penetrance, in *wox2 mp*, *wox2 wox8*, and *wox1 wox2 wox3*, some cells of the octant-stage embryo proper

do not divide tangentially, so that a "continuous" protodermal layer is not formed (10, 40). How *WOX* genes and MP-dependent auxin signaling mediate the proper orientation of these cell-division planes is not known.

An early difference between protodermal and inner cells is the divergence of transcriptional activities. The GLABRA 2 (GL2) family homeodomain transcription factors *ARABIDOPSIS THALIANA MERISTEM LAYER 1* (*ATML1*) and *PROTODERMAL FACTOR 2* (*PDF2*) are initially expressed throughout the early embryo proper, but immediately after the tangential divisions have occurred their expression becomes confined to the protodermal cells (1, 88) (**Figure 3a,b**). Conversely, the expression of ZWILLE [ZLL, also called *ARGONAUTE 10* (*AGO10*)], which is expressed in the apical cells from the four-cell stage on and is involved in shoot meristem maintenance, becomes confined to the inner cells (91, 104) (**Figure 3a,b**). Remarkably, in *Z. mays* and *O. sativa*, where the cell-division planes after the zygotic division appear randomly oriented, the expression of *ATML1* homologs also becomes confined to the protoderm, and these homologs might serve a similar function during protoderm development as their *A. thaliana* counterparts (52–54, 167).

In *atml1 pdf2* double-mutant seedlings, cotyledons seem devoid of an epidermis and the shoot apex lacks distinct cell layers (1). The *ATML1* promoter and the *PDF2* promoter each contain a potential binding site for WUS, the founding member of the WOX family (1, 40, 143), and thus the expression of *ATML1* and *PDF2* could be directly regulated by WOX transcription factors, including those involved in the tangential divisions of the octant-stage embryo (**Figures 2a** and **3c**). Furthermore, both the *ATML1* promoter and the *PDF2* promoter contain an eight-nucleotide sequence termed the L1 box, which is also present in the promoters of other epidermally expressed genes such as *PDF1*, *FIDDLEHEAD* (*FDH*), *LIPID TRANSFER PROTEIN 1* (*LTP1*), and—almost perfectly matching—the *O. sativa* *ATML1* homolog *Oryza sativa transcription*
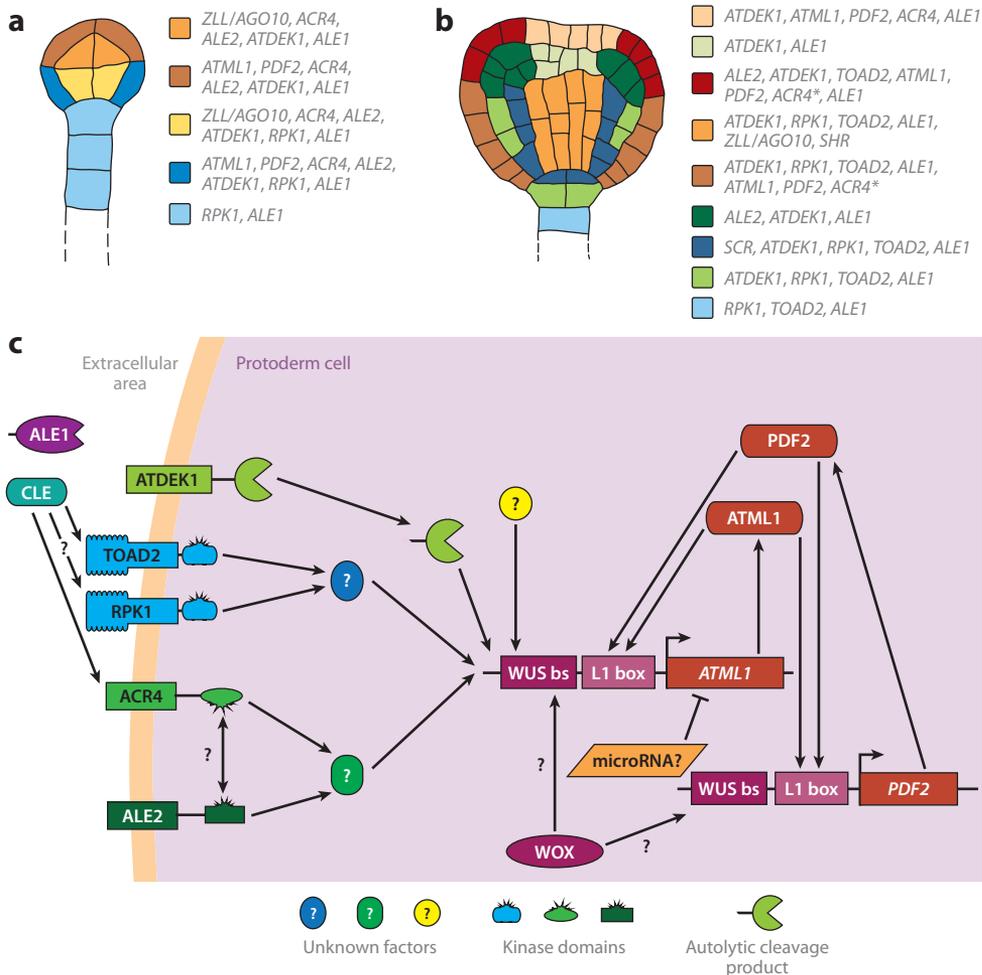
Figure 3

Radial patterning in early embryogenesis of *Arabidopsis thaliana*. (*a,b*) Expression patterns of genes important for radial patterning. Panel *a* shows the dermatogen stage; panel *b* shows the transition stage. Asterisk indicates that weak *ACR4* expression was detected ubiquitously in embryos. (*c*) Signaling pathways maintaining protoderm identity. Abbreviation: WUS bs, WUS binding site. Embryos not drawn to scale.

factor 1 (*OSTF1*) (1, 2, 167). Because ATML1 and PDF2 bind to the L1 box in vitro, it is conceivable that these two transcription factors establish a positive feedback loop that regulates the transcription of genes expressed in the epidermis (1, 2) (**Figure 3c**). In the case of *ATML1*, however, the L1 box and the WUS binding site do not appear to be the only important regulatory regions. Although the L1 box is essential for the expression of *PDF1*, this is not the case for *ATML1* (2, 143). Rather, the

L1 box controls expression redundantly with the WUS binding site in the *ATML1* promoter, but even when both elements are deleted, a hexameric copy of an *ATML1* promoter fragment still confers weak expression (143). In addition, *ATML1* is still expressed in the *atml1 pdf2* and *wox8 wox9* double-mutant backgrounds (10, 143). Thus, although these two "pathways" might converge on *ATML1* expression, other factors are probably involved in the regulation of this gene. Because the *ATML1* promoter

confers expression in the suspensor but the messenger RNA (mRNA) is detected there only in the *dicer-like 1* (*dcl1*) mutant, a microRNA might regulate the *ATML1* expression in the suspensor (111, 143) (**Figure 3c**).

The inner cells of the *A. thaliana* embryo give rise to the various concentric tissue layers that have been described in the root and are laid down during embryogenesis (126, 127). The GRAS transcription factor SHR is one of the best-described players involved in radial patterning. It is expressed in the provasculature and moves out to the neighboring cell layer, where it activates the transcription of another GRAS transcription factor gene, *SCR* (46, 106). *SCR* is expressed in the ground tissue and the hypophysis at the globular stage of embryogenesis. When the cells of the ground tissue of the hypocotyl and the embryonic root pole divide periclinally between the triangular stage and the heart stage to generate the inner layer of endodermis and the outer layer of cortex cells, *SCR* continues to be expressed in the inner layer (164) (**Figure 3b**). These periclinal cell divisions depend on both *SHR* and *SCR* (46, 164). SHR and SCR activate microRNA165/166 in the endodermis of the mature root, from where the microRNAs feed back onto the vasculature to control its patterning. Because the two microRNAs are already expressed during embryogenesis, they might contribute to embryonic patterning as well (14).

Maintenance of Radial Patterning

RECEPTOR-LIKE PROTEIN KINASE 1 (RPK1) and TOADSTOOL 2 (TOAD2), two closely related leucine-rich-repeat receptor-like kinases (LRR-RLKs), are redundantly required for the maintenance of radial patterning (112) (**Figure 3c**). The protoderm marker *ATML1* as well as the central domain markers *ZLL/AGO10* and *SHR* are correctly expressed only initially in *rpk1 toad2* embryos, which have enlarged protoderm cells (112). At the late-globular stage of embryogenesis, the expression of *ATML1* is (almost) lost, and the expression of *ZLL/AGO10* and *SHR* extends over the

entire basal domain in *rpk1 toad2*, suggesting that *RPK1* and *TOAD2* play an essential role in the maintenance but not the establishment of the radial pattern in *A. thaliana* (112).

The ligands binding to RPK1 and TOAD2 during embryogenesis are unknown, although it was recently suggested that the signaling peptide derived from CLAVATA 3 (CLV3) binds to TOAD2 (71). Because this signaling peptide is functionally similar to other signaling peptides of the CLV3/ESR-RELATED (CLE) family (109), any of these might be the endogenous ligand for RPK1 and TOAD2 (**Figure 3c**). Hence, at least some of these signaling peptides might play a role during early embryogenesis, an assumption that receives support from the analysis of the RLK ARABIDOPSIS CRINKLY 4 (ACR4). ACR4 might bind the signaling peptide CLE40, which is the closest homolog of CLV3, and is involved in protoderm specification, where it acts together with ABNORMAL LEAF-SHAPE 2 (ALE2), another RLK (138, 145) (**Figure 3c**). Although neither the single mutants nor the double mutant appear to show severe protodermal defects during embryo development, in mutant combinations with *ale1* the protoderm is misspecified (36, 145). Accordingly, *ale1 ale2* and *ale1 acr4* double mutants do not properly express *ATML1* (145). *ALE1* encodes a protease that is predominantly expressed in the endosperm, and thus ALE2 and ACR4 might perceive a signal from the endosperm to ensure proper protoderm specification (144, 145) (**Figure 3c**). However, toxin-dependent endosperm ablation rather suggests that the endosperm is not involved in embryo patterning, and the feasibility of somatic embryogenesis also argues against essential peptide signals from the endosperm (158; reviewed in 168). In addition to its expression in the endosperm, *ALE1* is weakly expressed in the early embryo itself (144), and this might be relevant for embryogenesis.

Protoderm formation and *ATML1* expression are prevented in *arabidopsis thaliana defective kernel 1* (*atdek1*) mutant embryos, which arrest at the globular stage (60, 81, 150).

ATDEK1 encodes a calpain protease that undergoes autolytic cleavage (**Figure 3c**) and is expressed in the embryo (60, 61, 81). In *ATDEK1* knockdown lines, seedlings show a transformation of epidermal to mesophyll-like cell fate in the cotyledons, similar to what has been observed in *atml1 pdf2* double mutants (1, 60). In conclusion, although a number of key players have been analyzed, the overall genetic program of setting up the radial pattern or only the protoderm is still largely unexplored.

SHOOT MERISTEM SPECIFICATION AND COTYLEDON INITIATION

The Organizing Center

The *A. thaliana* shoot meristem can be morphologically delineated for the first time during embryogenesis at the late-torpedo stage (6, 78). In the mature embryo, the shoot meristem consists of a few small cells with big nuclei and small vacuoles, and its first molecular mark is the onset of *WUS* expression in the four inner cells of the apical embryo region at the dermatogen stage (78, 97) (**Figure 2a**). *WUS* encodes a homeodomain transcription factor, and its expression remains confined to a subset of cells close to the shoot apex during later stages of development (**Figure 4a**), defining an organizing center that keeps the neighboring stem cells in a pluripotent state (97). The *wus* mutation results in the lack of a functional shoot meristem and the formation of a flat and enlarged shoot apex consisting of aberrant cells (78). *WUS* orthologs seem to play similar roles in dicots like *Petunia hybrida* and *Antirrhinum majus*, but possibly not in monocots like *O. sativa* and *Z. mays* (70, 107, 140).

Despite considerable efforts to identify regulators and downstream targets of this master regulator (11; reviewed in 24), our knowledge is scant about the mechanism(s) of initiation and early confinement of *WUS* expression and about the identity of the *WUS*-dependent non-cell-autonomous signal(s) maintaining stem cell fate in the shoot meristem. In postembryonic
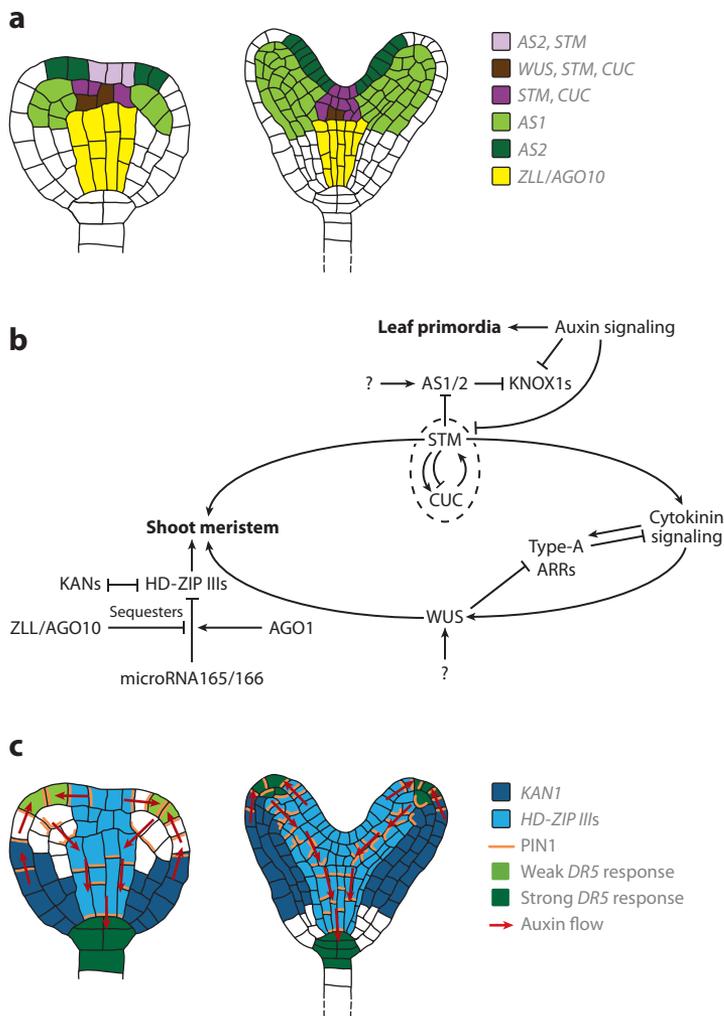
Figure 4

Shoot meristem and cotyledon initiation in *Arabidopsis thaliana*. (*a*) Expression patterns of genes important for establishment of the shoot meristem and initiation of cotyledons in *A. thaliana* during the transition stage and the heart stage. *CUC1–3* expression is generalized as *CUC*. (*b*) Pathways and hormonal regulation in shoot meristem and cotyledon initiation. (*c*) Expression patterns of *KAN1* and *HD-ZIP III* genes (exemplarily shown for *REV*, which includes domains of all other members), auxin flow mediated by PIN1 (idealized representation), and *DR5* response. Embryos not drawn to scale.

development, however, cytokinin signaling activates *WUS* expression (37). Because WUS inhibits the expression of several type-A *ARR*s that are negative regulators of cytokinin signaling, a positive feedback mechanism involving *WUS* and cytokinin signaling might thus operate in the shoot meristem to maintain

its integrity (37, 79) (**Figure 4*b***). This crosstalk may already operate during embryogenesis. In *O. sativa*, the *LONELY GUY* (*LOG*) gene, which encodes a cytokinin-activating enzyme and is specifically expressed in the shoot meristem region, is important for shoot meristem maintenance (75).

Shoot Meristem Indeterminacy and the Shoot Meristem–Cotyledon Boundary Region

The class I KNOTTED-like homeodomain transcription factor SHOOT MERISTEM-LESS (STM) might indirectly activate *WUS* expression via its induction of cytokinin biosynthesis and signaling (37, 57, 85, 166) (**Figure 4*b***), and in addition to its cytokinin-related effects, it restricts gibberellic acid levels (45, 57). Similar to its *Z. mays* ortholog *KNOTTED 1* (*KN1*) and its *O. sativa* ortholog *Oryza sativa homeobox 1* (*OSH1*), *STM* is expressed in the presumptive shoot meristem from the globular stage onward (85, 124, 134) (**Figure 4*a***); in addition, in the oil palm *Elaeis guineensis* an *STM* ortholog is expressed in the shoot meristem, at least during vegetative development (64). Together with WUS, STM is required to maintain the shoot meristem: WUS acts as the instructor of the organizing center, and STM acts as a repressor of differentiation across the entire shoot meristem (80). In differentiated tissue, simultaneous expression of WUS and STM can induce meristematic activity, with WUS non-cell-autonomously triggering divisions in *STM*-expressing tissue (35).

Being a transcription factor, STM functions in the nucleus, and this localization depends on BEL1-like homeodomain transcription factors (22, 121). Shoot meristem initiation is consistently inhibited in the *stm* mutant and the *arabidopsis thaliana homeobox 1* (*ath1*) *pennywise* (*pny*) *pound-foolish* (*pnf*) triple mutant, and also in the *cup-shaped cotyledon 1* (*cuc1*) *cuc2* double mutant, which fails to express *STM* in the presumptive shoot meristem (4, 6, 121). The NAC transcription factors CUC1–3 are redundantly required for shoot meristem establishment as well as cotyledon separation. At early embryonic stages, their expression domains partially overlap with the *STM* expression domain (**Figure 4*a***), whereas *CUC1–3* expression domains in general surround the *STM* expression domain at later stages (4, 47, 142, 152). How this expression pattern evolves is not clear. However, there appears to be mutual regulation involving positive and negative feedback loops (**Figure 4*b***): Not only are the CUCs required for *STM* expression, but STM regulates the expression of *CUC1–3* and the expression of microRNA164, which in turn targets *CUC1* and *CUC2* transcripts for degradation (4, 77, 92, 137). The *P. hybrida* and *A. majus* CUC orthologs *NO APICAL MERISTEM* (*NAM*) and *CUPULIFORMIS* (*CUP*) are also expressed at organ boundaries, and they are important for both boundary establishment and shoot meristem development (136, 159). In *Z. mays*, the putative *CUC1/2* orthologs *Zea mays NO APICAL MERISTEM 1/2* (*ZmNAM1/2*) and the *CUC3* ortholog *Zea mays CUP-SHAPED COTYLEDON 3* (*ZmCUC3*) are in part initially coexpressed with a shoot meristem marker, and later in a ringlike pattern around the shoot meristem (173), hinting at a strong conservation of *CUC* gene function at least among flowering plants.

Meristem Establishment

A general prerequisite for shoot meristem identity seems to be the presence of class III HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP III) transcription factors. This family consists of PHABULOSA (PHB), PHAVOLUTA (PHV), REVOLUTA (REV), ARABIDOPSIS THALIANA HOMEOBOX 8 (ATHB8), and ATHB15. Expression of all but *ATHB8* is already detectable from early embryonic stages onward, and in part there is overlap with the future site of the shoot meristem, whereas especially *PHB*, *REV*, and *ATHB15* expression domains partially coincide with the *ZLL/AGO10* provascular expression domain as well; ATHB8 mRNA is detectable from the heart stage onward (26, 91, 100, 116). Conversely, expression domains of members of the *KANADI* (*KAN*)

gene family could be regarded as complementary to those of the *HD-ZIP III*s, which they are supposed to antagonize (26–28, 69) (**Figure 4c**). The *phb rev* double, *phb phv rev* triple, and other loss-of-function mutant combinations involving *athb8* and *athb15* lack the embryonic shoot meristem and in severe cases fail to establish bilateral symmetry (26, 116). The dominant mutation *phb-1d* leads to ectopic meristems that express the shoot meristem marker *STM* on the lower side of leaves, and also causes an enlarged embryonic shoot meristem and partially suppresses the *stm* mutant phenotype (99). Two recent findings further support a pivotal role for HD-ZIP III transcription factors in shoot meristem formation. First, exclusion of HD-ZIP III proteins from the embryonic root pole is necessary for its proper establishment (38). Second, dominant *HD-ZIP III* mutants suppress the *tpl-1* double-root phenotype, possibly by excluding PLT1 and PLT2 from the future shoot meristem cells. Conversely, misexpression of dominant *HD-ZIP III*s can lead to (homeotic) root-pole-to-shoot-pole transformations during embryogenesis (135). It is not clear at present whether the HD-ZIP IIIs directly regulate *STM* and/or *WUS* in ectopic shoot meristem formation.

HD-ZIP III transcripts are targeted by microRNA165/166, and the dominant *HD-ZIP III* mutations reside in the microRNA pairing sites, rendering the HD-ZIP mRNAs resistant to degradation (93, 119, 146, 160, 171). The microRNA-dependent degradation involves the AGO proteins AGO1 and ZLL/AGO10, which both bind microRNA165/166 (172). It was suggested that ZLL/AGO10 and AGO1 act in an antagonistic fashion (**Figure 4b**), with ZLL/AGO10 positively regulating HD-ZIP III transcript levels through competition with AGO1—possibly by sequestering microRNA165/166. Such a sequestration could ensure sufficiently high HD-ZIP III levels during shoot meristem establishment and maintenance (172). Given that *ZLL/AGO10* expression in the provasculature is necessary for embryonic shoot meristem maintenance, a non-cell-autonomous signal could, in

principle, instruct the shoot meristem from the cells underneath (149). In this scenario, the two primary meristems of shoot and root would be initiated as WUS- and WOX5-positive cell groups, respectively, in response to inductive signals, at the opposite ends of the provasculature in early embryogenesis.

Initiation of Cotyledon Primordia

When the cotyledon primordia start to emerge in *A. thaliana*, the embryo organization shifts from radial to bilateral symmetry. The sites of cotyledon initiation correlate with auxin accumulation at subapical foci in the protoderm, as indicated by the auxin response reporter *DR5* (8) (**Figure 4c**). Auxin might therefore directly cause cotyledon initiation in the apical margins of the globular embryo (8). In addition, *STM* and *CUC* expression have to be excluded from those sites (see below). Auxin transport toward the incipient primordia is mediated by PIN auxin efflux regulators, probably mainly by PIN1 (8) (**Figure 4c**). PIN1 is apically localized in the protoderm, and the apical localization of PIN proteins is generally brought about by PID and its homologs PID2, WAG1, and WAG2, three of which have been shown to directly phosphorylate PINs (20, 23, 31, 49, 102). For example, the *pid wag1 wag2* triple mutant and the *pin1 pid* double mutant lack cotyledons (20, 33), as does the *pid enhancer of pinoid* (*enp*) double mutant (148). *ENP/MACCHI-BOU 4* (*MAB4*) encodes an NPH3-like protein that is involved in the regulation of PIN1 localization (32, 148). It is noteworthy that in both double mutants (*pin1 pid* and *pid enp*) the expression domains of *CUC* genes and *STM* are enlarged, and that cotyledon formation is partially restored when *CUC* genes or *STM* are knocked out in *pin1 pid* (33, 148); this highlights both the importance of directional auxin transport to the cotyledon initiation sites and the requirement to exclude specific transcripts/proteins from there. This view is supported by cotyledon formation defects in the auxin response mutants *mp* and *bdl* (9, 42). However, it might also be relevant in this context that MP directly activates the

expression of *DRN*—especially because *DRN* and *DRNL* redundantly act in cotyledon formation (16, 21). Additionally, DRN and DRNL are involved in the establishment and maintenance of boundary and shoot meristem gene expression domains, and they act together with PIN1 and PID (16, 18, 72). Auxin-related processes might be involved in cotyledon initiation in other flowering plant species as well, including monocots, but this has barely been investigated so far (reviewed in 15).

Another factor involved in cotyledon development, *ASYMMETRIC LEAVES 1* (*AS1*), which encodes a MYB domain protein and orthologs of which are present in *Z. mays* and *A. majus*, is initially expressed mainly subepidermally in the incipient cotyledon primordia, whereas *AS2*, which encodes a LATERAL ORGAN BOUNDARY (LOB) domain protein, is expressed protodermally before cotyledon outgrowth and later at the adaxial cotyledon side (12, 55, 84, 129, 153) (**Figure 4*a***). The loss of *AS1* or *AS2* makes *STM* dispensable for shoot meristem initiation and maintenance, suggesting that STM negatively regulates *AS1* and *AS2* (12, 13). Studies in primarily adult leaves suggest that *KNOX* genes are negatively regulated by AS1/2 and that AS1/2 possibly converge with auxin signaling to repress the *KNOX* member *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 1* (*KNAT1*)/*BREVIPEDICELLUS* (*BP*) (12, 39, 44) (**Figure 4*b***). The expression of *AS2* itself is negatively regulated by KAN1 and positively by BLADE-ON-PETIOLE 1/2 (BOP1/2), the expression of the latter in turn being directly or indirectly repressed by *STM* (65, 161). How exactly *AS1* and *AS2* are linked to auxin, however, has not been resolved.

PERSPECTIVES

Considerable progress has been made in the analysis of mechanisms underlying specific events in early embryogenesis, notably in *A. thaliana*. For example, we now have a clear conceptual framework for the initiation of the root meristem in the early embryo. However, although the main regulators have been identified and characterized, it is still rather obscure how these early events relate to the establishment of the molecular system for self-maintenance of the functional root meristem at the heart stage of embryogenesis. The initiation and establishment of the self-maintenance system are even less clear for the shoot meristem. Large-scale approaches combining expression profiling of specific embryo regions with functional characterization of putative developmental regulators might contribute to closing the gap.

Another unsolved problem is the origin of the apical-basal pattern. Although genes encoding developmental regulators are expressed in either the apical or the basal daughter cell of the zygote, it is not known how the expression of these regulators is ultimately established. This also relates to the mode of division of the zygote: Is it truly unequal, reflecting an intrinsic polarity of the zygote before division? Alternatively, the division might be equal, and only the two daughter cells would be exposed to different environments and thus might perceive different signals.

The contribution of the gametes to early embryogenesis still needs to be assessed. Although differentially regulated genes have been identified, their role in early patterning has not been clarified. And the significance of epigenetic regulation of patterning is still an open question.

Finally, most studies have focused on a few species, notably *A. thaliana*. Considering the differences in cell-division patterns between early embryos from different species, exploring orthologous developmental regulators might reveal to what extent their actions and regulatory networks are conserved among the flowering plant species when the cellular contexts of developmental events are not.

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