In vitro fertilization – a tool to dissect cell specification from a higher plant zygote

Takashi Okamoto[1] and Erhard Kranz[2]*

[1]Department of Biological Sciences, Tokyo Metropolitan University, Minami-Osawa 1-1, Hachioji, Tokyo, 192-0397, Japan
[2]Biozentrum Klein Flottbek und Botanischer Garten, Entwicklungsbiologie und Biotechnologie, Universität Hamburg, Ohnhorststr. 18, D-22609 Hamburg, Germany

Generally, the life of sexual organisms starts from a single cell, the fertilized egg cell or zygote. By cell division and growth this single cell finally gives rise to the mature organism, which contains different cell types, tissues and organs. The zygote, the progenitor cell is the origin for the formation of cells of different developmental fates and the main body axes.

In flowering plants (angiosperms) little is known about the underlying mechanisms of these processes and, in spite of its fundamental importance, regulation of early embryonic development is only poorly understood. New data suggest that the asymmetric division of the zygote separates determinants of apical and basal cell fates and that programmes of transcription are initiated in the domains of single cells of the early embryo. In this context, we describe results obtained by the use of powerful tools of *in vitro* fertilization and micromanipulation techniques for the elucidation of mechanisms of early embryonic patterning in higher plants.

Keywords: Apical cell, basal cell, embryogenesis, gene expression, *in vitro* fertilization.

MANY of developmentally regulated mechanisms and events appear during early embryogenesis. The embryo arises from the fertilized egg, the zygote. The processes of transition from the maternal to the zygotic state and the subsequent establishment of an embryo-specific developmental path underlie dramatic changes in gene expression programmes. Therefore the timing of these changes and the paternal and maternal contribution to this switch are of fundamental interest. In animals, the timing of zygotic gene activation varies considerably[1]. Generally, a delay between fertilization and the maternal to zygotic switch occurs at the two-celled stage in mice, not earlier than the three- to four-cell stage in *Caenorhabditis elegans* and later at the mid-blastula stage in *Xenopus* and zebra fish embryos, consisting of thousands of cells and early embryonic development largely depend on maternal mRNA and proteins[2–6]. In fertilized mouse eggs, nascent transcripts are not translated until the two-cell stage leading to a delay of expression of zygotic genes by uncoupling translation from transcription[7,8].

Among angiosperms only fragmentary data are available in *Arabidopsis* and maize. The early stages of embryo and endosperm development in *Arabidopsis* have been proposed to be largely under maternal control[9]. However, there might be no general maternal control during early embryogenesis, since the phenotypes of numerous embryo lethal mutants of *Arabidopsis* segregate with a typical sporophytic 3 : 1 ratio, suggesting no apparent maternal effect[10]. In addition, some paternal alleles are expressed early during development and are sufficient for normal development in this species[11,12], and gfp-mRNA – from a paternally inherited transgene – appeared as early as four hours after *in vitro* fertilization to coincide with male chromatin decondensation followed by translational activity six hours after fertilization in the maize zygote[13]. Expression analyses of 16 genes during early seed development in maize revealed that only maternally inherited alleles were detected during three days after fertilization[14]. Also, *de novo* zygotic genome activity has been shown in maize by expression analyses of cyclin genes[15]. Depending on individual genes, considerable variations can occur regarding the contributions of paternal and maternal alleles to early embryo and endosperm development and timing of their expression. It is generally accepted that a wide range of developmental events are epigenetically controlled. A characteristic feature of such underlying processes is that it is not associated with changes in DNA sequences but is under the control of heritable changes in gene expression. Importantly, RNA molecules are involved in epigenetic gene regulation by providing sequence specificity for the targeting of developmentally relevant genes. This RNA-based regulated gene expression can occur during development at the transcriptional level by modifying chromatin structure and/or DNA methylation and by interfering with transcript stability or translation[16].

The underlying mechanisms of the specification of cells originating from zygotes with different developmental fates are widely unknown. However, different expression profiles of specific cells suggest that they are established during early embryonic development. For example, the ATML1 gene (*Arabidopsis thaliana* meristem L1 layer), which encodes a homeodomain protein, starts expression

*For correspondence. (e-mail: ekranz@botanik.uni-hamburg-de)

in the apical cell after zygotic division and is expressed in all proembryo cells until the eight-cell stage. In the 16-celled embryo, mRNA expression is restricted to the protoderm. In the mature embryo, expression is restricted to the L1 layer of the shoot apical meristem. It is assumed that ATML1 is involved in the specification of cells and pattern formation by setting up morphogenetic boundaries of positional information[17].

Cell fate depends on positional signal molecules, and among them, graded concentrations of auxin play an important role in pattern formation of the young embryo[18,19]. The apical-basal axis of *Arabidopsis* is established by efflux-dependent auxin gradients[20,21]. Vesicle transport mediated by GNOM/EMB30 (GN) activity localizes putative auxin transporters of the PINFORMED (PIN) family in a polar fashion in the cell, suggesting a directional flow of auxin[22–25]. Apical-basal pattern formation in the embryo can be influenced by changes of PIN 1 location generated by mutations occurring in the *GN* gene[26]. A specific auxin response is also of particular importance for pattern formation of the young embryo. This is demonstrated by the *Arabidopsis BODENLOS* (*BDL*) gene which encodes an auxin response protein inhibiting MONOPTEROS (MP)-mediated embryo patterning[27]. Both are needed for normal root development. The mutant monopteros lacking function of ARF5, a transcription factor of the ARF (auxin response factor) family that activates auxin-responsive target genes[28,29], and the auxin-insensitive mutant fail to initiate the root meristem during early embryogenesis. The orientation of the division plane of the apical daughter of the zygote is influenced by *bdl* and *mp* mutations. The formation of the hypophysis is influenced by mutations in *MP* and *BDL* genes in the manner that the putative hypophysis fails to divide asymmetrically to form the precursors of the quiescent center and the lower-tier stem cells of the root meristem[30,31].

The above-mentioned genes which are involved in early embryogenesis have been identified through mutant analyses using *Arabidopsis*, since the plant lends itself to studies on embryogenesis in addition to being widely used as a genetic model organism. The fixed pattern of embryo formation in *Arabidopsis* makes it possible to trace the origin of seedling structures back to the region in the early embryo[32–34]. Another reason why mutant analyses have been employed for embryogenesis research is the difficulty associated with directly addressing the female gamete, zygote and early embryo in the embryo sac, deeply embedded in the ovular tissues. In the late 1980s, technical advances led to successful isolation of viable gametes[35,36]. An *in vitro* fertilization (IVF) system was developed whereby maize zygotes produced *in vitro* by electrical fusion of an isolated egg cell with an isolated sperm cell are able to develop into an asymmetrical two-celled embryo, proembryo and transition-phase embryo via zygotic embryogenesis in a similar manner to that *in planta*[37–39]. A major benefit of the *in vitro* gamete fusion and subsequent culture of zygotes is that the first unequal division of zygotes can be observed directly, and the zygote and two-celled embryo can be used as materials for further analyses[13,40,41]. Recently, a procedure for isolating the apical and basal cells from two-celled maize embryos was established, and these isolated cells were used as starting points for detecting genes that are up- or down-regulated in the apical or basal cell[42]. In this review, single cell manipulation and IVF techniques are outlined, genes expressed in apical and basal cells or zygotes of maize and *Arabidopsis* are described, and finally prospects for further investigations of early higher plant embryogenesis are outlined.

Single cell manipulation to dissect cell specification from a zygote

An experimental system has been established in maize to isolate, handle, and fuse single gametes, which enables studies of events that occur immediately after gamete fusion. Starting with gamete fusion or isolated zygotes, the development of a single zygote into a two-celled and multicellular embryo and finally into a higher plant can be followed *in vitro*. With experimental access to single gametes, to gamete fusion, and to early events after *in vitro* fertilization, the consequences and significance of such events as cell specification on pattern and plant formation can be studied at the single cell level[38,42].

In vitro gamete fusions are performed without surrounding cells of mother tissue or other cells of the embryo sac. IVF includes the combination of three basic microtechniques: (i) the isolation and selection of male and female gametes, (ii) the fusion of pairs of gametes, and (iii) single zygote culture.

The female gametes, zygotes and early embryos are located in the embryo sac which is deeply embedded in the ovule in most angiosperms. Therefore, for experimental access and single cell manipulation, for example, gamete fusions and cell analyses, these cells and small tissues have to be isolated. To overcome the difficulties of direct observation and analysis of these cells, methods were developed for the isolation of embryo sacs, egg cells, central cells and zygotes in a wide range of higher plant species (for review see refs 35, 43). In maize, routinely 20–40 egg cells and zygotes can be isolated by one experienced person per day; and under optimal conditions up to 60 of these cells can be obtained[44]. This amount of cells is sufficient, for example, for DNA-, mRNA-isolation, cDNA library construction and PCR-based molecular analyses. Despite the extremely small amount of plant material available, recent advances in proteomics technologies provide the possibility of identifying proteins in such cells. Therefore, biochemical analyses using maize egg cells were carried out to determine abundant proteins in the egg cells[41].

Electrical pulse-mediated gamete fusion is the method of choice for subsequent molecular analyses of cells to

study early events exactly timed after fusion. This technique by far exceed the yield of zygotes gained by other *in vitro* fusion methods and provides the required number of zygotes which is necessary for further analyses[13,40–42]. Sometimes there is no need to fuse gametes to obtain zygotes. A sufficient number of zygotes (comparable to that of isolated egg cells) can also be isolated. Monitoring the development of individual zygotes is possible with this system to study the early events of plant development[45,46].

As is the case of fertilized eggs *in planta*, maize zygotes produced by IVF of an egg cell with a sperm cell and isolated zygotes develop into an asymmetric two-celled embryo that consists of a small plasma-rich apical cell traditionally termed ca and a larger vacuolized basal cell (cb) (Figure 1). The completion of the division of the *in vitro*-produced maize zygotes occurs generally 42–46 h after gamete fusion[39] (Figure 2). Interestingly, the first division of single maize and wheat zygotes, cultured without mother tissue and without endosperm, is laid in the transverse plane similar to that *in planta*. With access to fuse gametes *in vitro*, it is possible to combine heterologous gametes which naturally do not fuse *in vivo*, for example, the fertilization of maize egg with wheat sperm. By using this approach, the consequences of such hybridizations on cell specification and pattern formation can be studied. It was observed that the egg cell is the predominant cell in heterologous egg–sperm fusions and determines the plane of the first cell division when fusion combinations of maize egg + maize sperm, maize egg + wheat sperm, wheat egg + wheat sperm and wheat egg + maize sperm were used (Figure 3). When a maize egg is fused with a wheat sperm cell, the first division in the fusion product is unequal as in *in vitro* zygotes of maize (Figure 3 *a* and *b*). Wheat *in vitro* zygotes do not show a pronounced asymmetrical first cell division (Figure 3 *d*). This can also clearly be observed after heterologous gamete fusion of a wheat egg and a maize sperm cell in the fusion product[47] (Figure 3 *c*).

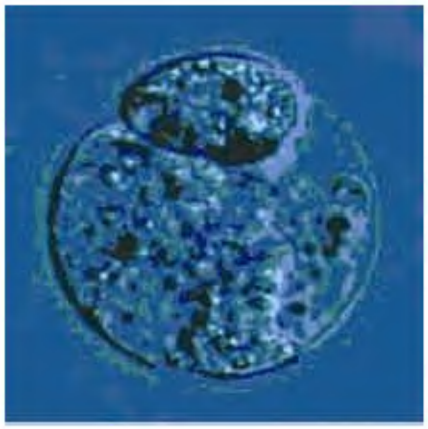

Figure 1. Two-celled embryo of maize developed after *in vitro* gamete fusion consisting of a small apical and a larger basal cell.

A procedure was established for the isolation of apical and basal cells from two-celled embryos derived from *in vitro* fertilized egg cells[42]. The use of *in vitro* gamete fusion and subsequent culture of zygotes was necessary to obtain these cells, because so far their manual isolation from ovules was not possible in maize. Thus, *in vitro* fertilized egg cells were cultured, harvested shortly after the division of the zygote and treated with a mixture of cell wall degrading enzymes. After a 20 min treatment with this mixture, a protoplast of the apical cell and a protoplast of the basal cell appear. The two protoplasts were mechanically separated by using a thin glass needle under an inverted microscope. This procedure resulted in single apical (ca) and single basal (cb) protoplasts (Figure 4).

Genes that are up- or down-regulated in the apical or basal cell

The establishment of a procedure for isolating apical and basal cells made it possible to identify genes that are up- or down-regulated in the apical or basal cell. Procedures for detecting such genes are presented in Figure 5. cDNAs were synthesized from 5 cell/embryo types: egg cells, apical cells, basal cells, two-celled embryos and multicellular embryos, and randomly amplified polymorphic DNA (RAPD) PCR was conducted using these cDNAs as templates. Based on the patterns of the DNA-bands detected in the gels, we categorized the expression patterns into six groups as below.

Group 1: A DNA-band, which was detected in the apical cell, two-celled embryo and multicellular embryo, but not in the egg cell or basal cell (arrow 1 in Figure 5), is assumed to be derived from a gene transcript that was up-regulated only in the apical cell after fertilization.

Group 2: A DNA-band, which was detected in the basal cell, two-celled embryo and multicellular embryo, but not in the egg cell or apical cell (arrow 2 in Figure 5), is assumed to be derived from a gene transcript that was up-regulated only in the basal cell after fertilization.

Group 3: A DNA-band observed in the apical cell, basal cell, two-celled embryo and multicellular embryo, but not in the egg cell (arrow 3 in Figure 5) was assumed to be derived from a gene transcript newly synthesized in both the apical and basal cells after fertilization.

Group 4: A DNA-band observed in the egg cell, basal cell, two-celled embryo and multicellular embryo, but not in the apical cell (arrow 4 in Figure 5) was thought to be derived from a gene transcript that was down-regulated only in the apical cell after fertilization.

Group 5: A DNA-band present in the egg cell, apical cell, two-celled embryo and multicellular embryo, but not

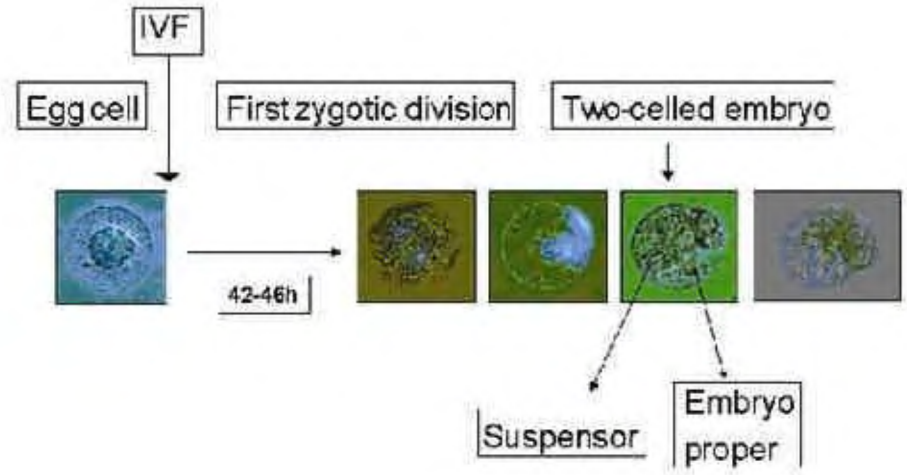

Figure 2. Development of a polar *in vitro* produced maize zygote into a two-celled and a polar multicellular embryo during culture[39].

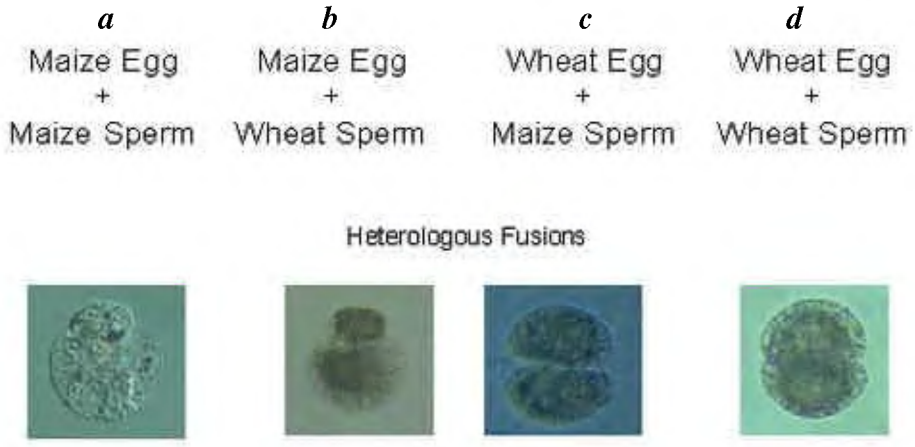

Figure 3 *a–d*. First cell division of *in vitro* zygotes and of fusion products after gametic homologous and heterologous *in vitro* fusions. ***a***, Distinct unequal cell division after fusion of maize egg and maize sperm. ***b***, Distinct unequal cell division after fusion of maize egg and wheat sperm. ***c***, Equal cell division after fusion of wheat egg and maize sperm. ***d***, Equal cell division after fusion of wheat egg and wheat sperm[47].

in the basal cell (arrow 5 in Figure 5) was assumed to be from a gene transcript that was down-regulated only in the basal cell after fertilization.

Group 6: A DNA-band detected in all cell/embryo types (arrow 6 in Figure 5) was judged to be amplified from a gene transcript that was constitutively expressed in all cell/embryo types.

After sequence determination of the DNA-band and verification of expression pattern using specific primers, BLAST search was conducted. The BLAST search results are summarized in Table 1, which contains a list of the cDNA clones (amplified DNA-bands) showing similarity to genes whose functions have been investigated. Clone 72 had strong similarity to the farnesyltransferase (FTase) β-subunit, which is involved in the prenylation of proteins. The *Arabidopsis* FTase β-subunit, encoded by the *ERA1–WIGGUM* gene, is expressed in the embryo proper throughout embryogenesis and is essential for meristem development[48,49]. An *Arabidopsis* ubiquitin-specific protease, similar to clone 1, is known to function in embryo development[50]. Although the similarity was relatively low, clone 97 was similar to the B subunit of CCAAT-binding factor, which has been identified as a critical regulator of embryogenesis in *Arabidopsis*[51–53]. The phytohormone auxin has been considered to be one of the key molecules that controls cell division/fate during embryogenesis[20,54,55], and an *Arabidopsis* ubiquitin-related protein, termed TIR1 protein, functions in the auxin response via the COP9 signalosome[56–58]. Clone 29, which showed similarity to the ubiquitin-related protein, was suppressed only in the apical cell after fertilization, suggesting that the ability to respond to auxin may differ between

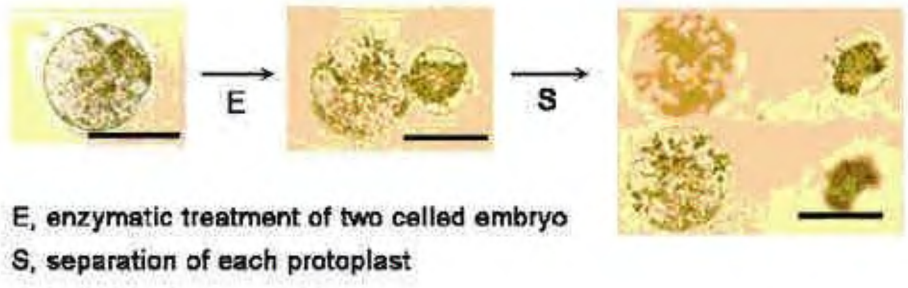

Figure 4. Isolation of an apical and a basal cell from a two-celled maize embryo, developed after *in vitro* fertilization. ***a***, Two-celled embryo developed in *in vitro* culture. ***b***, Two connected protoplasts derived from the two-celled embryo, which was treated with cell wall degrading enzymes. ***c***, Apical cell protoplast (right in focus, upper image) and basal cell protoplast (left in focus, bottom image)[42]. Bars, 50 μm.

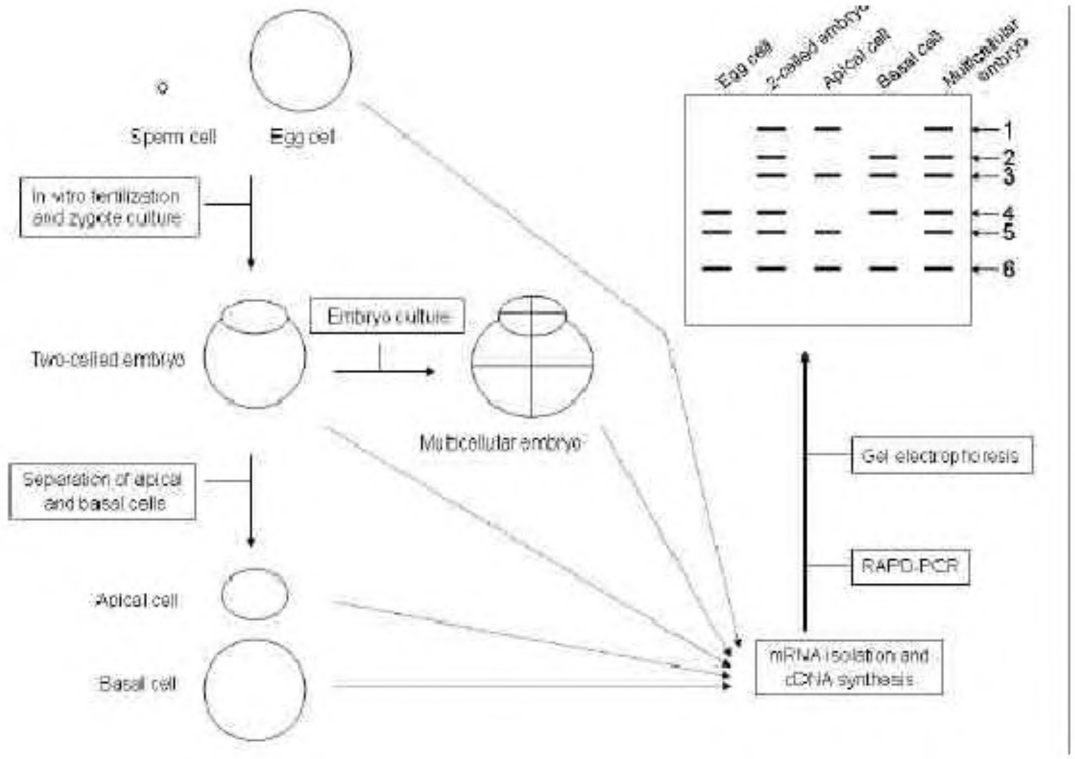

Figure 5. Procedures for detecting and isolating up- or down-regulated genes from isolated apical and basal cells from a two-celled maize embryo. Two-celled and multicellular embryos were produced by *in vitro* fertilization of maize gametes and subsequent culture of the zygotes. The apical and basal cells were isolated from the two-celled embryo, and cDNAs were prepared from the egg cell, apical cell, basal cell, two-celled embryo and multicellular embryo. Using RAPD primers, PCR was conducted using each cDNA preparation as a template, followed by separation of the amplified DNA by 1.2% agarose gel electrophoresis. The patterns of DNA bands detected in the gel were then compared among the 5 cell/embryo types. Based on the detection patterns of the DNA-bands showing the same mobility in the gel, the expression profiles were categorized into 6 groups as described in text.

the apical and basal cells. The existence of putative embryogenesis-related genes among the genes identified in the present study provides the potential for identifying embryogenesis-related genes that have not yet been characterized.

As for *Arabidopsis* genes that are expressed in apical cells, but not in basal cells, *MP, BDL, WOX2* and *AtML1* are well known. *PIN7, WOX8* and *WOX9* are reported to express in basal cells but not in apical cells. Extensive investigations for *Arabidopsis* mutants whose apical-basal axis was disturbed or lost have been carried out by Jürgens's group. The results suggested that auxin is a key molecule for the formation of embryonal axis and embryonic root meristem[20,27,28]. In the basal cell of *Arabidopsis* two-celled embryos, *PIN7,* encoding a putative auxin efflux carrier protein, is expressed, and the PIN7 protein localizes at the apical region of the cell. The specific *PIN7* expression and PIN7 localization in the basal cell results in directional transport of auxin from basal to apical cells, and the acropetal auxin transport increases the

auxin level within apical cells[20]. PIN7 protein dependent high auxin level in apical region cells continues until 8-celled stage embryo. This auxin surge in apical cells is thought to be a determinant of apical cell specification[20]. In globular embryos, auxin transport becomes bispetal via PIN1, 4 and 7 proteins from the apical part of the embryo to the hypophysis, and thereafter, in the heart stage embryo, auxin is accumulated at regions which form root meristems, cotyledon primordia and provascular bundles[27,28,59–63]. Among these, formation of root meristems through auxin dependent molecular mechanisms is best characterized. ARF5/MP, a transcription factor that activates auxin-responsive genes[29] *IAA12/BDL*, a putative inhibitor of MP[27] are probably central to auxin response during embryogenesis. Loss of MP or gain of BDL function interferes with specification of the apical cell, and prevents the formation of an embryonic root[27,31]. MP and BDL are co-expressed throughout embryogenesis and form heterodimers. Auxin-dependent degradation of BDL results in the release of MP and MP-dependent expression of auxin responsive genes. Auxin facilitates degradation of Aux/IAA proteins which are transcriptional regulators. Degradation of these proteins and auxin-regulated transcription is mediated by an F-box protein, TIR1, that has been recently shown to be an auxin receptor[64,65]. Besides TIR1, three additional F-box proteins interact with BDL and mediate auxin responses during plant development[66].

One of the putative targets of MP is WOX (WUSCHEL RELATED HOMEOBOX) 9, since WOX9 expression is altered in *mp* and *bdl* mutant embryos[67]. A homeodomain (HD)-containing transcription factor, WUSCHEL, is expressed throughout embryogenesis from early stages, and is required for embryonic shoot meristem formation[68,69]. Haecker *et al.*[67] demonstrated that WOX2 and 8 are expressed in the zygote, and that expression of WOX2 and 8 are restricted to apical and basal cells of the two-celled embryos, respectively. *WOX* genes might have roles in regulating specific functions of cells in which they are expressed, because WUS is required for the establishment of the shoot apical meristem. Gene expression in cells of the basal lineage of tobacco and pea is directed by a promoter element involved in asymmetric division[70].

Genes that are up- or down-regulated in the zygote

cDNAs were synthesized from maize zygotes cultured for 0.5, 1, 3, 6, 12, 24 or 36 h after fertilization and used as PCR templates to investigate when the expressions of the 12 up-regulated genes (groups 1–3 in Table 1) were initiated in zygotes after *in vitro* fertilization. Notably, the genes that are up-regulated after fertilization seemed to be expressed in early stages of zygote development. Amplified DNA-bands derived from two gene transcripts were detected in zygotes at 0.5 h after fertilization, and four genes were expressed in zygotes at 1 h. All genes tested were expressed in zygotes during the first 12 h of culture[42], despite that the first unequal cell division of zygotes produced by IVF occurs during 40–46 h of culture[39]. This suggests that fertilization induced gene expression in zygotes will start at early developmental stages of zygotes. The specific DNA-band amplified with the primer set for clone no. 1 was detected in zygotes at 3, 6 and 24 h after fertilization, but not at 12 or 36 h, indicating that this gene may be up- and down-regulated during zygote development in maize.

Expression of WOX2 and 8 are restricted to apical and basal cells, respectively[67]. However, it is still unclear whether these two homeobox genes are already expressed in egg cells, or are induced by fertilization.

Conclusion and direction of further lines of research

In flowering plants the polarity of the zygote is generally derived from the egg cell and is handed to the young embryo by the laying down of a transverse wall asymmetrically into a richly cytoplasmic smaller apical cell and a larger vacuolated basal cell. The former gives rise to the embryo proper and the latter to the progenitor of the suspensor and hypophysis[32,71–75]. The asymmetrical division of the angiosperm zygote is widely assumed to be a conserved process, although there are also symmetrical divisions of these zygotes[76].

It has been widely accepted that auxin appears to be essential for apical cell specification. For the basal cell specification, an *Arabidopsis* MAPKK kinase gene, named YODA, was identified. YODA promotes extra-embryonic cell fates in the basal cell lineage[77]. The zygote does not elongate properly in loss-of-function mutants, and the cells of the basal lineage cells that are incorporated into the embryo failed to differentiate the suspensor. In addition, gain-of-function alleles cause exaggerated growth of the suspensor and suppressed development of the embryo proper which was derived from the apical cell. The phenotype was similar to that of *pin7* mutant, in which the initial auxin flow from basal cell to apical cell is disturbed[20]. These findings suggest that MAPKKK and auxin signal pathways function in basal and apical cell specification in the two-celled embryos, respectively. These two independent mechanisms for cell specification between apical and basal cells will support the view that unequal cell division along zygote polarity is the first step of axis formation in the embryo, and that zygotic polarity is crucially important for axis formation. Many ultrastructural studies have revealed that reorganization of organelles in zygotes occurs after fertilization and during zygote development (reviewed in Raghavan[78]), suggesting that polarity of the zygote is not simply derived from that of the egg cell. Since IVF is a most suitable method for continuous observation of zygote development and

Table 1. Expression patterns, sequence features and BLAST searches

Expressed cell/embryo	Group no.	Clone no.	Similarity	BLASTN ScoreE-value	BLASTN Accession no.	BLASTN Plant
2, A, M	1	22	Polypyrimidine tract RNA-binding protein	248 3e-95	AF076924	*A. thaliana*
2, A, M	1	97	CCAAT-binding factor, B-subunit	68 2e-10	AY087696	*A. thaliana*
2, B, M	2	1	Ubiquitin-specific protease	312 3e-84	AF302665	*A. thaliana*
2, B, M	2	26	tRNA methyltransferase	305 9e-82	AY080697	*A. thaliana*
2, B, M	2	33	Receptor kinase	133 5e-30	AF285172	*P. vulgaris*
2, B, M	2	98	L-methionine S-methyltransferase	206 9e-53	AF144079	*Z. mays*
2, B, M	2	104	Mitotic checkpoint protein	279 5e-74	AB024032	*A. thaliana*
2, A, B, M	3	4	Putative outer envelope protein	291 1e-77	AC020666	*O. sativa*
2, A, B, M	3	43	DNA-binding protein	127 1e-28	X98744	*P. sativum*
2, A, B, M	3	65	GTPase-activating enzyme	182 6e-65	AC013483	*A. thaliana*
2, A, B, M	3	72	Farnesyltransferase, β-subunit	221 6e-57	AP003218	*O. sativa*
2, A, B, M	3	83	Sterol glucosyl transferase	317 1e-85	AP003745	*O. sativa*
E, 2, B, M	4	29	Ubiquitin-related protein	238 1e-61	AY062568	*A. thaliana*
E, 2, B, M	4	40	Zn finger protein	220 1e-56	AC120506	*O. sativa*
E, 2, B, M	4	70	DNA-binding Zn finger protein	213 7e-60	AP005249	*O. sativa*
E, 2, B, M	4	74	Phospholipase D	419 e-116	D73410	*Z. mays*
E, 2, A, M	5	52	RNA-binding protein	155 2e-46	AC091811	*O. sativa*
E, 2, A, B, M	6	86	Heat shock protein	353 6e-96	U55859	*T. aestivum*
E, 2, A, B, M	6	87	Ribosomal protein	323 3e-87	U64436	*Z. mays*

E, egg cell; 2, two-celled embryo; A, apical cell; B, basal cell; M, multicellular embryo.

transition to a two-celled embryo, examination of zygotes containing organelles which are labelled with fluorescent proteins will give a clue to study underlying mechanisms of polarity changes.

With proteins that regulate auxin response available to us, *in vitro* systems become all the more valuable to study subcellular localization of such proteins with immunocytological methods (developed for single egg cells and zygotes[40]) or by a transgenic approach using green fluorescent proteins[79].

A possible mechanism involved in the establishment of zygotic polarity is subcellular localization of mRNAs in zygotes. In unicellular organisms, animal and plant tissues and developing embryos from a variety of animal phyla, subcellular localization of RNA has emerged as a key mechanism through which cells become polarized, and the localization of RNA is known to be a widespread and efficient way to target gene products to a specific region of a cell or embryo[80–82]. Genes that are up-regulated in the apical or basal cell after fertilization were revealed to be expressed in the early zygote, providing the possibility that transcripts from these genes are localized to the putative apical or basal region of the zygote, or that the transcripts might be degraded in one of the daughter cells immediately after zygotic cell division[42,67]. In addition to the genes up-regulated in the zygote and two-celled embryos, down-regulated genes in the apical or basal cell were also identified (Table 1). This indicates that the transcripts from these genes may exist uniformly in the egg cells and become distributed to the apical and basal regions of the zygote after fertilization, or that subcellular localization of these transcripts may already be present in the egg cell. Alternatively, rapid degradation of the transcripts might occur in one of the daughter cells after zygotic cell division. Subcellular localization of mRNAs in the zygote may represent a crucial mechanism for asymmetrical development of the zygote and early embryogenesis in higher plants. Visualizing the putative subcellular localization of mRNAs by microinjection of fluorescence-labelled RNA into zygotes or an *in situ* hybridization approach will provide a cue for understanding the mechanisms of asymmetrical division.

1. Yashuda, G. K. and Schubinger, G., Temporal regulation in the early embryo: is MBT too good to be true?. *Trends Genet.*, 1992, **8**, 124–127.
2. Flach, G., Johnson, M. H., Braude, P. R., Taylor, R. A. and Bolton, V. N., The transition from maternal to embryonic control in the 2-cell mouse embryo. *EMBO J.*, 1982, **1**, 681–686.
3. Newport, J. and Kirchner, M., A major developmental transition in early *Xenopus* embryos: I. Characterisation and timing of cellular changes at the midblastula stage. *Cell*, 1982, **30**, 675–686.
4. Seydoux, G., Mello, C. C., Pettit, C., Wood, W. B., Priess, J. R. and Fire, A., Repression of gene expression in the embryonic germ line of *C. elegans*. *Nature*, 1996, **382**, 713–716.
5. Zamir, E., Kam, Z. and Yarden, A., Transcription-dependent induction of G1 phase during zebra fish midblastula transition. *Mol. Cell. Biol.*, 1997, **17**, 529–536.
6. Newman-Smith, E. D. and Rothman, J. H., The maternal-to-zygotic transition in embryonic patterning of *Caenorhabditis elegans*. *Curr. Opin. Genet. Dev.*, 1998, **8**, 472–480.
7. Matsumoto, K., Anzai, M., Nakata, N., Takahashi, A., Takahashi, Y. and Mayata, K., Onset of paternal gene activation in early mouse embryos fertilized with transgenic mouse sperm. *Mol. Reprod. Dev.*, 1994, **39**, 136–140.
8. Nothias, J.-Y., Miranda, M. and De Pamphilis, M. L., Uncoupling of transcription and translation during zygotic gene activation in the mouse. *EMBO J.*, 1996, **15**, 5715–5725.

9. Vielle-Calzada, J.-P., Baskar, R. and Grossniklaus, U., Delayed activation of the paternal genome during seed development. *Nature*, 2000, **350**, 241–243.
10. Tzafrir, I. *et al.*, Identification of genes required for embryo development in *Arabidopsis*. *Plant Physiol.*, 2004, **135**, 1206–1220.
11. Weijers, D., Geldner, N., Offringa, R. and Jürgens, G., Early paternal gene activity in *Arabidopsis*. *Nature*, 2001, **414**, 709–710.
12. Kohler, C., Page, D. R., Gagliardini, V. and Grossniklaus, U., The *Arabidopsis thaliana* MEDEA Polycomb group protein controls expression of PHERES1 by parental imprinting. *Nature Genet.*, 2005, **37**, 28–30.
13. Scholten, S., Lörz, H. and Kranz, E., Paternal mRNA and protein synthesis coincides with male chromatin decondensation in maize zygotes. *Plant J.*, 2002, **32**, 221–231.
14. Grimanelli, D., Perotti, E., Ramirez, J. and Leblanc, O., Timing of the maternal-to-zygotic transition during early seed development in maize. *Plant Cell*, 2005, **17**, 1061–1072.
15. Sauter, M., Von Wiegen, P., Lörz, H. and Kranz, E., Cell cycle regulatory genes from maize are differentially controlled during fertilization and first embryonic cell division. *Sex. Plant Reprod.*, 1998, **11**, 41–48.
16. Steimer, A., Schob, H. and Grossniklaus, U., Epigenetic control of plant development: new layers of complexity. *Curr. Opin. Plant Biol.*, 2004, **7**, 11–19.
17. Lu, P., Porat, R., Nadeau, J. A. and O'Neill, S. D., Identification of a meristem L1 layer-specific gene in *Arabidopsis* that is expressed during embryonic pattern formation and defines a new class of homeobox genes. *Plant Cell*, 1996, **8**, 2155–2168.
18. Lyndon, R. F., *Plant Development* (ed. Lyndon, R. F.), Unwin Hyman, Boston, 1990, p. 12.
19. Jürgens, G., Apical-basal pattern formation in *Arabidopsis* embryogenesis. *EMBO J.*, 2001, **20**, 3609–3616.
20. Friml, J. *et al.*, Efflux-dependent auxin gradients establish the apical-basal axis of *Arabidopsis*. *Nature*, 2003, **426**, 147–153.
21. Friml, J. and Wisniewska, J., Auxin as an intercellular signal. In *Intercellular Communication in Plants* (ed Fleming, A.), Ann. Plant Rev., **16**, Blackwell Publishing, 2005.
22. Busch, M., Meyer, U. and Jürgens, G., Molecular analysis of the *Arabidopsis* pattern formation gene GNOM: Gene structure and intragenic complementation. *Mol. Gen. Genet.*, 1996, **250**, 681–691.
23. Geldner, N., Friml, J., Stierhof, Y. D., Jürgens, G. and Palme, K., Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature*, 2001, **413**, 425–428.
24. Shevel, D. E., Leu, W.-M., Gilimor, C. S., Xia, G., Feldmann, K. A. and Chua, N.-H., EMB30 is essential for normal cell division, cell expansion, and cell adhesion in *Arabidopsis* and encodes a protein that has similarity to Sec 7. *Cell*, 1994, **77**, 1051–1062.
25. Steinmann, T. *et al.*, Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. *Science*, 1999, **286**, 316–318.
26. Meyer, U., Büttner, G. and Jürgens, G. Apical-basal pattern formation in the *Arabidopsis* embryo: studies on the role of the *gnom* gene. *Development*, 1993, **117**, 149–162.
27. Hamann, T., Benkova, E., Bäurle, I., Kientz, M. and Jürgens, G., The *Arabidopsis* BODENLOS gene encodes an auxin response protein inhibiting MONOPTEROS-mediated embryo patterning. *Genes Dev.*, 2002, **16**, 1610–1615.
28. Hardtke, C. S. and Berleth, T., The *Arabidopsis* gene *MONOPTEROS* encodes a transcription factor mediating embryo axis formation and vascular development. *EMBO J.*, 1998, **17**, 1405–1411.
29. Ulmasov, T., Hagen, G. and Guilfoyle, T. J., Activation and repression of transcription by auxin-response factors. *PNAS*, 1999, **96**, 5844–5849.
30. Berleth, T. and Jürgens, G., The role of the MONOPTEROS gene in organizing the basal body region of the *Arabidopsis* embryo. *Development*, 1993, **118**, 575–587.
31. Hamann, T., Mayer, U. and Jürgens, G., The auxin–insensitive bodenlos mutation affects primary root formation and apical-basal patterning in the *Arabidopsis* embryo. *Development*, 1999, **126**, 1387–1395.
32. Mansfield, S. G. and Briarty, L. G., Early embryogenesis in *Arabidopsis thaliana*. II. The developing embryo. *Can. J. Bot.*, 1990, **69**, 461–467.
33. Jürgens, G. and Mayer, U. *Arabidopsis*. In *A Colour Atlas of Developing Embryos* (ed. Bard, J.), Wolfe Publishing, London, 1994, pp. 7–21.
34. Laux, T., Wurschum, T. and Breuninger, H., Genetic regulation of embryonic pattern formation. *Plant Cell*, 2004, **16**, S190–202.
35. Theunis, C. H., Pierson, E. S. and Cresti, M., Isolation of male and female gametes in higher plants. *Sex. Plant Reprod.*, 1991, **4**, 145–154.
36. Weterings, K. and Russell, S. D., Experimental analysis of the fertilization process. *Plant Cell*, 2004, **16**, S107–118.
37. Kranz, E., Bautor, J. and Lörz, H., *In vitro* fertilization of single, isolated gametes of maize mediated by electrofusion. *Sex. Plant Reprod.*, 1991, **4**, 12–16.
38. Kranz, E. and Lörz, H., *In vitro* fertilization with isolated, single gametes results in zygotic embryogenesis and fertile maize plants. *Plant Cell*, 1993, **5**, 739–746.
39. Kranz, E., Von Wiegen, P. and Lörz, H., Early cytological events after induction of cell division in egg cells and zygote development following *in vitro* fertilization with angiosperm gametes. *Plant J.*, 1995, **8**, 9–23.
40. Hoshino, Y., Scholten, S., von Wiegen, P., Lörz, H. and Kranz, E., Fertilization-induced changes in the microtubular architecture in the maize egg cell and zygote – an immunocytochemical approach adapted to single cells. *Sex. Plant Reprod.*, 2004, **17**, 89–95.
41. Okamoto, T., Higuchi, K., Shinkawa, T., Isobe, T., Lörz, H., Koshiba, T. and Kranz, E., Identification of major proteins in maize egg cells. *Plant Cell Physiol.*, 2004, **45**, 1406–1412.
42. Okamoto, T., Scholten, S., Lörz, H. and Kranz, E., Identification of genes that are up- or down-regulated in the apical or basal cell of maize two-celled embryos and monitoring their expression during zygote development by a cell manipulation and PCR-based approach. *Plant Cell Physiol.*, 2005, **46**, 332–338.
43. Kranz, E. and Kumlehn J., Angiosperm fertilization, embryo and endosperm development *in vitro*. *Plant Sci.*, 1999, **142**, 183–197.
44. Kranz, E., *In vitro* fertilization with isolated single gametes. In *Methods in Molecular Biology, 111, Plant Cell Culture Protocols* (ed. Hall, R.), Humana Press Inc., NJ, 1999, pp. 259–267.
45. Kumlehn, J., Lörz, H. and Kranz, E., Differentiation of isolated wheat zygotes into embryos and normal plants. *Planta*, 1998, **205**, 327–333.
46. Kumlehn J., Lörz, H. and Kranz, E., Monitoring individual development of isolated wheat zygotes: a novel approach to study early embryogenesis. *Protoplasma*, 1999, **208**, 156–162.
47. Kranz, E., Kumlehn, J. and Dresselhaus, T., Fertilization and zygotic embryo development *in vitro*. In *Fertilization in Higher Plants* (eds Cresti, M., Cai, G. and Moscatelli, A.) Springer, Berlin, 1999, pp. 337–349.
48. Yalovsky, S., Kulukian, A., Rodriguez-Concepcion, M., Young, C. A. and Gruissem, W., Functional requirement of plant farnesyltransferase during development in *Arabidopsis*. *Plant Cell*, 2000, **12**, 1267–1278.
49. Ziegelhoffer, E. C., Medrano, L. J. and Meyerowitz, E. M., Cloning of the *Arabidopsis* WIGGUM gene identifies a role for farnesylation in meristem development. *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 7633–7638.
50. Doelling, J. H., Yan, N., Kurepa, J., Walker, J. and Vierstra, R. D., The ubiquitin-specific protease UBP14 is essential for early embryo development in *Arabidopsis thaliana*. *Plant J.*, 2001, **27**, 393–405.
51. Lotan, T. *et al.*, *Arabidopsis* LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. *Cell*, 1998, **93**, 1195–205.
52. Lee, H., Fischer, R. L., Goldberg, R. B. and Harada, J. J., *Arabidopsis* LEAFY COTYLEDON1 represents a functionally specialized

subunit of the CCAAT binding transcription factor. *Proc. Natl. Acad. Sci. USA,* 2003, **100**, 2152–2156.
53. Kwong, R. W., Bui, A. Q., Lee, H., Kwong, L. W., Fischer, R. L., Goldberg, R. B. and Harada, J. J., LEAFY COTYLEDON1-LIKE defines a class of regulators essential for embryo development. *Plant Cell*, 2003, **15**, 5–18.
54. Liu, C., Xu, Z. and Chua, N. H., Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. *Plant Cell*, 1993, **5**, 621–630.
55. Hadfi, K., Speth, V. and Neuhaus, G., Auxin-induced developmental patterns in *Brassica juncea* embryos. *Development*, 1998, **125**, 879–887.
56. Ruegger, M., Dewey, E., Gray, W. M., Hobbie, L., Turner, J. and Estelle, M., The TIR1 protein of *Arabidopsis* functions in auxin response and is related to human SKP2 and yeast grr1p. *Genes Dev.*, 1998, **12**, 198–207.
57. Schwechheimer, C. *et al.*, Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCFTIRI in mediating auxin response. *Science*, 2001, **292**, 1379–1382.
58. Schwechheimer, C., Serino, G. and Deng, X. W., Multiple ubiquitin ligase-mediated processes require COP9 signalosome and AXR1 function. *Plant Cell*, 2002, **14**, 2553–2263.
59. Aida, M., Vernoux, T., Furutani, M., Traas, J. and Tasaka, M., Roles of PIN-FORMED1 and MONOPTEROS in pattern formation of the apical region of the *Arabidopsis* embryo. *Development*, 2002, **129**, 3965–3974.
60. Furutani, M., Vernoux, T., Traas, J., Kato, T., Tasaka, M. and Aida, M., PIN-FORMED1 and PINOID regulate boundary formation and cotyledon development in *Arabidopsis embryogenesis*. *Development*, 2004, **131**, 5021–5030.
61. Baima, S., Nobili, F., Sessa, G., Lucchetti, S., Ruberti, I. and Morelli, G., The expression of the Athb-8 homeobox gene is restricted to provascular cells in *Arabidopsis thaliana*. *Development*, 1995, **121**, 4171–4182.
62. Baima, S., Possenti, M., Matteucci, A., Wisman, E., Altamura, M. M., Ruberti, I. and Morelli, G., The *Arabidopsis* ATHB-8 HD-Zip protein acts as a differentiation-promoting transcription factor of the vascular meristems. *Plant Physiol.*, 2001, **126**, 643–655.
63. Ohashi-Ito, K. and Fukuda, H., HD-Zip III homeobox genes that include a novel member, ZeHB-13 (Zinnia)/ATHB-15 (*Arabidopsis*), are involved in procambium and xylem cell differentiation. *Plant Cell Physiol.*, 2003, **44**, 1350–1358.
64. Dharmasiri, N., Dharmasiri, S. and Estelle, M., The F-box protein TIR1 is an auxin receptor. *Nature,* 2005, **435**, 441–445.
65. Kepinski, S. and Leyser, O., The *Arabidopsis* F-box protein TIR1 is an auxin receptor. *Nature,* 2005, **435**, 446–451.
66. Dharmasiri, N. *et al.*, Plant development is regulated by a family of auxin receptor F box proteins. *Dev. Cell.*, 2005, **9**, 109–119.
67. Haecker, A., Groß-Hardt, R., Geiges, B., Sarkar, A., Breuninger, H., Herrmann, M. and Laux, T., Expression dynamics of *WOX* genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*. *Development*, 2004, **131**, 657–668.
68. Bowman, J. L. and Eshed, Y., Formation and maintenance of the shoot apical meristem. *Trends Plant Sci.*, 2000, **5**, 110–115.
69. Sharma, V. K., Carles, C. and Fletcher, C., Maintenance of stem cell populations in plants. *Proc. Natl. Acad. Sci. USA*, 2003, **100**, 11823–11829.
70. Weterings, K., Apuya, N. R., Bi, Y., Fischer, R. L., Harada, J. J. and Goldberg, R. B., Regional localization of suspensor mRNAs during early embryo development. *Plant Cell*, 2001, **13**, 2409–2425.
71. Pritchard, N. H., A cytochemical study of embryo development in *Stellaria media*. *Am. J. Bot.*, 1964, **51**, 472–479.
72. Schulz, R. and Jensen, W. A. *Capsella* embryogenesis: the egg, zygote and young embryo. *Am. J. Bot.*, 1968, **55**, 807–819.
73. Tykarska, T., Rape embryogenesis: I. The proembryo development. *Acta Soc. Bot. Pol.*, 1976, **45**, 3–16.
74. Schel, J. H. N., Kieft, H. and van Lammeren, A. A. M., Interactions between embryo and endosperm during early developmental stages of maize caryopses (*Zea mays*). *Can. J. Bot.*, 1984, **62**, 2842–2853.
75. Lindsey, K. and Topping, J. E., Embryogenesis: a question of pattern. *J. Exp. Bot.,* 1993, **259**, 359–374.
76. Maheshwari, P., *An Introduction to the Embryology of Angiosperms*, McGraw-Hill, New York, 1950.
77. Lukowitz, W., 1 Roeder, A., Parmenter, D. and Somerville, C., A MAPKK kinase gene regulates extra-embryonic cell fate in *Arabidopsis*, *Cell*, 2004, **116**, 109–119.
78. Raghavan, V., *Molecular Embryology of Flowering Plants,* Cambridge University Press, 1997.
79. Scholten, S. and Kranz, E., *In vitro* fertilization and expression of transgenes in gametes and zygotes. *Sex. Plant Reprod.,* 2001, **14,** 35–40.
80. Choi, S. B., Wang, C., Muench, D. G., Ozawa, K., Franceschi, V. R., Wu, Y. and Okita, T. W., Messenger RNA targeting of rice seed storage proteins to specific ER subdomains. *Nature,* 2000, **407**, 765–767.
81. Kloc, M., Zearfoss, N. R. and Etkin, L. D., Mechanisms of subcellular mRNA localization. *Cell*, 2002, **108**, 533–544.
82. Hamada, S. *et al.*, Dual regulated RNA transport pathways to the cortical region in developing rice endosperm. *Plant Cell*, 2003, **15**, 2265–2272.