

RESEARCH PAPER

Exine dehiscing induces rape microspore polarity, which results in different daughter cell fate and fixes the apical–basal axis of the embryo

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Abstract

The roles of cell polarity and the first asymmetric cell division during early embryogenesis in apical–basal cell fate determination remain unclear. Previously, a novel *Brassica napus* microspore embryogenesis system was established, by which rape exine-dehisced microspores were induced by physical stress. Unlike traditional microspore culture, cell polarity and subsequent asymmetric division appeared in the exine-dehisced microspore, which finally developed into a typical embryo with a suspensor. Further studies indicated that polarity is critical for apical–basal cell fate determination and suspensor formation. However, the pattern of the first division was not only determined by cell polarity but was also regulated by the position of the ruptured exine. The first division could be equal or unequal, with its orientation essentially perpendicular to the polar axis. In both types of cell division, the two daughter cells could have different cell fates and give rise to an embryo with a suspensor, similar to zygotic apical–basal cell differentiation. The alignment of the two daughter cells is consistent with the orientation of the apical–basal axis of future embryonic development. Thus, the results revealed that exine dehiscing induces rape microspore polarization, and this polarity results in a different cell fate and fixes the apical–basal axis of embryogenesis, but is uncoupled from cell asymmetric division. The present study demonstrated the relationships among cell polarity, asymmetric cell division, and cell fate determination in early embryogenesis.

Key words: Asymmetric cell division, *Brassica napus*, cell fate, cell polarity, embryogenesis, exine-dehisced microspore.

Introduction

During the course of zygotic embryogenesis, a single cell gives rise to a multicellular organism with a distinct body shape and a specific pattern of different cell types (Mansfield *et al.*, 1991; Raghavan, 2003). Generally, the zygote divides asymmetrically into a two-celled proembryo consisting of an apical and a basal cell. The establishment of zygotic polarity

and apical–basal cell fate determination are among the most critical events in early embryogenesis.

Cell polarity can usually be recognized by the differential distributions of cellular components, mRNA, and proteins, vesicle trafficking, and directional relocation of organelles, or different characters of cell morphology and distinct

Abbreviations: AGP, arabinogalactan protein; CW, Calcofluor White; DAPI, 4',6-diamidino-2-phenylidole; EDM, exine-dehisced microspore; ex-axis, exine-dehisced microspores expanded long axis; ru-plane, the spatial plane indicated by the end of the broken exine between the exine-covered part and exine-detached part in exine-dehisced microspores.

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developmental patterns (Pruyne and Bretscher, 2000; Jansen, 2001; Fischer *et al.*, 2004). The role of cell polarity in multicellular system development and differentiation, especially during embryo development, has attracted increasing attention (Jurgens *et al.*, 1997; Kropf, 1999; Scheres and Benfey, 1999; Grebe *et al.*, 2002; Ekici and Dane, 2004). Analyses in easily accessible model plants, such as mosses and algae, indicated that polarity could be influenced by environmental factors, such as light and gravity, and these studies suggested important roles for ion fluxes, plasma membrane, and cytoskeleton in the establishment and maintenance of cell polarity (Schnepf, 1986; Cove, 2000). To achieve polarity, the cell must integrate a series of extrinsic and intrinsic cues through a signalling cascade leading to the specific intracellular localization of macromolecular complexes along the axis (Xu and Scheres, 2005; Bothwell *et al.*, 2008). The generation of such cellular asymmetry not only affects the subsequent development of the cell but also potentially affects the fate of its daughter cells. Therefore, it is critical to cell differentiation and cell fate determination. However, our understanding of the mechanisms underlying such polarity induction is still limited.

Arabinogalactan proteins (AGPs) are extracellular glycoproteins that play important roles in the process of plant development (Knox, 1996; Nothnagel, 1997). JIM is an anti-AGP monoclonal antibody that has been used to study the locations of AGPs (Knox, 1997). Pennell *et al.* (1991) found that JIM8 could only label the suspensor at the globular embryo stage. AGP can be a molecular marker for the cell fate variation in the embryo. In addition to cytological observations of asymmetric cleavage of plant zygotes, the mRNAs of two homeotic genes, *WOX2* (*WUSCHEL* related homeobox2) and *WOX8* (*WUSCHEL* related homeobox8), were detected in the egg cell and zygote before they became restricted to the apical (*WOX2*) and basal (*WOX8*) daughter cells by zygotic division (Haecker *et al.*, 2004), suggesting that the two daughter cells may possess different transcripts from the zygote. After zygote division, *WOX8* is expressed in the basal cell lineage (Minako *et al.*, 2011), and *WOX8* can be used as a marker for the basal cell lineage (Haecker *et al.*, 2004). Furthermore, following asymmetric division of the zygote, PIN7, an auxin efflux carrier protein, is expressed specifically in the basal cell and its derivatives, whereas PIN1 is expressed in the apical cell and its derivatives (Friml *et al.*, 2003). cDNA libraries of apical and basal cells have been constructed, and their transcript profiles have been compared. Generally, transcript profiles of the apical and basal cells were similar, but some embryogenesis-related genes were expressed differentially between the two cells (Ma *et al.*, 2011). It has yet to be confirmed whether zygote asymmetric cell division apporitions different transcripts into the two daughter cells. The relationships between cell polarity or asymmetric cell division and cell fate remain to be elucidated.

Microspore culture is a convenient tool for studying embryogenesis. Microspores initially determined to form male gametes can be reprogrammed to form embryos, and these develop similarly to their zygotic counterparts (Mordhorst *et al.*, 1997; Maluszynski *et al.*, 2003; Joosen *et al.*, 2007; Supena *et al.*, 2008; Wedzony *et al.*, 2009; Dubas *et al.*, 2011; Prem *et al.*, 2012). In

general, the replacement of the normal asymmetric cell division with symmetric cell division appears to be a general phenomenon in microspore embryogenesis (Zaki and Dickinson, 1990, 1991; Indrianto, 2001; Seguí-Simarro *et al.*, 2003; Maraschin *et al.*, 2005). The microspore is composed of three parts: the exine, intine, and protoplast. It was reported previously that the microspore exine could be completely removed by physical stress in *Nicotiana* (Xia *et al.*, 1996) and in *Brassica* (Xu *et al.*, 1997). Later, a similar technique was used to prepare exine-dehiscenced microspores (EDMs) in which the exine was broken but still partially covered the microspore. Such EDMs could develop into embryos with well-developed suspensors (Tian and Sun, 2003; Tang *et al.*, 2006). Further studies indicated that the first division of the EDMs, unlike that in intact microspores, could be asymmetric. It was also reported that after mild heat stress treatment, cultured microspores could develop into embryos with a suspensor after slightly asymmetrical mitotic division (Supena *et al.*, 2008; Dubas *et al.*, 2011). This may provide a unique opportunity to investigate the role of asymmetric cell division in cell fate determination and its relationship to cell polarity in early embryogenesis.

Based on previous work, an experimental system was established for this purpose using the microculture technique, and development was individually monitored to trace the cell division pattern of each EDM. Extensive studies indicated that exine dehiscing induced rape microspore polarity, and this polarity resulted in different cell fate, specifically, and the differentiation of an apical and a basal cell that developed into an embryo with a typical suspensor. This polarity also fixed the apical–basal axis of early embryogenesis. Interestingly, the EDM first division plane was controlled by the dehiscenced exine. Both symmetric and asymmetric microspore division resulted in an embryo with a suspensor as long as polarity was established, suggesting that asymmetric cell division is not a prerequisite for cell fate determination in EDM embryogenesis.

Materials and methods

Plant material

Plants of *Brassica napus* cv. Topas were grown in a greenhouse at the College of Life Sciences at Wuhan University. Conditions for cultivation were as described previously (Sun *et al.*, 1999).

Microspore isolation and culture

Microspores were obtained from buds 2.8–3.2 mm in length. Developmental stages of microspores were determined with 4 µg ml⁻¹ 4',6-diamidino-2-phenylidole (DAPI). Culture was performed according to Sun *et al.* (1999). Briefly, microspores were isolated in B5 medium (Gamborg *et al.*, 1968) and cultured in NLN13 medium (Lichter, 1982) in the dark at 32.5 °C continuously.

Aliquots of 1 ml of the microspore suspension were plated in 3 cm Petri dishes for culture. The microspores were incubated at a density of 40 000 ml⁻¹ in NLN13. Cultures were kept continuously in NLN-13.

EDM preparation and culture

EDMs were prepared as described by Tian *et al.* (2003). Briefly, the microspores were cultured in the high osmotic medium (the basic

constituents of NLN medium with 0.7 M sucrose and 0.4 M mannitol) and kept at 32.5 °C for 4 d. The EDMs were then collected for further culture. A co-culture system was used in this experiment. A Millicell (Millipore PICM 01250) was placed in a Petri dish 3.5 cm in diameter. About 2–10 EDMs were selected and placed into the Millicell using a hand-made micropipette. The microspore embryos were cultured for 1 week and used as a feeder layer. The development of EDMs was photographed with an inverted microscope equipped with a cooled charge-coupled device (CCD) camera (Roper Scientific), at 1 d intervals from day 1 to the day needed during culture. There were variations of the intervals according to different aspects of the culture. For each of the series, 3–20 images were collected for tracing the developmental process.

Cytological staining and morphological analyses

A stock solution of 0.25% (w/v) rhodamine 123 was prepared. Working solutions were made by dissolving 250 µl of the stock solution in 10 ml of NLN13 medium. Microspores were stained for 30 min. Then, the stained microspores were washed with NLN13 medium. Samples were observed by confocal laser scanning microscopy (Leica TCS 4D CLSM).

Organelle DNA in the microspores of *Brassica* was examined according to [Sodmergen et al. \(1998\)](#). Briefly, microspores were placed on a glass slide and immersed in a drop of phosphate-buffered saline (PBS) supplemented with 1% glutaraldehyde and 1 µg ml⁻¹ DAPI (Molecular Probes Co.) for ~10 min. The incubated cells were stained with 1 mg ml⁻¹ Calcofluor White (CW) for 30 min at room temperature to detect the cell division plane. Samples were observed under an epifluorescence microscope (Leica).

Electron microscopy

The ultrastructural sectioning was performed according to [Yan et al. \(1989\)](#), except that EDMs were immobilized in 1% (w/v) low melting temperature agarose before treatment of the sample (SeaPrep, Catalogue no. 50302, FMC). Ultrathin sections were cut on an ultramicrotome (Ultracut, Leica), stained for 15 min in 2% (w/v) uranyl acetate in 50% (v/v) ethanol and 10 min in 0.4% (w/v) lead citrate, and washed in deionized filtered water. Sections were observed using a Jeol 100 CX electron microscope. Electron micrographs were taken at 80 kV on Kodak 5302 FGP-film.

Immunofluorescent labelling with monoclonal antibody JIM8, and PIN protein immunolocalization

AGP distribution was investigated using the rat monoclonal antibody JIM8 as described previously ([Tang et al., 2006](#)).

PIN immunolocalization was performed according to [Supena et al. \(2008\)](#). Controls were performed according to the same procedure but without the first antibody.

RNA extraction and real-time RT-PCR

A Dynabeads mRNA DIRECT Micro Kit (DynaL Biotech) was used for mRNA isolation. mRNA was isolated from 25 microspores, 30 symmetrically divided microspores, 30 EDMs; 20 small globular embryos with a suspensor, and 20 small globular embryos without a suspensor at similar stages (4–8 µm in semi-diameter, see [Supplementary Fig. S1](#) available at *JXB* online); 10 large globular embryos with a suspensor, and 10 large globular embryos without a suspensor at similar stages (12–24 µm in semi-diameter, see [Supplementary Fig. S1](#)). First-strand cDNA was synthesized using oligo(dT)₂₅ (DynaL Biotech) and Superscript III Reverse Transcriptase (Invitrogen). A Super SMART™ cDNA PCR Synthesis Kit (Clontech Laboratories) was used for cDNA amplification.

Gene-specific primers were designed with primer3. Real-time PCR was performed according to [Ma et al. \(2011\)](#). For each gene examined, the expression levels were normalized relative to that of the

reference gene glyceraldehyde phosphate dehydrogenase (GAPDH). The primers used were: Bnwox8-S, CCGGGTCAAAAACAATGAGTT; Bnwox8-AS, CCAAAAACCAAACGCTGATT; GAPDH-S, GAAGGTGCTTCCACAGCTC; and GAPDH-AS, GTCGCAGCTTTCTCGAGTCT.

Results

Observation of different types of EDMs

After high temperature treatment, the exine of some microspores was broken at the germinal furrow, where it is much thinner. The exine was broken at two or three germinal furrows in some microspores, but most were broken at one germinal furrow ([Supplementary Fig. S2](#) at *JXB* online). The microspore exines dehiscenced at one germinal furrow showed two parts: the exine-covered part and the exine-removed part. The exposed intine and exine appeared distinct under light microscopy, and it was easy to differentiate the two parts of the EDM. To facilitate observation of the developmental course, the EDMs in which the exine was broken at only one germinal furrow were used. To evaluate fully the effects of the ruptured exine on microspore polarity induction and subsequent development, three types of EDMs were identified according to the proportion of exine coverage. In type 1, more than half of the EDM volume was covered by the ruptured exine ([Fig. 1A](#)); in type 2, about half of the microspore volume was covered by the ruptured exine ([Fig. 1B](#)); and in type 3, less than half of the EDM volume was covered by the ruptured exine ([Fig. 1C](#)).

Both symmetric and asymmetric EDM division could result in embryos with a suspensor

The EDM could divide both symmetrically and asymmetrically, leading to the formation of an embryo with a suspensor ([Fig. 1D](#)). The suspensor varied, appearing as a long filament ([Fig. 1E](#)) or redundant cells, visualized as an irregular protuberance at the radical pole of the embryos ([Fig. 1F, G](#)). In the majority of embryos, the suspensor structure consisted of 3–9 cells ([Fig. 1E](#)). Suspensor cell division ceased when the embryo developed to the late globular stage; this is similar to the zygote embryo.

In exine-intact unicellular microspores, a large vacuole occupies most of the cell, the nucleus is embedded in a thin layer of cytoplasm close to the microspore wall, and the nucleus moves to the centre of the cell during culture ([Fig. 2A](#)). The first division is typically symmetric ([Fig. 2A](#); [Zaki and Dickinson, 1990, 1991](#); [Jong et al., 1993](#); [Indrianto, 2001](#); [Seguí-Simarro et al., 2003](#); [Maraschin et al., 2005](#)). Later, a series of irregular cell divisions soon resulted in an embryonic cell cluster, which was still confined by the exine of the microspore ([Fig. 2B](#)), and then the embryonic cell cluster developed into a globular embryo ([Fig. 2C](#)). The embryo was released from the exine, followed by heart and cotyledon stage embryo formation. This is the traditional pathway of microspore embryogenesis, leading to the formation of the embryo without a suspensor ([Telmer et al., 1995](#); [Yeung et al., 1996](#)).

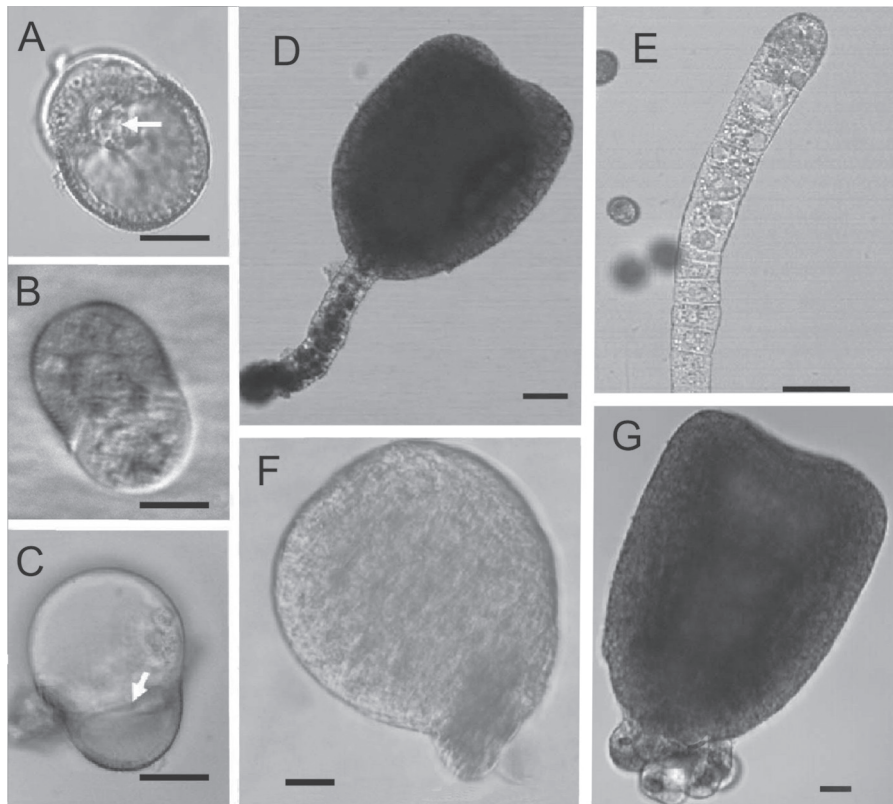


Fig. 1. EDM embryogenesis. (A) Type 1 EDM. (B) Type 2 EDM. (C) Type 3 EDM. The arrows indicate the division planes. (D) Suspensor-like structure. (E) Embryo with a suspensor. (F, G) Embryos with irregular suspensors. Bars=5 μ m.

To monitor EDM development carefully in comparison with the wall-intact microspores, cultures of three types of EDM were observed under an inverted microscope and recorded using a cooled-CCD camera. The total number of EDMs was 1623; the numbers of each type of EDM are shown in [Supplementary Table S1](#) at *JXB* online. All microspores could be followed during development using this system. For type 1 EDMs, 711 microspores were monitored, and 34.6% underwent the first division. The first cell division was asymmetric, and 25.6% of these divided EDMs subsequently developed into mature embryos ($n=246$). About 52.0% of the total number of embryos had a suspensor, whereas 47.0% did not have typical suspensors ($n=63$). After the first division, one of the daughter cell continued to divide transversely ([Fig. 2E](#)). Then, the other daughter cell swelled and divided vertically, resulting in the formation of a globular embryo with a suspensor ([Fig. 2D–F](#)). These observations were similar to those in *B. napus* embryogenesis described by [Tykarska \(1979\)](#). Additionally, one of the daughter cells ceased to divide or only divided once or twice, whereas the other cell continued to divide, resulting in the formation of an embryo without a typical suspensor ([Supplementary Fig. S3A–C](#)).

For type 2 EDMs, ~38.8% ($n=480$) of cells underwent the first division, which was a typical symmetric division. Of the divided microspores, 22.6% developed to mature embryos ($n=186$). About 64.1% of the embryos had a suspensor, whereas 35.8% showed no obvious suspensor ($n=42$). During the course of embryo development, one daughter cell first

underwent several transverse divisions, and formed a filament suspensor. The distal exine-covered cell then began to divide longitudinally, resulting in formation of the embryo proper ([Fig. 2G–I](#)). Another developmental pattern was similar to that of the exine-intact microspores described above. Both daughter cells contributed to the formation of the embryo proper ([Supplementary Fig. S3D–F](#) at *JXB* online). Furthermore, a few type 2 EDMs ($n=9$) continued to develop as a single file of cell filaments consisting of at least 10 cells through multiple transverse divisions, whereas the putative apical cell seemed to cease dividing ([Supplementary Fig. S3G–I](#)).

The developmental pattern of type 3 EDMs was very similar to that of type 1. Among the EDMs, 29.2% could initiate the first division ($n=432$), which was also asymmetric. Of the divided microspores, 26.2% developed into embryos ($n=126$); 45.4% of the mature embryos had a suspensor, whereas the others did not have typical suspensors ($n=33$). During the course of embryogenesis, the large naked daughter cell underwent several divisions, resulting in formation of a globular embryo, while the small exine-covered cell underwent several transverse divisions, resulting in a suspensor ([Fig. 2J–L](#)). It was also observed that the large naked daughter cell showed random cell divisions and eventually formed the embryo proper, while the small exine-covered daughter cell divided transversely and formed a small suspensor ([Supplementary Fig. S3J–L](#) at *JXB* online). These observations indicated that exine dehiscing modified the microspore

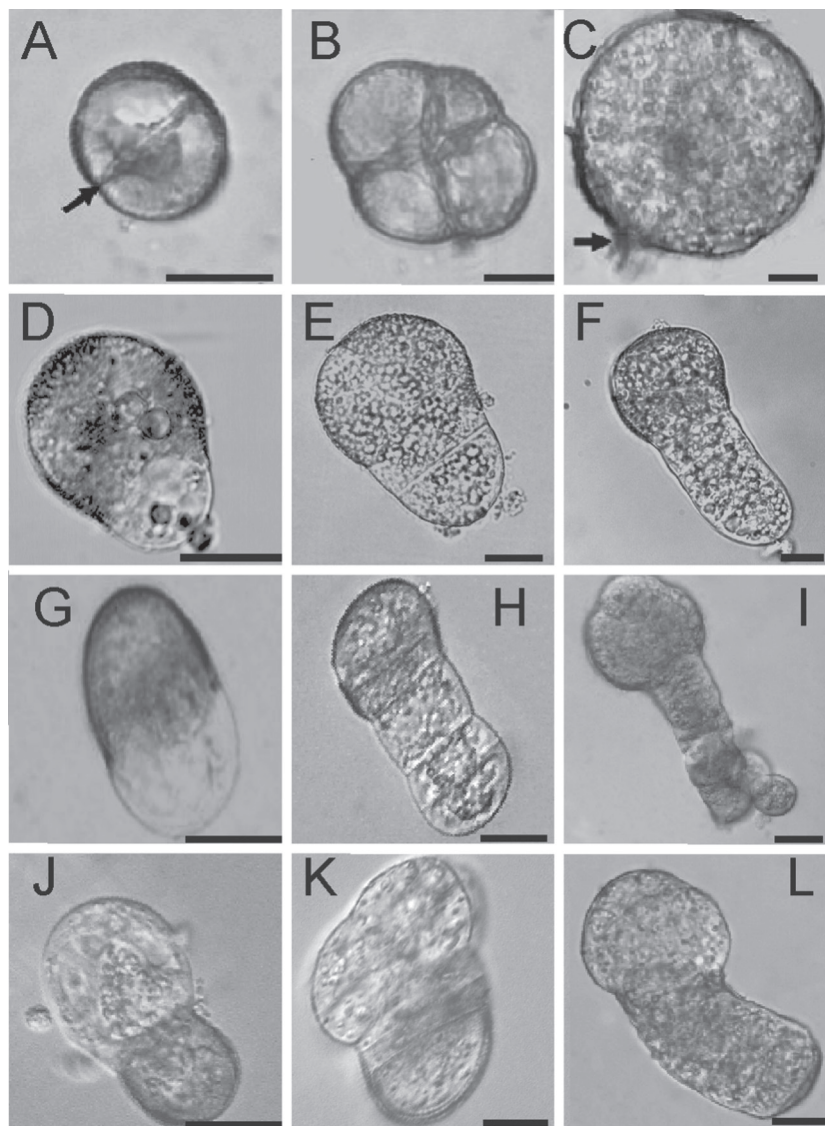


Fig. 2. The early embryogenesis course of intact-exine microspore and EDMs. (A–C) Intact-exine microspore embryogenesis. (A) Symmetric first cell division. The arrow indicates the division plane. (B) Four-celled embryo. (C) Globular embryo with no suspensor. The arrow indicates the exine. (D–F) Type 1 EDM embryogenesis. (D) Type 1 EDM. (E) The naked cell divided transversely, while the other cell divided vertically. (F) A globular embryo with a suspensor. (G–I) Type 2 EDM embryogenesis. (G) Type 2 EDM. (H) Transverse divisions of the naked daughter cell to form a suspensor. (I) Globular embryo with a suspensor. (J–L) Type 3 EDM embryogenesis. The larger naked cell divided both transversely and vertically, while the smaller exine-covered cell divided only vertically, resulting in the formation of a globular embryo with a suspensor. The images shown here were taken at day 1, 3, and 6 of culture. Bars=5 µm.

division pattern, resulting in the formation of an embryo with a suspensor. There were a few exceptions to embryogenesis described above, and twin embryos were occasionally found (Supplementary Fig. S4).

The embryo proper derived from both daughter cells of an EDM could finally develop into mature embryos with similar morphology.

Early cell division pattern in EDM-derived embryos with a typical filament suspensor

To confirm further where and how the embryo suspensor was generated, the division plane of the EDMs was checked with

CW during early embryogenesis. Two basic patterns were observed. In the first pattern, both daughter cells underwent transverse divisions after the first division of the EDM. Then, the apical cell of the structure underwent a longitudinal division and finally developed into the embryo proper, while the other cell continued to undergo transverse divisions to form the suspensor (Fig. 3A–C1). In the other pattern, after the first division of the EDM, one daughter cell underwent a longitudinal division, while the other underwent transverse division (Fig. 3D, D1). The transversely divided cell later formed the suspensor, whereas the longitudinally divided cell immediately divided several times (Fig. 3E, E1) and eventually gave rise to the embryo proper. Both the exine-covered cell and

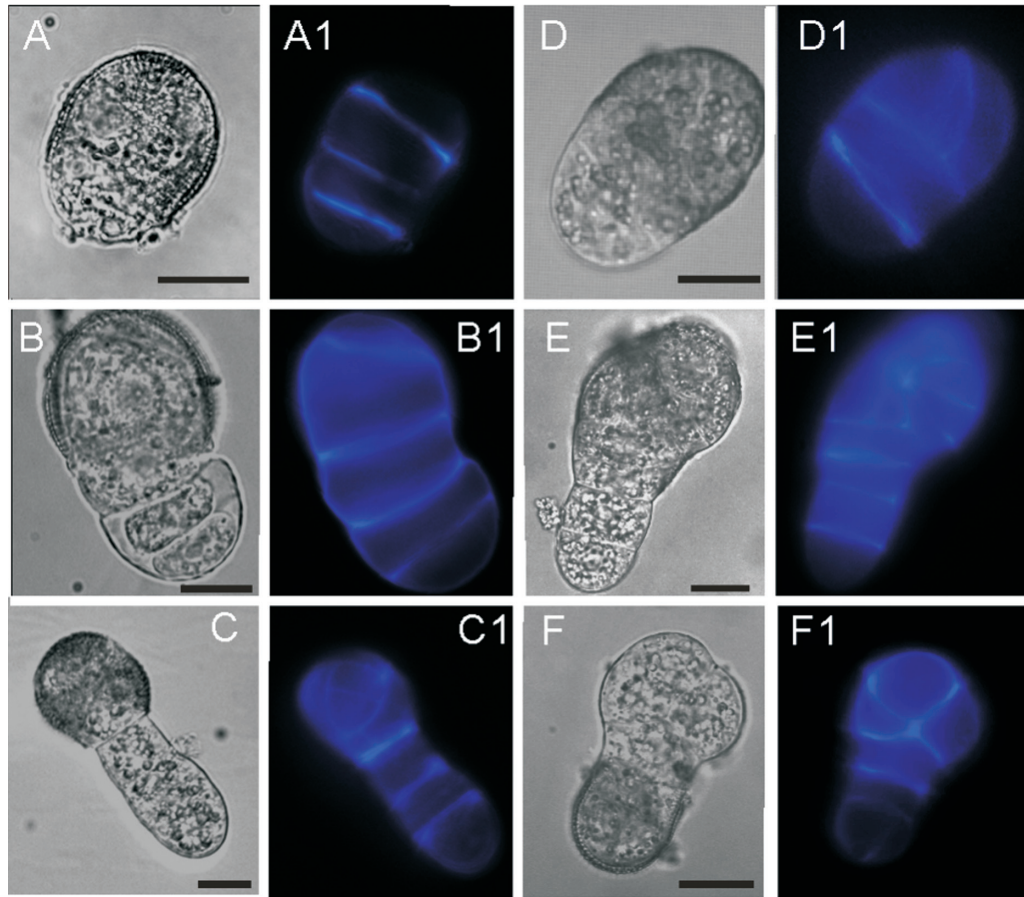


Fig. 3. Division pattern of the EDM-derived suspensors. (A–F) Bright field images. (A1–F1) Fluorescence microscopic images. Cells were stained with CW. (A–C1) Both daughter cells of the EDM underwent transverse divisions at first. The exine-covered cell underwent several divisions and developed into an embryo proper, while the naked cell kept transverse division and developed into a suspensor. (D–E1) The exine-covered cell underwent longitudinal cell division and later developed into an embryo proper. The naked cell kept transverse division and developed into a suspensor. (F, F1) The exine-covered cell divided transversely, giving rise to a suspensor. Bars=5 μ m.

the exposed cell can develop into the embryo proper (Fig. 3F, F1). These observations further confirmed that the suspensor is derived from one of the daughter cells of the EDM and basically follows the cell division pattern of the zygote embryo suspensor.

Exine dehiscing induces polarity of the EDM

To understand further why exine dehiscing could result in different cell fates of the two daughter cells of the first divided EDMs, cytological features of EDMs were compared with those of exine-intact microspores. At the unicellular microspore stage, the nucleus was near the pollen wall, and the organelle DNA was around the nucleus, representing a polar distribution (Fig. 4A, A1). After heat shock, organelles of the exine-intact microspores became evenly distributed, and the nucleus became centrally located (Fig. 4B, B1). However, in different types of EDMs, DAPI staining showed that the nucleus was always located near the plane indicated by the end of the broken exine between the exine-covered part and exine-detached part (Fig. 4C–E, C1–E1). Following

movement of the nucleus, organelles were also unevenly distributed (Fig. 4C1–E1). Rhodamine 123 was used as a marker for mitochondria (Gambier and Mulcahy, 1994). The mitochondria showed a polar distribution in exine-intact microspores (Fig. 4F, F1) and became evenly distributed after heat shock (Fig. 4G, G1). However, the mitochondria in EDM still showed a polar distribution (Fig. 4H–J1). Furthermore, electron microscopy indicated that the intine became much thicker on the exine-covered side of the EDMs (Fig. 4K). The organelles were highly concentrated around the nucleus (Fig. 4L), but one end of the EDMs was more vacuolated than the other (Fig. 4M). Obviously, the exine rupture results in the reorganization of the cytoplasm and induces morphological and structural polarity in EDMs.

The position of the first division plane is confined by the broken exine

EDMs were expanded, forming a long axis, designated as the ex-axis for convenience. The spatial plane indicated by the end of the broken exine between the exine-covered part

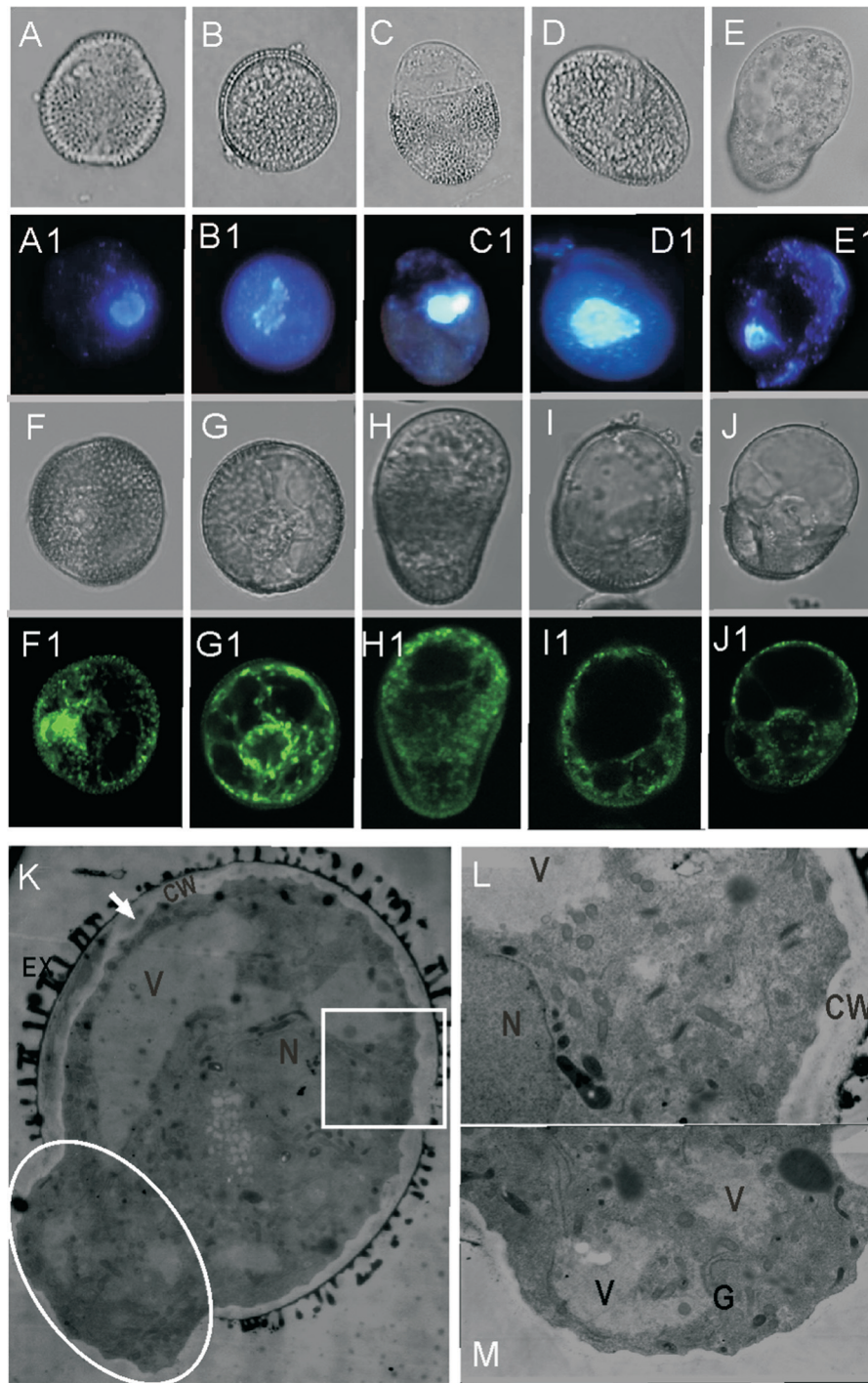


Fig. 4. Cytological characters of EDMs. (A–J) Light microscope images. (A1–J1) Fluorescence microscope images. (A, A1) An exine-intact microspore, showing that the nucleus locates near the pollen wall. (B, B1) An exine-intact microspore after heat shock, showing that the nucleus is located centrally. (C–E1) The nucleus locates at the plane between the exine-covered part and the exine-detached part. Organelle DNA is usually distributed around the nucleus in all type 1 (D, D1), type 2 (E, E1), and type 3 EDMs. (F, F1) An exine-intact microspore, showing that mitochondria distribute near the pollen wall. (G, G1) A exine-intact microspore after heat shock, showing that mitochondria are located centrally around the nucleus. (H–J1) Polar distribution of mitochondria in all type 1 (I, I1), type 2 (J, J1), and types 3 EDMs. Bars=5 μm . (K–M) Ultrastructural observation of EDMs. (K) Low magnification micrograph showing the entire cytological features; the intine became much thicker on the exine-covered side of the EDM. (L) Amplification of the boxed part in K, showing more organelles, especially mitochondria, in the exine-covered part. (M) Amplification of the ellipsoid part in K, showing more vacuolation and more endoplasmic reticulum in the naked part. EX, exine; CW, cell wall; V, vacuole; G, Golgi body; N, nucleus. Bars=0.5 μm .

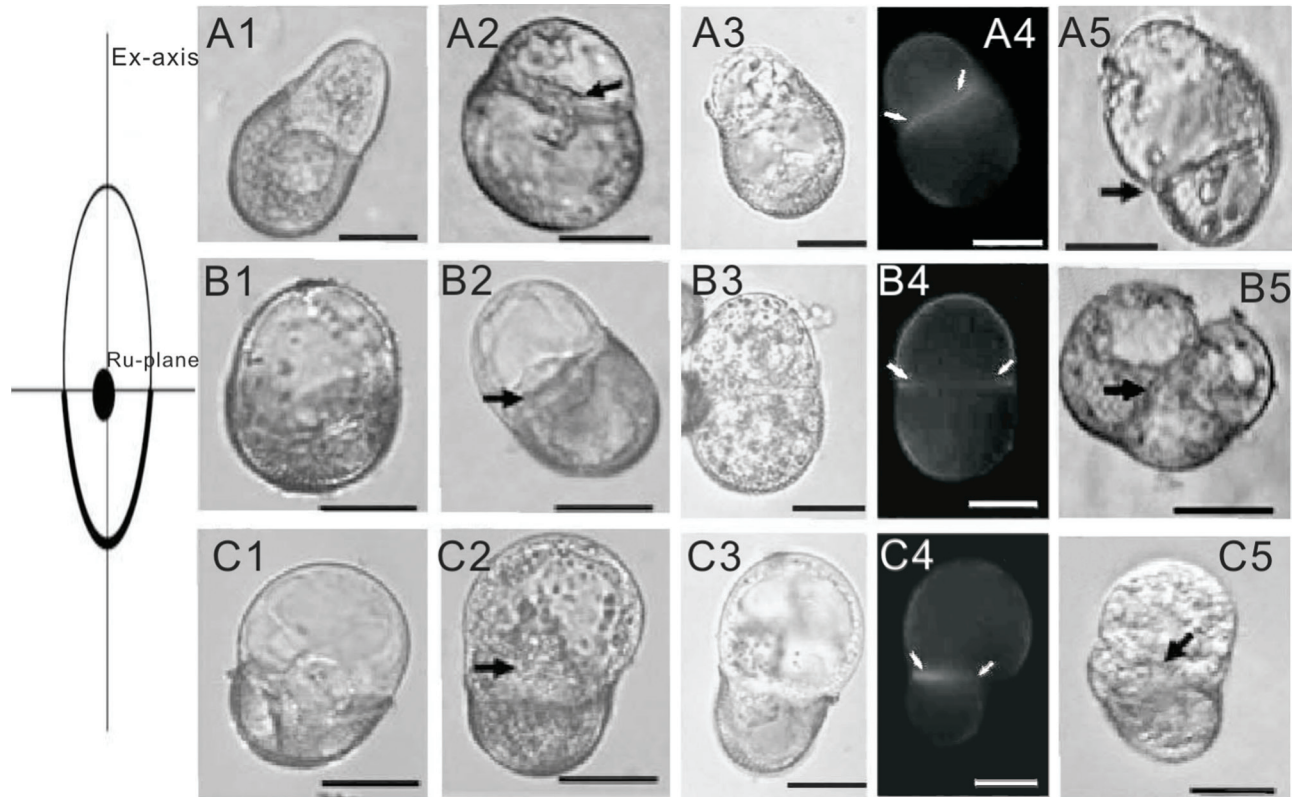


Fig. 5. The orientation of the first cell division in the three types of EDMs. The sketch indicates the ex-axis and ru-plane of EDMs. The expanded long axis of the EDM is named the ex-axis. The spatial plane at the border between the exine-covered part and exine-detached part is named the ru-plane. (A1–A5) Asymmetric divisions of type 1 EDMs. Interference contrast, bright field (A2, A3) and fluorescent image (A4), showing the overlap between the first cell division plane and the ru-plane. (A5) The division plane is parallel to the ru-plane as an alternative. (B1–B5) Symmetric division of type 2 EDMs. The division plane is also overlapping the ru-plane. (B5) The division plane is vertical to the ru-plane as an exception. (C1–C5) Asymmetric divisions of type 3 EDMs, showing that the first division plane is overlapping the ru-plane. (C5) The division plane is oblique to the ru-plane as an alternative. (A4, B4, C4) CW staining. Arrows indicate the division plane. Bars=5 μ m.

and the exine-detached part is named the ru-plane (Fig. 5). A total of 1,623 EDMs were traced. The first-division plane in type 1 EDMs (Fig. 5A1) was perpendicular to the ex-axis in 73.2% of cases (Fig. 5A2–A4; $n=711$), oblique to the ex-axis in 20.7%, and parallel to the ex-axis in 6.1% (Fig. 5A5). For type 2 EDMs (Fig. 5B1), 75.8% were perpendicular to the ex-axis (Fig. 5B2–B4; $n=480$), 19.4% were oblique to the ex-axis, and 4.8% were parallel to the ex-axis (Fig. 5B5). For type 3 EDMs (Fig. 5C1), 76.2% were perpendicular to the ex-axis (Fig. 5C2–C4; $n=432$), 19.0% were oblique to the ex-axis (Fig. 5C5), and 4.8% were parallel to the ex-axis. These data indicated that the majority of the EDM division planes were perpendicular to the ex-axis and overlapped with the ru-plane, suggesting that the position of the first division plane was dependent on the broken exine but uncoupled from EDM polarity.

Cytological characters of two EDM daughter cells with different cell fates

By tracing the early developmental process, it was found that after the first asymmetric division, in type 1 EDMs,

65% of embryos proper developed from the large cell, and 5% from the smaller cell ($n=63$). In type 3 EDMs, 77.6% developed from the large cell, and 4.45% from the small cell ($n=33$). These observations suggest that the large cell is probably fated to develop into the embryo proper, whereas the small cell is probably fated to develop into the suspensor. In type 2 EDMs, 40.7% of the embryos proper developed from the naked cell, and 6.78% developed from the exine-covered cell ($n=42$). Although it is not yet clear how this is determined, it is clear that the two cells have distinct developmental cell fates. Therefore, a comparison between the two daughter cells was performed to investigate their possible different features. Electron microscopic observations showed that one of the daughter cells was more vacuolated, whereas the other had more cell organelles (Fig. 6A–C). The vacuolated cell was fated to develop into the suspensor, and the other would divide vertically and give rise to the embryo proper (Fig. 6D, D1, E). More organelle DNA was usually distributed in the large daughter cell than in the small cell (Fig. 6F; $n=14$).

The distribution of JIM8 labelling in the EDMs and the just-divided EDMs was tested. In 60.8% of sister cells derived

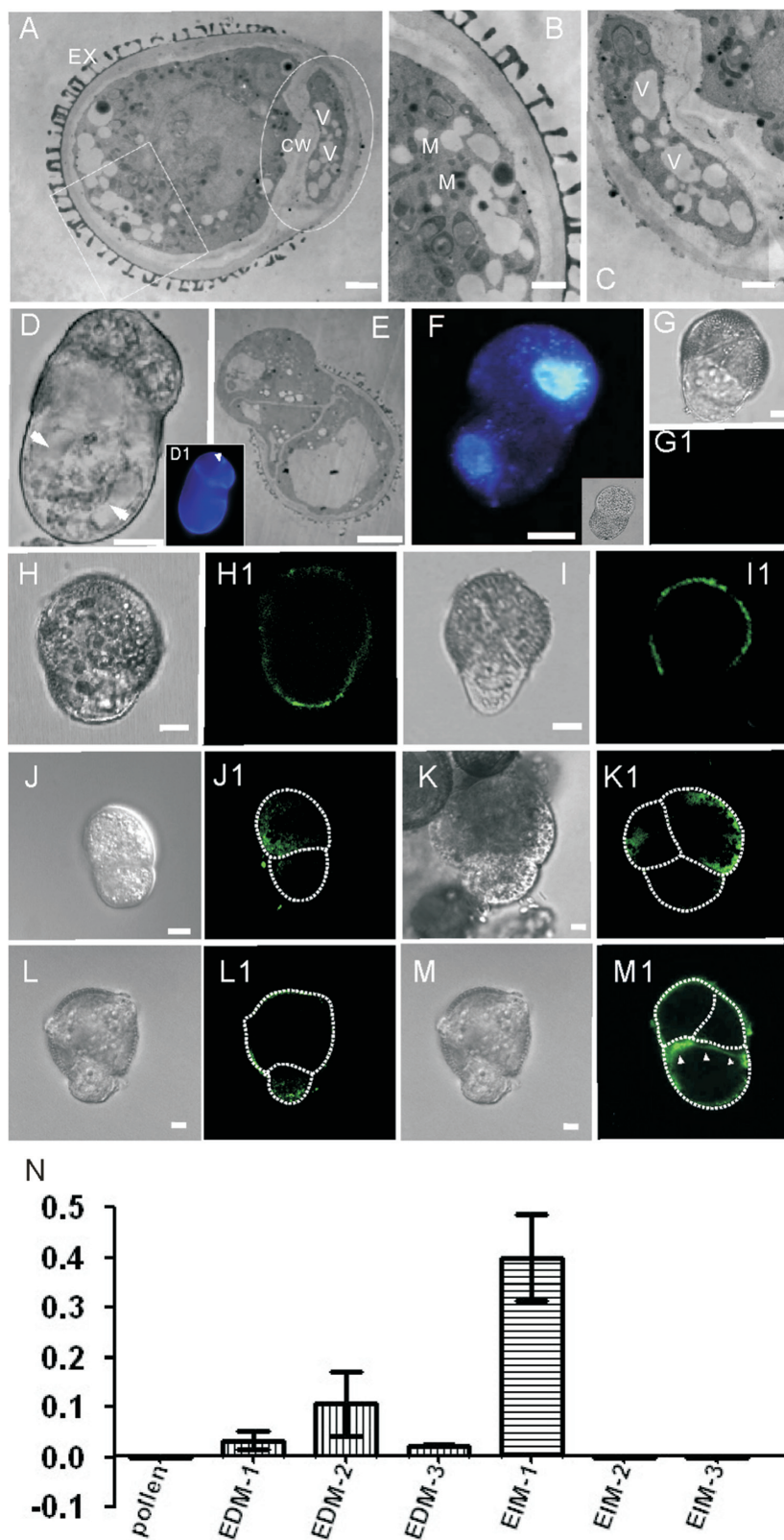


Fig. 6. Comparisons between two daughter cells of EDMs. (A–C) Ultrastructural observations of a divided EDM. (A) Low magnification micrograph shows the entire cytological features. (B) Amplification of the square area in (A); the larger cell has condensed cytoplasm and more mitochondria. (C) Amplification of the ellipsoid area in (A), showing that the small cell is more vacuolated. EX, exine; CW, cell wall; V, vacuole; M, mitochondria. Bars=0.5 μ m. (D) Vacuoles were observed in the larger naked cell after asymmetric cell division; the arrows indicate vacuoles. (D1) CW staining of the cell in (D). The arrowhead indicates the division plane. (E) Thin section of a symmetrically divided EDM, showing that an exine-covered cell contains a large vacuole. (F) A symmetrically divided EDM (indertion), showing that organelle DNA differentially distributes in its two daughter cells. (G–I1) Immunolocalization of JIM8. (G, G1) Control, showing no

from type 1 EDMs, JIM8 labelling was preferentially localized in the small cell (Fig. 6H, H1), with 39.2% in the large cell (Fig. 6I, I1; $n=74$). These observations suggested that the AGPs are distributed unevenly in the two sister cells after the first division of the EDM.

PIN7 was reported to be expressed in the basal cell in the two-celled proembryo, whereas PIN1 mRNA was mainly localized in the apical cell (Friml *et al.*, 2003; Weijers *et al.*, 2006). It was also confirmed that PIN are also expressed in *Brassica* microspore embryos (Supena *et al.*, 2008). Therefore, PIN distribution in the EDMs was investigated using antibodies targeting *Arabidopsis* PIN1 and PIN7 proteins. After the first unequal division, PIN1 was peripherally localized in the large cell in 80% of two-celled proembryos derived from the EDMs (Fig. 6J, J1; $n=25$). After the second division, PIN1 was mainly distributed in the two embryo-proper cells in 76.4% of EDM-derived embryos (Fig. 6K, K1; $n=17$). PIN7, on the other hand was localized in the small cell in 77.8% two-celled proembryos (Fig. 6L, L1; $n=27$) and, after the second division, it was mainly located in the initial suspensor cell in 66.7% of EDM-derived embryos (Fig. 7M, M1; $n=18$). Both PIN proteins were distributed in a non-polar manner in the cells, although PIN1 typically showed a polar localization in the cells of globular and heart stage microspore embryos (Supplementary Fig. S5 at *JXB* online).

It was also reported that *WOX8* can be used as a marker for the basal cell lineage (Haecker *et al.*, 2004). The sequence of *BnWOX8* was found in a genomic survey sequence (gss) that has the same homeodomain as *AtWOX8* and shows 82% identity at the DNA sequence level with *AtWOX8*. Its predicted protein sequence has 93% identity with *AtWOX8*. Its expression level was examined in microspores and embryos of different stages by quantitative real-time RT-PCR (RT-qPCR). *BnWOX8* was not expressed in the unicellular microspores but was detected in the EDM after the first cell division. Its expression was also detected in small (~4 μm in diameter) and large (~12 μm in diameter) EDM-derived globular embryos with a suspensor (Fig. 6N), but not in similar exine-intact microspore-derived globular embryos or large globular embryos without a suspensor (Fig. 6N). These observations suggested that *BnWOX8* can also be used as a marker of the suspensor lineage in EDM-derived proembryos, and *BnWOX8* is already expressed as early as the first division of EDMs. All of these differences between the two cells of EDM-derived two-celled proembryos suggest that cell differentiation occurs immediately after the first EDM division, leading the two cells to their distinct developmental cell fates.

Exine rupture determines the orientation of the developmental axis of the embryo

As described above, the microspores with an intact exine develop into non-polar globular embryos, and there is no visible axis before the embryo is released from the microspore exine. However, the EDM could develop into embryos with suspensors and thus with obvious polarity and apical–basal axes. It was found that in 84.1% of cases, the polar axis was consistent with the ex-axis formed by rupture of the exine in all types of EDMs ($n=69$; Fig. 7, types 1–3; Fig 2, 3). A few EDMs could divide parallel to the ex-axis; in this case, the two daughter cells could form the two embryos. However, the apical–basal axis of the two embryos was still parallel to the ex-axis (Fig. 7, small part of EDMs; Supplementary Fig. S4 at *JXB* online). Clearly, the axis of embryos derived from EDMs is determined by the rupture of the microspore exine and probably by its induced cell polarity.

Discussion

EDM embryogenesis is a convenient and peculiar system for studying the early events of embryogenesis

Microspore embryogenesis is a convenient model system to understand the general principles of plant embryogenesis. It was widely accepted that microspore embryogenesis typically starts with symmetric cell division, which gives rise to two cells of equal size. Usually the embryos derived from these symmetrically divided microspores lack suspensors. Therefore, the early process of microspore embryogenesis is distinct from zygotic embryogenesis (Hause *et al.*, 1994; Maraschin *et al.*, 2005). Ten years ago, a procedure was established to prepare EDMs, and it was found that EDMs could develop into embryos with a suspensor (Tian and Sun, 2003; Tang *et al.*, 2006). In fact, a rudimentary suspensor-like structure was occasionally observed (Hause *et al.*, 1994; Ilić-Grubor *et al.*, 1998; Yeung, 2002; Supena *et al.*, 2008) and, later, this structure could also be highly induced (Straatman *et al.*, 2000; Dubas *et al.*, 2011; Prem *et al.*, 2012). However, the reason for the generation of the suspensor remains unknown.

In the present study, the full sequence of development events in early embryogenesis was elucidated from the different types of EDMs tracked using individual monitoring techniques. The results showed that during the first 4–6 d in culture, three types of EDMs could be identified morphologically, all of which could give rise to embryos. It is easy to observe the relationships between asymmetric cell division

fluorescence on the exine. (H, H1) JIM8 locates more on the small daughter cell. (I, I1) JIM8 locates more on the large daughter cell. (J, J1) PIN1 mainly localizes in the large daughter cell. (K, K1) The cell with PIN1 signal divided longitudinally and will probably develop into an embryo proper. (L, L1) PIN7 appears in the small daughter cell, which will probably develop into a suspensor. (M, M1) The cell without PIN7 signal divided vertically. (N) *BnWOX8* expression level in different microspore embryos. (EDM-1) The first divided EDMs. (EDM-2) Small embryo with suspensor (4 μm in diameter). (EDM-3) Large embryo with suspensor (12 μm in diameter). EIM, exine-intact microspores. EIM1, the first divided EIM; EIM2, small globular embryos without a suspensor (4 μm in diameter); EIM3, large microspore embryos without a suspensor (12 μm in diameter). Data are the mean of three independent experiments. Bars indicate standard errors.

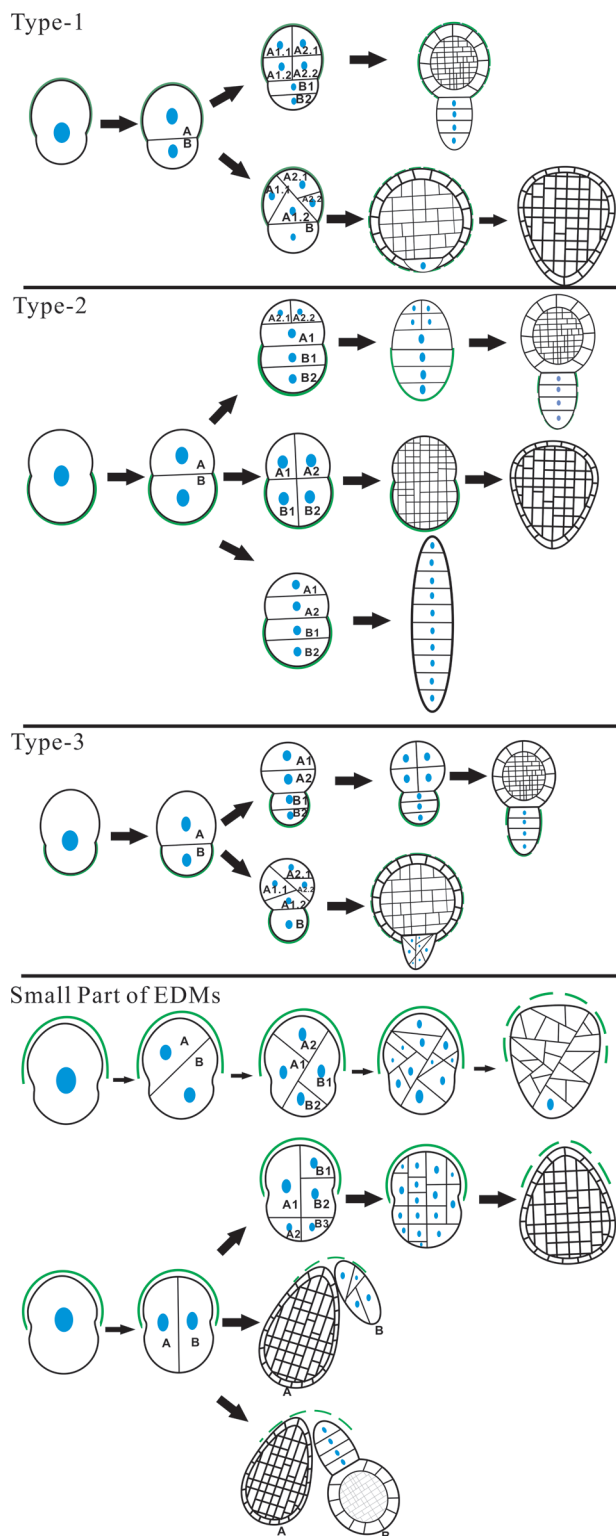


Fig. 7. Diagram of the embryogenesis in different types of EDMs. The polar axis was always consistent with the ex-axis. The small part of EDMs: the exine was not so tightly covered in some EDMs. In this case, some of them went through random divisions and grew into embryos without suspensors. Occasionally, the two daughter cells could develop into two embryos, respectively, but the apical-basal axis of the two embryos is still parallel to the ex-axis. All embryo proper with and without a suspensor could develop into mature embryos. (This figure is available in colour at JXB online.)

and cell developmental fate and to distinguish the orientation of the embryo body axis in relation to EDM polarity by this technique. This method provides a unique opportunity to investigate these important developmental events during early embryogenesis. Although zygote culture systems have been established previously for the same purpose (He *et al.*, 2007), the EDM system has a number of advantages as it is convenient to collect a number of cells for statistical analysis, and it is highly efficient for embryogenesis.

Exine dehiscing induces microspore polarity, resulting in different daughter cell fate and fixing the orientation of the embryo body axis

In exine-intact microspore embryogenesis, both of the daughter cells after the first symmetric cell division take part in embryo formation. The first sign of the establishment of polarity appears at the stage of transition from a globular to a heart-shaped embryo after rupture of the pollen wall (Hauser, 1994; Indrianto *et al.*, 2001). In the present study, it was shown that in EDMs, the polarity appeared immediately after microspore exine rupture and preceded the first cell division (i.e. the organelles were unevenly distributed). Additionally, one end of the EDM was more vacuolated, and the intine became much thicker on one side of the EDM. These characters indicated that cytological reorganization occurs after exine rupture and cell polarity is established. After the first cell division, the two daughter cells showed distinct characters—the small daughter cell was more vacuolated and was fated to develop into the suspensor, while the large daughter cell had more organelles and was fated to develop into the embryo proper. *BnWOX8*, which marks the suspensor cells, was expressed immediately after the first EDM division. This observation suggests the establishment of polarity in the EDMs due to exine dehiscing and daughter cell differentiation, with different developmental fates at the two-celled proembryo stage.

Individual cell tracing indicated that the suspensor was generated from one of the daughter cells of the EDM, indicating that exine dehiscing resulted in different cell fates at the two-cell stage, similar to the process of zygotic embryogenesis. Two different mechanisms have been proposed for cell fate diversity. In the first, intercellular communication provides information for position-dependent cell fate determination, whereas the second involves unequal cell division of a polarized cell, generating two daughter cells that assume different fates (St Johnston and Nusslein-Volhard, 1992; Jacobs and Shapiro, 1998; Laux, *et al.*, 2004). In animals, it was reported that special molecules located in the maternal cytoplasm can be unequally divided into the two daughter cells, thus determining the different cell fates (Ephrussi and Lehmann, 1992; St Johnston and Nusslein-Volhard, 1992). Gallagher and Smith (2000) reported that the fate of subsidiary cells was dependent on the polarity of subsidiary mother cells (SMCs) and inheritance of daughter cell nuclei in maize. However, whether this is the case in early embryogenesis remains unknown. In normal microspore embryogenesis there is no cell fate diversity at the early stage. However,

in EDM embryogenesis, the two daughter cells showed different cell fates and produced embryos with a suspensor. These observations indicate that cell polarity plays a critical role in the determination of the fates of the daughter cells. Thus, the results here indicate a close link between EDM polarity and different fates of daughter cells in early embryogenesis, suggesting that polarity is necessary for cell fate diversity during early embryogenesis.

EDM embryogenesis also showed that the apical–basal axis of the embryo body is in line with the orientation of EDM polarity. Following exine dehiscing, the microspores elongated and formed a long growth axis. After the first EDM division, one of the daughter cells developed into a suspensor, while the other developed into the embryo proper. Interestingly, when the first cell division of EDMs was vertical; that is, perpendicular to the ru-plane, each of the two cells could divide into the embryo, and the apical–basal axis of both embryos was still in line with the orientation of EDM polarity (Fig. 7). This suggests that the establishment of the apical–basal axis is probably dependent on EDM polarity. It was reported that the polar secretion of Golgi-derived vesicles is required for polarity fixation in the *Fucus* embryo (Kropf *et al.*, 1999; Belanger and Quatrano, 2000). The reasons why exine rupture induces polarity and finally defines the apical–basal axis of embryo development remain unclear. It is possible that the ruptured exine directs cytoplasm reorganization and movement of the nucleus to establish the polarity of EDMs, which pre-defines the apical–basal axis of the embryo.

The orientation and spatial location of the first cell division plane are modulated by the ruptured exine, but uncoupled from cell polarity

In the present study, it was shown that the first division plane of EDMs, whether symmetric or asymmetric division, was in line with the ru-plane. It seems that positioning of the cell division plane is not controlled by internal cell polarity, but is dependent on the external exine envelope. In fact, it has been reported that the cell division plane may be strongly guided by extrinsic clues (Lynch and Lintilhac, 1997; Scheres and Benfey, 1999; Smith, 2001). Force stress could induce alignment of the cell division plane (Lintilhac and Vseckky, 1984). It was also shown that a gentle touch is sufficient to provide a signal to the cells and cause the nucleus to move to the site of stimulation (Qu *et al.*, 2008). Yeoman (1971) proposed that mechanical stress can result in the formation of a protein complex that directs formation of the phragmoplast in the corresponding position. The present data showed that the first division plane of most EDMs was parallel to or in line with the ru-plane, showing a close correlation between the plane of cell division and the position of the dehiscence. The EDM nucleus always moved to the border of the broken exine, where cell division then occurred. It is likely that the dehiscence could generate mechanical pressure, and the position of the ru-plane is the site where the pressure is most intense. Therefore, the first cell division plane may be adjusted by mechanical stress caused by the ruptured exine. Although the cell polarity may be correlated with the

cell division plane, the present data support the suggestion that the division plane of EDMs is mainly modulated by extrinsic cues.

In animal cells, a model has been defined to explain how extracellular mechanical signals are transmitted to the nucleus via conformational changes in the cytoskeleton (Wang *et al.*, 1993). This model suggests that cytoskeletal association with the nucleus may be capable of direct signalling of gene expression via mechanical signals, such as integrin-like proteins, which are specialized transmembrane proteins with cytoskeleton associations. These proteins have been found in plants (Schindler *et al.*, 1989; Gens *et al.*, 1996). It was reported that there is abundant cytoskeleton in microspores, and the cytoskeleton changed after cell culture (Pierson and Cresti, 1992). This model may explain how the EDM perceives mechanical signals of the exine and then translates them into oriented division.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. The stages of the embryos used for RNA extraction.

Figure S2. The different aspects of microspore exine rupture.

Figure S3. Variations of developmental fate of EDM-derived cells.

Figure S4. The specific EDM embryo development: the axis of the embryos.

Figure S5. PIN1 polar localization in the cells of the globular and heart stage embryo, which is similar to the *Arabidopsis* zygotic embryo at the globular and heart stage.

Figure S6. The negative control for the immunofluorescent labelling of PIN.

Table S1. The number of EDMs in tracing the development of the three types of EDMs.

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