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RESEARCH PAPER

Regeneration of zygotic-like microspore-derived embryos suggests an important role for the suspensor in early embryo patterning

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Abstract

The inaccessibility of the zygote and proembryos of angiosperms within the surrounding maternal and filial tissues has hampered studies on early plant embryogenesis. Somatic and gametophytic embryo cultures are often used as alternative systems for molecular and biochemical studies on early embryogenesis, but are not widely used in developmental studies due to differences in the early cell division patterns with seed embryos. A new *Brassica napus* microspore embryo culture system, wherein embryogenesis highly mimics zygotic embryo development, is reported here. In this new system, the donor microspore first divides transversely to form a filamentous structure, from which the distal cell forms the embryo proper, while the lower part resembles the suspensor. In conventional microspore embryogenesis, the microspore divides randomly to form an embryonic mass that after a while establishes a protoderm and subsequently shows delayed histodifferentiation. In contrast, the embryo proper of filament-bearing microspore-derived embryos undergoes the same ordered pattern of cell division and early histodifferentiation as in the zygotic embryo. This observation suggests an important role for the suspensor in early zygotic embryo patterning and histodifferentiation. This is the first *in vitro* system wherein single differentiated cells in culture can efficiently regenerate embryos that are morphologically comparable to zygotic embryos. The system provides a powerful *in vitro* tool for studying the diverse developmental processes that take place during the early stages of plant embryogenesis.

Key words: *Brassica napus*, microspore embryogenesis, pattern formation, polarity, suspensor, zygotic embryogenesis.

Introduction

Genetic approaches have proven to be a very powerful tool for dissecting processes that control embryogenesis in plants. Mutant screens in *Arabidopsis*, rice, and maize have led to the identification of genes that function in regulatory pathways that specify early developmental processes, such as the initial asymmetric division of the single-celled zygote (Mayer *et al*., 1993), the first division in the apical daughter cell of the zygote (Hamann *et al*., 1999), the determination of apical–basal organization (Berleth and Jürgens, 1993; Lu *et al*., 1996), the differentiation of the shoot (Barton and Poethig, 1993; Clark *et al*., 1993, 1995; Laux *et al*., 1996; Kayes and Clark, 1998) and root apical meristems (Di Laurenzio *et al*., 1996; Willemsen *et al*., 1998), and the interaction

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between the embryo and suspensor (Marsden and Meinke, 1985; Schwartz *et al*., 1994; Vernon and Meinke, 1994; Zhang and Somerville, 1997; Haecker *et al*., 2004; Lukowitz *et al*., 2004). Genetic perturbation of hormone homeostasis, and cell ablation using developmentally regulated promoters, have also provided powerful tools for embryo development studies (Gallois, 2001; Weijers *et al*., 2003, 2005, 2006). However, molecular and biochemical studies on early embryo development from the zygote stage onwards have proven difficult due to the small size of the embryo and its inaccessibility within the seed coat and endosperm tissues.

In vitro cultures, either starting from excised zygotic embryos (Liu *et al*., 1993*a*, *b*; Fischer and Neuhaus, 1996), fertilized ovules (Sauer and Friml, 2004), or isolated zygotes (He *et al*., 2007), have been used as an alternative source of material for studies on early embryogenesis. However, these systems have their limitations due to the high frequency of embryo mortality when very young embryo stages are concerned and developmental abnormalities induced by the isolation and/or culture procedures. Non-zygotic embryos derived from *in vitro* cultures of somatic or gametophytic cells (Ferrie *et al*., 1995; Merkle *et al*., 1995), are more frequently used as an alternative to zygotic embryos for research on the early stages of plant embryogenesis (De Jong *et al*., 1993; Schmidt *et al*., 1997; Hecht *et al*., 2001; Zhang *et al*., 2002; Ikeda-Iwai *et al*., 2003). Large numbers of non-zygotic embryos can be produced in culture, and since maternal and filial tissues do not envelop them, they are highly amenable to biochemical and physiological manipulation (Zimmerman, 1993). Although non-zygotic embryogenesis has many features in common with zygotic embryogenesis, there are also some differences in the way the embryos originate and develop (Dodeman *et al*., 1997; Mordhorst *et al*., 1997). Most somatic embryogenesis systems use hormones to trigger callus development, from which single cells or clusters of cells are induced to form pro-embryogenic masses, followed by embryos upon culture on hormone-free medium (Nomura and Komamine, 1985; De Vries *et al*., 1988). This dependence on exogenous hormones constitutes the major disadvantage associated with the use of somatic embryogenesis for studying the natural course of plant embryogenesis. Another disadvantage of such systems is that somatic embryos, being derived from unorganized pro-embryogenic masses, suffer from the absence of well-defined early embryo stages (Mordhorst *et al*., 1997).

Gametophytic systems for non-zygotic embryo formation, in particular, microspore embryogenesis, more closely mimic zygotic embryogenesis than do somatic embryogenesis systems. They generally do not rely on hormones for embryo induction, but rather on a transient (heat) stress treatment that is crucial for promoting the developmental switch from gametophytic development to embryo development (Touraev *et al*., 1996; Zoriniants *et al*., 2005). Large amounts of embryos can be regenerated from individual single-cell microspores without going through an intermediate callus phase. In this system, multicellular proembryo structures with up to 40–60 cells are formed through random cell divisions, which then develop through a hypothetical self-organizing mechanism into recognizable globular embryos with a distinct protoderm that subsequently form heart-shape embryos (Mordhorst *et al*., 1997). A very ordered early cell division pattern as is characteristic for zygotic embryogenesis, however, is absent during microspore embryogenesis, and the primordia for the seedling organs are formed later than in zygotic embryos. Furthermore, suspensor development is absent in microspore embryos, although a rudimentary suspensor-like structure is occasionally observed (Hause *et al*., 1994; Ilić-Grubor *et al*., 1998*a*; Yeung, 2002).

Brassica napus microspore embryogenesis is being used as model for studying the molecular mechanisms controlling embryo initiation and early development. These studies revealed mRNAs and proteins specifically accompanying the induction of embryogenesis (Cordewener *et al*., 1995, 2000; Custers *et al*., 2001; Joosen *et al*., 2007), and genes have been identified that control embryo induction and cell differentiation processes (Boutilier *et al*., 2002; Fiers *et al*., 2004, 2005). In the course of these studies, we became interested in polarity establishment and early differentiation of the young microspore-derived embryos. One marker of early embryo polarity is the occasional occurrence of suspensor-like structures attached to microspore-derived embryos. Ilić-Grubor *et al*. (1998*a*) showed that these structures originated as protrusions from the future basal pole of the heart-shape embryo stage, and exhibited a variety of shapes, ranging from short uniseriate filaments to irregular shaped multicellular protuberances. These suspensor-like structures were also observed in our *B*. *napus* microspore embryo cultures, but it was noted that the frequency of their occurrence varied between different experiments. Recently, it was found that a milder heat-stress treatment and the use of a more narrow range of microspore developmental stages than are used in conventional *B*. *napus* microspore embryo cultures contributed to both higher frequencies of embryos with suspensor-like structures and to development of embryos bearing long, uniseriate filaments. Based on these observations, a culture procedure was developed for the reproducible formation of filament-bearing microspore-derived embryos (Joosen *et al*., 2007).

A cytological analysis of the early cell division and patterning events that accompany the development of filament-bearing embryos in *B*. *napus* microspore embryo cultures is presented here. It is shown that development of the embryo proper of microspore-derived embryos with a long, uniseriate suspensor-like filament parallels zygotic

embryo development from the initial cell divisions onward, while histodifferentiation in conventional microspore-derived embryos possessing no or only rudimentary suspensor-like structures is strongly delayed. This new *B. napus* microspore embryo culture system provides a unique starting point for molecular- and cell biological analysis of early embryo initiation and development in plants. Moreover, our results suggest an important role for the suspensor in regulating pattern formation in early zygotic embryo development, and provide additional support for the role of the embryo proper in prohibiting cell proliferation in the suspensor (Weijers *et al*., 2003).

Materials and methods

Growth conditions of the donor plants

Plants of *Brassica napus* L. cv. Topas line DH 4079 were used for the experiments and they were grown according to Custers (2003). When plants just started to flower, flower buds of 3.2–3.5 mm in length were selected and divided over three bud length classes (3.2–3.3, 3.3–3.4, and 3.4–3.5 mm), and from each class 15–20 buds were used for the isolation of microspores.

Microspore isolation and culture

Microspore isolation and culture were performed according to Custers (2003) with modifications by Joosen *et al*. (2007). NLN-13 medium (Lichter, 1982) was used for both isolation and culture of the microspores. In order to prevent fluctuation in temperature during the isolation procedure, the bleach solution used for sterilization, the water used for rinsing, and the isolation media were taken from a 4 °C refrigerator and were used immediately. The microspores were incubated at a density of 40 000 ml^{-1} in NLN-13 culture medium at room temperature. Aliquots of 1 or 3 ml microspore suspension were plated in 3 cm or 6 cm Petri dishes, respectively, for culture. Embryogenic cultures producing microspore-derived embryos with long, uniseriate suspensor-like filaments were obtained by applying 24 h 32±0.2 °C heat stress followed by transfer to 25 °C, while conventional microspore-derived embryos without suspensors developed in cultures continuously at 32±0.2 °C. All the cultures were kept in darkness. Cultures were continuously kept in NLN medium with 13% sucrose (NLN-13) or were transferred from NLN-13 to NLN medium containing 1% sucrose and 22% polyethylene glycol (PEG 4000) after 5 d of culture to improve the embryo quality (Ilić-Grubor *et al*., 1998*b*).

Cytological and morphological analyses

The developmental stage of the microspores at the start of culture was determined using 4′,6-diamidino-2-phenylindole (DAPI) epifluorescence staining according to Custers *et al*. (1994). Microspore populations consisting of 50–60% late unicellular microspores and 30–40% early bicellular pollen were most responsive in the development of microspore-derived embryos with long, uniseriate suspensor-like filaments (Joosen *et al*., 2007), and such a microspore composition was mostly found in the bud length class of 3.3–3.4 mm. Microspore embryo cultures from this particular class were then used for further analysis.

Early embryo development from the microspores was observed using either an inverted microscope or after DAPI staining using a Zeiss Axioskop microscope equipped with epifluorescence and Nomarski optics. Microspore embryo culture samples (75–100 μl) were collected by centrifugation at 3000 rpm in an Eppendorf centrifuge, and incubated overnight in DAPI staining solution. Cell tracking experiments were performed with non-immobilized cultures that were left on the inverted microscope stage for the entire experiment. To facilitate morphological observation, specific embryo types were collected after 10 d of culture and incubated on top of NLN medium solidified with 0.6% Plant Agar (Duchefa).

PIN protein immunolocalization

PIN immunolocalization was performed according to Friml *et al*. (2003*a*) with modifications (Szechynska-Hebda *et al*., 2006). Suspensor-like filaments collected at days 6–8 of culture were immediately fixed in a freshly prepared prefixative mixture containing 1% paraformaldehyde (PFA) and 0.025% glutaraldehyde (GA) in a microtubule stabilizing buffer [MTSB, 50 mM 1.4-piperazinediethane sulphonic acid (PIPES, Sigma), 5 mM EGTA (Sigma), 5 mM $MgSO_4$, pH 7.0, adjusted with KOH] for 15 min at room temperature. The main fixation was done with 3% PFA/0.025% GA for 30 min at room temperature. Samples were washed with MTSB/0.025% Triton X-100 for 10 min, treated with a 0.05 M NH_4Cl and 0.05 M $NaBH_4$ for 5 min, and washed again. Cell walls were partly digested in a mixture of 1% cellulase ('Ozonuka R-10' from *Trichoderma viride*, Serva), 0.8% pectinase (from *Rhizopus*, Sigma), 0.02% pectolyase (from *Aspergillus japonicus*, Sigma), and 0.3% macerozyme (R-10 from *Rhizopus lyophil*, Serva) in MTSB for 1 h at 37 °C, and then washed with MTSB/0.025% Triton X-100 five times for 10 min each. To enhance permeability further, the material was incubated in 10% DMSO/3% Nonidet P-40 in MTSB for 50 min at room temperature. After rinsing, a blocking step was performed with 2% BSA in MTSB at 30 °C. PIN1, 4 and 7 antibodies [raised in rabbit and kindly provided by J Friml (Friml *et al*., 2003*a*)] were applied in 3% BSA/MTSB overnight (anti-PIN1 1:1000, anti-PIN4 1:200, and anti-PIN7 1:50 diluted). Finally, the suspensor-like filaments were incubated in secondary antibody GaR/IgG/Alexa 488 (Molecular Probes, final dilution 1:100), and in propidium iodide PI 1 mg ml^{-1} H_2O) for DNA counterstaining. Microscopic observation was done with a CELL MAP IC Bio-Rad (Hemel Hempstead, UK) confocal laser-scanning microscope, mounted on a Nikon Eclipse TE 2000-S inverted microscope.

Results

Development of suspensor-like filaments in Brassica napus *microspore embryo cultures*

In *B. napus*, the late unicellular microspore and early bicellular pollen microgametophyte stages can be efficiently switched to embryogenesis. Here, both stages are collectively referred to as microspores for the sake of convenience. Under the culture conditions that were usually applied (continuous 32 °C), embryogenesis begins with a series of unordered cell divisions in the microspores (Fig. 1A). The resulting embryonic cell clusters are released from the constraints of the microspore exine wall and develop into globular embryos with a recognizable protoderm (Fig. 1B), followed by heart- and cotyledon-stage embryo formation. This is the most commonly observed pathway of microspore embryogenesis, and leads to embryo development without suspensor formation (Telmer *et al*., 1995; Yeung *et al*., 1996). Two distinct regions were observed in 20–30% of these initial embryonic cell clusters (Fig. 1C). These regions were

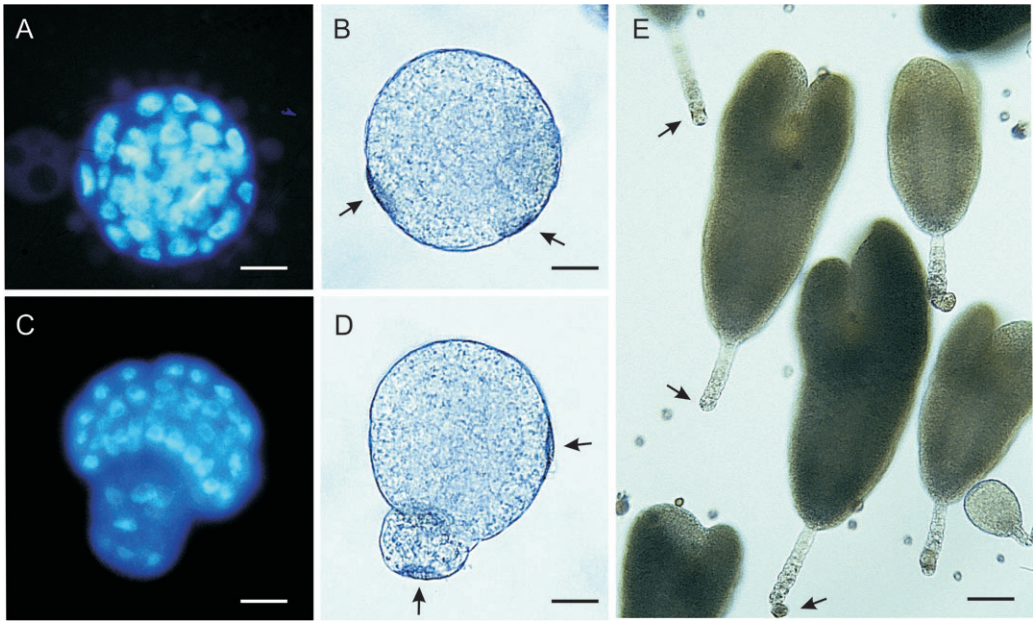


Fig. 1. Embryo structures observed in *Brassica napus* microspore embryo cultures kept continuously at 32 °C (A–D), or cultured for 24 h at 32 °C followed by transfer to 25 °C (E). (A, B) An embryonic cell cluster after 5 d of culture, giving rise to a globular embryo at day 7 of culture (arrows indicate remnants of the microspore exine wall). (C, D) An embryonic cell cluster with two distinct regions, the larger gives rise to the embryo proper while the smaller region with fewer cells forms a rudimentary suspensor-like structure (pictures taken at the same culture time-points as (A) and (B). (E) Heart-shape embryos with long, suspensor-like filaments and remnants of the microspore walls attached to the filament tips (arrows), after 16 d of culture. Bars=10 μm for (A) and (C), 20 μm for (B) and (D), and 50 μm for (E). (A) and (C) are epifluorescence microscopy images of DAPI-stained material, (B) and (D) are Nomarski optics images, and the image in (E) was taken using an inverted microscope.

described earlier by Ilić-Grubor *et al*. (1998*a*), who observed that the larger region gives rise to the embryo proper, whereas the region containing fewer cells forms a rudimentary suspensor-like structure, visualized as an irregular protuberance at the future radicle pole of the embryo (Fig. 1D). The occurrence of embryos with long, suspensor-like structures in *B. napus* microspore embryo cultures has been reported sporadically in the literature (Pechan *et al*., 1991; Hause *et al*., 1994; Ilić-Grubor *et al*., 1998*a*; Straatman *et al*., 2000). Recently, it was shown that formation of long, uniseriate suspensor-like filaments in these cultures can be attributed to the application of a shorter or milder heat-stress treatment than is usually applied (Joosen *et al*., 2007). High yields of microspore-derived embryos with long, suspensor-like filaments can reproducibly be obtained by applying only 12–24 h of 32 °C heat stress followed by transfer to 25 °C (Figs 1E, 2). Remarkably, these long filament-bearing microspore-derived embryos no longer initially develop by random cell division but rather follow the highly regular cell division pattern typical for *B. napus* zygotic embryos (Joosen *et al*., 2007).

Cell tracking reveals the similarity between microspore-derived embryo filaments and zygotic suspensors

Cell tracking and time-lapse photography of individual *B. napus* microspores was carried out to follow the origin and development of embryos with long, suspensor-like filaments (Fig. 3). Cell tracking demonstrated that these embryos originate by the formation of a filament, followed by embryo proper development from the distal cell (opposite to the remnant microspore) of the filament. The filamentous structures emerged from microspores at days 6 or 7 of culture (Fig. 3A), and developed as single files

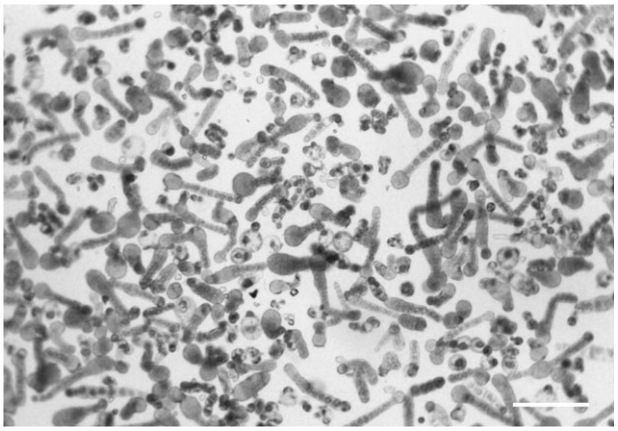


Fig. 2. A population of *Brassica napus* microspore-derived embryos with suspensor-like filaments obtained from a culture enriched by sieving after 10 d of culture. The microspores were treated at 32 °C for 24 h and then transferred to 25 °C. Under this condition, the yield of embryos with long, suspensor-like filaments reached 1000–2000 ml^{-1}. Bar=125 μm.

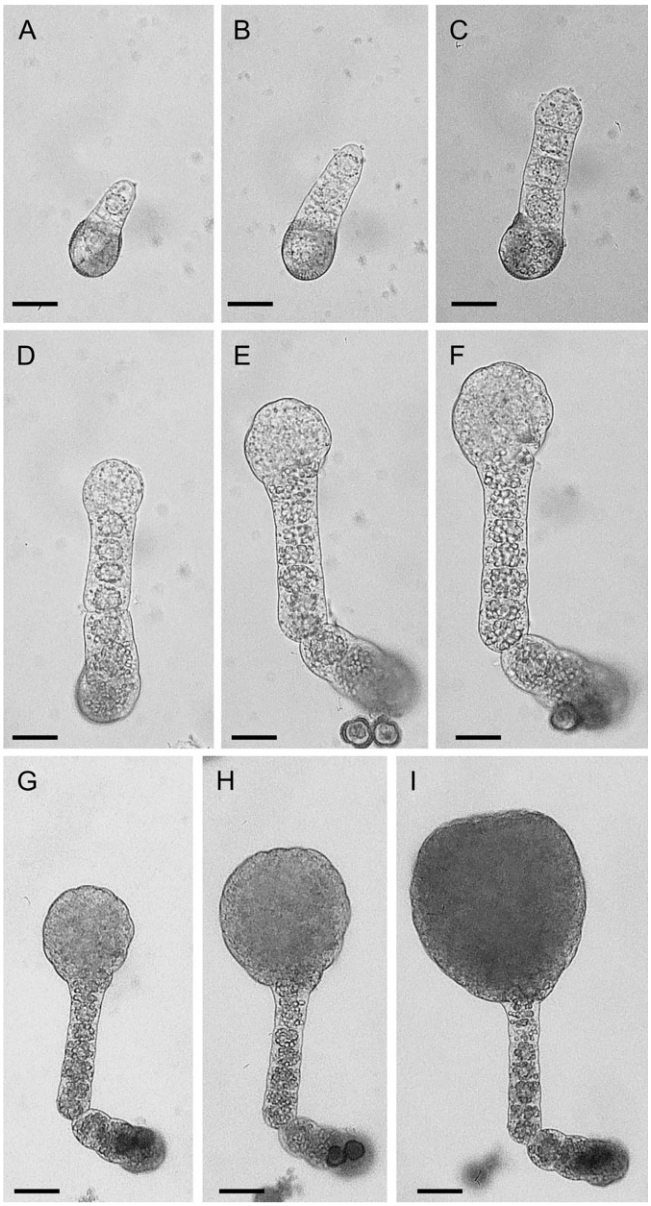


Fig. 3. Representative time-lapse photographs showing the development of an embryo with a long, suspensor-like filament in *Brassica napus* microspore embryo culture. (A) Filamentous structure emerging from a microspore. (B, C) Growth of the filamentous structure through transverse divisions. (D) Swelling of the distal cell opposite to the microspore. (E–I) Globular embryo developing into an early heart-shaped embryo. Embryo stages (D) and (E) were accompanied by cell division in the suspensor-like structure, while cell division in this structure ceased from the (F) stage onwards. Photographs were taken at 1 d intervals from day 6 to day 14 of culture, during which period the culture remained on the inverted microscope stage. Cultures were continuously in NLN-13 medium. Bars=30 μm for (A–F), and 40 μm for (G–I).

of cells, elongating by transverse divisions, with one end connected to the microspore (Fig. 3B, C). Most of the filaments comprised 3–8 cells at day 8 of culture (Fig. 3C). Thereafter, the distal cell swelled (Fig. 3D) and produced a globular embryo (Fig. 3E), while the number of cells in the filament continued to increase. The globular embryos developed into early heart-shape embryos (Fig. 3F–I). Cell division in the filament generally ceased when the embryo reached the late globular stage. These observations reveal that the series of developmental events leading to the formation of microspore-derived embryos with long filaments is highly similar to those that take place during the development of the *B. napus* zygotic embryo proper and its suspensor *in planta* as described earlier by Tykarska (1976, 1979). This suggests that the filament attached to the microspore-derived embryos is comparable with the zygotic suspensor.

The microspore-derived embryos with long, suspensor-like filaments matured in a similar way as conventional *B. napus* microspore-derived embryos without a filament, and normal plantlets were obtained upon germination. Improved germination was obtained when, after 5 d of culture, the initial NLN medium with 13% sucrose was replaced for NLN medium with 1% sucrose and 22% PEG. It was also found that PEG-containing medium led to a decrease in the amount and timing of the onset of starch accumulation in the embryo and filament. As a result, both these structures became more translucent, allowing more detailed examination of early cell division and pattern formation in the embryo (compare images in Fig. 3 and Fig. 6A, B).

The early cell division pattern in microspore-derived embryos with long, suspensor-like filaments mimics that of zygotic embryos

In our new system of *B. napus* microspore embryogenesis, the distal cell of the filamentous structures develops into the embryo proper. To study the pattern of cell division that takes place during early embryo formation, samples were taken from cultures at regular intervals and observed with 4′,6-diamidino-2-phenylindole (DAPI) staining to visualize the nuclei. As shown in Fig. 4, the filaments consisted of a single row of cells, formed through transverse divisions (Fig. 4A, B). At the 3–8 cell stage, the distal cell of the filament underwent a longitudinal division (Fig. 4C), immediately followed by a second one, giving rise to a 4-cell embryo proper. This change in division plane is considered a landmark in zygotic embryogenesis of all crucifer species (Tykarska, 1976). The embryo proper then divided transversely, producing an upper and a lower tier of 4 cells (Fig. 4D). Thereafter, another important division occurred, in which all 8 cells in the embryo proper underwent a periclinal division, to produce 8 protodermal cells and 8 inner cells (Fig. 4E). The cell just under the distal tip cell of the original filament developed into a lens-shaped, hypophysis-like cell (Fig. 4F, G). This cell divided once more and the upper hypophysal daughter cell was incorporated in the radicle zone of the embryo proper (Fig. 4H) and thereafter divided longitudinally (not shown). Continued cell

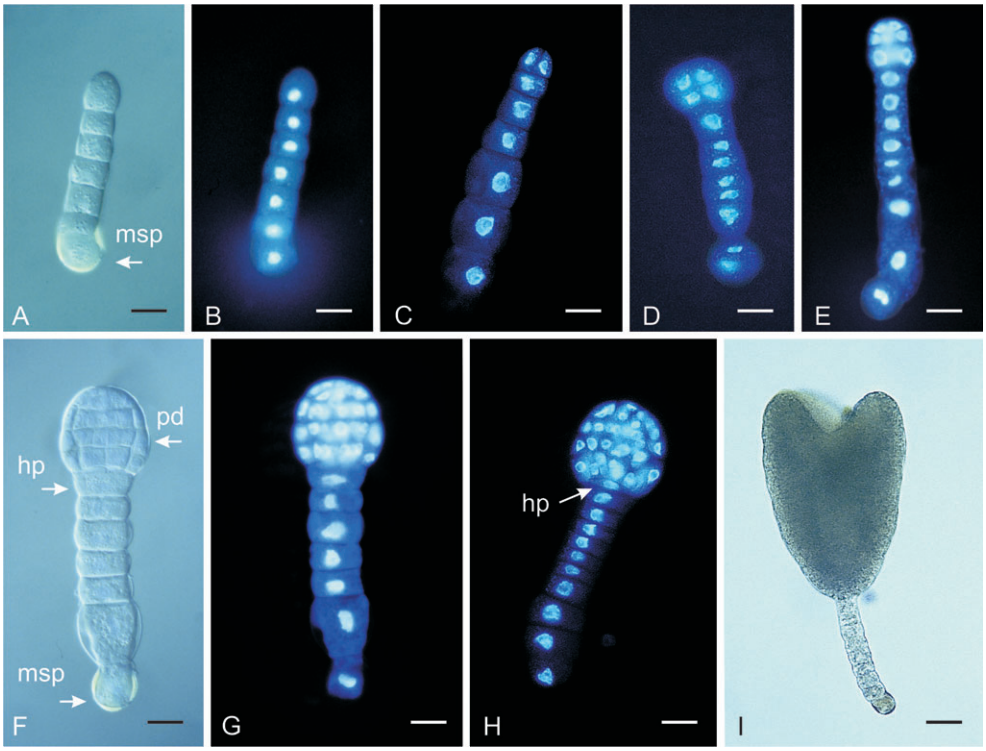


Fig. 4. Initial cell patterning during the development of embryos with a long suspensor-like filament in *Brassica napus* microspore embryo culture, observed with Nomarski optics (A, F, I), or examined with an epifluorescence microscope after staining with DAPI (B–E, G, H). (A, B) Initial filamentous structure emerging from a microspore (msp). (C) First longitudinal division in the distal cell opposite to the microspore, leading to a 2-cell embryo proper. (D) Octant stage proembryo (another four nuclei are present behind the four nuclei in focus). (E) Periclinal cell divisions in the upper part of the embryo proper, forming the protoderm. (F, G) Early globular stage embryo with protoderm (pd) and hypophysal cell (hp). (H) Globular embryo with an upper hypophysal daughter cell (hp) incorporated in the radicle zone of the embryo proper. (I) Late heart-shape embryo with a suspensor-like structure attached. Photographs were taken from individuals after 7 (A, B), 8 (C), 9 (D), 10 (E–G), 11 (H), and 15 d (I) of culture. Bars=30 μm for (A–G), 35 μm for (H), and 55 μm for (I).

division in the embryo proper led to the formation of globular and heart-shape embryos with a normal-looking suspensor (Fig. 4I). These observations indicate that microspore-derived embryos with long, suspensor-like filaments undergo the same early cell division pattern as *B. napus* zygotic embryos developing in seeds. This highly ordered and regular pattern of cell division stands in stark contrast to the initial random cell divisions observed in microspore-derived embryos formed in conventional microspore embryo cultures that lack or produce abnormal suspensor-like structures.

The suspensor-like filament specifies the embryonic identity of the distal cell of the filament, and later directs the apical–basal polarity in the embryo

Long, filamentous structures were transferred from liquid cultures to solid medium to facilitate observation of the developmental fate of the embryo proper at the tip of the cell files. The majority of long filaments began to bend soon after they were transferred to solid media (Fig. 5A, B). This bending appeared to be caused by the physical constraint imposed by the medium on the cell file as it continued to elongate on the solid surface. Embryo development was initiated at several places along the bent filaments, leading to the development of a string of embryos (Fig. 5C, D). This suggests that subfiles were formed due to breaks or kinks in the original cell file, each with the ability to specify embryonic identity in its newly formed distal cell.

Occasionally, development of an embryo proper in the middle of filaments in the original liquid cultures was observed, and the question arose as to where the apical meristem would develop in such embryos. Therefore, the position of the filament relative to the embryo proper in late-heart and torpedo stage embryos (after 15–18 d of culture) was studied. In the majority of embryos (93%) a single suspensor-like filament was attached to the radicle pole of the embryo (Table 1). The opposite situation, in which the suspensor-like filament was attached to the apical pole of an embryo, was never observed. In approximately 4% of the microspore-derived embryo population, the embryo proper initiated from the middle of a relatively long filamentous structure, such that the embryo initial contained two filament arms (Table 1; Fig. 6A, B). Approximately half of these embryos continued to grow and were able to produce heart- and torpedo-stage

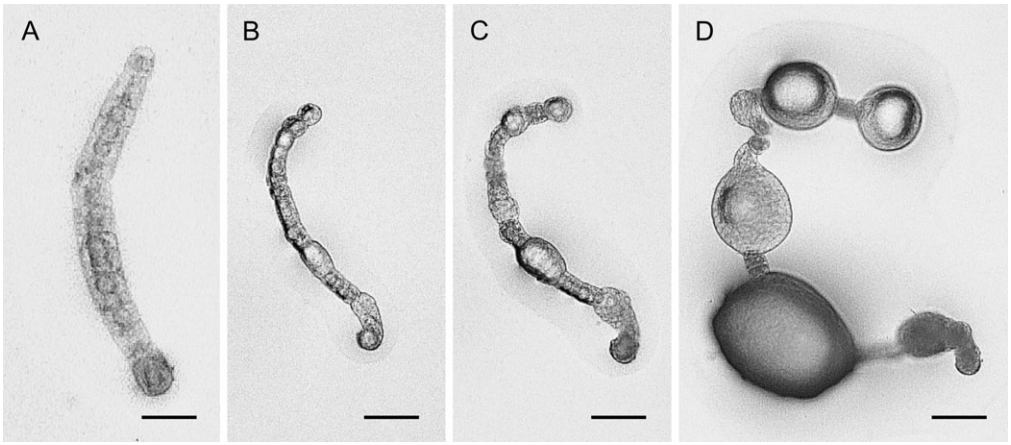


Fig. 5. Developmental fate of a long, filamentous structure from a *Brassica napus* microspore embryo culture after subculture on solid medium. (A) Cell file with approximately 15 cells from a 10-d-old culture, just upon subculture. (B–D) Disappearance of the smooth cell surface and formation of several nodules along the cell file, each of which formed an individual embryo. Five cotyledon-stage embryos were eventually obtained from the suspensor-like structure shown. Photographs were taken at 4 (B), 7 (C), and 10 d (D) after subculture on solid medium. Bars=40 μm for (A), and 100 μm for (B–D).

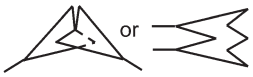
embryos (Table 1). Normal embryos with a single basal suspensor-like filament developed from the two-celled stage to the late heart-shape stage within 5–6 d (Fig. 4), whereas embryos that developed from the middle of a filamentous structure took 2–3 weeks to reach this stage (see legend to Fig. 6). In addition, embryos that developed from the middle of the filament initially followed an abnormal cell division pattern such that one or even two putative apical zones were formed approximately perpendicular to the direction growth of the two filament arms (Fig. 6C, D). Eventually, apical meristems were formed opposite to the point of attachment of the filament arms. Single embryos were formed that have one filament arm connected to the radicle pole and a second arm attached to the side of the embryo (Fig. 6E), or twin embryos were formed with a filament connected to each of the two radicle poles (Fig. 6F, G). Strikingly, in embryos that possess multiple suspensor-like filaments, the filaments never developed at the eventual shoot apex, even when two filament arms were attached to two opposing sites on the initial embryo. Our finding that the embryo apical dome never forms at the point of filament attachment suggests a role for the suspensor-like filament in defining the apical–basal axis of the embryo proper.

Basal-to-apical auxin transport in the young suspensor-like filaments

In *Arabidopsis* polar transport of the plant hormone auxin plays a crucial role in the specification of the apical–basal axis of the zygotic embryo (Friml *et al*., 2003*b*; Weijers *et al*., 2006; Tanaka *et al*., 2006). The direction of auxin flow in the embryo is correlated with asymmetric localization of the PIN auxin efflux carriers (Friml *et al*., 2003*b*; Petrasek *et al*., 2006; Wisniewska *et al*., 2006). Cellular auxin transport is initiated from the suspensor cells toward the embryo proper cell around the time that the zygote has divided to form an apical and a basal cell, and the basal cell itself has divided to produce 2–3

Table 1. *Frequencies of embryo–filament combinations observed in a* Brassica napus *microspore embryo culture pretreated at 32 °C for 24 h and then transferred to 25 °C*

Classification was based on the position in the suspensor-like cell files where embryos were initiated (in the proembryo stage), and later on the position of the suspensor-like filament (or filament arms) relative to the apical and radical poles of the embryos (in the heart-/torpedo-stage). The group indicated with an asterisk (*) ceases development and an embryo will not successfully differentiate as long as the two filament arms remain opposite to another. More complex embryo-filament combinations, occurring at a frequency of ⩽1%, are not presented.

Various embryo-filament combinations		Frequency (%)
Proembryo stage	Heart- / torpedo-stage	
		93.3
		0.9
		0.9
		2.1*
		1.7
		0.2

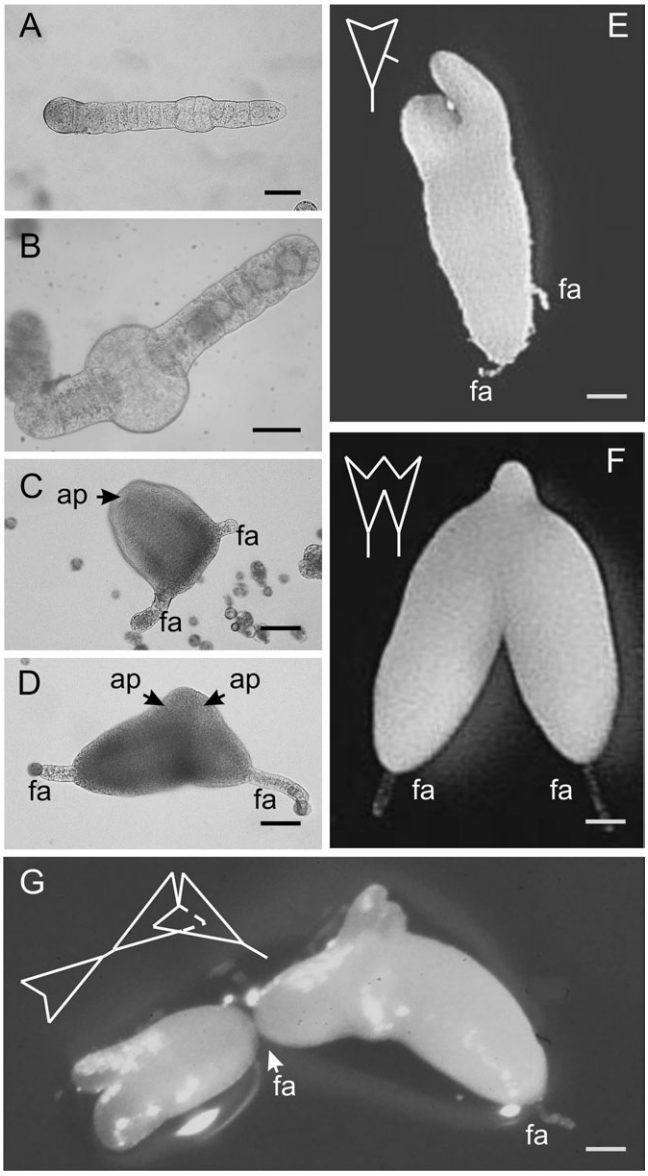


Fig. 6. Developmental fate of embryo initials formed in the middle of long, filamentous structures in *Brassica napus* microspore embryo culture. (A) Embryo initial in the middle of a cell file. (B) Globular embryogenic structure in between two filament arms. (C) Apical pole (ap) with potential embryo proper formation perpendicular to the direction growth of the two filament arms (fa). (D) Embryonic structure with two putative apical poles (ap) perpendicular to the direction growth of the two filament arms (fa). (E) Embryo with one filament arm (fa) attached to the radicle pole and another to the hypocotyl area. The apical pole with cotyledons is opposite to the radicle pole. (F) A twin embryo with two filament arms (fa) connected to the radicle poles, and potential shoot meristem development at opposite apical poles. (G) Normal embryo (at the left) and a twin (at the right) developed from one filamentous structure. The root poles are connected to the filament arms (fa) and cotyledons are formed at the opposite apical poles. Insets in (E), (F), and (G) are schematic diagrams from Table 1, showing the assumed apical–basal orientation of the embryos. Photographs were taken after 9 (A), 11 (B), 18 (C, D), and 28 d (E, F, G) of culture. Images (A) and (B) are from cultures wherein the initial NLN-13 medium containing 13% sucrose was changed for NLN medium with 1% sucrose and 22% polyethylene glycol (PEG 4000) after 5 d of culture. Bars=40 μm for (A), 60 μm for (B), 90 μm for (C, D), and 110 μm for (E, F, G).

suspensor cells. This transport is mediated by apical localization of the PIN7 auxin efflux carrier in the suspensor cells. The transport of auxin upward from the suspensor generates an auxin maximum in zygote apical daughter cell that is thought to specify the identity of the early embryo proper. A reversal of the auxin gradient and maximum is observed from the 32-celled stage of embryo development onward, and correlates with the basal cellular localization of the PIN1, PIN4, and PIN7 auxin efflux carriers (Friml *et al*., 2003*b*). It was investigated whether a similar basal-to-apical auxin flow takes place in the suspensor-like cell files in our *B. napus* microspore embryo cultures by examining the localization of antibodies targeted against *Arabidopsis* PIN1, PIN4, and PIN7 proteins. Only limited information is available on PIN proteins in *Brassica* species (Ni *et al*., 2006), thus our results can only be used to infer the direction of auxin flow, rather than the contribution of individual PIN proteins to this gradient. Only the *Arabidopsis* PIN1 and PIN4 antibodies detected *Brassica* PIN proteins, and both of these antibodies were concentrated between the cells of the filamentous structures (Fig. 7A, C), indicating vertical auxin transport between those cells. It was often difficult to distinguish whether the PIN signal was localized to the upper or lower cell membranes of adjacent cells. Closer observation regularly revealed that the PIN signal was localized to a downward facing shoulder of a cell (Fig. 7B), indicating apical localization of PIN proteins. At places where the membranes of the upper and lower cell could be distinguished, more PIN antibody was localized to the apical surface of the lower cell than to the basal surface of the upper one (Fig. 7B, D). Together these observations suggest that, as in zygotic embryos, the direction of auxin flow is toward the distal cell of the filament. Notably, apically-localized PIN1 and PIN4 was also observed in the distal most cells of the uniseriate cell files (Fig. 7A, C).

Discussion

Microspore embryogenesis provides an efficient way to produce a large quantity of developmentally synchronized embryos, and is therefore frequently used as a model system to study embryogenesis in plants (Touraev *et al*., 1996). *B. napus* microspore embryogenesis is considered to be one of the best models for this purpose due to the speed and efficiency with which embryos develop (Telmer *et al*., 1993; Custers *et al*., 1994; Yeung, 2002). However, as in other non-zygotic embryo culture systems, differences in the development of *B. napus* microspore-derived embryos and zygotic embryos are routinely observed (Mordhorst *et al*., 1997; Thorpe and Stasolla, 2001), in particular with regard to the regularity of the initial cell divisions. The initial cell divisions in *B. napus* zygotic embryos are extremely regular (Tykarska, 1976), whereas

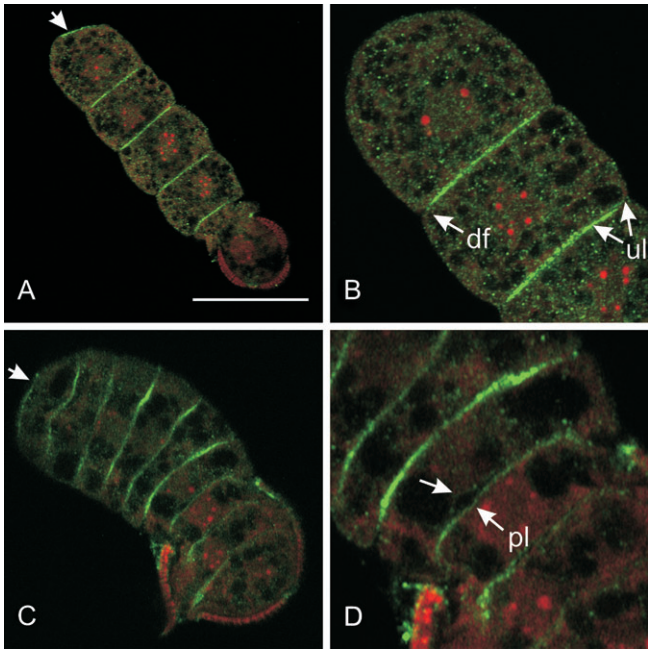


Fig. 7. Immunolocalization of PIN proteins in suspensor-like filaments developed from *Brassica napus* microspores in culture, using anti-AtPIN1 (A, B) and anti-AtPIN4 (C, D) antibodies. PIN protein signals are in green and propidium iodide DNA counter stain is in red, while microspore exine walls show red autofluorescence. (A, C) Surveys of whole filaments, showing that most PIN protein signal is localized between adjacent cells. (B, D) Specific regions in different optical sections of (A) and (C), respectively, showing that the PIN signal is mainly localized to the apical cell surface of cells in question; downward facing shoulder of PIN signal, separated from the cell above it (df in B), separately visible upper and lower membranes of two connected cells (ul in B), and separation of the two membranes due to plasmolysis (pl in D). Arrow heads in (A) and (C) point to apically-localized PIN protein in the distal most cells of the filaments. Bar=50 μm.

microspore-derived embryos initially develop through a series of unorganized cell divisions that only become ordered upon establishment of the protoderm. A second difference between microspore-derived embryos and zygotic embryos is the partial or complete absence of a suspensor. In a previous study, suitable culture conditions were established for the formation of long, suspensor-like filaments attached to *B. napus* microspore-derived embryos that mimic the development of zygotic embryos in seeds (Joosen *et al*., 2007). Using this system, it was observed that the regularity of the initial cell divisions in the long filament-bearing microspore-derived embryos and their subsequent early patterning are more similar to that of zygotic seed embryos than to that of conventional (filament-less) microspore-derived embryos. Extrapolating these data to seed embryos suggests a new role for the zygotic suspensor in controlling ordered cell division and patterning during early plant embryogenesis. Previous key functions attributed to the suspensor are (i) the positioning of the embryo in close proximity to sources of nutrition in the embryo sac, (ii) the absorption and transport of nutrients to the embryo, and (iii) the synthesis of hormones to support embryo growth (Raghavan, 2001). The importance of the suspensor in driving early embryo pattern formation has recently been postulated in several invasive studies with *Arabidopsis* embryos (Friml *et al*., 2003*b*; Weijers *et al*., 2005, 2006; Tanaka *et al*., 2006), and has now been corroborated by our cytological studies on microspore-derived embryos. However, our data do not provide definitive proof that suspensor formation alone is fully responsible for driving the early ordered cell divisions in the embryo proper. Firstly, although the morphological similarity between the suspensor-like filament and the zygotic suspensor is striking, the identity of cells in the microspore-derived filament needs to be confirmed. Secondly, an additional role for the embryo proper in directing tissue patterning cannot be excluded. Reporter gene studies and laser ablation in *B. napus* microspore embryo cultures will provide the necessary insight into the sequence of events and factors controlling suspensor-driven embryo patterning.

Suspensor-like structures attached to microspore-derived embryos were occasionally reported in *B. napus* microspore embryo cultures, but their origin was interpreted differently from that interpreted here. Ilić-Grubor *et al*. (1998*a*), who related suspensor-like structure formation to an established polarity in the original microspores, mainly described these structures as cellular protrusions attached to the radicle pole of globular to heart-shape embryos. They proposed that the cells from which the suspensor-like structures emerged are initially quiescent during microspore-derived embryo formation. Straatman *et al*. (2000) interpreted the formation of microspore-derived embryos with suspensor-like structures as a deviant form of the normal pathway of microspore embryogenesis. They suggested that the aberrant development of suspensor-like structures is due to an early rupture of the microspore exine wall. In our new system, it was clearly observed that suspensor-like filament formation is an autonomous process, in which a linear file of cells initially emerges from the embryogenic microspores, followed by development of the embryo proper from the distal tip cell.

This new pathway of microspore embryogenesis is strikingly similar to the zygotic embryogenesis pathway with *B. napus* in that a short suspensor-like filament is formed prior to the first division of the embryo proper cell, followed by development of the embryo proper from the apical cell (Tykarska, 1976). Such microspore-derived embryos subsequently follow the same ordered pattern of cell division as the *B. napus* zygotic embryos. The similarity of the newly established microspore embryogenesis system with zygotic embryogenesis is particularly evident when the microspore-derived embryos bear suspensor-like filaments with a similar number of cells as in zygotic embryos (3–5 cells at the first division of the embryo proper). Microspore-derived embryos with even longer filaments (6–12 cells or more at the first division of

the embryo proper) were also observed. The signal to initiate formation of the embryo proper is clearly delayed in these embryos. The extra long filament formation might be due to a weak embryo-inducing signal in the initial cell of the embryo proper. Alternatively, the suspensor-like filament may have a function in determining embryonic identity in the distal cell of the filamentous structure, and when the suspensor-like filament fails in this, it causes the continuation of its own elongation. With respect to this, Friml *et al*. (2003*b*) reported that, in *Arabidopsis*, zygotic embryos polarity is established from the two-celled stage of embryogenesis onward. This polarity is thought to be built up via an auxin gradient, in which auxin is transported by the PIN7 efflux carrier from the suspensor to the embryo proper. As proposed by the authors, this basal-to-apical auxin gradient specifies the identity of the apical embryo proper. Following this hypothesis, and based on our observation of preferentially apically-localized PIN proteins in the suspensor-like cells of microspore-derived embryos, it is presumed that a similar auxin gradient is built up in these suspensor-like cell files, and that this auxin gradient contributes to the embryonic identity specification of the distal cells in the files. Interestingly, *pin7* mutants sometimes fail to establish the embryo proper leading to formation of long filamentous structures resembling the suspensor (Friml *et al*., 2003*b*). Accordingly, it is concluded that our results show for the first time the functional significance of the suspensor in plant embryogenesis, because microspore-derived embryos with suspensor-like filaments exhibit the initial ordered cell divisions and histodifferentiation seen in zygotic embryos, whereas filament-less microspore-derived embryos lack this early regularity and initially develop through a series of unorganized cell divisions.

In addition to microspore-derived embryos with suspensor-like filaments attached to the radicle pole, a low frequency of additional embryo-filament relationships, specifically embryo initiation from the middle of a filament, the formation of twin embryos, and the development of multiple embryos along a long filament was also observed. Our experiments showed that the suspensor-like filaments have embryogenic potential and can be induced to form embryos. These observations are in a broad sense comparable to observations on the *twin* (*twn*) and *twn2* mutants of *Arabidopsis*, where it was shown that growth defects in the embryo proper facilitated adventitious embryo formation from the suspensor cells (Vernon and Meinke, 1994; Zhang and Somerville, 1997). The common interpretation of these mutant phenotypes is that the embryonic potential of the suspensor cells is always subordinate to the embryo proper, and that the suspensor cells can only express this potential after release from inhibition by the embryo proper (Meinke, 1995; Schrick and Laux, 2001). Our new microspore embryogenesis system provides additional information, which is not observed with the *twn* and *twn2* mutants. In the case of embryo initiation from the middle of a suspensor-like cell file, it was found that the entire embryogenic process took much longer than when an embryo with one suspensor-like filament was formed. It appears as if the two filament arms compete with each other in directing the progressive development of the embryo initial. The final result of this competition is that either a single embryo or a twin embryo was formed, with the filaments arms connected to the radicle poles or sides of the embryo, but never to the apical poles. Taken together, our observations and observations on early embryo development in *Arabidopsis*, suggest an active role for the suspensor in guiding apical–basal pattern formation during the early stages of embryo proper formation (Friml *et al*., 2003*b*; Tanaka *et al*., 2006).

The most interesting aberrant form of embryo formation in our cultures was seen when long suspensor-like cell files, after subculture on solid medium, gave rise to the formation of several embryos. This ‘embryos on a string’ phenotype may be caused by the occurrence of physiological breaks along the suspensor-like cell file, and thereafter each subfile produces its own embryo. Our experiments provide the first experimental proof under *in vitro* conditions for the concept first proposed by Vernon and Meinke (1994) that the zygotic apical cell actively inhibits embryo formation from the basal cell lineage during embryogenesis. In our system, a kink in the suspensor-like filament may release the cell file from the inhibitory effect of its apical cell allowing underlying cells to become embryogenic. Alternatively, it is also plausible that the filament itself specifies the embryonic identity of the cell at its distal tip, through an auxin gradient built up in the short cell file. The latter hypothesis fits well with the basal-to-apical auxin gradient model proposed by Friml *et al*. (2003*b*).

To conclude, a refined *B. napus* microspore embryo culture system is presented, in which embryo development resembles zygotic embryogenesis. This is the first *in vitro* system that can efficiently produce high frequencies of morphologically normal embryos from single differentiated cells in culture. This unique system enables plant embryologists to manipulate and study early embryogenesis under non-invasive conditions.

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