Journal of Plant Biology, September 2002, 45(3) : 119-133 **REVIEW**

The Canola Microspore-Derived Embryo as a Model System to Study Developmental Processes in Plants

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The ability of microspores to undergo embryo development after a successful induction treatment provides a unique experimental system to study a variety of developmental processes in plants. Recent published results focus on the cellular and molecular aspects of the early induction process. In this review, besides summarizing the current findings, the advantages of using the MDE system to study other aspects of embryo development are emphasized. The continual improvement of culturing procedures, media components, and molecular methods guarantees exciting new findings in the near future.

Keywords: *Brassica*, embryo development, microspore-derived embryos

The reports by Guha and Maheshwari in 1964 and 1966 demonstrated that microspores of a flowering plant, *Datura*, are capable of developing into haploid plants. This discovery clearly illustrates the added developmental potential of microspores as they can switch from gametophytic to sporophytic development under appropriate conditions. As a result, this observation has enabled further development of haploid plant technology. Since the original reports by Guha and Maheshwari, successes have been reported for different species (see Jain et al., 1996-1997). A majority of studies have been focused on tobacco, *Brassica* species, and different varieties of barley, rice and wheat. This is primarily due to their economic importance or their ease of manipulation. Although a large amount of information is available in the literature, emphasis is usually placed on the pre-culturing conditions, the stress treatments, the media components and the early cellular, biochemical and molecular events related to the induction process. The purpose of this review is to summarize recent findings and to draw attention to the usefulness of the microspore-derived embryo (MDE) system in the study of other processes related to embryo development and maturation. Since not every aspect of MDE development will be discussed, readers are urged to consult reviews by Ferrie et al. (1995), Palmer et al. (1996), Raghaven (1997), Smykal (2000), Custers et al. (2001), Datta (2001), Pechan and Smykal (2001), and Touraev et al. (2001) for additional information. Some comments are speculative and I hope that they might stimulate further research in the future.

In this review, the *Brassica* MDE system will be emphasized. This is because a large amount of information related to *Brassica* zygotic embryo development is available to serve as control studies (e.g. Tykarska, 1976, 1979, 1980, 1982, 1987; Yeung et al., 1996; see additional references in other sections). The methodology of generating MDEs is clearly detailed and a large number of MDEs can be obtained readily (Telmer et al., 1992; Ferrie and Keller, 1995). In addition, negative control cultures are available (Simmonds and Keller, 1999; Custer et al., 2001). Furthermore, the genus *Brassica* belongs to the same family as *Arabidopsis* and, therefore, the molecular genetics information for *Arabidopsis* may also be applicable for the study of the *Brassica* MDEs (Custer et al., 2001). Hence, the *Brassica* MDE system is an excellent model for the study of microspore embryogenesis in plants.

Success in MDE production depends on a number of factors (for a more detailed discussion, see Smykal, 2000; Datta, 2001). It is well established that different genotypes of the same species can respond differently to experimental treatments. The identification of appropriate stages of microspore development for an optimal response is essential for perceiving the necessary treatment. Pretreatment procedures such as growing plants at a lower day/night temperature serve as priming steps for allowing optimal response to subsequent

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Abbreviations: ABA, abscisic acid; MDE, microspore-derived embryo; PEG, polyethylene glycol; HSP, heat shock protein; PPB, preprophase band; SAM, shoot apical meristem; TIBA, tri-iodobenzoic acid.

treatment procedures. The selection of an induction procedure, such as starvation and/or heat shock, which enables the switching of gametophytic development into embryo development is key to microspore embryogenesis. In general, some form of stress treatment has to be employed that enables the termination of the gametophytic program of development and initiates the embryo development program. The media components are also critical in MDE development. In addition to providing the necessary nutrients for embryo development, media components are important in the maintenance of the proper osmotic environment and some additives could have a morphogenetic function.

A Stress Treatment is Essential for Successful Development of MDEs by Redirecting Developmental Pathways

It is generally agreed that an appropriate stress treatment is essential to the success of MDE development. Because of the sessile nature of plants, they react to external stimuli by developmental changes in order for them to survive the changing environment. This innate genetic property of the plant cell is best illustrated in MDE formation. Under normal conditions, microspores will undergo the process of microgametogenesis leading to the formation of a vegetative and a generative cell. Depending on the species, the generative cell may divide further and give rise to two sperm cells. However, when stresses are imposed at the microspore stage of pollen development, the embryo development program takes precedence over the process of microgametogenesis. The idea of atavism put forward by Bonet et al. (1998) is a plausible one. The stress treatment provides the necessary signal to terminate the gametophytic program and "reset" the cells into a default embryogenic pathway.

Starvation and low/high temperature treatments are the most commonly employed stresses during the induction process for different species (Ferrie and Keller, 1995; Touraev et al., 1996). Other methods such as the chemical treatment by colchicine (Zaki and Dickinson, 1995; Zhao and Simmonds, 1996a, 1996b) and osmotic shock (Wei et al., 1986) are also useful. Targeting a major biochemical or physiological event in pollen development may be the key in designing a treatment for MDE induction. For example, in cereal grains, starch accumulates during late stages of microspore formation. A starvation treatment alters starch biosynthesis and accumulation, and this may provide the signal for the termination of pollen development. Pollen development is sensitive to a variety of stresses (Saini, 1997) including heat stress (Mascarenhas and Crone, 1996). A heat shock treatment serves to terminate the gametophytic program and these microspores are "rescued" by culturing them in an appropriate nutrient medium that enables the microspores to express their innate potential of embryo development. Microtubules have multiple functions within cells. Hence, disruption of this important organelle using microtubule inhibitors at a specific cell cycle event will result in disrupting normal pollen development. This can also serve as a stress signal for MDE induction.

Protein and RNA Synthesis during the Induction Phase of MDE Formation

The induction phase of MDE formation is the main focus of today's research. Understanding this process is key to the success of microspore embryogenesis and to the understanding of cell development. The stress induction treatment serves a dual function. Besides terminating the gametophytic program, it also induces the synthesis of macromolecules that are essential to the initiation of the embryogenic pathway and early stages of embryo development. In *Brassica* species, 8 hours of heat shock is sufficient for the induction process (Pechan et al., 1991). Thus, in order to study events related to the induction process, changes that occur during the first 8 h should be considered to be key induction events. During the early induction period, qualitative and quantitative differences have been found in proteins and RNA biosynthesis. A recent review by Custers et al. (2001) clearly summarizes the changes in protein synthesis and early embryogenesis-associated genes during early microspore embryogenesis.

Protein Synthesis

One of the most notable findings in protein synthesis is the detection of heat shock proteins (HSPs), especially the HSP20 and 70 families. These HSPs appear to be essential to the induction process (Smykal and Pechan, 2000; Custer et al., 2001; Pechan and Symkal, 2001). Through the use of immunostaining procedures, changes in the cellular localization of HSP70 were found where the nucleus of the vegetative cell showed intense staining after 8 h at 32°C. This pattern of staining was not detected in cultures incubated at 18°C (Cordewener el al., 1995). Similarly, the appearance of the smaller HSP transcripts correlated well with embryo induction (Smykal and Pechan, 2000). A mutant form of *Brassica*

napus cv. Topas which produces substantially fewer MDEs, also has a substantially reduced expression of the small HSPs (Smykal and Pechan, 2000). It is interesting to note that colchicine treatment also induces the appearance of small HSP transcripts albeit at a lower level (Smykal and Pechan, 2000).

Although HSPs have been identified and appear to be a consistent feature observed during the induction phase of MDE development, the importance of these proteins in the induction process is still unclear. During normal pollen development, certain HSP genes or heat shock cognate genes are activated in the absence of heat stress (Mascarenhas and Crone, 1996). Thus, the ability to synthesize HSPs in response to the heat shock treatment is an inherent property of developing pollen grains. The fact that HSP genes are present during normal development in the absence of heat stress indicates that these genes have a developmental function during the course of pollen development (Mascarenhas and Crone, 1996). Pechan and Smykal (2001) suggest that HSPs function to prevent the formation of "phenocopies" and may have chaperone-like activity (Smykal et al., 2000). Sung et al. (2001) suggest that HSP70 could function to "mitigate aggregation of stress-denatured proteins and to refold non-native proteins restoring their biological function". Irrespective of HSP function in MDE induction, HSPs are certainly useful markers for *Brassica* microspore embryogenesis when heat is used as the stress signal. The temperature differential between donor plant growth appears to be a means to maximize synthesis of the heat shock proteins.

Changes in protein synthesis and phosphorylation during the early stages of microspore induction and embryogenesis in *B. napus* were noted (Cordewener et al., 1994, 1995, 2000; Hause et al., 1995). One of the HSP70 isoforms becomes highly phosphorylated and may serve to regulate HSP functions (Cordewener et al., 2000). Recently, the potential function of the phosphorylation of smaller molecular weight HSPs has been discussed (Gaestel, 2002). Other phospho-proteins have been identified which can also play a role in the dedifferentiation of tobacco pollen grains cultured in vitro (Kyo et al., 2000). In tobacco, the phospho-protein shows moderate homology with copper-binding glycoproteins. At present, the significance of protein phosphorylation is still unclear and awaits further studies. It is also important to note that many cell cycle regulation proteins are activated by phosphorylation. Additional information on the phosphorylation event during induction and early embryo development is essential to our understanding of MDE development.

Another interesting observation regarding protein synthesis is the observation that newly synthesized proteins appear in the medium of a 32°C culture within 2 days of culture initiation. These proteins are absent from the non-induced cultures (Cordewener et al., 2000). Extracellular proteins have been found to play a role in plant somatic embryogenesis and they could serve as signaling molecules in embryo development (Vroemen et al., 1999; Souter and Lindsey, 2001). Further characterization of the extracellular proteins and their synthesis will provide important insights into the early induction process.

In relation to the signal transduction pathway, G-protein-like sequences have been identified in *B. napus* microspores (Chan and Pauls, 2001; personal communication). G-proteins have been shown to play a pivotal role in signaling pathways (Ma, 2001). The Rho GTPase may also have effects on cytoskeletal elements (Hollenbeck, 2001). Since changes in cytoskeletal elements have been noted during the early inductive phase, G-proteins may help in bringing about the observed switch in the developmental pathway of microspores by influencing microtubule organization and function.

Besides HSPs, additional proteins have been found during the induction phase and early embryo development (Yoon et al., 1993; Cordewener et al., 2000). Judging from their expression pattern, some of these proteins are essential to embryo development and histodifferentiation (Cordewener et al., 2000).

RNA Biosynthesis

With continual improvements in gene isolation and amplification techniques, e.g. the differential-display reverse-transcriptase polymerase chain reaction (DD-PCR), it is now possible to identify cDNAs that are unique to microspore embryogenesis (see Custers et al., 2001 for details). In *B. napus*, a number of DD-PCR clones have been isolated and sequenced. Based on sequence information, some of the genes have tentatively been identified. Among some of the genes identified were the receptor-like kinases similar to *CLAVATA2* and the transcription factors similar to *SCARECROW* and *AGAMOUS*-like15. In other species, additional genes have also been found. In barley, 3 different clones appear to be specific to early stages of development (Vrinten et al., 1999). In wheat, an early-labeled metallothionein gene has been used as a marker for pollen embryogenesis (Reynolds, 2000). Calcium ions and abscisic acid influence the expression of this gene. Starvation treatment during tobacco pollen embryogenesis stimulates new mRNA production and changes in protein kinases were also detected (Garrido et al., 1993). The

latter observation indicates that protein phosphorylation is important to MDE formation.

The cell sorting methods as reported by Paul's laboratory (Schulz and Pauls, 1998, 2002) enable the sorting of embryogenic cells from non-embryogenic ones. Together with the improvements in the identification of proteins using proteomic techniques and the more sensitive methods in gene amplification and sequencing, the structure and function of other minor proteins and the less abundant genes can now be determined. They may indeed provide new insights into the inductive process.

Structural Events Associated with the Induction Process

Although there are a number of reports dealing with cytological and ultrastructural changes during the induction phase of MDE formation, the fact that only a small percentage of microspores continue to develop into MDEs does not allow one to draw definitive relationships between structural changes and the induction process. Thus, it is no surprise that controversies exist in this area of research regarding the early stages of pollen embryo formation. A detailed discussion of the ultrastructural changes that occur during MDE formation can be found in an earlier review (see Yeung, 1995). In *B. napus*, a number of changes have been observed in association with the high temperature treatment. A loss of cellular polarity in terms of organelle distribution and an increased division symmetry occur after 24 h of treatment. The symmetrical division is preceded by the formation of a preprophase band (PPB) (Hause et al., 1993; Simmonds and Keller, 1999). The formation of the PPB can serve as a marker for sporophytic development as a PPB did not form in heat-treated microspores of a non-embryogenic line (Simmonds and Keller, 1999). Furthermore, the reappearance of the PPB clearly indicates changes to cell cycle regulatory processes upon a heat treatment. Cytoplasmic granules also appear in the cytoplasm and they are most likely heat shock proteins (Telmer et al., 1995). However, the formation of these granules is not an absolute marker of embryogenic development, as some microspores fail to develop into pollen embryos. In addition, some electron dense deposits and vesicle-like structures were present in the newly formed walls of potentially embryogenic cultured microspores but their functional significance remains to be determined.

New techniques of investigation promise to provide additional structural information in relation to the early induction of MDEs. In a recent report, changes in the wheat embryogenic microspores could be studied by a cell tracking procedure (Indrianto et al., 2001). Three types of microspores were identified based on their cytological features. These features ranged from microspores having a large vacuole with a parietal location of the nucleus to cytoplasmic microspores with a centrally located nucleus. Using the cell tracking method, it was determined that these microspores were not distinct classes; instead, these microspores simply represented different stages in the formation of MDEs. The cell sorting procedure as detailed by Schulze and Pauls (1998, 2002) can also provide an added tool to study the enriched population of embryogenic microspores for structural investigations.

The First Mitotic Division during Microspore Embryogenesis

The first mitotic division is considered to signal the completion of the induction event and that the microspore is committed to the embryo development program (Pechan and Smykal, 2001). Using the cell tracking method, it was determined that the first cell division of the wheat embryogenic microspores was always symmetric (Indrianto et al., 2001). For *Brassica* species, the first cell division is also symmetrical (Zaki and Dickinson, 1991; Yeung et al., 1996; Nitta et al., 1997). The majority of vegetative nuclei of the cultured *B. napus* microspores begin to re-enter the cell cycle after 12 h of culture and begin DNA replication (Binarova et al., 1993).

Asymmetric division in plant cells is a unique process as it involves polarization of cytoplasm. The resulting daughter cells tend to adopt different developmental fates. Since embryogenic microspores usually divide in a symmetric manner after a stress treatment, the process of asymmetric division may be sensitive to stress treatments. The symmetric division in plant cells most likely represents the default pathway of cell division. In the case of *B. napus* microspore induction, a change in the symmetry of division can be considered as an "androgenesis-related process" (Pechan and Smykal, 2001). It is important to note that in the tobacco microspore culture, gametophytic development continues even after a symmetrical division (Touraev et al., 1995) indicating that changes in cell division plane alone is not sufficient to cause a switch in developmental pathway. Furthermore, it has been shown that mannitol pretreatment of wheat microspores results in a symmetric division while cold pretreatment results in an asymmetric first nuclear division (Hu and Kasha, 1999). Hence, different stress treatments can elicit different

responses from the microspores during induction.

Cytoskeletal elements are essential to the cell division process. Both microtubules and microfilaments are present during normal pollen development in *B. napus* (Hause et al., 1992, 1993; Gervais et al., 1994). A PPB is not present prior to the first microspore mitosis (Hause et al., 1992) and a detailed pattern of changes was illustrated by Hause et al. (1993) for *B. napus*.

During the induction phase, microtubules exhibit new arrangements (Hause et al., 1993). The repositioning of the microspore nuclei prior to the first division appears to be a result of the "temperature induced disappearance or disturbed synthesis of microtubules" (Hause et al., 1993). Furthermore, a PPB of microtubules is observed just prior to the first division (Simmonds and Keller, 1999). Although the plane of division is determined by a PPB, the position and orientation of the mitotic spindle varied (Telmer et al., 1993, 1995). Actin filaments, on the other hand, appear to play a lesser role during the induction phase. A pulse treatment of cytochalasin B appears to have little influence on MDE formation (Hause et al., 1993). Further studies on factors regulating microtubule dynamics and function within the microspores during induction are necessary. In *B. napus*, it is important to note that a majority of microspores having symmetric divisions do not undergo embryogensis (Simmonds and Keller, 1999) indicating other intrinsic events are essential to the process. Irrespective of the fate of microspores after the first division, in *B. napus*, the symmetric division is a first cytological marker towards MDE formation.

Polarity Establishment

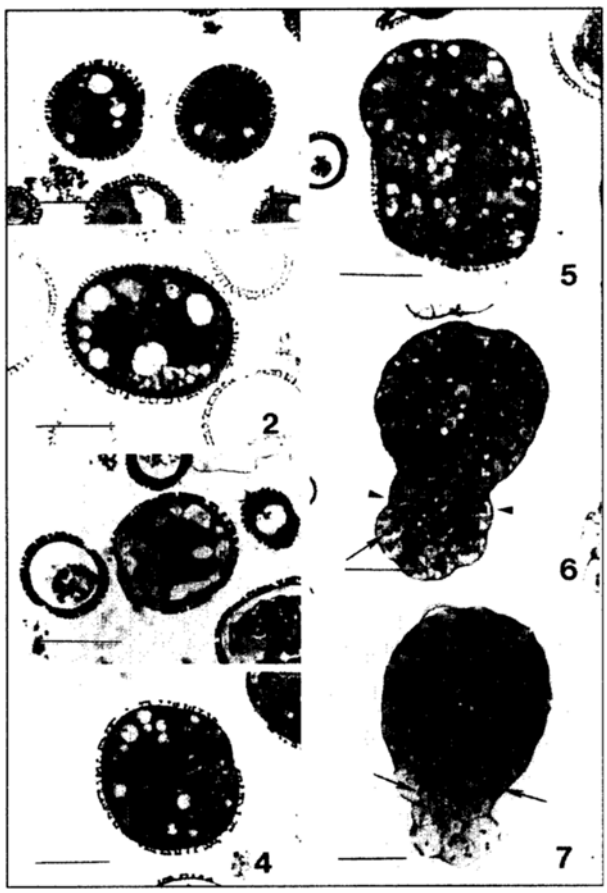
Polarity is central to the development of all living organisms. Cells with different polarities have different fates that allow for subsequent morphogenetic events to take place. During MDE induction, the existing polarity of pollen development is disrupted and a "new" polarity has to be re-established for continual embryo development. At present, the process of polarity establishment during MDE formation is not clear. In wheat, using the cell tracking procedure, starch appears to be a clear indicator of polarity establishment as the suspensor and/or future root pole emerged from it (Indrianto et al., 2001). Is there an earlier signal for the establishment of polarity? Ilic-Grubor et al. (1998b) suggest that the presence of the generative cell may serve as the potential signal for polarity establishment. During normal pollen development, an asymmetric division results in the formation of the vegetative cell and the generative cell. Are there cytoplasmic determinants that pre-determine the future polarity of the MDEs? A careful structural analysis may enable us to answer this question in the future.

Could the cell wall play a role in determining the site of future growth? In recent years, cell wall components have been identified and shown to play a morphogenetic role in plant development. Cell wall components may provide positional information for organized development (Souter and Lindsey, 2000). In *Fucus*, it has been suggested that targeted secretion of cell wall material may provide positional cues for orienting subsequent divisions (Vroemen et al., 1999). As the microspore enlarges during the induction phase, the pollen wall splits open. Part of the wall often sticks to the tip of the suspensor cell. This could be due to the fact that the wall attaches to a region of the cell which does not enlarge and hence adheres to it better. However, one cannot discount the fact that certain areas of the pollen wall are unique and aid in the establishment of polarity. With respect to cell wall rupture, Hause et al. (1994) also suggested that "the rupture of pollen wall may play an important role in the local activation of cell metabolism and thus in the determination of the polarity axis in MDEs". As indicated earlier, Telmer et al. (1995) reported some electron dense deposits and vesicle-like structures were present in the newly formed walls of potentially embryogenic cultured microspores. The newly formed wall with new wall components may play a key role in polarity establishment. A series of monoclonal antibodies have been generated that recognizes cell wall epitopes, especially the arabinogalactan proteins (Knox et al., 1991; Pennell et al., 1991, see also Souter and Lindsey, 2000). Through the use of these antibodies, one may be able to determine whether heterogeneity in wall component distribution exists within the pollen intine and the newly formed walls of the dividing microspores.

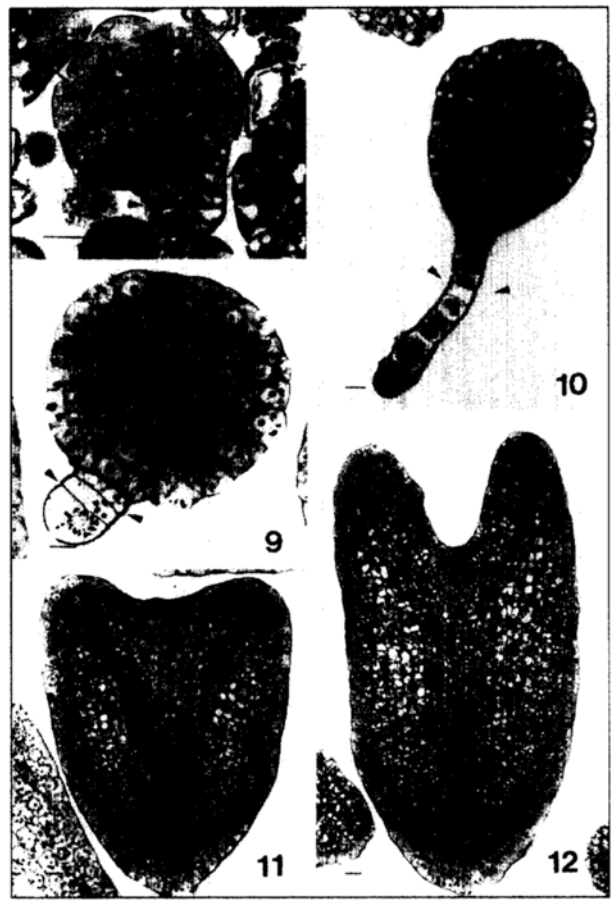
Histodifferentiation

The process of histodifferentiation in *Brassica* MDEs has been detailed by Yeung et al. (1996). Figures 1-18 illustrate the structural pattern of MDE development beginning with the microspores soon after isolation. At the time of culture, the microspores are about 20 - 25 µm in diameter (Fig. 1). After 1 day in culture, the microspores increase in size to 30 - 35 µm and begin to divide. By day 3 in culture, some microspores have divided. The first mitotic division results in the formation of two daughter cells with more or less equal size (Fig. 2). At about day 5, pre-globular stage embryos with several

cells can be found in culture and some begin to break out of the microspore wall (Figs. 3 - 5). By day 8 in culture, various developmental stages starting from microspores up to 30-celled globular embryos can be observed in the same culture (Figs. 7 - 10). The early cell division pattern varies (Figs. 5 - 10). Differences in the distribution of starch grains can be found in early developing proembryos. A higher concentration of starch is usually present in the suspensor end of the



Figures 1-7. Microspore embryogenesis in *B. napus* cv Topas. **1.** Light micrograph illustrating the features of the microspores at the time of culture. The microspores are uni nucleate with prominent vacuoles. **2.** Microspore, 3 d after culture (DAC). Microspores have expanded in size, and some microspores have begun to divide. The first mitotic division is symmetrical giving rise to two daughter cells of similar size. **3.** Microspore 4 DAC. Additional division takes place within the microspore wall. **4.** Microspore 5 DAC. Multicellular structures become abundant within the culture medium. However, cell division appears to be random. **5.** Microspore 7 DAC. Continual mitotic activity results in the formation of a globular cell mass. **6.** Microspore 8 DAC. An early proembryo having a globular-shaped suspensor (arrowheads). More starch grains (arrow) are present in the suspensor than the developing embryo proper. **7.** A more advanced stage of MDE development. More starch grains (arrows) are present in the suspensor end of the embryo. All scale bars = 20 μm.



Figures 8-12. 8. Microspore 8 DAC showing a developing embryo with a suspensor (arrowheads) similar to its zygotic counterpart. Tissue differentiation begins with the formation of the protoderm by periclinal divisions (arrows) in the outermost cell layer. **9.** Expansion in size continues prior to further histodifferentiation. The protoderm cells are large, and the inner cell mass is homogeneous in size and shape. A short suspensor (arrowheads) is present in this microspore embryo. Prominent starch grains are present within the suspensor and adjoining cells. **10.** A microspore embryo showing a long filamentous suspensor (arrowheads). **11.** Early heart stage. The tissue pattern is better defined and the cotyledon primordia are present. The procambium (*P*) is centrally located, and the cells are elongated with a dense cytoplasm. Surrounding it is the ground tissue, which is more vacuolated. The apical meristems are developing and occupy opposite poles of the embryo. **12.** Microspore embryo 12-14 DAC. The embryo begins to elongate and takes on a torpedo shape. This is primarily due to vacuolation in the ground tissue (*). The procambium (*P*) has bifurcated underneath the shoot apical meristem and enters into the cotyledons. The shoot apical meristem is located at the apical notch. All scale bars = 20 μm.

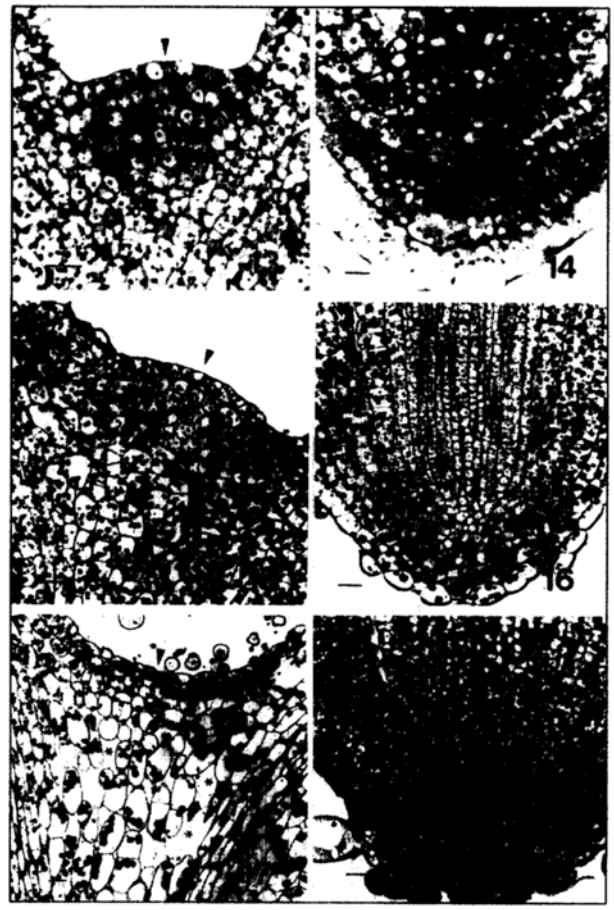
embryo (Figs. 6 - 10). This observation is also reported for wheat pollen embryos (Indrianto et al., 2001). The starch distribution pattern clearly indicates physiological differences within the proembryo.

A rudimentary suspensor is often present in microspore embryos (Figs. 6 - 10). The morphology of the suspensor can differ between embryos. It can appear as a small projection near the future root pole of the embryo (Figs. 6 and 7) or as a uniseriate structure similar to the zygotic embryo (Figs. 8 - 10). It is interesting to note that for those embryos having a "zygotic embryo-like" suspensor, the cell profile of the embryo proper also resembles its zygotic counterpart (Figs. 8 and 9). Recently, Custers et al. (2001) have succeeded in producing suspensor-bearing embryos with high frequency. Judging from their micrographs, the formation of the proembryo closely resembles the zygotic counterparts. This new system will enable further studies on suspensor-embryo proper interaction (Custers et al., 2001).

Globular embryos can be observed in 10-day-old cultures. Once the globular mass of cells is formed, periclinal divisions occur leading to the formation of the protoderm (Fig. 8). Concomitant with the formation of the protoderm, tissue differentiation begins. The body plan is established at the late globular to the early heart stages with the formation of the apical meristems, cotyledon primordia, procambium, and the ground tissue (Fig. 11). The morphology of the suspensor appears to have little influence on tissue differentiation. By day 14, the embryo gradually elongates into a torpedo shape (Fig. 12). This is primarily due to vacuolation and expansion of cells in the cortex. It is important to note that the general tissue pattern of MDEs from the heart-torpedo stages of development is similar to their zygotic counterpart.

Although basic structural information is available for *B. napus* MDEs, information on the genetic regulation of the histodifferentiation process is limited. At present, a number of genes such as *WUSCHEL* and *SHOOT MERISTEMLESS* have been characterized and have been shown to play important roles during *Arabidopsis* zygotic embryo development (see Souter and Lindsey, 2001). The *Brassica* MDE system would be an excellent system to study factors regulating gene activity during histodifferentiation as embryos are readily produced in large quantities for functional analysis.

One of the important events in embryo morphogenesis is the change of symmetry from radial to bilateral. Auxin has been shown to control symmetry changes in zygotic embryos (Liu et al., 1993). When *B. napus* MDEs are treated with the auxin transport inhibitor, triiodobenzoic acid (TIBA), one cup shaped cotyledon appears indicating that auxin transport is essential to the establishment of a bilateral symmetry (Iwanowska et al., 1997; Ramesar-Fortner, 1999).



Figures 13-18. 13. Cotyledon stage (20 DAC). The shoot meristem (arrowhead) has expanded forming a dome-shaped apical meristem. **14.** An enlarged view of the root apical meristem (arrowheads) at the late heart stage. This organization of the root apical meristem is maintained till the cotyledon stage. **15.** Microspore embryo beyond 35 DAC. The shoot apical meristem cells (arrowhead) gradually change into parenchyma cells with abundant starch deposits. **16.** Microspore embryo beyond 40 DAC. Procambial cells (*) begin to change into storage parenchyma cells. The root apical meristem initials are difficult to discern. **17.** Microspore embryo 50 DAC. Intercellular spaces (*) begin to appear between cells in the shoot pole. The original meristem cells become highly vacuolated with prominent starch grains within their cytoplasm. This completely obliterates the shoot meristem organization. **18.** Microspore embryo beyond 40 DAC. As more procambial cells differentiate into storage parenchyma cells, the organization of the root pole is disrupted, and the root meristem initials have lost their structural identity. All scale bars = 20 μm.

The formation of the apical meristems is one of the key

events in the histodifferentiation of an embryo (Yeung and Stasolla, 2000). From the study of *B. napus* zygotic embryos, the process of shoot apical meristem (SAM) initiation appears to be labile (Ramesar-Fortner and Yeung, 2001). When TIBA is applied at the globular stage of embryo development, SAM formation is disrupted leading to low conversion. However, once the embryo is at the heart stage or later, TIBA cannot alter the SAM morphology and one hundred percent conversion is observed. This observation indicates that SAM formation and determination occurs within a narrow window in the time course of embryogeny. In the *B. napus* MDEs, SAM formation resembles the process that occurs in their zygotic counterparts. However, upon prolonged culture, i.e. beyond 30 days, the SAM begins to revert to storage parenchyma indicating that it is not fully determined (Figs. 13 - 18, Yeung et al., 1996). This abnormality leads to a low percentage of pollen embryo conversion. A similar observation was reported for *Brassica juncea* MDEs for their inability to germinate like normal zygotic embryos (Sharma and Bhojwani, 1989).

The study of *B. napus* embryo development indicates that once the process of embryo development is initiated, histodifferentiation proceeds similar to their zygotic counterparts. The failure for "fixation" of the tissue pattern lies in the embryonic environment. The work of Ilic-Grubor et al. (1998a, 1998b) clearly illustrates the importance of media components on MDE development. A zygotic embryo develops in an environment with very negative water potential (Yeung and Brown, 1982). The addition of an osmoticum such as sucrose is beneficial to the rearing of young developing embryos during in vitro culture (see Yeung et al., 2001). But, in recent years, polyethylene glycol 4000 (PEG) has become the compound of choice especially when liquid suspension is used (Attree and Fowke 1993). The inclusion of 22% PEG and 0.1% sucrose results in "normal" embryo development (Ilic-Grubor et al., 1998a, 1998b). The morphological pattern and storage product deposition of MDEs are similar to their zygotic counterparts. A one hundred percent germination was observed. The addition of ABA to the "conventional" culture medium also proved to be beneficial to MDE development. ABA prevents deterioration of the SAM over time (Ramesar-Fortner, 1999). Hence, a proper design of culture medium is essential to allow for "normal" embryo development.

The fact that *B. napus* MDEs can be cultured in an extremely low sucrose concentration suggests that the early proembryos may not have a high nutritional demand, and instead the proper osmotic environment is more important for proper development (Yeung et al., 2001). Although PEG and ABA elicit a similar response in terms of SAM development, it has been shown that ABA and osmoticum have different functions during embryo development (Xu et al., 1990). Future biochemical and molecular studies during the histodifferentiation will provide additional information as to the function of PEG and ABA in pollen embryogenesis.

Hormonal Control of MDE Development

The subject of hormonal control of embryo development has recently been discussed by Fischer-Iglesias and Neuhaus (2001). Although all major classes of growth regulators have been detected during zygotic embryo development, only the functions of auxin, cytokinin, and ABA are better understood (Fischer-Iglesias and Neuhaus, 2001).

In recent years, MDEs have served as an experimental system and have extended our understanding concerning the function of growth regulators during embryo development. Through the use of MDEs, the role of auxin in the control of embryo symmetry has been confirmed (Ramesar-Fortner, 1999). As discussed in the previous section, auxin levels play a role in symmetry formation and most likely serve as the patterning signal molecules in the establishment of the body plan of the embryo (Souter and Lindsey, 2001).

GA content in developing seeds is high; however, its role during embryo development is still unclear. In *B. napus* MDEs, at the globular stage of development, GA_1 levels increase rapidly. Uniconazole, an inhibitor to GA biosynthesis, inhibited axis elongation and its effect could be reverse by the addition of GA_1 (Hays et al., 2002). This work indicates that GA is essential to axis elongation. The elongating axis of MDEs could serve as a useful tool to study the cellular function of gibberellins in developing embryos, especially in the cell elongation process.

Ethylene is an endogenous gaseous hormone which has been extensively studied in relation to different aspects of plant growth and development. In *B. napus*, a large amount of ethylene is produced by the developing seeds (Johnson-Flanagan and Spencer, 1994). The ethylene produced may play a role in seed degreening (Johnson-Flanagan and Spencer, 1994) as well as regulating silique growth and seed dispersal (Meakin and Roberts 1990; Child et al., 1998). However, its function during zygotic embryo development is still unclear. Through the use of MDEs in culture, ethylene production by the embryo has been shown to control cotyledon expansion (Hays et al., 2000). This study

clearly demonstrates that ethylene is involved in normal MDE development. It is likely that ethylene also plays a similar role during zygotic embryo development in canola.

Abscisic acid plays an important role during seed development, especially during seed maturation and storage product biosynthesis (Fischer-Iglesias and Neuhaus, 2001). Similar to its action during zygotic embryogenesis, ABA has been shown to stimulate storage protein and oil body protein production in MDEs (see next section). Besides functioning during late embryo development, a recent study indicates that ABA is essential to early somatic embryo development of *Nicotiana plumbaginifolia* (Senger et al., 2001). In *B. napus*, the inclusion of ABA during the early stages of MDE development results in a stably determined SAM (Ramesar-Fortner, 1999). Although few studies are available concerning the hormonal control of microspore embryogenesis, the usefulness of this system is clearly demonstrated by the published studies available. The MDEs can certainly serve as a useful system to assay for hormone functions during embryo development.

Storage Product Deposition Pattern

Storage product accumulation is a key program in seed development of flowering plants. From an evolutionary point of view, the lower vascular plants germinate as soon as the process of histodifferentiation is completed. The gametophyte functions to provide the necessary nutrients for the early growth of the young sporophyte. In seed plants, food reserve deposition is an added program that has occurred over the course of evolution which enables the embryo to accumulate food reserves in order that germination can be delayed until a suitable environment presents itself. Since seed storage reserves provide essential nutrients to man, it is not surprising to find numerous studies focusing on the structural, biochemical and molecular biology of storage reserve deposition with the aim to further improve their quality. Studies are continuing to unravel the events related to the physiology, biochemistry and molecular biology of seed storage products and how these products are packaged within the maturing embryo. A recent issue of the Journal of Plant Physiology highlighted the recent advances in this area of research (Volume 158, number 4, 2001). From the standpoint of cellular, biochemical and molecular biology, the MDEs can be an excellent system to study storage product biosynthesis and deposition.

In *B. napus*, the types of storage product and their deposition pattern are strongly influenced by medium components and the methods of culture. When MDEs are cultured using the "conventional" Lichter's medium (Lichter, 1982) with 13% sucrose, starch is the primary storage product found in developing MDEs (Yeung et al., 1996). At the globular stage of development, cells in the future root pole tend to contain starch grains of larger size. Starch deposition varies in different tissues. The meristem poles and the procambium tend to contain less starch grains while the protoderm, the ground tissue and the cotyledons are abundant in starch. The 20-day old MDEs contain numerous starch grains, which continue to increase in 30-day old embryos. Storage lipids are present within MDEs. The lipid bodies appear as irregularly shaped large globules and are not packed into smaller lipid bodies as in their zygotic counterparts. Storage protein bodies are generally absent from MDEs (Yeung et al. 1996, unpublished results). The culture environment may play an important role in protein body formation as Sharma and Bhojwani (1989) reported the presence of protein bodies in anther culture derived microspore embryos of *B. juncea* using agar-gelled medium.

When the *Brassica* MDEs are cultured in 21% PEG with 0.1% sucrose, the pattern of storage product deposition is similar to their zygotic counterparts (Ilic-Grubor et al., 1998a, 1998b, and personal communication). The addition of ABA also has a profound influence on storage product synthesis (see next section). The information at hand clearly indicates that the physical and chemical environments play an important role in storage product deposition. Further studies on the role of the osmoticum in storage product deposition are definitely needed.

Storage Protein

The major storage proteins in *Brassica* zygotic embryos are napin and cruciferin and their distribution have been detailed by Höglund et al. (1992). The amount of storage proteins and their corresponding transcripts of napin and cruciferin are low in MDEs at the torpedo stage of development. However, in the presence pf ABA, storage protein transcripts increased (Taylor et al., 1990). It is interesting to note that the napin seed storage protein gene but not cruciferin, is also expressed early during MDE formation (Boutilier et al., 1994). Thus, it appears that besides ABA, stress treatment may also play a role in storage protein gene expression. Additional studies are needed on the regulation of biosynthesis, assembly and processing of storage proteins in cruciferous species (Delseny and Raynal, 1999).

Lipid Bodies

Canola is a major oil seed crop in Canada and in other parts of the world. Oil quality is a determining factor for further growth in this industry. The pattern of lipid biosynthesis and accumulation during normal seed development has been detailed by Murphy and Cummins (1989). The ease of production of MDEs provides "attractive alternatives in both biochemical studies and biotechnology programs for the improvement of oil seed cultivars and the development of novel oilseed crops" (Taylor and Weber, 1994). Biochemical analyses indicate that although some differences are noted in fatty acids between MDEs and their zygotic counterparts, the overall fatty acid composition is comparable between the two systems (Pomeroy et al., 1991; Wilberg et al., 1991). Abscisic acid treatment of MDEs strongly stimulates fatty acid biosynthesis (Zou et al., 1995). Since ABA also stimulates the production of oil body proteins (oleosins), the coordination of lipid and oleosin biosynthesis in lipid body formation can be studied using MDEs (Taylor et al., 1990; Holbrook et al., 1992). As many MDEs can be obtained easily using a liquid suspension system, this is a convenient system to study the regulation of lipid biosynthesis, especially enzymes related to lipid biosynthesis (Taylor et al., 1991). Furthermore, as we are dealing with a double haploid, it is possible to screen for oil quality in genetically altered seeds (Wilberg et al., 1991). Thus, the *Brassica* MDE system is ideal for the study of storage lipid biosynthesis and regulation.

Oil Body Protein- Oleosins

The storage lipid in seeds usually appears as oil bodies with triacylglycerol forming the core and the surface covered by oil body associated proteins known as oleosins. The structure, properties and biological roles of the seed oleosins have recently been summarized by Napier et al. (2001). Their function is believed to maintain the size and stability of the oil bodies within the cytoplasm to prevent them from coalescing with one another (Frandsen et al., 2001; Napier et al., 2001). Recently, another oil body related protein, caleosin has been described (Chen et al., 1999). It appears to function in calcium mediated fusion of oil bodies (for details, see Frandsen et al., 2001). Through the use of the MDEs, the regulation of oleosin gene expression and biosynthesis has been studied (Moloney, 1999). Oleosin mRNA can be detected as early as the heart stage using Northern blot analysis (van Rooijen et al., 1992). Furthermore, the synthesis of oleosin is stimulated by ABA, osmotic stress and jasmonic acid treatment (Holbrook et al., 1991; van Rooijen et al., 1992).

The published information as indicated above clearly indicates the usefulness of the MDE system in the study of seed storage product biology.

Biotechnology Potential

In recent years, more reports are becoming available describing methods of refining the culture conditions for increased induction efficiency and improved embryo development (e.g. Hu and Kasha 1997; Hofer et al., 1999; Indrianto et al., 1999; Guo and Pulli, 2000; Kunz et al., 2000; Kasha et al., 2001; Li and Devaux, 2001; Ritala et al., 2001; Zheng et al., 2001, 2002; Liu et al., 2002). Besides providing new insights into the process of pollen embryogenesis, the information is essential to the genetic manipulation of microspores and MDEs.

The microspore can be used as a source of plant material for the generation of mutants (Castillo et al., 2001) and transgenic plants (Huang, 1992). The advantage of this system is that any genetic variation introduced will permit recovery of diploid mutant/transgenic plants that are homozygous for mutant/trans- genes after spontaneous or chemical chromosome doubling (Kott et al., 1996; Yao et al., 1997). Recently, the topic of "microspore-based transformation" has been reviewed by Touraev et al. (2001). Different transformation procedures have been used such as microinjection (Jones-Villeneuve et al., 1995), polyethylene glycol mediated DNA transfer (Kuhlmann et al., 1991), electroporation procedure (Fennell and Hauptmann, 1992), and *Agrobacterium*-mediated transformation (Pechan, 1989; Huang, 1992). The biolistic procedure appears to be the method of choice in the generation of stable transgenic plants (see Yao et al., 1997). Stable transgenic plants albeit at low frequency were obtained using isolated microspores of barley by the biolistic procedure (Yao et al., 1997; Carlson et al., 2001). An additional protocol for *Antirrhinum majus* microspores has just been published (Barinova et al., 2002). Future improvements in the transformation techniques promise increased efficiency in the generation of stable transgenic plants. Selected new genes could be added to further improve characteristics of different cultivars.

Conclusion and Perspectives

The inaccessibility of the zygotic embryo makes the MDE an ideal system in the study of plant embryo-

genesis. Although the pattern of MDE development is not identical to its zygotic counterpart, by altering culture conditions, the development of the MDEs can closely mimic that of the zygotic embryo and thus provide for unique experimental opportunities. Thus, the information obtained would provide insights into embryo development as a whole.

There is little doubt that stress treatments are essential to the induction process. Although different cellular and molecular events have been described for the induction process and early embryo development, many questions remain. In *B. napus*, one of the key components in the induction process is the osmotic environment. It has been shown to play a morphogenetic role in plant growth and development (Yeung et al., 2001) and is essential that the function of osmotic stress on MDE induction be studied. There may be a synergistic effect with the heat shock treatment.

The HSPs appear to be essential to the induction of microspores for *B. napus* but other, yet undiscovered, proteins may also play a causal role in the induction process. In *Arabidopsis*, the *LEAFY COTYLEDON* and *WUSCHEL* genes both encode transcription factors that play key roles in early embryo development (Harada, 2001; Harada et al., 2002; Zuo et al., 2002). The over expression of *WUSCHEL* results in high frequency somatic embryo formation (Zuo et al., 2002). Are these genes and their gene products produced during the early induction phase? Future improvements in gene detection and amplification procedures used with functional genomics (Colebatch et al., 2002) will enable us to identify additional proteins and genes that control the induction process. The ability to sort embryogenic from non-embryogenic cells using flow cytometric methods (Schulz and Pauls, 1998, 2002) will provide us with a powerful tool in the study of the early events related to microspore induction. The induction step is a complex event that involves many cellular changes. Some of these changes must be well coordinated to bring about morphogenetic changes leading to embryo formation. Besides focusing on the early induction process, the MDE is an ideal experimental system that can be used to study different aspects of embryo development. Currently, there are few studies using MDEs to study gene regulation of embryo development. With a better appreciation of the MDE system, exciting progress will be made in the near future.

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