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Exine-dehisced Microspores: A Novel Model System for Studying Embryogenesis

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ABSTRACT

Upon stress of high temperature, *Brassica* microspores can be switched from their gametophytic development to an embryogenenic pathway. It provides a powerful system for understanding the biological basis of embryogenesis. However, embryos derived from symmetrically divided microspores usually lack a suspensor, which is distinct from normal zygotic embryogenesis. This obviously limits the application of the system in the investigation of the early developmental events of embryogenesis. We have now established a novel system for inducing and studying embryogenesis: the exine-dehisced microspore, in which the exine is ruptured but still envelopes the microspore. Evidence shows that the exine-dehisced microspore shares all the advantages of the traditional androgenesis system and offers a unique chance to examine the role of polarity, asymmetric division, and the cell wall in cell fate determination and apical-basal axis selection in embryos. Thus, the exine-dehisced *Brassica* microspore system provides a novel and useful alternative model for studying these early events of embryogenesis. In this mini-review, we mainly introduce the system and outline its potential applications.

Keywords: axis formation, *Brassica*, cell division, cell fate, polarity
Abbreviations: **ABA**, abscisic acid; **AGP**, arabinogalactan protein; **GA**, gibberellic acid; **MD**, microspore-derived; **NLN**, Nitsch & Nitsch medium modified by Lichter

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INTRODUCTION

In plant seeds, embryogenesis begins with the fertilized egg cell, which gives rise to a multicellular organism through cell growth and development processes characterized by polarity induction, cell division, expansion, and differentiation along the body axis. Genetic studies screening for embryo mutants have resulted in the identification of genes involved in basic developmental processes, such as axis establishment, pattern formation, and organogenesis (Jürgens 1996; Weijers and Jürgens 2005). However, progress has been more limited in our understanding of the developmental events that take place during fertilization and early embryo induction, because of the inaccessibility of the egg cell and zygote (Márton *et al.* 2005; Mori *et al.* 2006).

For many years, the unique biological process known as microspore embryogenesis, or androgenesis, has been used as an important tool in plant breeding to obtain double-haploid plants (Wang *et al*. 2000; Maraschin *et al*. 2005). In this process, the microspore or immature pollen grain, upon exposure to suitable medium and stress treatment, can be shifted from normal gametophytic development toward proliferation, leading to the production of whole haploid plants. In recent years, such production of embryos from isolated and *in vitro*-cultured microspores of several species, including tobacco, rapeseed, barley, and wheat, has become efficient and reproducible (Touraev *et al*. 1997; Wang *et al*. 2000; Maraschin *et al*. 2005).

From a developmental point of view, the study of microspore embryogenesis, or androgenesis, may result in a greater understanding of the general principles of plant embryogenesis (Honys *et al*. 2006). It is a useful alternative system for the study of embryogenesis. Androgenesis typically starts with a symmetric cell division, which produces two cells more or less equal in size. However, embryos derived from symmetrically divided microspores usually lack a suspensor, which is distinct from normal zygotic embryogenesis (de Jong *et al*. 1993; Hause *et al*. 1994; Maraschin *et al*. 2005). This limits the application of the system in the investigation of the early developmental events of embryogenesis such as the role of polarity in the first embryonic division pattern and the relationship be-

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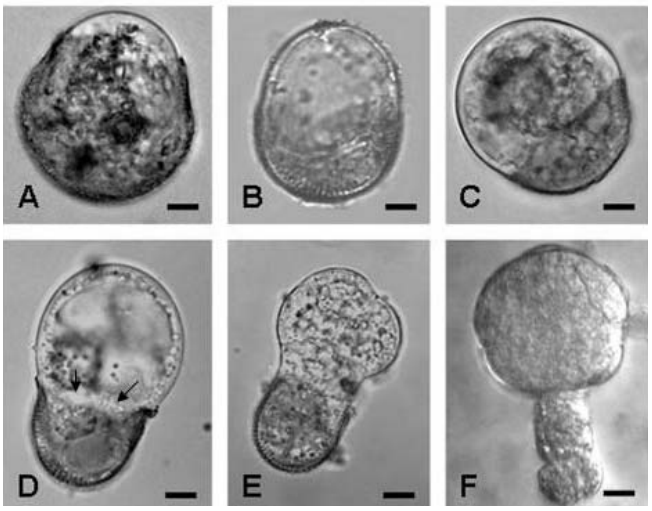

Fig. 1 Exine-dehisced microspore regeneration in rapeseed. (**A**) Part-exine-dehisced microspore; (**B**) Half-exine-dehisced microspore; (**C**) Largely exine-dehisced microspore; (**D-F**) Exine-dehisced microspore embryogenesis: (**D**) First division. The arrows indicate the new cell wall; (**E**) Early globular embryos; (**F**) Globular embryo with a suspensor. Bar = 12 μm in A-D, 15 μm in E, 25 μm in F.

tween asymmetric cell division and cell fate determination.

We sought to establish techniques for preparing different types of microspores with different cell wall layers and to test their developmental fate under culture conditions to reveal the role of different cell wall layers in microspore development. The microspore types developed include the microspore protoplast, in which the exine and intine are totally removed, the de-exined microspore, in which only the exine is removed, and the exine-dehisced microspore, in which the exine is ruptured but still envelopes the microspore (Xia *et al*. 1996; Xu *et al*. 1996; Sun *et al*. 1999; Tian and Sun 2003). The results suggested that the exine plays an important role in the germination of pollen and the embryogenic development of the microspore. Based on these techniques, we established a novel system for studying embryogenesis: the exine-dehisced microspore. In this mini-review, we introduce the system and outline its potential applications.

PREPARATION OF THE EXINE-DEHISCED MICROSPORE

The microspore wall consists of the exine, made primarily of sporopollenin, and the intine, consisting primarily of polysaccharides (Knox *et al*. 1986). After hydration, heat shock, and osmotic shock, the exines of some microspores or immature pollen grains can be broken to form so-called "exine-dehisced" microspores. The available methods for obtaining exine-dehisced microspores were investigated in *Brassica napus* (Tian and Sun 2003). The frequency of exine dehiscence is highly dependent on the growth conditions and physiological status of donor plants, the developmental stage of the microspores, and the treatment method used. Microspores from buds grown under natural conditions in the proper season were the best choice for preparing exine-dehisced microspores. The age of the donor plant can also influence the preparation efficiency. The exine dehiscence frequency of microspores from the first emerging flowers can be 10% higher than that of microspores isolated from late flowers. Generally, the more mature the pollen grain is, the higher is the exine dehiscence frequency.

The late uninucleate to early binucleate stages are the most suitable for microspore embryogenesis, with the exine dehiscence frequency being higher in early binucleate pollen grains than in late uninucleate microspores. Cold treatment for 2-3 d before culture can improve both the frequency of exine dehiscence and subsequent embryo induction. In addition, high temperature treatment at 32°C for longer than the normal 2-d treatment is better, as is 32°C over 25°C.

MORPHOLOGICAL CHARACTERISTICS OF EXINE-DEHISCED MICROSPORES

Exine-dehisced microspores have been studied in tobacco and rapeseed, particularly in *Brassica napus*. The exine usually breaks at one of the microspore furrows, although it still tightly covers the microspore. Only part of the protoplast enveloped by the intact intine protrudes from the exine, which is quite different from the total removal of the exine as in a de-exined microspore (Zhou and Yang 2000). The refractive index between the naked intine and exine appears distinct by light microscopy. It is easy to recognize the broken and intact exine ends of the microspore; thus, the broken exine acts as a marker for orientating the microspore elongation and following the developmental fate of the two daughter cells after the first cell division in culture. Exine-dehisced microspores can be subdivided into three types: part-exine-dehisced, half-exine-dehisced, and largely exine-dehisced microspores (**Fig. 1A-C**).

After a brief culture period, exine-dehisced microspores usually expand and elongate, forming a long axis, termed the exine-axis (ex-axis). The nucleus is typically located in the exine ruptured plane (ru-plane) (**Fig. 2**). Such morphological characteristics are convenient for recognizing the position of the cell division plane and the orientation of the embryo developmental axis.

EXINE-DEHISCED MICROSPORE CULTURE AND PLANT REGENERATION

The efficiency of androgenesis varies greatly depending on the stress treatment and the plant species and genotype (Touraev *et al*. 1997; Wang *et al*. 2000; Maraschin *et al*. 2005). Rapeseed has been considered a model species in which to study developmental events, because of its high regeneration efficiency. Procedures for treating microspores in medium to induce androgenesis have been well summarized (Datta 2005), which are also suitable for exine-dehisced microspore culture. However, because of the limited number of exine-dehisced microspores obtained, a co-culture system should be used, as was demonstrated in culturing *Brassica* microspore protoplasts (Sun *et al*. 1999). Exine-dehisced microspores were selected with a micropipette under an inverted microscope and transferred to a millicell with ~100 μL NLN medium. The millicell was placed in a Petri dish containing dividing microspores as feeder cells (**Fig. 3**). Feeder cells can sustain the embryonic development of microspore cells (van Hengel *et al*. 1998;

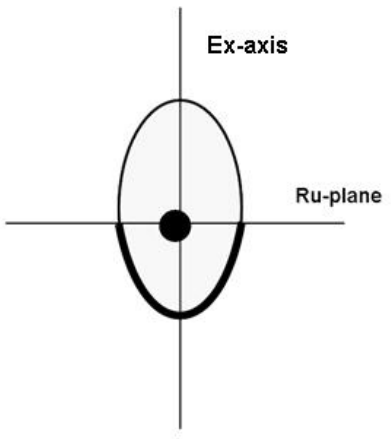

Fig. 2 Scheme of the exine-dehisced microspore. The dark dot indicates nucleus. The thin line shows intine of the microspore and the thick line shows exine that covers half of the microspore here.

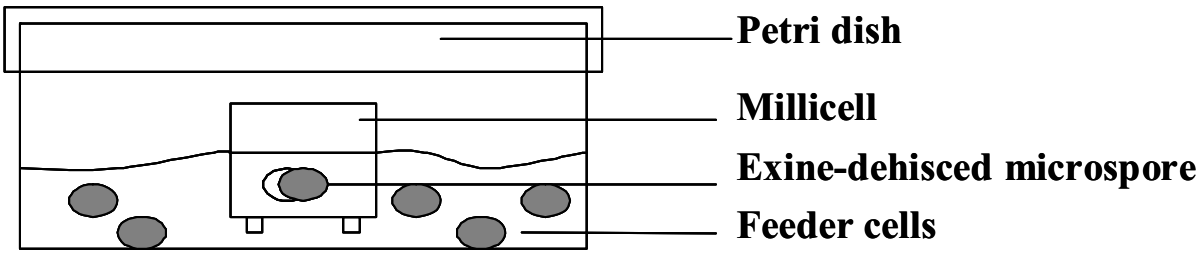

Fig. 3 Microculture device.

Paire *et al*. 2003; Borderies *et al*. 2004). The establishment of co-culture systems has played an important role in revealing the developmental pathways of induced exine-dehisced microspores and in tracking the main morphological characteristics of the early androgenetic process.

Haploid and double-haploid plants regenerated from microspore embryos are invaluable breeding tools. The primary bottleneck, however, for the practical application of microspore-derived (MD) embryos in breeding is improving germination efficiency (Baillie *et al*. 1992). Seed development is terminated by maturation drying, with a gradual loss of water and acquisition of desiccation tolerance. Many attempts to improve plantlet conversion in *Brassica* have focused on the induction of desiccation tolerance in MD embryos by thermal stress (Anandarajah *et al*. 1991), ABA treatment (Senaratna *et al*. 1991; Brown *et al*. 1993; Takahata *et al*. 1993), or a combination of ABA and high osmotic pressure conditions (Wakui *et al*. 1994). Additionally, Polsoni *et al*. (1988) reported that, by combining treatment with gibberellic acid (GA_3) and increasing the sucrose concentration in the culture medium, 40-60% of embryos developed into plants directly from the primary shoot apex. The conversion frequency of these microspore embryos was relatively low. The majority of plants recovered from the embryos arose either from secondary embryogenesis or from a complex tissue mass (Swanson *et al*. 1987). Some abnormalities were observed, including the absence of meristems, early necrosis, and polycotyly (Cao *et al*. 1994). Tian *et al*. (2004) developed a new method to improve the conversion frequency by supplementing with calcium and vitamins, suggesting that calcium may play an important role in the conversion of embryos into plantlets. With the protocol of Tian *et al*. (2004), embryos derived from exine-dehisced microspores can develop directly into plants at a high frequency and without obvious morphological variation. Since the intact and rigid exine is the main barrier for transferring foreign genes into microspores by any known method, the exine-dehisced microspore could greatly facilitate gene transformation (Wang *et al*. 1998). Thus, techniques for exine-dehisced microspore culture and embryo conversion are important for both the study of embryogenesis and crop breeding.

APPLICATIONS OF EXINE-DEHISCED MICROSPORES IN THE STUDY OF EMBRYOGENESIS

Polarity induction and cell division patterns

Polarity is an important feature in an organism's development. In higher plants, the nucleus and much of the cytoplasm are located in the chalazal pole, and a large vacuole is present at the micropylar end of the egg cell. Additionally, there are differences in thickness and composition between the chalazal and micropylar end cell walls (Schulz and Jensen 1968; Russell 1993; West and Harada 1993). Following fertilization, the redistribution of the endoplasmic reticulum, plastids, and mitochondria accentuate the polar organization seen in the egg cell before embryogenesis (Jensen 1968; Russell 1993). This is believed to be in preparation for the first unequal division and apical and basal cell differentiation.

Another useful model is that of fucoid algae. Polarization in the fucoid egg cell can be triggered in response to a variety of environmental stimuli, including fertilization and unidirectional light (Quatrano 1997; Brownlee *et al*. 2001). The process involves localization or redistribution of plasma membrane components such as ion channels, redistribution of calcium to the basal shaded pole, localization of microfilament networks, asymmetric distribution of total mRNA and actin mRNA (Kropf *et al*. 1989; Hable and Kropf 2000), and polarized secretion of Golgi-derived cell wall toward the rhizoid pole (Shaw and Quatrano 1996; Quatrano and Show 1997; Belanger and Quatrano 2000a, 2000b). Following the induction of polarity, asymmetric division results in two daughter cells with different cytoplasm. This strictly determined cell division pattern plays an important role in defining and maintaining the body regions in zygotic embryogenesis (Jürgens 1996, 2001; Willemsen and Scheres 2004).

It is widely accepted that the first morphological evidence of the embryonic pathway is the symmetric division of the microspore, and not the asymmetric division occurring during normal gametophytic development (Zaki and Dickinson 1991; Hause and Hause 1996). Although asymmetric division patterns have been reported in cultured bicellular pollen of pepper (*Capsicum annuum*), these embryogenic divisions were the result of a response of either the vegetative or generative nuclei of the bicellular pollen (Kim *et al*. 2004). The first sign of polarity expression is the accumulation of starch grains in the future root pole in multicellular globular embryos derived from microspores (Hause *et al*. 1994; Indrianto *et al*. 2001). In this case, no unequal division or cell differentiation is observed in normal, early microspore embryogenesis. As a result, it is not comparable to zygotic embryogenesis with regard to early developmental events. However, exine-dehisced microspores appear morphologically polarized. Cytological evidence also indicated a redistribution of organelles during the process of exine dehiscence, suggesting that a kind of polarity is induced by the rupture of the pollen wall in exine-dehisced microspores (unpublished data). Different division patterns, including asymmetric division (**Fig. 1D**), have been observed in exine-dehisced microspores. The orientation and spatial position of the division plane varied according to the degree of exine rupture. This system offers an opportunity to compare normal and intact microspore embryogenesis, to examine the role of polarity in the initiation of embryogenesis, and to determine the relationship between cell polarity and cell dividing patterns. We found that dehiscence of the exine is important in inducing polarity during early microspore embryogenesis, and thus the wall could play a key role in polarity establishment. Ilic-Grubor (1998) proposed that the polarity of MD embryos was inherited from the young gametophyte, which became structurally polarized as the nucleus moved laterally toward the pollen wall in the late uninucleate microspore. Using the broken exine as a marker of microspore orientation at this stage could provide a useful way of addressing the question.

Cell fate determination

Different cell types originate from asymmetric cell division. During early embryogenesis in higher plants, a zygote gene-

rally produces a small apical cell and a larger basal cell after the first asymmetric division. The fates of the two daughter cells are clearly distinct; the small cell develops into an embryo proper, whereas the large one develops into a suspensor. How cell fate is determined remains unclear, but it is believed that cell fate is related to zygote polarity and unequal cell division (Jürgens 1996; Dodeman *et al*. 1997; Jürgens 2001). Distinct gene expression patterns between the embryo and the suspensor provide evidence in favor of this (Haecker *et al*. 2004; Lukowitz *et al*. 2004). Much evidence supports the general rule that cell fate is directed by cell position rather than lineage (Schers 2000; Scheres and Browse 2001), suggesting that positional information is more important than differential inheritance of determinants in the specification of cell fate following asymmetric division. Studies with *Arabidopsis twin* mutants (Vernon and Meinke 1994; Zhang and Somerville 1997) showed that the apical cell actively inhibits embryonic development of the basal cell, strongly implicating the participation of intercellular communication in cell fate determination. In addition, laser ablation experiments on eight-celled *Fucus* embryos have also indicated that communication between the two cells might determine their different fates (Bouget *et al*. 1998). Thus, the positions of the apical and basal cells and possible interactions between them could provide new clues about developmental fate determination. Furthermore, it was found that the cell wall could play a critical role in the determination of cell fate (Berger *et al.* 1994). When the whole basal cell of a two-celled embryo was destroyed by laser beam, the apical cell could only develop into an embryo proper. In contrast, if the basal cell protoplast was destroyed but the cell wall remained, both embryo proper and suspensor were developed. Even if only part of the basal cell wall remained, one of the embryo proper cells, which was adjacent to the remaining cell wall could develop into a suspensor. The experiment indicates that positional information that regulates cell fate might act in the cell wall.

Although previous studies have reported differences between zygote and microspore embryogenesis systems, androgenesis can be used as a model system for exploring developmental events. Suspensor formation in MD embryos has been previously reported (Pechan *et al*. 1991; Rahman 1993; Yeung *et al*. 1996; Ilic-Grubor 1998). Based on studies of microspore embryogenesis in several species, Touraev *et al*. (1997) emphasized that stress in various forms, rather than the first symmetric division, was the key factor in determining embryonic cell fate. Ilic-Grubor (1998) proposed that, upon heat shock, the first division in embryonic microspores, although apparently structurally symmetrical, actually formed two cells with different developmental fates, one giving rise to the embryo proper, and the other dividing to produce the suspensor. It seems cell fate determination may be uncoupled from asymmetric cell division, but further studies are needed.

As depicted above, both symmetric and asymmetric divisions can be observed in exine-dehisced microspore cultures. Both division patterns can result in exine-dehisced microspore embryogenesis, giving rise to an embryo with a suspensor **(Fig. 1E-F)**. Immunochemical analysis of MD embryogenesis with a suspensor revealed that there is differential AGP expression (as recognized by monoclonal antibody JIM 8 and Jim 13) between the embryo proper and the suspensor. Jim 8 signal mainly appeared on the embryo proper, but the Jim 13 signal concentrated on the suspensor (Tang *et al*. 2006). This indicates that, although the cells forming the embryo proper or suspensor may derive from symmetric or asymmetric cell division, they have similar characteristics, apart from morphology. In this case, the exine-dehisced microspore can provide a unique experimental system for studying cell fate determination in relation to cell division patterns and is comparable to the zygote embryogenesis system. By selecting both symmetrically and asymmetrically divided exine-dehisced microspores and individually tracing their developmental fate in the same culture conditions, the issue can be examined. Exine-dehisced microspores are much easier to collect than zygotes, and their microculture technique is well established. This model system could also be used to study how the cell division pattern influences cell developmental fate. In addition, this system offers an opportunity for testing the potential role of cell-cell interactions in determining cell fate, given that a two-celled proembryo derived from exine-dehisced microspores can be separated and cultured in the same conditions. Furthermore, laser ablation techniques in combination with the system may provide insight into the mechanism of apical-basal cell interactions (Bouget *et al*. 1998).

Axis formation

In higher plants, the mechanism by which egg cell polarity is translated into the embryo axis has remained elusive (Weijer and Jürgens 2005). In the Fucus zygotic embryo, body axis development involves two processes, axis selection and axis amplification. The polar secretion of Golgi-derived vesicles is required for polar fixation (Kropf *et al*. 1999; Belanger and Quatrano 2000a). The importance of localized secretion in generating intracellular asymmetry has also been reported in plants seeds (Scheres and Benfey 1999; Vroemen *et al*. 1999). Evidence indicates that the establishment of the apical-basal axis of an embryo involves the accumulation of auxins at specific position in early zygotic embryogenesis in higher plants (Jürgens 2001; Friml *et al*. 2003; Weijers and Jürgens 2005). However, the possible role of egg and zygote polarity in apical-basal axis selection in globular embryos is still unknown. During androgenesis, the establishment of an apical-basal axis takes place from the globular stage onward (Hause *et al*. 1994; Maraschin *et al*. 2003). Embryo axis development also involves auxins (Hay *et al*. 2000, 2002), although there is no evidence for any influence of microspore polarity. Following dehiscing of the exine, however, the microspores become elongated and form a long growth axis, created by exine rupture. Alignment of the growth axis is consistent with cell polarity and the future body axis (unpublished data), emphasizing the relationship between cell polarity and growth axis selection. Why the ruptured exine may regulate the orientation of the growing microspore and finally confine the apical-basal axis of embryo development is an interesting question and raises the issue of the role of microspore cell wall in androgenesis.

It is possible that components in the exine may serve as signals to regulate the embryo body axis. It may be that mechanical pressure from the ruptured exine on the growing cells directs the orientation of the apical-basal axis of embryos. Whatever the mechanism, the exine-dehisced microspore system, as compared with the typical intact microspore androgenesis system, offers a model system for gaining insight into the process.

CONCLUDING REMARKS

Recently, the early events of embryogenesis have attracted much attention from developmental biologists. Although some systems, such as *in vitro* zygotic embryogenesis, somatic embryogenesis, and microspore embryogenesis, have been established for studying developmental mechanisms, it is still difficult to apply these systems to the study of early embryo developmental events. The exine-dehisced *Brassica* microspore system provides a novel and useful alternative model for studying these early events. As a single-cell developmental system, the exine-dehisced microspore shares all the advantages of the traditional androgenesis system and offers a unique chance to examine the role of polarity, asymmetric division, and the cell wall in cell fate determination and apical-basal axis selection in embryos. As increasing data on the molecular mechanisms of embryogenesis become available for *Arabidopsis*, a close relative of *Brassica*, the exine-dehisced microspore system could be

more useful in related studies mentioned above.

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