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Morphology and Protein Pattern During Microspore-derived Embryogenesis of *Brassica napus*

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유채 소포자 유래 배의 발달과정에 따른 형태와 단백질 양상

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ABSTRACT

Developmental pattern of *in vitro* embryogenesis from microspore of *Brassica napus* L. cv. Topas was studied by histological analysis and two dimensional gel electrophoresis of protein. Developmental stage of responsive microspore for embryo induction was determined after staining the cells with 4,6-diamidino-2-phenylindole (DAPI). Initial viability of the microspores was 63.9% and these different viabilities of microspores resulted in microspore dimorphism in the early stage of culture. The first cell division switching to the embryogenic pathway took place to form two identical daughter cells inside the pollen wall followed by suspensor development. Rapid embryo development from globular to torpedo-shape was accompanied with various tissue differentiations. For two dimensional gel electrophoresis, 5 phases of the microspore embryogenesis were recognized as follows; 1) freshly isolated microspores, 2) 3-day cultured cells, 3) globular-/heart-shaped embryos, 4) torpedo-shaped embryos, and 5) mature cotyledonous embryos after 21-day culture. Twenty-three proteins disappeared in the early stage of embryogenesis. In 3-day cultured cells, eight proteins were induced specifically or lastingly. Forty-two proteins in the range of 20~50 kD were induced rapidly or progressively in the course of the later embryo development.

INTRODUCTION

Many attempts have been made to characterize the cellular and biochemical changes occurring in the course of plant embryogenesis (Meinke, 1991). For many years emphasis was restricted on comparative morphology and cytochemical analysis using electron microscopy. The fact that manipulation of zygotic embryos is limited by the presence of surrounding maternal tissues made it difficult to elucidate detailed mechanism of embryogenesis. Attention, then, shifted to experimental studies of somatic embryogenesis. Morphological and biochemcal similarities between somatic and zygotic embryos, particularly in later development, were reported (Perez-Grau and Goldberg, 1989; Sterk *et al.*, 1991). In the very early stage of embryo development, however, significant differences existed between embryos derived from somatic cells and those from gamete fusion. Furthermore, in suspension culture, it was difficult to isolate early stage of somtic embryos from undifferentiated cell clusters. So recent advances in molecular biology have not contributed much to studies on nonzygotic embryogenesis as a model of totipotency.

When cultured *in vitro*, microspore can be diverted from the normal developmental pathway to undergo repeated divisions which lead to the formation of embryo-like structure. Since this phenomenon was first reported in *Datura* (Blakeslee *et al.*, 1922), great interest has developed in anther culture and microspore culture to produce haploid and homozygous diploid plants for plant

breeding purposes (Sangwan and Sangwan-Norreel, 1990). Especially microspore culture system in *Brassica napus*, since first report (Lichter, 1982), has improved continuously (Chuong and Beversdorf, 1985; Pechan and Keller, 1988; Polsoni *et al.*, 1988; Kott and Beversdorf, 1990), now recognized as a model system in microspore embryogensis. Using this system as a tool for biochemical studies on early embryogenesis, many limitations previously placed on zygotic embryos or somatic embryos have been overcome (Pechan *et al.*, 1991).

Proper stage of microspore development for undergoing embryogenesis was determined (Pechan and Keller, 1988), and underlying cytochemical changes in this conversion were studied using light and electron microscope (Zaki and Dickinson, 1990). But consistent model for sporophytic pathway of microspore has not been suggested. Although, recently, a number of mRNA specifically induced during the commitment phase of microspore embryogenesis were identified (Pechan *et al.*, 1991), no more data have been presented afterwards.

The purpose of this work is to characterize cell division patterns and tissue differentiation during embryo development, and to analyze total protein patterns correlated to the morphological development. Ultimately this study should provide us systemic recognition of microspore-derived embryogenesis and identification of corresponding change of proteins which are developmentally regulated.

MATERIALS AND METHODS

Plant materials and microspore culture. Seeds of *Brassica napus* L. cv. Topas (kindly supplied by Dr. Bang, Rural Development Administration) were sown in sterilized filter paper under dark at 27℃. Once established the plants were transferred to pots and grown in a conditioned environment growthroom under 16-h photoperiod (approximately 150 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 27℃ for 3 weeks and subsequently transferred to 24-h photoperiod (300 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 12℃. Isolation and culture of microspores mostly followed the procedure outlined by Polsoni *et al.* (1988). Microspores were plated at a density of 2×10^4 microspores/mL and distributed to 60×15 mm petridish as 4 mL aliquot.

Plant regeneration. After 3-week culture normal-shaped, mature embryos were selected and air-dried to 40~60% moisture content. Alternatively, final concentration of 25 μM ABA was added to 3 week-cultured suspension and incubated for 1 more week. The embryos were then air-dried to below 10% moisture content. Dry embryo were transferred to petri plates containing solid B5 medium (1% sucrose) with 0.1 mg/mL GA to test survival. The embryos were incubated at 27℃ with 16 h photoperiod. After 21 days of the desiccation treatment, embryos producing both roots and shoots were considered as having converted into plants.

Viability assay and nuclear observation. For determining viability of microspores, 2 μL of fluorecein diacetate (FDA) stock solution (2 mg/mL in acetone) was diluted in 1 mL microspore culture medium. A small aliquot of microspore suspension was mixed with a drop of FDA diluted medium. The reaction was allowed to proceed for 10 min. Cells were examined by Nikon Epifluorescence microscope (B2 filter, main wavelength 495 nm). For identifying nuclei, microspores and developing embryos were stained for 10~20 min with 1 μg/mL of DAPI in 1% Triton X-100. The material was examined by Nikon Epifluorescence microscope (UV-2 filter, main wavelength 365 nm).

Anatomical analysis. Plant material was fixed in 5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.0) overnight, post-fixed in 1% osmium tetroxide overnight, dehydrated in graded ethanol series and transferred into low viscosity Spurr resin (Spurr, 1969). Semi-thick sections (1 μm) of resin embedded material were stained with 1% methylene blue. Alternatively large, mature embryo was fixed in FAA (formalin : glacial acetic acid : ethanol : water=2 : 1 : 10 : 7), and embedded in paraffin. Thick-sections (10 μm) were stained with hematoxylin.

Protein extraction and gel electrophoresis. Soluble protein extraction followed the procedure of Hurkman and Tanaka (1986). To synchronize early developmental stage of embryos, 7~15-day suspension culture solution was sieved sequentially through a 375 μm and a 43 μm mesh. Early globular/heart-shaped embryos were collected on the 43 μm mesh. Torpedo-shaped embryos were collected on the 395 μm mesh after 15~18-day culture. Mature cotyledonous embryo was hand-selected under stereomicroscope after 21-day culture.

Two dimensional gel electrophoresis was done according to O'Farrell (1985) with modifications. The isoelectric focusing (IEF) rod gels [9 M urea, 2% NP-40, 5% ampholyte (4% 5~8, 1% 3~10)] were 12 cm long and had a diameter of 1.6 mm. After prerunning, isoelectric focusing of equivalent of 200 μg BSA were conducted for 15 h at 400 V plus 1 h at 800 V. Following focusing, the pH gradient of a gel was measured by the method of Bollag and Edelstein (1991). Ten percent polyacryla-

mide gels were used for the second dimension. Two-dimensional gels were fixed and silver stained by method of Heukeshoven and Dernick (1985) with an exception that concentration of sodium bicarbonate was reduced to half.

RESULTS

Microspore culture and plant regeneration. Microspores from buds, 2.7~3.3 mm in length having single marginally located nuclei were in uninucleate stage (Fig. 1A). So 3~5 buds in this length (Fig. 1B) were harvested from each racemy and microspores for culture were isolated from them.

After 10-day culture, many early embryos were identified by naked eye. After 21-day culture, most embryos developed to mature cotyledonous stage. Conversion test was done after ABA and/or drying treatment on 21-day old embryos (Table 1, Fig. 2). Partial desiccation (40~60 % water content) resulted in 90.1% of whole plant conversion rate and showed little difference between shoots and roots production rates. In case of drying to less than 10% water content, ABA pretreatment had significant effect on regeneration efficiency.

Anatomical analysis of microspore embryogenesis. We could not observe pollen dimorphism in freshly isolated microspores (Fig. 3A). Initial viability of freshly isolated microspores was 63.9% in determination with FDA staining. This indicated that quite a number of microspores had been dead before isolation. Microspore dimorphism appearing after 30 min *in vitro* culture (Fig. 3B) was caused by different viability of each microspore, which was recognized by FDA staining (Fig. 3C). Dead microspore did not swell in *in vitro* condition. Conseque-

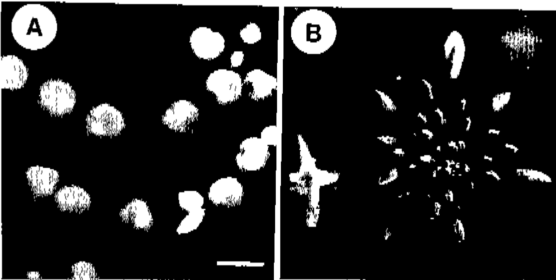

Fig. 1. Development of microspores *in vivo* (A) with terminally differentiated inflorescence (B) of *B. napus*. A, late uninucleate microspores found in bud of 3.2 mm; B, arrows indicate position and relative size of buds used for microspore isolation. Bar = 25 μm.

Table 1. Effect of ABA and air drying as pretreatment conditions on plant regeneration from *B. napus* cv. Topas microspore embryos[a]

	Water content[b] (%)	Shoot recovered (%)	Root recovered (%)	Plant conversion (%)
Partial drying*	40~60	90.1	91.9	90.1
ABA[c]+Drying**	<10	18.2	93.7	18.2
Control***	<10	0	0	0

[a]Normal shaped, 21-day old embryos (n = 161*, 444**, 307***), [b][(dry wt − final dry wt)×100]/fr wt, [c]25 μM ABA pretreated for 7 days.

Fig. 2. Plant regeneration from microspore-derived embryos. Plantlet regenerated after partial desiccation.

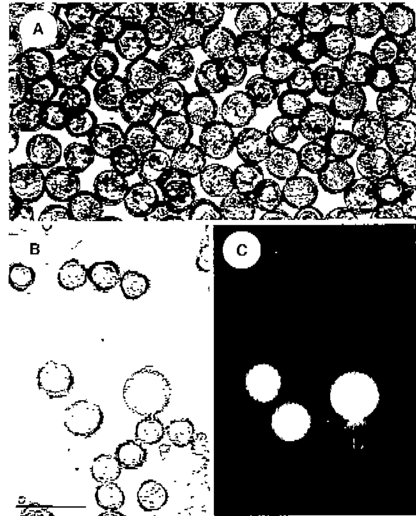

Fig. 3. Initial morphology of microspores in *in vitro* culture. A, freshly isolated microspores; B, microspores observed after 30 min. culture; C, the same phase as B, but stained with FDA, observed under blue light illumination. Bar = 50 μm.

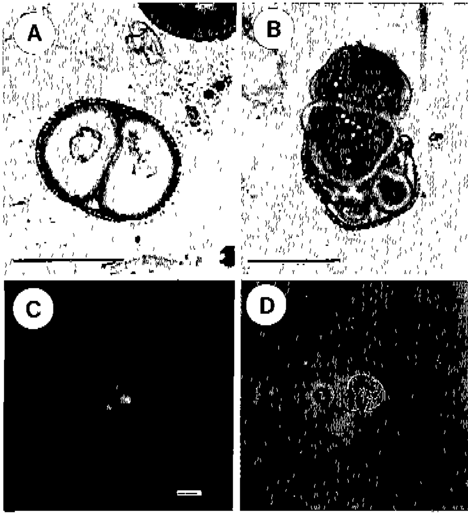

Fig. 4. Early stage of cell divisions after 3-day culture. A, first division of microspore inside pollen wall; B, early cell clusters bursting out pollen wall; C, two cell cluster bursting out pollen wall stained with DAPI under UV illumination; D, the same phase as C, but observed under bright field. Bar=25 μm.

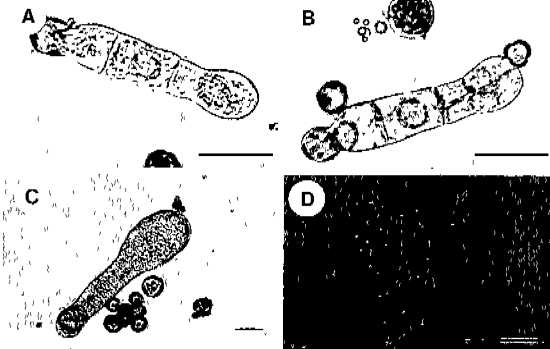

Fig. 5. Early development of embryos. A, suspensor resulted from unidirectional cell division from microspore; B, initial development of embryo by perpendicular cell division to suspensor cells; C, globular-shaped embryo with suspensor; D, semi-thick (1 μm) sectioned globular-shaped embryo without suspensor, observed by phase contrast microscopy, arrows indicate pollen wall debris. Bar=50 μm.

ntly, different viabilities of microspores caused pollen dimorphism and resulted in different embryogenic potentials.

Although the first cell division was reported to initiate in 8 h culture, we chose 3-day cultured cells to analyze initial cell division patterns because more cells participated in division within 3-day culture. The first cell division took place to form two identical daughter cells inside the pollen wall (Fig. 4A). Then cell cluster protruded pollen wall by unidirectional division and cells which remain inside pollen wall were degenereated (Fig. 4B, C, D). Unidirectional cell divisions resulted in suspensor-like structure (Fig. 5A). Perpendicular cell division plate to suspensor-like structure was led to initiation of globular shaped embryo development (Fig. 5B, C). However, a few embryos were not preceded by suspensor-like development, but held direct embryo development inside pollen wall (Fig. 5D). Early stage of embryo already showed protodermal layer (Fig. 5D), and accelerated cell divisions resulted in the formation of globular embryo in more

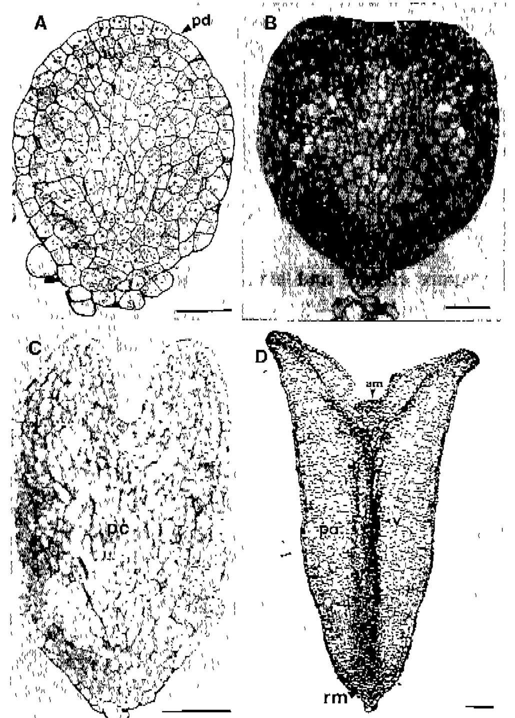

Fig. 6. Histological observation of embryo development. A, globular-shaped embryo showing protoderm, meristematic cells and longitudinal elongation; B, heart-shaped embryo showing cotyledonous differentiation; C, torpedo-shaped embryo; D, mature cotyledonous embryo after 21-day culture, proceeded by paraffin embedding method, stained with hematoxylin. am, apical meristem; pd, protoderm; rm, root meristem; v, vascular bundle; pc, procambium; pa, parenchyma cells. Bar in A, B=50 μm, Bar in C, D=100 μm.

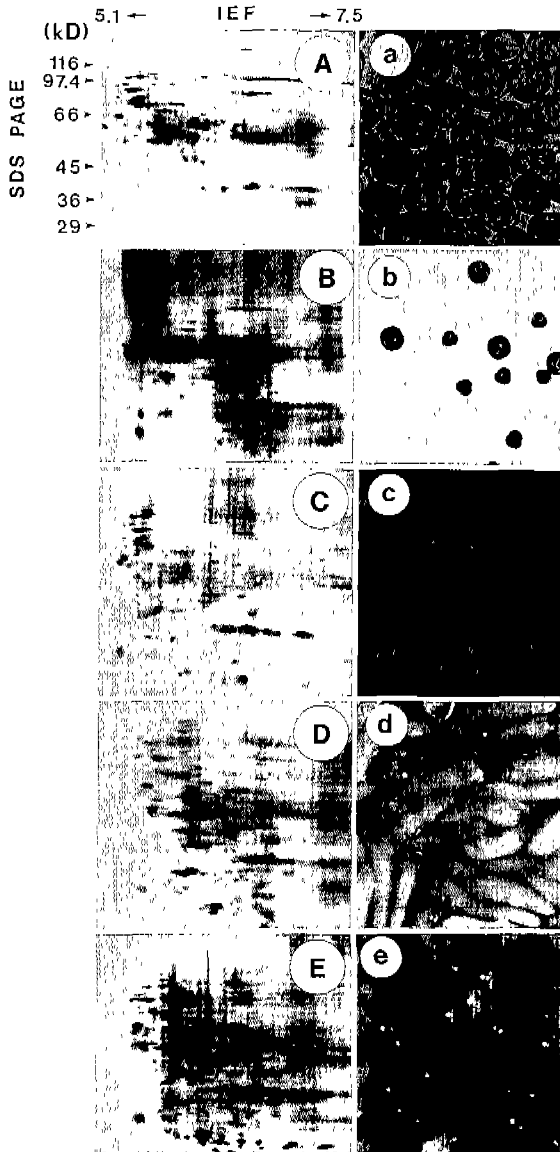

Fig. 7. Representative 2D gels of total protein extracted from 5 stages of microspore-derived embryo development. A, a, freshly isolated late uninucleate microspore; B, b, 3-day cultured cells; C, c, early globular/heart stage embryos; D, d, torpedo stage embryos; E, e, mature cotyledonous embryos. Eqivalents of 200 μg of BSA were fractionated.

than 100 μm length in 7-day culture. Globular embryo showed ordered differentiation of various tissues like protoderm and procambium (Fig. 6A), and were more densely packed than amorphous cell cluster which appeared on rare occasions. As globular embryo developed into heart-shaped stage, cell elongation and longitudinal cell divisions contributed to polar elongation of embryo (Fig. 6A,B). Cotyledon, protoderm, parenchymatous layer, apical/root meristem and procambial strand differentiated rapidly in the course of torpedo-shaped embryo development (Fig. 6C). Distinct tissue differentiation was observed in 21-day cultured cotyledonous mature embryo (Fig. 6D).

Protein pattern analysis. In an attempt to determine protein patterns (Fig. 7A~E) accompanied with morphological change, embryo development from microspore was distinguished into 5 phases as freshly isolated microspores, 3 day-cultured cells, globular/heart-shaped embryos, torpedo-shaped embryos, and mature cotyledonous embryos after 21-day culture (Fig. 7A~E). Two dimensional gel electrophoresis enabled us to analysis total 185 proteins in the range of MW 20~120 kD and pH 5.1~7.5. One hundred and seventeen proteins showed constitutive expressional pattern. Twenty-three microspore-specific proteins slowly decreased in early stage of culture. They lasted to 3-day culture and completely disappeared in globular/heart-shaped embryos. A protein pattern formed in torpedo-shaped embryos showed a little change during late embryo development.

Six microspore-specific proteins in the range of 50~66 kD rapidly disapeared in 3-day culture (Fig. 8A). An 150 kD protein, 15 proteins in the range of 50~66 kD, and a 40 kD protein which slowly disappeared in globular/heart-shaped embryos development also showed the correlation to microspore developmental pathway (Fig. 8A). A 45 kD protein and two proteins about 60 kD were expressed only in 3-day cultured cells (Fig. 8B). Four proteins about 45 kD and a 30 kD expressing constitutively from 3-day culture (Fig. 8B) showed the embryogenic potentials in 3-day cultured cells.

A number of proteins in the range of 20~50 kD appeared during globular/heart-shaped embryo development (Fig. 8C) and showed constitutive or strengthened expressions in the late embryo development. There were little difference in protein pattern of torpedo-shaped embryos and that of mature cotyledonous embryos except that three proteins about 40 kD markedly expressed in torpedo-shaped embryos (Fig. 9A) and three proteins about 50 kD and a 27 kD in cotyledonous embryos (Fig. 9B).

DISCUSSION

Despite of its advantages, microspore culture of *B. na-*

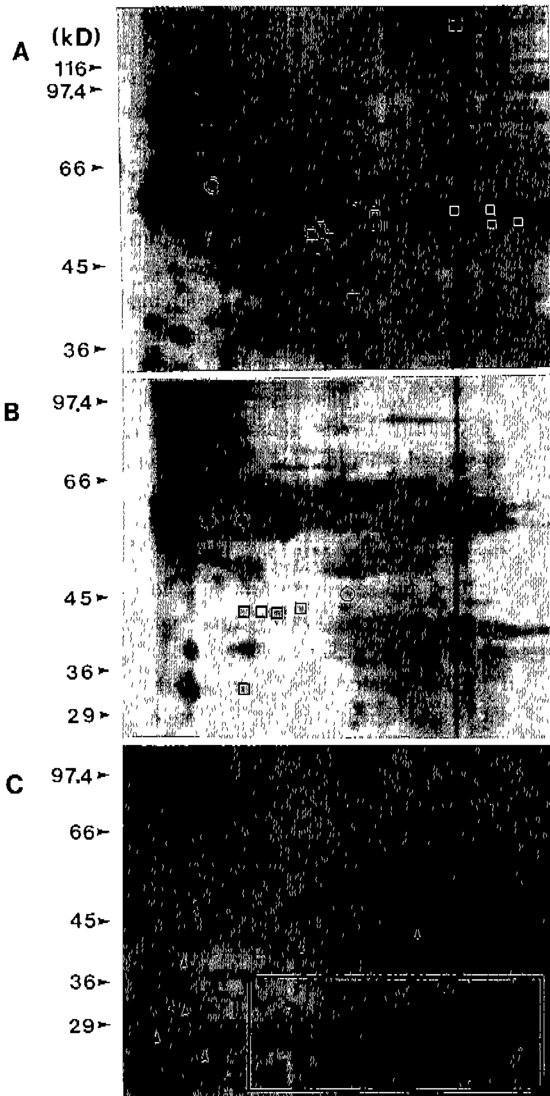

Fig. 8. Protein patterns during early embryo development. A, freshly isolated uninucleate microspore, circle indicates microspore specific protein disappeared in 3-day cultured cells, square indicate microspore specific protein lasted to 3-day cultured cell; B, 3-day cultured cells, circle indicate 3-day cultured cell specific protein, square indicate embryogenic protein existed to all stage of embryo development; C, early globular/heart stage proembryo. Arrows and box indicate embryogenic proteins not detected in previous stage.

pus is not widely used for biochemical purpose. The reason is that tissue culture technique for microspore-derived embryogenesis is still more difficult than that of so-

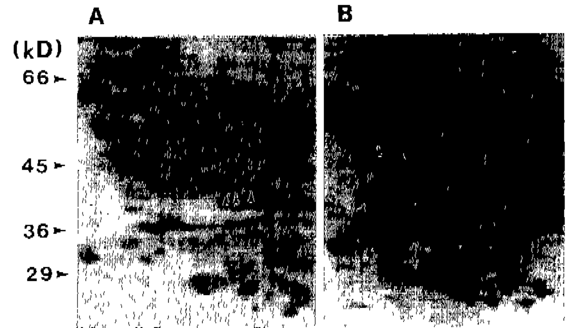

Fig. 9. Protein patterns during late embryo development. A, torpedo stage embryo; B, mature cotyledonous embryo after 21-day culture. Arrow indicates protein showing strengthened expression from corresponding stage.

matic embryognensis, that infeasibility of long term suspension culture of embryogenic cell line requires continuous cultivation of mother plants, and that well-controlled environment for mother plant growth is required. In culture of *B. napus* microspore, optimization of mother plant growth condition has more fundamental importance than selection of embryogenic microspore by pollen dimorphism (Heberle-Bors, 1985) as we recognized that feasibility of microspore division leading to embryogenesis was based on microspore viability (Fig. 3B, C) which was correlated to mother plant growth condition.

In case of *B. napus,* both late uninucleate stage and early binucleate stage are responsive for embryo induction (Pechan and Keller, 1988). However we used only late uninucleate microspore because it was difficult to recognize cell division patterns in binucleate state where only reproductive nucleus could divide for embryogenesis (Zaki and Dickinson, 1990).

In the early cell divsions, embryogenic development following suspensor formation resembled zygotic embryogenesis. But there were no typical patterns in embryogenic development, for example, proembryo formation without suspensor development (Fig. 5D) or great variation with regard to the number of component cells in suspensor. It has been suggested that the suspensor anchors the embryo to the embryo sac and pushes it deep into the endosperm so that the embryo lies in a nutritionally favorable environment (Bhojwani and Bhatnagar, 1979; Yeung, 1980). In recent *in vitro* culture of zygotic embryo, early embryo with suspensor removed efficiently developed into mature embryo (Liu *et al.*, 1993). These reports supported that suspensor be more important in the initiation of embryo than in embryo development.

In our experiment embryo development was preceded by suspensor development (Fig. 5B) and initiated in any position of suspensor layer, which forced us to suppose that all the component cells of suspensor have totipotency. As exact cell division pattern was reported in early zygotic embryogenesis (Bhojwani and Bhatnagar, 1979), embryo induction from single cell like microspore in liquid culture has an advantage in pursuing early cell division pattern. But there has been no reports explaining consistantly about cell division and differentiation pattern in the course of embryogenesis from microspore. We could neither suggest exact model for microspore embryogenesis.

Biochemical studies using somatic embryogensis system have difficulties in synchronizing embryos according to morphologically defined developmental stage. Using homogeneous late uninucleate microspores we could overcome synchronization problem in early state of cell division. To acquire synchronized globular/heart shaped embryos, two kinds of sieve which had different pore size were used and mostly globular embryos (mixed with some heart-shaped embryos) could be isolated (Fig. 4C).

Many embryo-specific genes recognized in the developmental procedure of somatic embryogenesis were turn out to be expressed in embryogenic mass (PEM) in auxin-added media, which intimated PEM already had embryogenic potential (Dayton *et al.*, 1988). Likewise, 5 proteins expressed from 3-day cultured cells proceeding suspensor-like development proved that 3-day cultured cells had obtained embryogenic potential. In culture of *B. napus* microspore, plant hormone were not used, but only high temperature culture condition was employed for switching to embryogenic pathway. In addition, comparing embryos to microspores lying in quite different developmental pathway, we could analyze protein pattern related to totipotency or embryo specificity more efficiently than in the case of somatic embryogenesis using 2,4-D (Sung and Okimoto, 1981).

Changes of protein patterns during embryo development from globular/heart- to torpedo-stage were more striking than during torpdedo to cotyledonous stage (Figs. 8C, 9). This result was coincident with anatomical analysis that tissue differentiation was rapidly achieved during the development of torpedo-shape embryo (Fig. 6). Consequently, changes of protein patterns during embryo development were significantly correlated to morphological differentiation.

In this study we confirmed superiority of microspore culture system showing high efficient embryogenesis from homologous microspores as a tools for biochemical studies of plant embryogenesis, and understood cell division and differentiation pattern in the course of early embryogenesis from the microspore. We also compared changes of protein patterns to morphological differentiation during the embryogenesis.

적 요

유채(*Brassica napus* L. cv. Topas) 소포자 배양에 의한 기내 배발생 과정을 관찰하고 발생과정에 따른 총단백질의 변화 양상을 이차원 전기영동을 통하여 분석하였다. 배양에 적합한 소포자의 발달 단계는 4,6-diamidino-2-phenylindole (DAPI) 형광염색으로 핵상관찰을 통하여 결정하였다. 최초의 소포자 생존율은 63.9%였으며 이러한 생존율의 차이로 배양초기에 소포자의 이형성이 나타났다. 배 발달 경로에 따른 최초의 세포분열은 화분벽 안에서 균등분열로 시작되어 배병의 발달이 선행된 후 배 발생이 일어났으며, 구형, 심장형, 어뢰형으로의 배 발달은 여러 조직의 분화와 더불어 빠르게 진행되었다. 소포자배 발달과정을 치상단계의 소포자, 배양 3일째의 초기 분열세포, 구형 및 심장형배, 어뢰형배, 성숙한 자엽단계의 배 등의 5단계로 나누어 각각 2차원 전기영동을 수행한 결과, 최초 소포자 단계에서 나타났던 23개의 단백질들은 배 발달 경로로 진행됨에 따라 사라지고, 배양 3일째에는 8개의 단백질이 특이적 또는 지속적으로 발현되었다. 배의 발달과 더불어 20~50 kD 사이에서 총 42개의 단백질이 급격히 나타나거나 또는 후기배로 진행하면서 점차적으로 발현되었다.

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