REGULATION OF THE INDUCTIVE PHASE OF MICROSPORE EMBRYOGENESIS IN *BRASSICA NAPUS*

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Abstract

In cultured microspores from *Brassica napus*, embryogenesis can be synchronously and irreversibly induced by elevating the culture temperature to 32°C for a minimum of 8 h. Culture at 18°C allows gametophytic development to continue, and results in the formation of pollen in vitro. This allows us to study the temperature controlled switch in developmental fate from gametophytic development to embryogenic development by molecular means.
Analysis of protein synthetic patterns by [^{35}S]-methionine incorporation and 2-dimensional gel electrophoresis, revealed that 25 proteins were differentially synthesized during the induction of microspore embryogenesis. Most of these proteins (17) appeared to belong to the class of heat shock proteins (HSPs). Four of these HSPs have been identified by Western blotting using antibodies raised against HSP17, HSP68 and HSP70. One protein that was only synthesized under embryogenic culture conditions, and did not belong to the heat inducible HSPs, is a candidate marker for early embryogenic development.

1. Introduction

Genetic and molecular studies have recently made important strides towards understanding of the development of angiosperm embryos (Goldberg et al., 1994). The advances mainly concern development in the already existing embryo, such as pattern formation (Mayer et al., 1991, 1993), and region- and organ-specific gene expression (Perez-Grau and Goldberg, 1989; Goldberg et al., 1994; De Jong et al., 1993). Due to the inaccessibility of the egg cell in angiosperms, progress has been far more limited in our understanding of the developmental events that take place during fertilization and early embryogenesis in vivo. Only very recently have these processes come under experimental control in vitro (Kranz and Lörz, 1993; Faure et al., 1994). We use *Brassica napus* microspore embryogenesis as a model for studying the initiation of embryogenic development in general. Understanding these events will have wide ranging implications to plant breeders, ranging from plant regeneration from microspores or pollen in species recalcitrant in haploid plant production, to production of artificial seeds and induced apomixis.

In the 1980s, it was demonstrated that high embryo yields could be obtained by culturing isolated microspores and pollen from *B. napus* in simple, hormone free media, without going through an intermediate callus phase (Chuong and Beversdorf, 1985; Keller et al., 1987). Under optimal conditions, up to 70% of the isolated cells can participate in embryogenic development (Pechan and Keller, 1988). Microspore embryogenesis in *B. napus* is routinely initiated by a high temperature (30°-32.5°C) stress treatment for two or more days (Gland et al., 1988; Polsoni et al., 1988; Telmer et al., 1993), but as stated by Pechan et al. (1991), eight hours of culture at 32°C is sufficient to synchronously and irreversibly induce the microspores to enter embryogenic development. As large quantities of microspores and pollen can easily be obtained for

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culture (about 100,000/flower bud), we adopted *B. napus* microspore culture as a model for our study.

A limiting factor for studying the induction of embryogenic development in *B. napus* microspores, was the lack of a good non-embryogenic control. Culture at 25°C, which was previously used as non-embryogenic culture condition (Pechan et al., 1991), yielded appreciable numbers of embryos in our hands. This led us to study lower culture temperatures, in order to find a more suitable non-embryogenic control. Here, we present cytological and morphological data of the development of microspores cultured at 18°, 25° and 32°C. We found that culture at 18°C allows the microspores to continue gametophytic development in vitro, and mature into pollen. This finding has allowed us to bring the induction of microspore embryogenesis under simple experimental control.

Making use of this ability to control the switch in developmental fate between gametophytic development (18°C culture) and embryogenic development (32°C culture) we set out to find differentially synthesized proteins that may serve as marker proteins for the induction of embryogenesis. Here, we describe 25 proteins that were differentially synthesized during the 8 h 32°C inductive heat treatment. We tried to determine whether these 25 proteins were differentially synthesized as a result of the increased culture temperature, and belonged to the family of heat shock proteins (HSPs), or whether they were differentially synthesized as a result of entry into embryogenic development. Differentially synthesized proteins that likely were HSPs, were further identified and analysed by Western-blotting using anti-HSP antibodies.

2. Material and methods

Plant material. Plants of *Brassica napus* L. cv Topas (seeds from doubled haploid line DH4079, kindly provided by Dr. P.M. Pechan, Max Planck Inst., München, Germany) were raised all year round in a phytotron room at 18°C under 16-h photoperiod of 150μmol.m^{-2}.s^{-1} HPI-T (Philips) light.

Microspore culture. Flower buds, 3.3-3.5 mm in length containing late unicellular microspores to early bicellular pollen, were collected and microspores were isolated as described by Duijs et al. (1992). They were cultured, at a density of 40,000 microspores.ml^{-1} in NLN-13 medium (Lichter, 1982), at 18°, 25° and 32°C continuously. Early development in culture was studied by using the DNA-specific fluorochrome DAPI. It was examined whether microspores/pollen developed sporophytically (beginning of the embryogenic development) or gametophytically under the different culture conditions (see Custers et al. (1994), for detailed criteria).

Analysis of protein synthetic pattern. Changes in protein synthesis associated with the 8 h 32°C inductive treatment were determined by using in situ [^{35}S]-methionine labeling, followed by 2-D gel electrophoretic analysis of the radiolabeled proteins (see Cordewener et al. (1994), for details). For this study, microspore cultures were kept at either 18°C (non-embryogenic condition) or 32°C (embryogenic condition). Qualitative and quantitative differences between the incorporation of [^{35}S]-methionine into individual polypeptides on 2-D gels from 18° and 32°C cultures were determined by using computer aided analysis of PhosphorImager data (Cordewener et al., 1994). Results from five independent microspore cultures were collected to allow statistical analysis of the differences and were used to build a database of the [^{35}S]-methionine labeled proteins.

Identification of heat shock proteins (HSPs). Freshly isolated microspores were cultured for 2 h at a sub-lethal, non-embryogenic temperature of 42°C in the presence of [^{35}S]-methionine; conditions under which heat shock proteins (HSPs) are the only proteins synthesized. Newly synthesized HSPs were analysed on 2-D gel as described previously (Cordewener et al., 1994). This protein synthetic pattern was matched to the database of [^{35}S]-methionine labeled proteins detected under embryogenic culture conditions (32°C). Further identification of the putative HSPs was performed by Western

blotting, using anti-tomato HSP17, HSP68 and HSP70 polyclonal antibodies (kindly provided by Dr. D. Neumann (Neumann et al., 1987; Neumann et al., 1993)), and anti-human HSP72/73 monoclonal antibody from StressGen (clone N27F3-4), as described previously (Cordewener et al., 1995).

3. Results

3.1. Refinement of the model

Microspore populations with 40-80% unicellular microspores and the remainder early bicellular pollen, were used for the analysis of sporophytic and gametophytic development under the 18°, 25° and 32°C culture conditions. The most characteristic differences were found between cultures at 18° and 32°C (Fig. 1). No developmental changes could be observed after 8 h of culture. This period has been reported to be sufficient to cause an irreversible commitment to entry into embryogenic development (Pechan et al., 1991). After a 24 h 32°C treatment clear differences could be observed. Microspores that had been in the late unicellular stage at the start of the culture had entered sporophytic development, characterized by a symmetric cell division, resulting in two large vegetative-like nuclei and an absence of chromosome condensation in the daughter cells. Similar microspores cultured at 18°C had undergone an asymmetric cell division, yielding one large cell with decondensed chromatin and a small cell with condensed chromatin, indicative for gametophytic development. In the early bicellular pollen, the 32°C embryogenic treatment induced a symmetric cell division of the large vegetative cell, as first morphological sign of the embryogenic development. Early bicellular pollen cultured at 18°C, never showed divisions of the vegetative cell. After 24 h under these conditions, the generative cell had moved into the cytoplasm of the vegetative cell in preparation for its division into two sperm cells (second pollen mitosis), but that stage had not been reached after 24 h of culture.

Table 1 shows the results of a representative microspore population after 48 h in culture, when the developmental fate of almost all microspores can be recognized and quantified. Very rarely a sporophytic pattern of division was scored (1%) in the 18°C cultures, but these events may not represent 'real' sporophytic development, as continued 'sporophytic' divisions were never observed. Large proportions of the microspores had divided sporophytically both at 25° and 32°C. Sporophytic microspores with up to four or six nuclei were already found after 48 h of culture. In contrast, at 18°C almost all microspores showed gametophytic development (up to the mid bicellular pollen stage), which significantly decreased when temperature was increased to 32°C. However, a sizable proportion of microspores with gametophytic characteristics could still be detected at 32°C.

After longer times in culture, striking morphological differences were observed at the different temperatures. After five to seven days of culture at 25°C and 32°C, globular embryos appeared (Fig. 2A), that developed into torpedo-shaped embryos 10 days later. Embryo yield at 32°C was much higher than the yield at 25°C, and no embryos were produced in the 18°C cultures (Table 2). In contrast, at 18°C, numerous oval bodies developed that resembled pollen after five days of culture (Fig. 2B). Application of the DNA stain DAPI to these oval bodies showed that they were pollen, all containing a large, decondensed vegetative nucleus accompanied by two highly condensed sperm nuclei (Fig. 2C). In 25°C cultures, pollen could also be observed, but numbers were low (Table 2). These pollen disappeared by collapsing on day 5 or 6 of culture. In 18°C cultures, the number of pollen increased till day 7 or 8 of culture, and then gradually disappeared on day 9 and 10 by a type of pollen germination. The pollen burst and formed large blisters 75-100μm in diameter, and rarely real pollen tubes were seen.

3.2. Changes in protein synthesis

The analysis of protein synthesis was performed by labeling 18°C and 32°C cultures in situ with [^{35}S]-methionine, for a period of 8 hours. This period was shown to be required for the irreversible commitment to embryogenic development (Pechan et al. 1991). Only data of those experiments were used that originated from cultures of which a sample cultured at 32°C yielded 5% or more embryo formation (≥2,000 embryos.ml^{-1}), and of which a sample cultured at 18°C yielded more than 60% pollen formation. On the 2-D gels a total of about 500 [^{35}S]-methionine labeled protein spots could be detected. For each spot, the amount of [^{35}S]-methionine incorporation was quantified by PhosphorImager technology, and computed relative to the total amount of radioactivity on the entire gel. Based on the data from five independent labeling experiments, 25 proteins were found with a significantly changed synthesis at 32°C as compared to 18°C (Table 3, first six columns). Six of these proteins were specifically synthesized under the 32°C embryogenic culture conditions, and were never detected under the 18°C non-embryogenic conditions. Eighteen proteins were detectable under both conditions, but synthesis at 32°C was significantly higher than that at 18°C. One protein (Table 3, no. 17) was found that showed a higher relative synthetic rate at 18°C.

3.3. Heat shock proteins

Differential synthesis of the 25 identified proteins might be due to (i) the elevated temperature, or (ii) the entry into embryogenic development. In order to separate these two classes, we studied the differentially synthesized proteins at a high, non-embryogenic temperature of 42°C. Elevation of the culture temperature to 42°C results in the sessation of normal protein synthesis, and the almost exclusive synthesis of the so called heat shock proteins or HSPs. Matching the (heat shock) protein synthetic pattern of a culture labeled with [^{35}S]-methionine for 2 h at 42°C, to the database of [^{35}S]-methionine labeled proteins, indicated that 17 of the 25 differentially synthesized proteins might be HSPs (Table 3, column 7). From six proteins that were exclusively synthesized under embryogenic culture conditions, only one (no. 13) was found not to be heat induced, and might be a potential marker for early embryogenesis (class ii).

As many of the differentially synthesized proteins appeared to be HSPs, we used 2-D immunoblotting to identify a number of them. So far we identified four of the differentially synthesized proteins to be members of the 17-kDa, 68-kDa and 70-kDa families of HSPs (Table 3, column 8).

4. Discussion

Microspore culture of *Brassica napus* produces high amounts of embryos (Chuong and Beversdorf, 1985; Pechan and Keller, 1988; Deslauriers et al., 1991), and is an excellent model system for studying the induction of the embryogenesis (Zaki and Dickinson, 1991; Pechan et al., 1991; Hause et al., 1992, 1993; Cordewener et al., 1994; Dickinson, 1994). Culture at 32°C causes the synchronous induction of sporophytic development. As a severe imperfection, however, we found that in 25°C cultures, that had standardly been used as non-embryogenic controls, significant proportions of the microspores showed sporophytic divisions, and quite high numbers of embryos were produced (Custers et al., 1994). As a solution to this, we showed 18°C culture to be a much better control. At this temperature, a high level of apparent gametophytic development was observed, leading to pollen maturation, and no embryos were formed. Through comparison of the 32°C and the 18°C cultures of *B. napus* we have now, for the first time, a convenient model for studying the molecular events causing the switch from gametophytic to sporophytic development during the first hours in culture. A further advantage is that contributions from general growth or general cell cycle related events

are eliminated, as both embryogenic and non-embryogenic cultures continue to divide (see Fig. 1).

We have also established a system for the quantitative detection of proteins that are induced or repressed during the very limited time period of 8 h 32°C, that irreversibly induces the microspores to enter embryogenesis. The *B. napus* microspore culture is one of the very few experimental systems in which such study can be done. Classical models for somatic embryogenesis, such as carrot or alfalfa cell-suspension cultures, are less appropriate for this kind of studies, as only a very small percentage of the cells (1-2%) in culture enter embryogenesis (De Vries et al., 1988; Dudits et al., 1991). Tobacco pollen culture, another model of embryogenesis from male gametophytes, is less appropriate because of the 4-8 days starvation needed and the long transition time from pollen to embryogenic cells (Vicente et al., 1992; Zarsky et al., 1992). The high percentage of isolated microspores (routinely about 40%) that become induced to embryogenesis, and the short period necessary to get irreversible commitment to sporophytic development make the *B. napus* system so excellent for biochemical and molecular research.

A series of 25 differentially synthesized proteins could be reproducibly identified to be associated with the 8 h 32°C embryogenic inductive treatment of the microspores. These changes in protein synthesis may be induced either as a consequence of the elevated temperature, or as a consequence of the induction of embryogenesis. A high percentage (almost 70%) of differentially synthesized proteins appeared to be HSPs. From the six proteins specifically synthesized under the embryogenic culture conditions, the synthesis of only one (Table 3, no. 13) may be due to the induction of embryogenesis. This protein is a candidate marker for early embryogenic development, and we are currently trying to isolate sufficient material for microsequencing.

The high number of proteins whose differential synthesis is associated with temperature elevation are also of potential interest, as they might be the cause of the primary steps of the induction process itself. Indicative of their potential role is the fact that elevation of the temperature is the only external factor that causes the switch in developmental fate from pollen development to embryogenic development. Recently, we found that specific changes in the synthesis and localization of members of the 70 kDa class of HSPs accompany the induction of embryogenesis in the *B. napus* microspores (Cordewener et al., 1995). We are currently investigating whether these HSPs are instrumental to the induction process.

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Table 1. Percentages sporophytic and gametophytic development, counted after 48 h, in *Brassica napus* microspore cultures at 18°C, 25°C and 32°C. The initial population consisted of 36% microspores and 64% pollen. Values are the means of three samples of 50 microspores from different Petri dishes.

Temperature (°C)	Sporophytic develop. (%)	Gametophytic develop. (%)
18	1 a*	84 b
25	9 b	77 b
32	38 c	46 a

* Means within a column followed by the same letter do not significantly differ at P=0.05.

Table 2. Percentages pollen and embryos, counted after 5 and 15 days respectively, in *Brassica napus* microspore cultures at 18°C, 25°C and 32°C. Data represent the range of percentages of four independent experiments with initial populations consisting of 40-80% microspores and the remainder pollen.

Tempereture (°C)	Pollen (%, after 5d)	Embryos (%, after 15d)
18	50.2 - 78.5	0.0
25	5.3 - 34.7	0.2 - 0.7
32	0.0	1.3 - 8.5

Table 3. Differentially synthesized proteins in embryogenic (8h 32°C) and non-embryogenic (8h 18°C) microspore cultures, accompanied by data on their synthesis at an elevated non-embryogenic temperature of 42°C (2h) and on their identification as heat shock proteins. Synthesis of the proteins was studied by [^{35}S]-methionine labeling, followed by 2-D gel electrophoretic analysis of the radiolabeled proteins. [^{35}S]-Methionine incorporation into the individual spots is expressed as a fraction (x10^3; ‰) of the total radioactivity measured in the 2-D gel. Data presented are the means ± standard deviation from 5 independent experiments. Protein synthesis at 42°C was examined only in one experiment, and identification of HSPs was performed by Western-blotting using antibodies raised against HSP17, HSP68 and HSP70.

Spot no.	M, (kDa)	pI	[^{35}S]-methionine incorporation(‰)		Ratio 32/18°C	Heat shock response, at 42°C	Identity
			18°C	32°C			
1	24.1	4.76	0.12 ± 0.14	1.94 ± 0.60	16.2**	++	
2	29.9	4.86	[a]	0.23 ± 0.12	-	+	
3	65.6	4.88	0.26 ± 0.11	0.55 ± 0.15	2.1**	-	
4	69.0	4.89	0.18 ± 0.05	0.39 ± 0.12	2.2**	-	
5	40.7	5.04	0.35 ± 0.08	0.84 ± 0.18	2.4**	+	
6	76.8	5.15	3.14 ± 1.42	18.9 ± 6.85	6.0**	++	HSP70
7	40.6	5.34	0.48 ± 0.16	0.99 ± 0.43	2.1*	-	
8	65.5	5.33	1.03 ± 0.25	2.63 ± 0.38	2.6**	±	
9	74.8	5.33	2.88 ± 1.42	8.61 ± 3.55	3.0*	+	HSP68
10	76.5	5.36	[a]	0.73 ± 0.51	-	+	
11	75.1	5.51	0.26 ± 0.11	0.97 ± 0.52	3.7*	++	
12	75.4	5.60	0.22 ± 0.12	0.97 ± 0.46	4.4*	++	
13	67.4	5.65	[a]	0.13 ± 0.07	-	-	
14	24.1	5.94	[a]	0.23 ± 0.22	-	++	
15	19.6	6.01	1.33 ± 0.31	5.21 ± 1.44	3.9**	++	
16	67.5	6.15	[a]	0.19 ± 0.07	-	±	
17	101.4	6.14	0.42 ± 0.16	0.21 ± 0.11	0.5*	-	
18	17.8	6.37	0.22 ± 0.19	2.32 ± 0.95	10.5*	++	HSP17
19	75.9	6.26	0.30 ± 0.12	1.03 ± 0.18	3.4**	-	
20	73.1	6.34	0.23 ± 0.12	0.69 ± 0.15	3.0**	-	
21	76.2	6.39	0.45 ± 0.05	0.72 ± 0.18	1.6*	-	
22	24.4	6.50	0.38 ± 0.17	0.82 ± 0.22	2.2*	+	
23	32.3	6.64	[a]	0.16 ± 0.03	-	±	
24	17.8	6.81	0.07 ± 0.08	0.81 ± 0.33	11.6*	++	HSP17
25	18.9	6.98	0.12 ± 0.14	0.92 ± 0.26	7.7**	+	

[a] Value below detection range (< 0.01). ** $P < 0.005$. * $P < 0.05$.

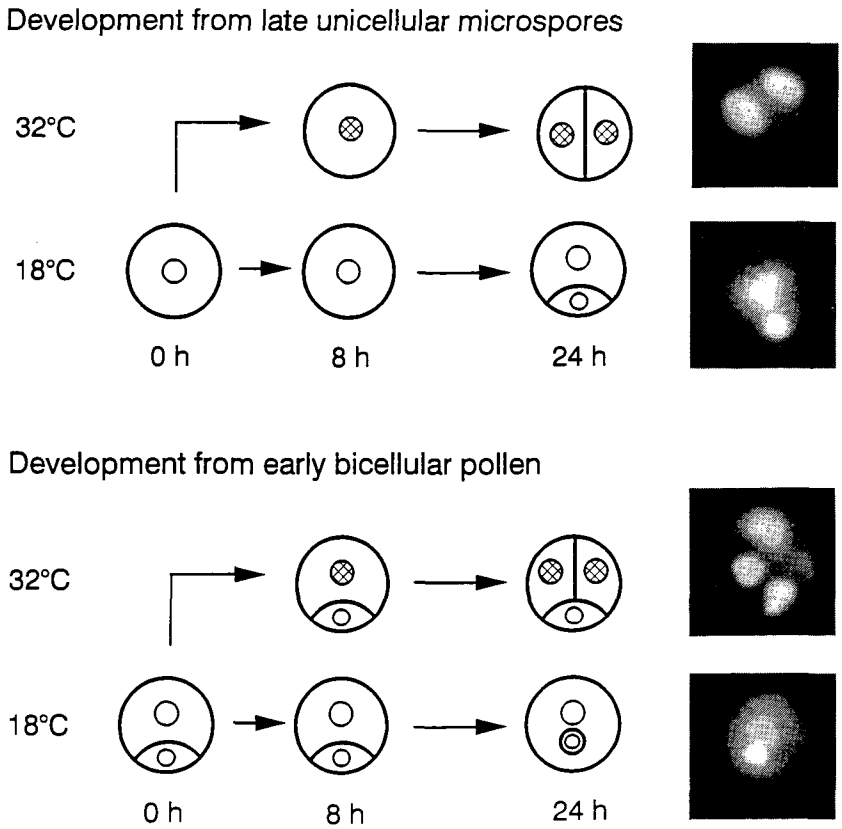

Figure 1. Diagram of sporophytic development (32°C) *versus* gametophytic development (18°C) during the first 24 h in *Brassica napus* microspore/pollen culture.

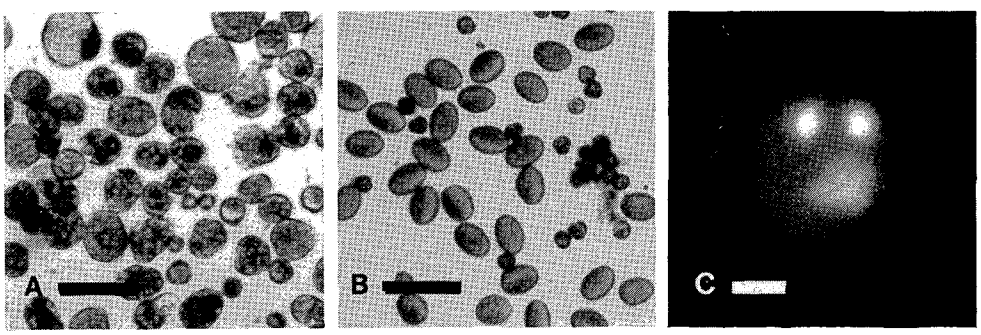

Figure 2. Development of embryos and pollen from *Brassica napus* microspores after eight days of culture. A. Globular embryos at 32°C, B. Pollen at 18°C. C. DAPI stained photograph of a pollen. Bars = 100 µm for photographs A and B, and 10 µm for C.