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Induction of microspore embryogenesis in *Brassica napus* L. is accompanied by specific changes in protein synthesis

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Abstract. Culture temperature determines the developmental fate of isolated microspores from *Brassica napus* L.. At 18°C, tricellular pollen develops, whereas culture at 32°C for 8 h leads to the quantitative and synchronous induction of embryogenesis, and ultimately to the formation of embryos. We investigated the changes in protein synthesis that are associated with this 8-h inductive period by using in-situ [^{35}S]methionine labeling, followed by two-dimensional (2-D) gel electrophoretic analysis of the radiolabeled proteins. Qualitative and quantitative computer analyses of 2-D [^{35}S]methionine protein patterns showed six polypeptides specifically labeled under embryogenic culture conditions. Eighteen polypeptides incorporated [^{35}S]methionine at a statistically significant higher rate under embryogenic culture conditions (32°C) than in the controls (18°C), whereas one protein was preferentially labeled under non-embryogenic culture conditions (18°C). These results indicate that only a limited number of proteins detectable in the 2-D gels of microspore extracts are associated with the early induction of embryogenesis. The reproducible identification of the differentially radiolabeled proteins in the 2-D gels allow the sequencing of representative peptides and the isolation of the corresponding cDNAs. This may lead to the identification and characterization of proteins associated with the very first stages of plant embryogenesis.

Key words: *Brassica* (microspore embryogenesis) – Cytoskeletal proteins – [^{35}S]Methionine labeling – Protein synthesis – Storage phosphor technology

Introduction

In rapeseed (*Brassica napus* L.) the developmental fate of isolated microspores can be controlled by culture temperature. Culture at 32°C causes the synchronous induction of sporophytic development, ultimately leading to the formation of torpedo-shaped embryos (Lichter 1982; Chuong and Beversdorf 1985). Cultures maintained at 18°C continue their normal gametophytic development and form pollen in the absence of sporophytic development (Custers et al. 1994). About 8 h of culture at 32°C is sufficient to cause an irreversible commitment to the sporophytic pathway (Pechan et al. 1991), and up to 70% of the microspores in the culture can participate in this sporophytic development (Pechan and Keller 1988). Because of this simple and quantitative switch in developmental fate, the *B. napus* microspore system provides an unique model system for studying embryogenesis and can be used for the biochemical and molecular analysis of changes that are associated with the induction of embryogenesis.

Induction of changes in development are characterized by the activation of specific transcription factors, which cause altered patterns of gene expression. By studying changes in the synthesis of proteins during the very first stages of embryogenesis in-situ, one can identify targets of the primary transcription factors controlling its induction. These proteins, and ultimately their genes, can then be used as reporters for studying the signal-transduction events leading to their expression. Changes in protein expression patterns during the induction of pollen and/or microspore embryogenesis have been investigated by two-dimensional (2-D) gel electrophoresis in rapeseed and tobacco (Pechan et al. 1991; Garrido et al. 1993). Garrido et al. (1993) found no changes in in-situ [^{35}S]methionine incorporation into tobacco pollen during the induction period (one week on starvation medium), but did find some changes in the synthesis of polypeptides from in-vitro-translated mRNAs over the same period. Pechan et al. (1991) did find differences in 2-D protein patterns between embryogenic and non-em-

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Abbreviations: 2-D = two-dimensional

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bryogenic *B. napus* microspore cultures by comparing silver-stained gels. However, up to now, this has not led to the identification of any proteins that can be used as early markers of embryogenesis.

With the introduction of immobilized pH gradients, storage phosphor technology, and sophisticated computer software, the overall reproducibility of 2-D protein patterns has been greatly improved over the past few years, and radiolabeled proteins on 2-D gels can now be automatically detected, quantified and compared. This has resulted in comprehensive, qualitative and quantitative databases of proteins from a variety of origins (Garrels et al. 1990; Celis et al. 1992; Giometti et al. 1992; Hochstrasser et al. 1992; VanBogelen et al. 1992; Yun et al. 1992). New technologies have also reduced the amount of protein required for amino acid sequence analysis and proteins can be microsequenced directly from 2-D gels (Rasmussen et al. 1992). These technical advances, together with the development of a good non-embryogenic control culture (Custers et al. 1994), have led us to initiate a new study of the changes in protein synthesis during the induction of embryogenesis in *B. napus*.

Here we report the computerized qualitative and quantitative analysis of 2-D protein synthesis patterns from *B. napus* microspores during the induction of embryogenesis in culture, as compared to those obtained from microspores that continue gametophytic development. This provides us with a starting point for the identification of proteins associated with the induction of microspore embryogenesis. The data from this computer analysis can also be used as a 2-D gel protein reference database for *B. napus* microspores, analogous to those already available for mammalian and bacterial cells.

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, Martinsried bei München, Germany) were raised all year round in a phytotron room at 18°C under a 16-h photoperiod at a photon fluence rate of 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Time from sowing till the onset of flowering was approximately ten weeks.

Microspore culture. Flower buds were sorted into appropriate size classes and surface-sterilized. The microspores of 10–20 buds of each size class were isolated and cultured in the dark in modified Lichter medium (NLN-13, Lichter 1982) at a density of 40 000 microspores$\cdot ml^{-1}$ (Custers et al. 1994). Cultures were maintained at either 18°C (non-embryogenic condition) or 32°C (embryogenic condition). Only material from isolations that yielded more than 5% embryos (2000 embryos$\cdot ml^{-1}$), after two weeks of culture at 32°C, were used for the experiments detailed below. Under these conditions, about 40% of the microspores show sporophytic divisions after 2 d in culture at 32°C (Custers et al. 1994).

Metabolic labeling of microspores with [^{35}S]methionine. Microspores were labeled for 8 or 24 h in 3 ml of medium containing 0.74 MBq L-[^{35}S]methionine (in-vivo cell-labeling grade from Amersham; >37 TBq$\cdot mmol^{-1}$). Incubations were terminated by addition of 0.3 ml ice-cold 80% trichloroacetic acid (TCA; w/v). Pellets were washed with 1 ml of 10% TCA, resuspended in 1 ml of 10% TCA and stored immediately at −70°C. Precipitates of TCA were thawed, pelleted and extracted once with 1 ml of ethanol/ether (1:1, v/v; −20°C). The dried pellets were resuspended in 50 µl isoelectric focusing sample buffer (8 M urea, 0.5% Triton X-100, 2% β-mercaptoethanol, 2% Pharmalyte 3–10 (Pharmacia, Roosendaal, The Netherlands), 1 $\mu g \cdot ml^{-1}$ bromophenol blue). The samples were frozen and thawed once prior to application to the isoelectric focusing gel strips. Aliquots (2 µl) were TCA-precipitated on Whatman 3MM filter paper (Merck, Amsterdam, The Netherlands) followed by scintillation counting to determine the total amount of ^{35}S incorporation.

Two-dimensional (2-D) gel electrophoresis was performed as described by Görg et al. (1988) making use of a MultiphorII (Pharmacia) horizontal electrophoresis unit for both isoelectric focusing and SDS-PAGE. First-dimension isoelectric focusing was performed at 15°C by using Immobiline DryStrips pH 4.0–7.0 (Pharmacia). Strips were loaded with either the total protein extract of a 3-ml culture or with fixed amounts of radiolabeled proteins. Separation in the second dimension was carried out at 15°C on precasted 8–18% gradient SDS ExelGels (Pharmacia).

Autoradiography and silver-staining. Following 2-D gel electrophoresis, gels were fixed in 7% acetic acid, transfered to preserving solution (10% glycerol), and wrapped in a cellophane sheet for drying at room temperature. For autoradiography the dried gels were used to expose a Kodak X-Omat AR film using a Kodak intensifying screen at −70°C. The dried gels were also used to expose a Molecular Dynamics (B&L Systems, Zoetermeer, The Netherlands) storage phospor plate (Johnston et al. 1990). After exposing for the desired time at room temperature, the storage phosphor plates were scanned with the use of a 400B PhosphorImager (Molecular Dynamics; B&L Systems). After obtaining the autoradiographs and PhosphorImager data the corresponding gels were rehydrated and subsequently silver-stained (Blum et al. 1987).

Image-analysis procedure. The 2-D autoradiographs of [^{35}S]methionine-labeled proteins were scanned using a Desk Top (light) Densitometer model DNA35 (Pharmacia; resolution 64 µm). Digitized autoradiographs or PhosphorImager data were transferred to a Sun IPC Sparc workstation for analysis with the PDQuest 4.1 software package developed by PDI (Pharmacia; Garrels 1989). Results from five independent labeling experiments were used for the building of databases of [^{35}S]methionine-labeled proteins. Data of each gel were displayed, spots were automatically detected, and images were visually reviewed by using the image editor. Before corresponding images were matched, 55 landmarks were entered within each image. Subsequently images were matched and all spots within a matchset were collected in the database. Two separate databases from the embryogenic and non-embryogenic cultures were compiled, matched to each other, and used for qualitative and semi-quantitative analyses. Quantification of selected spots was performed using the ImageQuant software (Molecular Dynamics; B&L Systems).

Statistical analysis. Quantitative data were statistically analyzed making use of the unpaired student *t*-test assuming Gaussian populations with equal standard deviations (GraphPad Instat v2.02 software; San Diego, USA).

Results and discussion

The induction of embryogenesis from microspores or pollen has to be accompanied by the initiation of a new program of gene expression. Eight hours of culture at 32°C is sufficient to irreversibly divert competent microspores from their normal gametophytic development into sporophytic development (Pechan et al. 1991). With our method for microspore isolation (using multiple buds), at least 40% of the cells divide sporophytically,

within the first 2 d in culture at 32°C, alongside a varying percentage of gametophytic-like divisions (estimated at 5–25%, depending on the amount of bi-cellular pollen at the start of the culture). To identify the earliest changes in protein synthesis that constitute the switch from gametophytic to sporophytic development, we investigated the differences in [^{35}S]methionine incorporation between microspores cultured under embryogenic (32°C) and non-embryogenic (18°C) culture conditions.

[^{35}S]*Methionine incorporation.* Figure 1 shows the result of a representative labeling experiment in which the overall rate of incorporation of [^{35}S]methionine into proteins was measured at 8-h intervals during the first day of culture. During the first 8 h of culture, an on average 2.25 $\pm$ 0.50 (n = 7)-fold higher [^{35}S]methionine incorporation was found in 32°C cultures than in 18°C cultures. Thereafter the [^{35}S]methionine incorporation was similar for both temperature treatments. This pattern of [^{35}S]me-

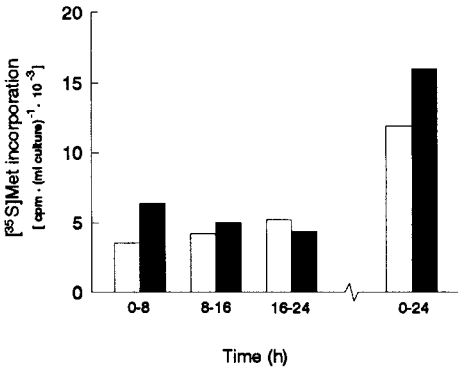

Fig. 1. [^{35}S]Methionine incorporation into embryogenic and non-embryogenic microspore cultures of *B. napus*. Isolated microspores were cultured under non-embryogenic (18°C; *open bars*) or embryogenic conditions (32°C; *closed bars*). [^{35}S]Methionine was added during the times indicated. At the end of the labeling period samples were taken and TCA-precipitable radioactivity was measured. A representative experiment is shown

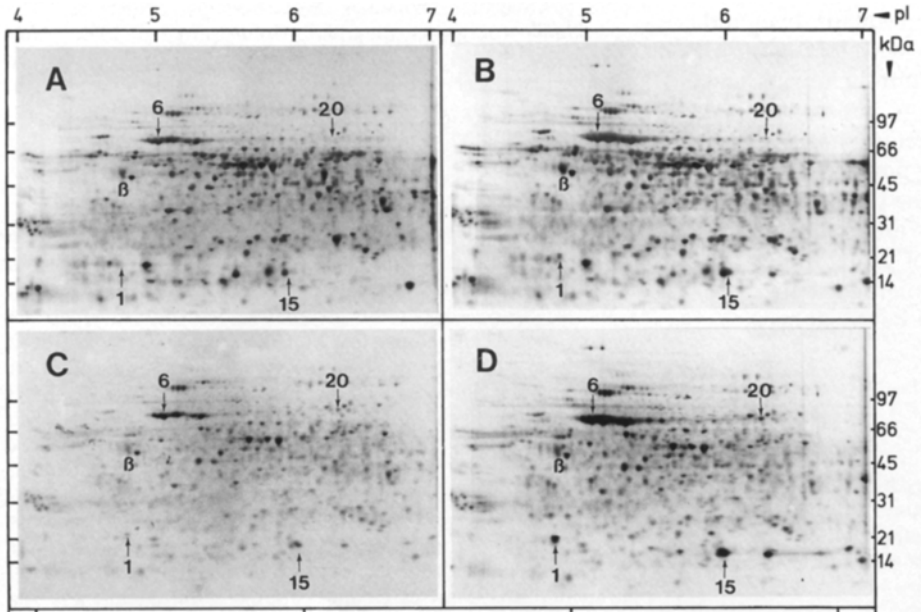

Fig. 2A–D. Two-dimensional gel electrophoretic separation of [^{35}S]-methionine-labeled polypeptides from embryogenic and non-embryogenic microspore cultures of *B. napus*. Microspores were incubated in the presence of [^{35}S]methionine during the first 8 h after initiation of the culture under non-embryogenic conditions (18°C; **A,C**) or embryogenic conditions (32°C; **B,D**). Equal amounts of microspores were used for protein extraction and subsequent 2-D gel analysis. Proteins were separated in the first dimension by isoelectric focusing between pH 4 and pH 7 (abscissa, pI) and then in the second dimension by gradient polyacrylamide gel electrophoresis (ordinate, kDa). After autoradiography, the [^{35}S]methionine gels were silver-stained. **A,B** Images of silver-stained gels. **C,D** Autoradiographs of [^{35}S]methionine-labeled polypeptides. In the *lower-panel* line drawing, the positions of the polypeptides described in Table 1, and the areas that have been enlarged for Fig. 3 are given for convenience (see below). Some of these spots have been marked with *arrows* to facilitate comparisons of the gels. The *open spot outlines*, numbered 1–25, refer to the spot numbers from Table 1; *hatched spot outlines* refer to cytoskeletal proteins that were identified by immunoblotting. a_{1-3}: actin isoforms; α_{1-3}: α-tubulin isoforms; β: β-tubulin cluster of at least four isoforms. Standard spot (SSP) number, relative molecular mass (M_r), isoelectric point (pI), and quantification of [^{35}S]methionine incorporation into the numbered polypeptides (open outlines) are given in Table 1

thionine incorporation resembles the pattern of nuclear DNA synthesis as shown by bromodeoxyuridine (BrdU) labeling (Binarova et al. 1993). The initial period of elevated [^{35}S]methionine and BrdU incorporation coincides with the period in which the microspores become committed to embryogenic development.

Two-dimensional gel electrophoresis. For a detailed comparison of protein synthesis during the first 8 h of culture, [^{35}S]methionine-labeled proteins from 18°C and 32°C cultures were separated by 2-D gel electrophoresis. Figure 2 presents silver-stained images (Fig. 2A,B) along with corresponding autoradiographs (Fig. 2C,D) from a representative labeling experiment. Equal amounts of biological material from the 18°C and 32°C cultures ($1.2 \cdot 10^5$ microspores) were used for protein extraction and subsequent 2-D gel electrophoresis. The overall 2-D patterns of the silver-stained and [^{35}S]methionine-labeled polypeptides looked very similar under both temperature conditions. The higher overall rate of [^{35}S]methionine incorporation under embryogenic culture conditions (see also Fig. 1) could therefore not be ascribed to the differential synthesis of a small subset of polypeptides in the 32°C culture. As changes in [^{35}S]methionine labeling of individual polypeptides in the 2-D pattern that are associated with the induction of embryogenesis were not immediately obvious, a comprehensive qualitative and quantitative analysis of the 2-D gels from a series of independent experiments was performed, making use of the latest technological innovations.

Qualitative and quantitative analysis of protein patterns. PhosphorImager data from 2-D gels of five independent [^{35}S]methionine labeling experiments were quantified and compared using the ImageQuant and PDQuest 4.1 software for construction of separate protein databases of embryogenic and non-embryogenic cultures. In two [^{35}S]methionine labeling experiments, equal amounts of biological material of an 18°C and 32°C culture were loaded (see e.g. Fig. 2), whereas in the three remaining sets equal amounts of radioactive material were loaded, thus avoiding the possibility that protein spots with low radioactivity, just above the detection limit of the 2-D gel, would only be found in the 32°C cultures. The protein database from embryogenic cultures was then compared with the database of non-embryogenic cultures using the PDQuest 4.1 computer program. Spots were selected that were (i) detectable in all five 32°C gels, while never detectable in any of the 18°C gels and vice versa, and (ii) [^{35}S]methionine labeled to a relatively higher level under one of the two culture conditions. Table 1 gives a summary of this analysis listing all the

Table 1. Differentially synthesized proteins in embryogenic and non-embryogenic microspore cultures of *B. napus*. Protein spots that were differentially labeled with [^{35}S]methionine during the first 8 h of microspore culture, under either embryogenic (32°C) or non-embryogenic (18°C) culture conditions, were selected and quantified as described under *Materials and methods*. [^{35}S]Methionine incorporation into the individual spots is expressed as a fraction ($\times 10^3$; ‰) of the total radioactivity measured in the corresponding 2-D gel, and is therefore corrected for the overall change in labeling due to the temperature difference. Data presented are the means ± SD from five independent experiments

Spot number	SSP number[a]	M_r (kDa)	pI	[^{35}S]methionine incorporation (‰)		Ratio 32°/18°C
				18°C	32°C	
1	1107	24.1	4.76	0.12±0.14	1.94±0.60	16.2**
2	1208	29.9	4.86	[b]	0.23±0.12	-
3	1707	65.6	4.88	0.26±0.11	0.55±0.15	2.1**
4	1708	69.0	4.89	0.18±0.05	0.39±0.12	2.2**
5	2407	40.7	5.04	0.35±0.08	0.84±0.18	2.4**
6	2709	76.8	5.15	3.14±1.42	18.9 ±6.85	6.0**
7	3411	40.6	5.34	0.48±0.16	0.99±0.43	2.1*
8	3704	65.5	5.33	1.03±0.25	2.63±0.38	2.6**
9	3711	74.8	5.33	2.88±1.42	8.61±3.55	3.0*
10	3712	76.5	5.36	[b]	0.73±0.51	-
11	4705	75.1	5.51	0.26±0.11	0.97±0.52	3.7*
12	4709	75.4	5.60	0.22±0.12	0.97±0.46	4.4*
13	5709	67.4	5.65	[b]	0.13±0.07	-
14	6112	24.1	5.94	[b]	0.23±0.22	-
15	6116	19.6	6.01	1.33±0.31	5.21±1.44	3.9**
16	6716	67.5	6.15	[b]	0.19±0.07	-
17	6802	101.4	6.14	0.42±0.16	0.21±0.11	0.5*
18	7107	17.8	6.37	0.22±0.19	2.32±0.95	10.5*
19	7708	75.9	6.26	0.30±0.12	1.03±0.18	3.4**
20	7711	73.1	6.34	0.23±0.12	0.69±0.15	3.0**
21	7712	76.2	6.39	0.45±0.05	0.72±0.18	1.6*
22	8104	24.4	6.50	0.38±0.17	0.82±0.22	2.2*
23	8209	32.3	6.64	[b]	0.16±0.03	-
24	9101	17.8	6.81	0.07±0.08	0.81±0.33	11.6*
25	9106	18.9	6.98	0.12±0.14	0.92±0.26	7.7**

[a] Standard spot (SSP) number derived from the [^{35}S]methionine-labeled protein database of *B. napus* microspores
[b] Value below detection range (< 0.01). $^{**}P < 0.005$. $^{*}P < 0.05$

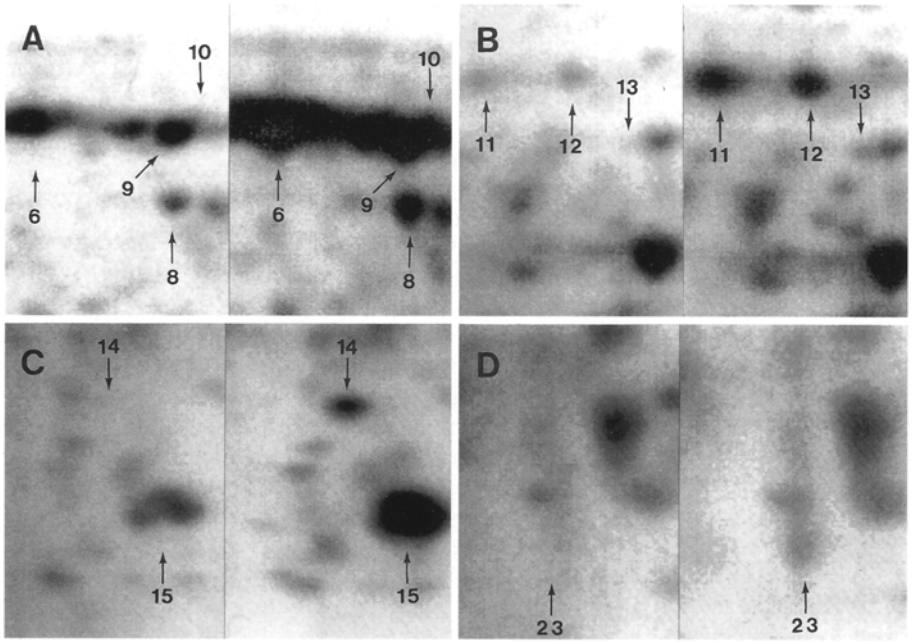

Fig. 3. Examples of [^{35}S]methionine-labeled polypeptides differentially induced in non-embryogenic (*left* side of each panel) or embryogenic (*right* side of each panel) microspore cultures of *B. napus*. The areas in the 2-D autoradiographs of Fig. 2C and D, indicated by boxes in the lower panel of Fig. 2, were enlarged by computer after digitalization of the autoradiographs to visualize some of the differentially synthesized proteins mentioned in Table 1

proteins in the two databases falling into one of the above-mentioned categories. Six [^{35}S]methionine-labeled polypeptide spots belonged to the first category, and were exclusively found in the five 2-D gels from the embryogenic cultures (Table 1, spot numbers 2, 10, 13, 14, 16, and 23; see also Figs. 2 and 3). Eighteen spots incorporated significantly more [^{35}S]methionine in the embryogenic cultures than in the non-embryogenic cultures, when expressed as a fraction of the total amount of [^{35}S]methionine detected on the corresponding gel (Table 1). One spot (Table 1, spot number 17) was labeled relatively stronger in non-embryogenic cultures, which may either be due to decreased synthesis as a consequence of the elevated temperature, or due to a specific repression as a consequence of the induction of embryogenesis. Because some gametophytic development can still be observed in 32°C cultures (Binarova et al. 1993; Custers et al. 1994), we would expect to detect only those proteins whose synthesis is enhanced or repressed as a consequence of the induction of embryogenesis or as a consequence of the elevated temperature. Proteins whose synthesis is specific to gametophytic development will therefore not be identified.

The standard spot (SSP) number used to assign each protein spot in the database is given in Table 1. The proteins are arranged by increasing SSP number, and an approximation of M_r- and pI-value is included. Outlines of all the spots from Table 1 are shown in the lower panel of Fig. 2 as an aid to the reader. Figure 3 shows the enlargements of selected regions indicated by boxes in the lower panel of Fig. 2. When viewing Fig. 3 one should keep in mind that the panels are enlarged from only one gel-set and that the data shown in Table 1 are based on the data of 5 such gel-sets. The enlargements highlight the power and detail of the computer analysis. In total, approximately 530 silver-stained (Fig. 2A,B) and 515 ^{35}S-labeled polypeptides (Fig. 2C,D) could be detected on each of the corresponding 2-D gels by computer analysis. About 75% of the proteins detected by silver-staining incorporated detectable levels of [^{35}S]methionine within the first 8 h of culture (data not shown).

Previously, a 2-D-gel analysis of silver-stained proteins during the induction of embryogenesis of *B. napus* microspores led to conclusions different from ours (Pechan et al. 1991). These authors found large differences in the staining patterns of 2-D gels from 8-h embryogenic cultures (32°C) and control cultures (25°C). Especially noticeable was the reduction in staining of a number of proteins in embryogenic microspores (32°C) as compared to the controls. Over the course of many experiments, we have never observed similar changes. The results presented here are more in line with the data published for changes in protein synthesis during the induction of embryogenesis in tobacco pollen (Garrido et al. 1993). These authors could not detect (by eye) any major changes in [^{35}S]methionine incorporation after a 7-d-starvation treatment, which causes the induction of embryogenesis in the tobacco microspore system. Comparison of in-vitro translation products of mRNA from embryogenic and non-embryogenic tobacco pollen did reveal differences between the two populations (Garrido et al. 1993).

Differentially synthesized proteins. The six proteins that were exclusively labeled in the embryogenic cultures exhibited a relatively low incorporation of [^{35}S]methionine; on average between 0.013% (spot number 13) and 0.073% (spot number 10) of the total amount of radioactivity detected on the gel (Table 1). To determine whether these six protein spots were specific for the 8-h induction period or would remain present thereafter, we performed a qualitative analysis of these protein spots on one set of 2-D gels from microspore extracts prepared after labeling for 24 h with [^{35}S]methionine during the second day in

culture at either 18°C or 32°C. Spot numbers 10 and 16 could not be detected under these conditions, and appear to be specific to the induction period. Spot numbers 2, 13, 14, and 23 continued to be synthesized during the second day in the embryogenic culture and remained absent in the non-embryogenic culture. These proteins are potentially specific to embryogenesis and may serve as early markers of embryogenesis from which the induction mechanism can be backtracked. We are currently studying the expression of these three proteins throughout embryogenesis, in an attempt to facilitate the isolation of enough material for microsequencing.

As the elevation of culture temperature is the only external factor affecting the switch from gametophytic to sporophytic development, proteins whose differential synthesis is associated with temperature elevation are also of potential interest, as they might be the cause for the primary steps of the induction process itself. Indicative of the existence of a set of temperature induced proteins in the embryogenic microspore cultures is the occurrence of a number of the differentially synthesized proteins in two clusters around the 20-kDa and 70-kDa areas (see lower panel Fig. 2). These two relative-molecular-mass values coincide with the location of the most-abundant stress-induced proteins of plants: the 17-kDa and 70-kDa families of heat-shock proteins (Nover et al. 1989). We are currently investigating by 2-D immunoblotting whether any of the proteins listed in Table 1 belong to one of these families of heat-shock proteins.

Identification and synthesis of cytoskeletal proteins. Microscopical observations of the microtubular and microfilamental cytoskeletons after labeling with specific probes revealed important differences in cytoskeletal organization and cell-division planes between microspores of *B. napus* cultured under non-embryogenic (18°C) and embryogenic (32°C) conditions (Hause et al. 1993). These cytological changes led us to investigate the synthesis of a number of key cytoskeletal proteins under the two culture conditions. Total protein extracts from microspore cultures were separated by 2-D gel electrophoresis, blotted onto nitrocellulose, and probed with well-characterized monoclonal antibodies specific to either α-tubulin (clone DM1A), β-tubulin (clone TUB2.1) or actin (clone KJ43A). Using the PDQuest 4.1 computer program, the location of the immuno-reacting proteins was matched to the 2-D gels of 8-h [^{35}S]methionine-labeled cultures (see hatched outlines in the lower panel of Fig. 2). Three actin, three α-tubulin, and at least four β-tubulin isoforms could be detected and matched to the database (data not shown). None of these proteins was found to be preferentially synthesized under embryogenic or non-embryogenic culture conditions ($P > 0.1$; data not shown). This indicates that the observed changes in microtubular organization occur without changes in the synthesis of tubulin isoforms during embryo induction, but are more likely regulated through post-translational modifications of the substituent tubulin subunits.

Conclusions. In the present study we have established a system for the quantitative detection of proteins that are induced or repressed during the induction of embryogenesis. The combination of highly reproducible 2-D gels, storage phosphor technology, and sophisticated software for matching and analysis of the various 2-D gel protein patterns, has made an accurate statistical analysis of the changes in protein synthesis possible. The *B. napus* microspore culture system is one of the very few experimental systems in which such studies can be carried out because a high percentage of the isolated microspores (about 40%) become committed to sporophytic development, synchronously, within 8 h after initiation of the culture. This compares to 1–2% in embryogenic carrot or alfalfa cell-suspension cultures (De Vries et al. 1988; Dudits et al. 1991), and the requirement of several days in a special starvation medium in embryogenic tobacco pollen cultures (Kyo and Harada 1986; Vicente et al. 1992). When the transition time from somatic cell to embryogenic cell is not well characterized and/or too long, the interpretation of the observed changes in protein pattern between embryogenic and non-embryogenic conditions becomes increasingly difficult. Since both qualitative and quantitative changes in protein synthesis may be of importance for the switch in development, the percentage of cells that enter the embryogenic pathway should be high enough for quantitative analysis. The *B. napus* microspore system fulfills all these requirements.

The reproducible identification of the differentially radiolabeled proteins will allow microsequencing of peptides derived from the more-abundant ones (Rasmussen et al. 1992). Such sequence information can be used for the raising of peptide antibodies and/or the isolation of corresponding cDNAs. This may lead to the identification and characterization of proteins associated with the very first stages of plant embryogenesis.

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