Induction of embryogenesis in microspores and pollen of *Brassica napus* L. cv. Topas

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Induction of embryogenesis in microspores and pollen
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To the memory of our friend and colleague Tomáš Havlický

Stellingen

1. The microtubular cytoskeleton plays an important role during the induction of microspore embryogenesis, but only as a tool, not as a trigger.
   (This thesis)

2. Prerequisites for the continuation of the cell cycle are preserved in the vegetative cell of angiosperm pollen.
   (This thesis)

3. Heat shock proteins of the 70kDa class are involved in the progress of the S-phase of the cell cycle.
   (This thesis)

4. Non-radioactive, cytological methods have not only the advantage to avoid the handling with radioactive chemicals, they are also most useful by being fast and giving a precise subcellular signal.
   (This thesis)

5. Induction of microspore and pollen embryogenesis is a process of two steps: The dedifferentiation of microspore or pollen development and subsequent embryo specific differentiation.
   (This thesis)

6. It is easier to find possibilities to investigate a biological problem with one of the dozen model organisms than to transfer the results obtained to organisms which are important for mankind.

7. „Growth of science is growth of ideas of the scientists." (Ernst Mayr: The Growth of the Biological Thought. 1984)

8. The creation of part time positions for scientists also in Germany and its strict realisation would not be only very helpful for the employment market, but also very beneficial for the family life.

9. A humane society is reflected by the attitude towards their children.

10. Opposite to other European countries, in the Netherlands exist some special traffic rules for bicyclists at crossings of streets of the same rank. The early information to foreigners about this would improve the security in the traffic.

Stellingen van Gerd Hause behorende bij het proefschrift getiteld
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STELLINGEN

1. The induction of symmetrical divisions is an essential, but not a sufficient prerequisite to get the formation of embryos from isolated microspores and pollen.
   (This thesis)

2. Configurational changes in the microfilamental cytoskeleton which occur during symmetrical divisions in cultured microspores and pollen are only a reflection of alterations caused by induction.
   (This thesis)

3. The direction of the longitudinal axis of embryos derived from microspores and pollen is determined by the site of the rupture of the original pollen wall that still covers the proembryo.
   (This thesis)

4. Embryogenic cultures can be started with microspores from late G1 to G2 phase by completing the current cell cycle, whereas in pollen the vegetative cell, being in G1, always has to re-enter the cell cycle before embryogenesis can occur.
   (This thesis)

5. Investigations of complex tissues by biochemical and molecular-biological methods need to be completed by cytological and histological techniques.
   (This thesis)

6. Among the methods used for biological research, cytological and morphological techniques allay aesthetic desires, too.

7. Capital investments given for research, but used for the construction of new buildings and not used for the creation of research jobs or the supply with laboratory materials, become rather a subsidy of the building trade than of the research itself.

8. Alles was der Mensch treibt, kultiviert ihn.
   J.W. von Goethe

9. Only having own children one can see the goodness of the own parents.
   Proverb from Japan.

10. By the help of a computer most things go faster, but need more time.

Stellingen van Bettina Hause behorende bij het proefschrift getiteld
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Contents

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Chapter 1

General Introduction

Induction of embryogenesis in microspores and pollen of *Brassica napus* L. cv. Topas

Bettina and Gerd Hause

GENERAL INTRODUCTION

1. Embryogenesis as a complex differentiation event

Embryogenesis is an initial phase of the life cycle of higher plants. It is preceded by a sequence of events summarized as plant sexual reproduction. For sexual reproduction higher plants have developed specialized parts, the flowers, in which male and female gametes are produced by gametophytes. Gametes are haploid cells and are able to fuse and form diploid zygotes, the first cell of the new generation, the sporophyte. Gametes are cells formed by the processes of sporogenesis and gametogenesis. Two types of gametes are developed, large female gametes, the egg cells, and small male gametes, the sperm cells.

The reduction of the diploid chromosome set to a haploid one takes place during meiosis. Microspore mother cells form the microspores, and megaspore mother cells give rise to megaspores. The unicellular micro- and megaspores mature during the process of sporogenesis. Both types of spores divide mitotically forming the gametophytes. The microgametophyte (the pollen grain) formed by the microspore, consists of a vegetative cell and a generative cell, which will form the two sperm cells. The megagametophyte (embryo sac) generally consists of 7 cells, the female gamete (the egg cell), two synergids, a central cell and three antipodal cells.

After pollination the mature pollen grain germinates at the stigma of the pistil and forms a pollen tube transporting the two sperm cells. The pollen tube grows through the style into the ovary of the pistil, penetrates the megagametophyte in the ovule, and discharges the sperms in one of the synergids. One sperm cell fuses with the egg cell forming the zygote, the other one fuses with the central cell forming the endosperm (for review see Van Went and Willemse 1984). After this process of "double fertilization" (Strasburger 1884) the arising zygote is the onset of the plant embryo.

Embryogenesis is the process by which the unicellular zygote develops into an organism which develops a stem, roots, and leaves. The aims of embryogenesis are (i) to establish meristems and the plant body pattern, (ii) to differentiate the primary plant tissue types, (iii) to generate specialized storage organs essential for seed germination and (iv) to enable the new plant to remain dormant until conditions are favourable for postembryogenic development (Goldberg et al. 1994). It starts with the unequal division of the zygote giving a large basal and a small apical cell. The asymmetry of this division is caused by the polar distribution of the zygote cytoplasm (Schaffner 1906). This first division predicts already the apical-basal-axis of the arising embryo (Johansen 1950, Jürgens 1995). The basal cell differentiates to the suspensor, which might function in anchoring and positioning the embryo, and in the uptake and transport of nutrients needed for embryogenesis (Natesh and Rau 1984). The apical cell is the origin of the embryo proper. In dicotyledons a number of defined cell divisions of the apical cell lead to the formation of a globular embryo proper. Further on, directed divisions in periclinal as well as in anticlinal planes lead to the first visible cell differentiation. This is the appearance of the protoderm, and later the formation of the procambium strands and the ground meristem in the globular stage. Just after the globular stage, cotyledons are formed, the hypocotyl region begins to elongate, and the root meristem becomes differentiated. The

8

result in dicotyledons is the formation of a heart-shaped embryo which develops via the torpedo-stage to the mature embryo.

2. Experimental systems for *in vitro* embryogenesis

Intensive investigations of embryogenesis started with the development of light microscopy and later on using electron microscopical techniques. Meanwhile, for numerous plant species detailed cytological observations exist regarding sporogenesis, gametogenesis, pollination, pollen tube growth, fertilization and embryo development (for review see Johri *et al.* 1992, Cresti *et al.* 1992). Embryogenesis proceeds within the former female gametophyte, which is covered by several layers of sporophytic tissue of the ovule necessary for the protection and nutrition of the developing embryo. Those tissues are barriers for the observations of the process of embryo formation as well as for the direct manipulation of the embryo and they influence embryogenesis. Several experimental systems were developed to overcome this problem.

Zygotic embryo culture
For the culture and rescue of zygotic embryos, already existing young embryos are dissected from the ovule and cultivated *in vitro* (for review see Monnier 1995). This technique is useful to investigate embryogenesis and gives the possibility to change the embryo development and metabolism by the variation of nutrition, environmental conditions, and phytohormones (Liu *et al.* 1993a, b). Using this system it is, however, impossible to analyse the very early events, for instance the formation of the globular embryo because of the small percentage of very early embryos which survive *in vitro*.

Fusion of isolated gametes
Separated from their surrounding maternal tissue isolated gametes can fuse and form embryos *in vitro* (Kranz and Lörz 1993). In *Zea mays* it is possible to follow the morphological and biochemical changes that occur at the transition from the egg cell to the zygote and during zygote development after the fusion of the gametes (Kranz *et al.* 1995). Studies on initial signalling events have become feasible, for instance the study of the involvement of calcium in these processes (Tirlapur *et al.* 1995). However, this system needs a lot of experience, is very time consuming and not applicable for large scale research necessary for molecular and biochemical analyses. Till now successful and reproducible *in vitro* fertilization is only described for *Zea mays*.

Somatic embryogenesis
Embryos can be formed in *in vitro* cultures starting from single somatic cells (review De Jong *et al.* 1993, Emons 1994). In general, plant cells are totipotent, i.e. already differentiated diploid cells are able to re-enter the cell cycle and start a process of regeneration. That can occur spontaneously (Taylor 1967) but in general a well balanced treatment with plant hormones in special culture media is necessary to induce cell divisions and embryogenesis. Direct somatic embryogenesis starts from single cells of e.g. immature zygotic embryos (*Trifolium repens* - Maheswaran and Williams 1985, *Linum usitatissimum* - Pretova and

9

Williams 1986). Cultured cells epigenetically far from the embryogenic state are used for indirect somatic embryogenesis. For some species it is shown that the induction of an unequal division plane during the first mitosis of regenerating protoplasts can lead directly to the formation of an embryo (*Medicago sativa* - Dijak and Simmonds 1988, *Nicotiana plumbaginifolia* - Tewes, personal communication). The majority of plant species, however, forms at first embryogenic calli or proembryogenic cell masses (PEMs) in which daughter cells remaining attached to each other are produced (*Daucus carota* - Reinert 1958, Steward *et al.* 1958). Certain cells of those calli or PEMs can be stimulated to form embryos. During the first stages those embryos develop comparable to the zygotic embryo proper (globular stage - heart shaped stage - torpedo shaped stage) including the synthesis of storage products, but later on they form directly plantlets without entering the stage of embryo dormancy which is common for zygotic embryogenesis *in vivo*.

For a number of plant species protocols exist to initiate somatic embryogenesis with high efficiency. Somatic embryogenesis is already widely used to transform plants, to multiply special cultivars and rare plants or to produce secondary metabolites (*Simmondsia chinensis* - Lee and Thomas 1985, *Digitalis lanata* - Kuberski *et al.* 1984).

Androgenesis

Microspores or immature pollen can be induced to leave the path of further gametophytic development and to enter sporophytic development (androgenesis). The induction can take place within a dissected anther on a solid medium (anther-culture) or in isolated microspores and pollen put in a liquid culture medium (microspore or pollen culture). The induction of androgenesis is realized by special pretreatments such as (i) cold shock, (ii) high temperature, (iii) cultivation in starvation medium, (iv) centrifugation, (v) ethanol treatment, (vi) irradiation with γ-rays or x-rays, and (vii) by reduced atmospheric pressure. Androgenesis has been successfully induced in about 200 species belonging to more than 50 genera and 25 families of dicots and monocots (for review see Sangwan and Sangwan-Norreel 1990, Ferrie *et al.* 1995). The origin of the embryo is a single haploid cell. That means the arising embryo is haploid or it diploidizes spontaneously by exogenous factors. In those cases the arising plants are homozygous. Such haploid as well as dihaploid embryos produced in large numbers are of great value for plant breeders. Because of the possibility to transform microspores or pollen to form transgenic material (Jähne *et al.* 1994), microspore and pollen culture is a very interesting tool for basic research as well as for plant biotechnology.

3. Microspore and pollen embryogenesis in *Brassica napus*

The most basic of all problems in anther and microspore culture is the proper understanding of the induction of androgenesis. To investigate this process a system easily to handle has to be used. Among the large amount of androgenic systems, the cultivation of *Brassica napus* microspores and pollen is a kind of model system. Embryos can be obtained at high frequencies using relatively simple culture conditions (Lichter 1982). The ability of the isolated microspores and pollen to form embryos is influenced by various factors such as plant growth conditions, genotype variations, culture conditions, and induction temperature,

10

and was optimized recently (Chuong and Beversdorf 1985, Keller *et al.* 1987, Charne and Beversdorf 1988, Chuong *et al.* 1988a, b, Gland *et al.* 1988, Huang *et al.* 1990, Ferrie *et al.* 1995). To obtain high yields of embryos late microspores and young bicellular pollen from the cultivar "Topas" have to be cultured in a modified Lichter-medium (NLN, Lichter 1982) for at least 8 hours at 32°C followed by cultivation at 25°C (Pechan and Keller 1988). The importance of the proper developmental stages of microspores and pollen is described in detail by Telmer *et al.* (1992). Some cytological aspects of microspores and pollen at the induction phase were investigated by Fan *et al.* (1988) and Zaki and Dickinson (1990, 1991).

4. Scope of the thesis

The aim of this thesis is the analysis of the process of embryo induction in microspore and pollen cultures of *Brassica napus* L. cv. Topas. The thesis represents the detailed investigations of cytological events happening in isolated and cultured microspores and pollen of *B. napus* cv. Topas in the early period of embryo induction. Especially those cytological events are investigated which are in strong relation to the stop of the gametophytic development and the initiation of sporophytic divisions. Since the cytoskeleton plays an important role in cell division and cell shaping, cytoskeletal configurations during microspore and pollen development are investigated. The changes in configuration of the microtubular cytoskeleton and the microfilamental cytoskeleton are presented in **Chapter 2** for the *in vivo* development. Cytoskeletal configurations and their impact on embryogenesis under inductive and non inductive *in vitro* conditions are described in **Chapter 3**. Changes in the replication pattern of the nuclear DNA are a sign of the cell cycle process and are investigated in **Chapter 4**. Cell regeneration and the re-entry in the cell cycle might be related with changes in protein phosphorylation. Therefore, the phosphorylation events occurring during the initial phase of the microspore and pollen culture were compared with the *in vivo* phosphorylation in **Chapter 5**. Microspore and pollen development were specifically turned into embryogenic development by heat pulse treatment. As heat treatment generally causes the formation of heat shock proteins (HSPs). We investigated in **Chapter 6** whether HSPs were formed, where they were localized, and to what extent they were related to the changing developmental pathway of the cultured microspores and pollen. In **Chapter 7** we investigated gene expression in microspores and pollen and present results of *in situ* hybridization methods for this system. **Chapter 8** presents an overview of the further development of the microspore derived embryos. Here the expression of polarity within the developing microspore derived embryo is compared to zygotic embryogenesis.
Although all the chapters of the thesis are established by close co-operation of both authors, the initiation and finalization of the **Chapters 3, 4,** and **8** are done by Bettina Hause and of the **Chapters 2, 5, 6** and **7** are done by Gerd Hause.

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Chapter 2

Microtubular and actin-filament configurations during microspore and pollen development in *Brassica napus* L. cv. Topas

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SUMMARY

The structures of the microtubular and microfilamental cytoskeletons were investigated during the development of microspores and pollen grains of *Brassica napus* L. cv. Topas. Microfilaments were observed directly with rhodamine-phalloidin and microtubules with FITC by indirect immunofluorescent staining and transmission electron microscopy. We observed microtubules in all developmental stages and noted several changes in the configuration of the microtubular cytoskeleton during microspore development, microspore mitosis, and pollen development. A preprophase band before microspore mitosis was not observed. The arrest of the microspore nucleus in an eccentric position is likely caused by microtubules as is the shape of the phragmoplast at microspore mitosis. Despite the application of various staining methods, i.e., labelling of fixed and unfixed fresh and cryosectioned microspores and pollen with rhodamine-phalloidin, microfilaments could not be observed in all developmental stages. Prominent microfilamental arrays were observed during cytokinesis of microspore mitosis and during the free generative cell stage. They mark the stages with different configurations.

Keywords: *Brassica napus*, immunolabelling, cytoskeleton, microspore and pollen development

INTRODUCTION

Pollen formation in the locules of anthers is preceded by several steps: (*i*) the formation of diploid sporogenous tissue through several series of mitotic divisions; (*ii*) the formation of haploid microspores through two meiotic divisions; and (*iii*) the formation of the microgametophytes containing sperms through two mitotic divisions. The developmental processes *in vivo* mostly proceed simultaneously within one anther. Development can be influenced *in vitro* by changing culture conditions resulting in the production of androgenic embryos (Nitsch and Nitsch 1969; Narayanaswamy and Chandy 1971; Wagner and Hess 1974; Thomas and Wenzel 1975). Many authors focus on *in vitro* development of the microspores of *Brassica napus* because this species is amenable to studies of artificial embryo induction in microspore cultures (Thomas and Wenzel 1975; Lichter 1982; Chuong and Beversdorf 1985; Keller et al. 1988; Pechan and Keller 1988, 1989; Charne and Beversdorf 1988; Pechan et al. 1991). It was found that the suitable stages for the initiation of artificial embryo induction are around the microspore mitosis (Pechan and Keller 1988). It is conceivable that such change from the normal microspore and microgametophyte development to androgenic embryogenesis coincides with alterations in the configuration of microtubules (MT) and microfilaments (MF) because cytokinesis proceeds differently (Zaki and Dickinson 1990, 1991). Introductory to the studies on artificial embryo induction, we report here the *in vivo* development of microspores and pollen with special attention to the MT and MF configurations.

MATERIALS AND METHODS

Plant material
Plants of *Brassica napus* L. cv. Topas were grown under green-house conditions at 18-23 °C. In a sequence from 2 to 7 mm, buds were harvested from the terminal raceme.

Fixation and immunofluorescent staining of MTs
Anthers were excised, cut into three parts and fixed in 3% paraformaldehyde (PFA) in microtubule stabilizing buffer (MSB) consisting of 0.1 M 1,4-piperazinediethanesulfonic acid (PIPES), 1mM ethylene glycol bis (2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM $MgCl_2$ and 0.4% polyethyleneglycol (PEG) 6000 (pH 6.9) for 2 h at room temperature (RT). Triton X-100 (0.05%) was added to the fixative. After fixation, samples were rinsed, dehydrated, embedded in PEG, and sectioned according to Van Lammeren et al. (1985). Sections were mounted on poly-L-lysine coated slides, treated with 0.1 M NH_4Cl, and then incubated with the polyclonal antibody R229 that was raised in rabbit against bovine tubulin and purified according to the method of Shelanski et al. (1973) (see also Van Lammeren et al. 1985). Sections were incubated with goat anti-rabbit IgG conjugated with FITC (Nordic, Tilburg, The Netherlands) and covered with 20% Mowiol in Citifluor (Citifluor Ltd., London) containing 0.01 μg/mL 4,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co., St.Louis, Mo.).

17

Staining of actin filaments in unsectioned microspores and pollen
Microspores and pollen were isolated at various developmental stages by gently pressing the anthers in a buffer containing 100 mM PIPES, 10 mM EGTA, 0.05 % (v/v) Nonidet P-40, 5% (v/v) dimethyl sulfoxide (DMSO), and the osmoticum sucrose (see also Traas et al. 1987). The sucrose concentration varied from 7.5% for early developmental stages to 15% (w/v) for later stages. Microfilaments were stained by incubating microspores with 0.25 units/mL rhodamine-phalloidin (Molecular Probes Inc., Eugene, Oreg.) in the isolation buffer for 20 h up to 3 days at 4 °C in the dark.

Three procedures were tested. (*i*) Isolated microspores were stained without prefixation; (*ii*) in some experiments Triton X-100 (0.1 - 0.2 % (v/v)) was added to the isolation buffer to permeabilize cell membranes; microspores were then stained without prefixation; (*iii*) microspores were fixed in 3% PFA in MSB for 15 min at RT; they were washed twice (10 min each) in MSB and then stained. All three procedures were concluded by adding 0.02 µg/mL DAPI and cells were examined within 2h.

Staining of actin filaments in cryosectioned microspores and pollen
Flower buds without sepals and petals were fixed in 3% PFA in MSB containing 0.1% Triton X-100 for 1 h at RT and washed twice in MSB for 20 min. Buds were rapidly frozen in liquid nitrogen, warmed up to -20 °C and cut into 6 µm sections with a Damon IEC/CTF microtome-cryostat. Sections were stuck onto poly-L-lysine coated slides at -20 °C, warmed up to RT, and incubated in 0.25 units/mL rhodamine-phalloidin and 0.02 µg/mL DAPI in the buffer described above for 2 h at 4 °C in the dark.

Fluorescence microscopy
Fluorescence of nuclei, MTs, and MFs was visualized with a Nikon Labophot epifluorescence microscope with a mercury lamp (Hg 100 W) using the following filters: (*i*) for DAPI: EX 365/ DM 400/BA 420; (*ii*) for FITC: EX 470-490 / DM 510 / BA 515 EF; and (*iii*) for rhodamine: EX 510-560 / DM 580 / BA 590. Black and white images were recorded on Kodak TMY 135-film.

Electron microscopy
Anthers were dissected, cut into three parts and fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0) for 2 h at RT. After rinsing with buffer, samples were postfixed with 1 % (w/v) osmium tetroxide in buffer for 1.5 h, rinsed again and dehydrated in a graded ethanol series. Uranyl acetate (1% (w/v)) was added to the 70% ethanol step and applied for 1 h. Ethanol was substituted by epoxy resin (Spurr 1969) and samples were polymerized at 70°C. Ultrathin sections, cut with glass knives on an LKB Bromma 2088 Ultrotome, were poststained with uranyl acetate and lead citrate in an LKB Bromma 2168 Ultrostainer Carlsberg System. Sections were observed with a JEOL JEM-1200 EXII electron microscope.

RESULTS

Microtubule patterns

The development of male gametophytes was investigated from early microspore to mature tricellular pollen stage. Representative images of the organization of the MT configurations are given in Figs. 1a-12a and the concomitant structure of the nuclei stained with DAPI in the same sections in Figs. 1b-12b. Control experiments in which the first antibody was omitted, or pre-immune serum was applied, revealed no fluorescent labelling (data not shown).

Microspores just released from tetrads exhibited MTs surrounding the nuclei and running throughout the cytoplasm (Figs. 1a and 1b). Hereafter microspores formed vacuoles and enlarged. Initially nuclei remained in the centre (Fig. 2b). Prominent MT staining surrounded the nuclei, and was also observed in the central and cortical cytoplasm (Fig. 2a). Electron microscopy revealed bundles of MTs containing 5-10 MTs each (data not shown).

All microspores at late unicellular stage had formed a large vacuole (Figs. 3a and 3b). Nuclei were found near the cell wall. Where they bordered the large vacuole they were somewhat indented (Fig. 3b, arrow). Microtubules were evident in the cytoplasm (Fig. 3a) but not in thick bundles, as observed in the previous stage. Single MTs, connected to the nucleus and the plasma membrane, were observed in the thin layer of cytoplasm between the nucleus and the cell wall (Fig. 13, arrows). Preprophase bands were not observed before mitosis.

At prophase (Fig. 4b) the nuclei were surrounded by numerous MTs arranged in a dense network (Fig. 4a). Up to the end of cytokinesis, MTs were only present in spindles and phragmoplasts (Figs. 5-8). Figure 5a shows the mitotic spindle during metaphase. Most fluorescent MTs ran from the unstained chromosomes towards the poles. Some MTs penetrated the equatorial plate running from one pole to the other. From metaphase to telophase (Fig. 6) the MTs running from kinetochore to pole shortened and disappeared at the onset of cytokinesis. The MTs running form pole to pole persisted in the centre of the spindle until the end of telophase (Fig. 6a). At this time a phragmoplast was formed between the generative and the vegetative nuclei (Figs. 7a and 7b). Electron micrographes show phragmoplast MTs connected with the nuclear membrane and running close to the vesicles of the cell plate (Fig. 14). After cell wall synthesis in the central region, the phragmoplast expanded towards the periphery of the cell (Fig. 8). At the final stage of cell wall formation, when the cell plate was attached to the parental wall, numerous MTs were observed in the phragmoplast located near the pollen wall (Fig. 15). In the generative cell we observed MTs running from the nuclear membrane towards the phragmoplast (Fig. 15). Finally the generative cell completely separated from the vegetative cell but remained attached to the parental wall (Fig. 9). The cytoplasm of the vegetative cell contained a random network of MTs (Fig. 9a). During this stage most MTs were found near the common wall between the generative and vegetative cells (Fig. 9a). Electron microscopy revealed that the MTs in the generative cell were predominantly ordered parallel to that common wall, whereas in the vegetative cell they were mainly attached to the cell membrane and radiated into the cytoplasm (Fig. 16).

During the progress of microgametogenesis the generative cell rounded up, separated from the parental wall and migrated to the centre of the pollen, there occupying a position near the vegetative nucleus (Fig. 10b). At this stage the pollen grain contained MTs in both cells. Most MTs in the cytoplasm of the vegetative cell were found near the plasma membrane that

19
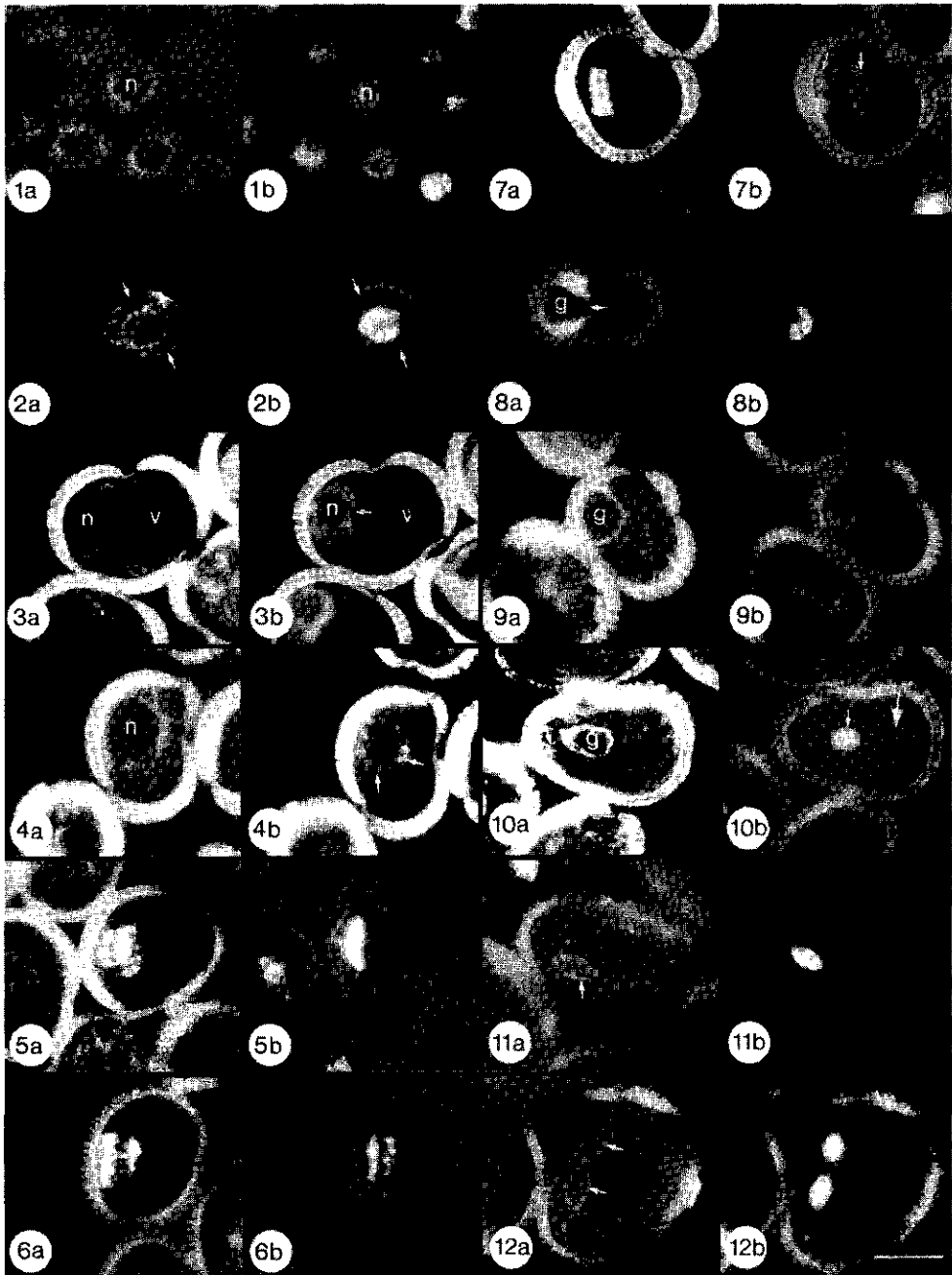
borders the pollen wall. In the thin layer of cytoplasm of the generative cell all MTs were arranged in bundles (Figs. 10a and 17).

At prophase and metaphase of pollen mitosis, MTs were only observed in the generative cell (for prophase see Fig. 11). The mature tricellular pollen grain exhibited MTs in the cytoplasm of the sperm cells (Fig. 12; electron microscopical data not shown). The cytoplasm of the vegetative cell exhibited diffuse fluorescence.

FIGS. 1 - 12. Survey of changes in microtubules (MTs) and nuclei observed in sectioned microspores and pollen of *Brassica napus*.

Figs. 1a-12a show MTs visualized immunocytochemically with FITC.

Figs. 1b-12b show the identical sections illuminated for the DAPI-stained nuclei.

Bar = 10 μm for all figures.

Fig. 1.   Stage of free young microspore. (a) MTs arranged in a crisscross pattern, especially around the nucleus (n). (b) Microspores with DAPI-stained nuclei (n).

Fig. 2.   Midmicrospore stage. (a) Thick bundles of MTs throughout the cytoplasm. Small vacuoles are formed (arrows). (b) The nucleus occupies the central position in the cell. Arrows point to the vacuoles.

Fig. 3.   Late microspore stage. (a) Cells are enlarged and contain a large vacuole (v). MTs are around the nucleus (n) and in the cytoplasm. (b) Nucleus that is near the wall is indented (arrow) by the large vacuole (v).

Fig. 4.   Microspore in prophase. (a) MTs form a dense network around the nucleus (n). (b) Chromosomes (arrows) become visible in the nucleus.

Fig. 5.   Microspore in metaphase. (a) MTs form a mitotic spindle with pole to pole MTs and chromosome to pole MTs. (b) The position of chromosomes coincides with the nonstained area in (a).

Fig. 6.   Microspore in telophase. (a) The mitotic spindle clearly shows pole to pole MTs. (b) Separation of chromosomes is evident.

Fig. 7.   Early stage of cytokinesis after microspore mitosis. (a) Numerous parallelly arranged MTs form the phragmoplast. Note the flat phragmoplast at this stage. (b) DAPI staining reveals karyokinesis, which has just finished because nuclei are still flattened (arrows).

Fig. 8.   Late stage of cytokinesis after microspore mitosis. (a) The phragmoplast extends centrifugally after wall synthesis between daughter nuclei and becomes hemispherical. Note the absence of MTs in the area of the newly formed cell wall (arrow; g, generative cell). (b) Only the generative nucleus is in the plane of the section.

Fig. 9.   Young bicellular stage. (a) Dense and specific staining along the common wall does not represent fluorescence of the wall itself but shows the presence of numerous MTs (see also Fig. 16, arrowheads and arrow). A random pattern of MTs is found in the vegetative cell (g, generative cell). (b) Only the generative nucleus is in the plane of the section.

Fig.10.   Bicellular pollen with a free generative cell. (a) Vegetative cytoplasm exhibits arrays of MTs radiating from the plasma membrane (arrow). The generative cell (g) becomes lens shaped and its cytoplasm is densely stained by fluorescent MTs. (b) Note the difference in staining of the generative nucleus (small arrow) and vegetative nucleus (large arrow) and position of the generative cell near the vegetative nucleus.

Fig.11.   Prophase of pollen mitosis. (a) MTs were only observed in the cytoplasm of the generative cell (arrow). (b) DAPI staining shows condensed chromosomes in generative cell. The vegetative nucleus is not in the plane of the section.

Fig.12.   Mature pollen grain. (a) MTs (arrows) are only observed in sperm cells that attach to each other. (b) Sperm nuclei are well stained; the vegetative nucleus is not in the plane of the section.

21
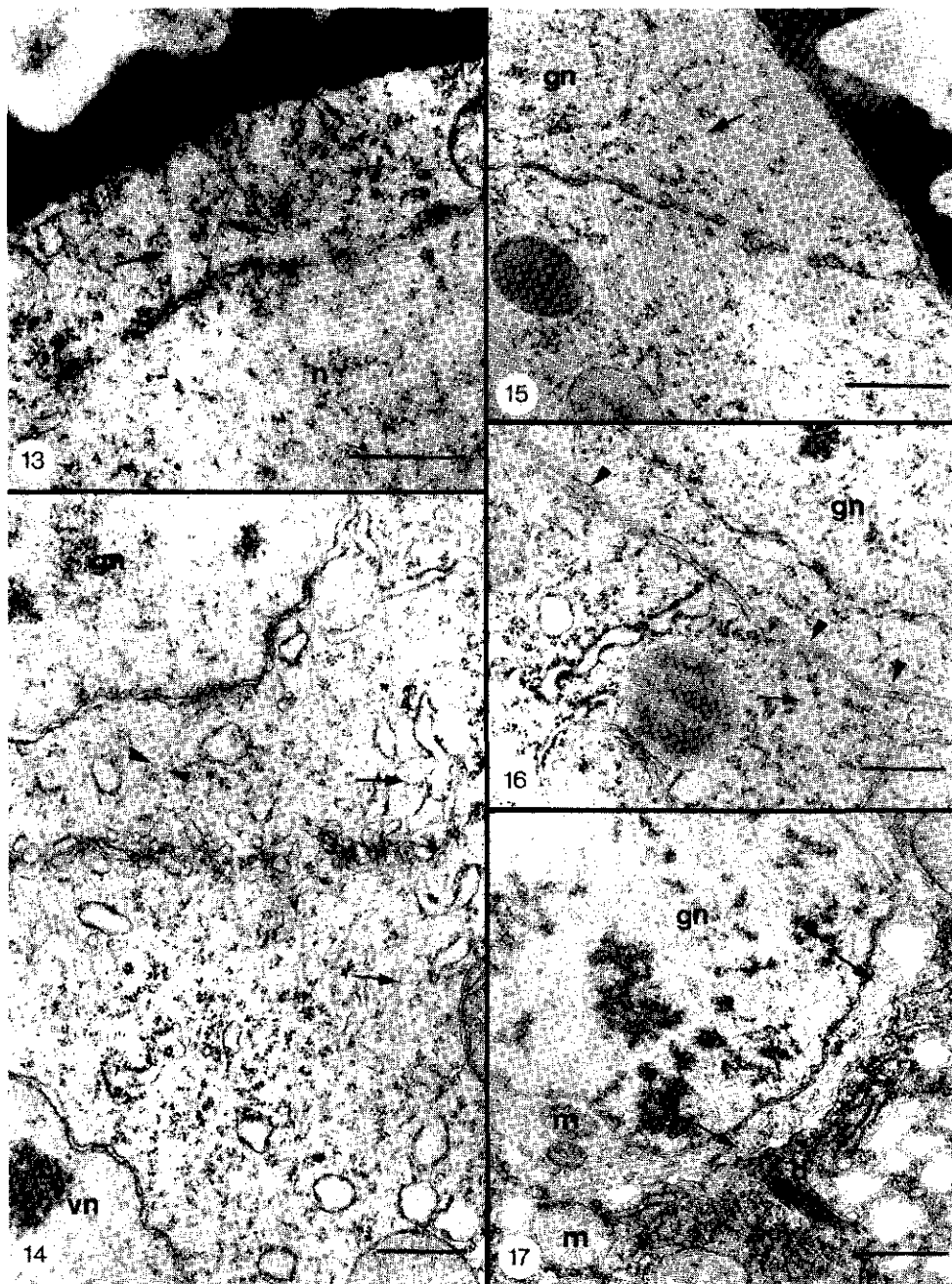
Microfilament patterns

The distribution of MFs in microspores and pollen was analyzed from the early microspore to the mature tricellular pollen stage. Figures 18-25 show representative images of MF configurations, visualized by rhodamine-phalloidin, and the concomitant nuclei as visualized by DAPI staining. The visualization of MFs by electron microscopy proved unsuccessful with the methods applied. Also, microfilaments could not be visualized light microscopically in released microspores (Fig. 18) despite the application of various staining methods. Only diffuse fluorescence was detected in the cytoplasm during microspore mitosis.

At the onset of cytokinesis MFs appeared in the phragmoplast between the two daughter nuclei (Figs. 19a and 19b). MFs expanded to the periphery of the cell as did the phragmoplast described before (Fig. 20a). At the end of cytokinesis, when the generative cell was completely separated from the vegetative cell and from the parental pollen wall, the generative cell was surrounded by or contained relatively thick bundles of MFs (Fig. 21). Simultaneously, MFs appeared in a small zone of the cortical cytoplasm of the vegetative cell and in a distinct orientation, i.e., hoop-like arrangements perpendicular to the imaginary axis between the two nuclei of the bicellular microgametophyte (Figs. 22 and 23). This banding pattern was at first limited to the equatorial plane (Fig. 23), but it progressively spread over most of the surface of the developing pollen grain at the end of the bicellular stage (Fig. 24). The overall orientation, however, was maintained. During pollen mitosis MFs were not detected.

The tricellular, mature pollen grain showed fluorescence in the cytoplasm of the vegetative cell particularly around the nucleus (Fig. 25a and 25b).

FIGS. 13-17. Transmission electron micrographs of microspores and pollen of *Brassica napus*. ABBREVIATIONS: d, dictyosome; gn, generative nucleus; m, mitochondrion; n, nucleus; vn, vegetative nucleus. Bar = 0.5 μm.

Fig.13. Late microspore stage (cf. Fig. 3). The nucleus located near the cell wall is connected with the cell membrane via MTs (arrows).

Fig.14. Cytokinesis of microspore mitosis (cf. Fig. 7). Phragmoplast MTs (arrows) are located in both cells. Note MTs connected with the generative nucleus running to vesicles of the cell plate (arrowheads).

Fig.15. Detail of bicellular pollen at end phase of cytokinesis with phragmoplast MTs in both cells (cf. Fig. 8). Note MT running from the generative nucleus to the phragmoplast (arrow).

Fig.16. Young bicellular pollen (cf. Fig. 9). Note MTs parallel to the wall in the generative cell (arrowheads) and MTs radiating from the common wall into the cytoplasm of the vegetative cell (arrow).

Fig.17. Detail of a free generative cell surrounded by vegetative cytoplasm (cf. Fig. 10). Its thin layer of cytoplasm contained bundles of MTs (arrows).

DISCUSSION

*The MT cytoskeleton during microspore development.*
After the release of the microspores from the tetrad, most MTs were found surrounding the nucleus. Some MTs radiated from that region into the cytoplasm. Such phenomenon is also described by Dickinson and Sheldon (1984) for *Lilium henryi* during the tetrad stage. According to the authors this configuration is responsible for the positioning of the nucleus and the transport of materials to the surface of the cell. Van Lammeren et al. (1985) suggested a scaffolding function. In our illustrations of *B. napus* microspores the fine distribution of MTs also points to such a stabilizing function. This configuration of MTs disappears during further growth of the microspore. In the midmicrospore stage, when the nucleus is still in a central position, some MT bundles still surround and may anchor the nucleus but others run throughout the cytoplasm. MTs might be involved in the movement of the nucleus to an eccentric position near the wall where the first karyokinesis will occur. In the nonvacuolated microspores of orchids (genus *Phalaenopsis*), Brown and Lemmon (1991a) found a so-called generative pole microtubule system that marks the path of nuclear migration prior to microspore mitosis. In *B. napus*, however, no such polar MT arrangement was observed, but a large central vacuole developed (Figs. 3a and 3b). The shift in nuclear position might therefore be induced by the developing vacuole: when the small vacuoles shown in Fig. 2 fuse in a controlled fashion, they form at least one large laterally positioned vacuole that influences the position of the nucleus (Figs. 3a and 3b). Electron and light micrographs of late microspores strongly indicate that the nucleus is pressed aside and even flattened or indented by pressure generated by that vacuole (Fig. 3b, arrow). Terasaka and Niitsu (1990) showed in *Tradescantia* that movement of the microspore nucleus was generated first by the growing vacuole and second by the MT cytoskeleton. Although in *B. napus* we did not observe a similar two-step mechanism of nuclear movement, both vacuoles and MTs might yet be involved simultaneously in the process of nuclear migration. Once the nucleus is forced to its peripheral position by the enlargement of the vacuole, MTs may maintain that position (Fig. 13) because we found MTs connecting the nucleus with the plasma membrane (Fig. 13; see also Van Lammeren et al. 1985). Thus the maintenance of the lateral position of the nucleus and the resulting positions of the future spindle and phragmoplast are most probably realized by MTs.

The MT cytoskeleton during microspore mitosis and pollen development.
Cell division in mitotic cells is often preceded by the formation of preprophase bands that determine the position of the future cell plates (Wada et al. 1980; Gunning 1982; Palevitz 1986). In microspores of *B. napus*, however, we never observed such preprophase bands. The absence of preprophase bands in microspore mitosis was also reported for *Gasteria* by Van Lammeren et al. (1985), for *Tradescantia* by Terasaka and Niitsu (1990), and for *Phalaenopsis* by Brown and Lemmon (1991a). Apparently preprophase bands do not determine the plane of division in these microspores as they do in other cell types.
During cytokinesis we observed MTs connecting the nuclei with phragmoplast vesicles (Fig. 14). Initially, when the phragmoplast between the two nuclei was a flat plate, MTs radiated from both nuclei. Thereafter the phragmoplast appeared hemispherical (Fig. 8a). Since MTs

FIGS. 18-25. Survey of changes in the microfilamental (MF) cytoskeletons and nuclei observed in microspores and pollen of *Brassica napus*. All a and b photographs are made from the same cell. Except for Figs. 18a and 18b, taken from a cryosection, all figures show whole cells. All left hand figures and Fig. 24 show rhodamine-phalloidin stained cells; the other photographs show DAPI staining. Bar = 10 μm.

Fig.18. Late microspore stage. (a) MFs are not visible, only the diffuse background (v, vacuole). (b) DAPI fluorescence indicates the position of the nuclei.

Fig.19. Cytokinesis of microspore mitosis. (a) MFs are observed in the region of the phragmoplast (arrow). (b) Positions of the generative (small arrow) and vegetative nuclei (large arrow) are indicated.

Fig.20. Late cytokinesis of microspore mitosis. (a) MFs stain in the region of the extending phragmoplast located at the periphery of the extending cell plate (arrows). (b) Positions of the generative (small arrow) and vegetative (large arrow) nuclei are indicated.

Fig.21. Midbicellular stage. MFs (arrow) are around the generative cell (g) and in cortical cytoplasm of the vegetative cell (arrowheads).

Fig.22. Midbicellular stage. The generative cell dissolved from the parental wall and located near the vegetative nucleus (large arrow). The small arrow indicates the nucleus of the generative cell. The white lines assign an imaginary axis through the generative cell and vegetative nucleus.

Fig.23. Midbicellular stage comparable with Figs. 21 and 22. Cortical MFs are arranged in hoop-like arrays perpendicular to the imaginary axis through generative cell and vegetative nucleus marked by white line (see also Fig. 22).

Fig.24. Late bicellular stage. Hoop-like cortical MFs spread over the whole surface of developing pollen.

Fig.25. Mature pollen. (a) MF staining around the vegetative nucleus (arrow). (b) Positions of the sperm cell nuclei (small arrows) and vegetative nucleus (large arrow) are indicated.

25
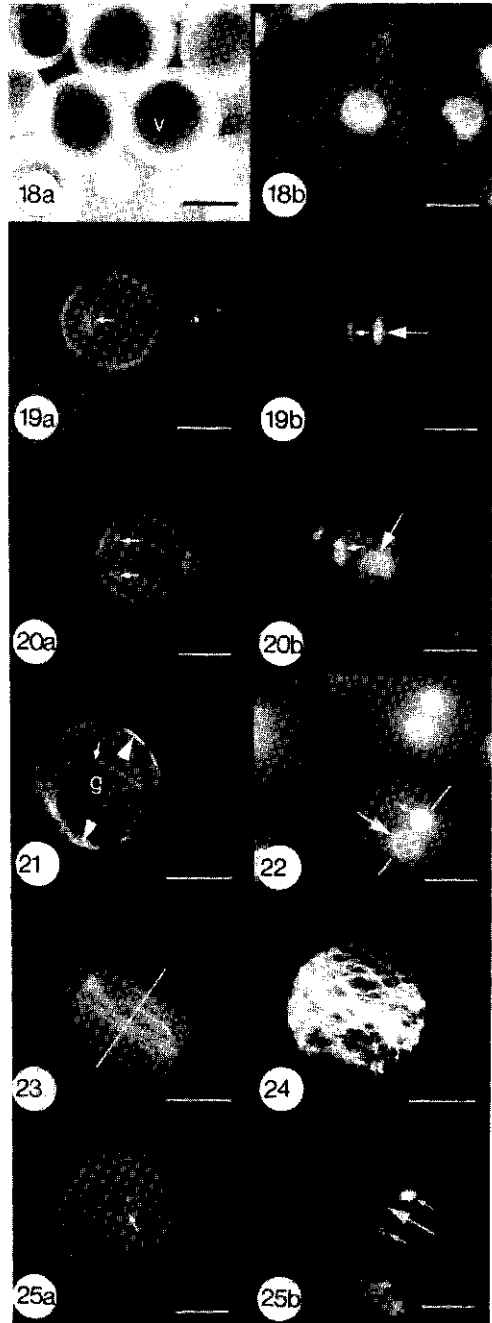
run only from the generative nucleus to the extended phragmoplast at that stage and not from the vegetative nucleus (Fig. 15), we suggest that this particular curving of the phragmoplast was caused by MTs. As MTs attached to the nucleus of the generative cell, we propose it to be the regulator. These findings are in agreement with the data reported for *Phalaenopsis* by Brown and Lemmon (1991b). Their statement, however, that the newly formed wall does not join the parental wall deviates from our results.

After cell division, but before the generative cell separated from the parental wall of the pollen grain, MTs were mainly observed along the common wall. This distribution changed during the free generative cell stage. Newly formed bundles of MTs in the generative cell may be responsible for the lens shape of the generative cell (Fig. 17; see also Sanger and Jackson 1971).

During the last developmental steps to maturity, MTs were always observed in both the generative and sperm cells. The high, diffuse fluorescence in the cytoplasm of the vegetative cell may be a pool of unpolymerized tubulin, to be used for MT polymerization at the onset of pollen germination.

The MF cytoskeleton during microspore and pollen development.

Prominent arrays of MFs were found in the cortical cytoplasm of the vegetative cell and around the generative cell in the mid- and late-bicellular stage. Based on the evident patterns of fluorescent arrays and control experiments previously published (Van Lammeren et al. 1989), the specificity of the staining was confirmed (see also Wulf et al, 1979). Although various staining methods were applied, we could not recognize MFs during microspore development. Either the methods applied are not suitable for the observation of all types of MF configurations or *B. napus* exhibits a particular type of pollen development. The latter explanation is preferable since the methods were successful for the detection of MFs during all stages of pollen development in *Gasteria* (Van Lammeren et al. 1989).

In *B. napus*, MFs were not detected in spindles of dividing microspores but present, though faintly stained, in the phragmoplasts. Schmit and Lambert (1990) did show the simultaneous presence of MFs and MTs in mitotic spindles and phragmoplasts of *Haemanthus* endosperm cells. Double-labelling of F-actin during the cell cycle showed, however, that MFs in the phragmoplast are newly assembled. The authors suppose a pool of G-actin during nuclear division that is induced to polymerize during anaphase. Because we did not find MFs during mitosis but observed them in the phragmoplast of the microspore and in the bicellular stage, a pool of G-actin is also likely to exist in *B. napus*. The pool of G-actin in the mature pollen will be used for the assembly of MFs during pollen germination.

The co-distribution of MFs and MTs in phragmoplasts found during microspore mitosis is also described for other species: (*i*) *Allium*, Clayton and Lloyd (1985) and Parke et al. (1986); (*ii*) *Equisetum*, Derksen et al. (1986); (*iii*) *Gasteria*, Van Lammeren et al. (1989); (*iv*) *Lilium*, Sheldon and Hawes (1988). Lloyd and Traas (1988) assume that MFs, in co-operation with MTs, cause the movement of the phragmoplast from the initially central position to the periphery of the cell. Additionally, it might be well possible that they function in the transport of vesicles towards the cell plate.

The orientation of the parallel-arranged hoop-like bundles of cortical MFs in the bicellular pollen grain was not observed in *Gasteria* with comparable techniques (Van Lammeren et al. 1989). The orientation clearly points to an expression of polarity within the pollen grain

26

because it is always fixed in relation to the two nuclei. Perhaps the cortical MFs function in the generation of cytoplasmic streaming or, more generally, maintain the polarity of the pollen grain and prepare the vegetative cell for pollen germination.

ACKNOWLEDGEMENTS

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Chapter 3

Cytoskeletal changes and induction of embryogenesis in microspore and pollen cultures of *Brassica napus* L.

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ABSTRACT

Microspores and pollen of *Brassica napus* were cultured under conditions leading to embryo formation. Concomitant changes in cytoskeletal configurations were analysed. The microfilamental cytoskeleton exhibited a loss of polarity in embryogenic cells but cytochalasin treatment revealed that microfilaments do not influence embryogenesis. Two embryogenic pathways started from microspores and were either characterized by turned division planes or by division when the nucleus was in the cell centre. In both cases microtubules clearly exhibited new arrangements and likely played a major role in newly induced symmetrical division. In pollen, embryogenic development started in the vegetative cell provided the generative cell was arrested near the pollen wall. The concomitant disappearance of defined microtubular arrays is likely to be responsible for the positioning of the cell.

INTRODUCTION

Embryogenic microspore cultures represent an excellent tool for basic research on the induction of regeneration in single cell systems. First reports of Thomas and Wenzel (1975) and Lichter (1982) about the initiation of embryo formation from microspores of *Brassica napus* encouraged several groups to optimise the frequency of regeneration. Today *B. napus* represents a model plant for regeneration. Yields of up to 70% embryos can be obtained (Pechan and Keller, 1988). Because of numerous difficulties in initiating embryogenic cultures from microspores of other species, it was decided to analyze the cytological, physiological and biochemical changes in cultures of *B. napus* in detail.

It is known that high temperatures induce embryogenic development in late microspores and young bicellular pollen of *B. napus* and that this property is acquired within 8 hours of elevated temperature (Pechan and Keller, 1988). The change in developmental pathway from gametophytic to sporophytic development coincides with changes in the expression of mRNA and protein synthesis (Pechan et al., 1991) as well as with changes in cell division patterns. As karyokinesis and cytokinesis depend on cytoskeletal configurations (Van Lammeren et al., 1985), we analyzed the changes of the cytoskeleton during the induction phase of embryo development in *B. napus* microspores and pollen under embryogenic (32°C) and non-embryogenic (18°C) conditions. This paper deals with the changes in the microfilamental (MF) and microtubular (MT) cytoskeletons during the first 24 h of cultivation. The data are compared with those found during microspore and pollen development *in vivo* (Hause et al., 1992), and are related to subcellular changes leading to embryo development to state the role of the cytoskeleton in regeneration.

MATERIALS AND METHODS

Plant material. Plants of *Brassica napus* L. cv. Topas were obtained from seeds, grown 4 weeks under greenhouse conditions at 18-23°C, and then kept at low temperature (10°C in the light at 300 μmol photons.$m^{-2}s^{-1}$ for 16 h and 5°C in the dark for 8 h) till the onset of flowering. Buds from 3.6 to 4.2 mm were harvested from the terminal raceme and used for the initiation of cultures. Experiments were repeated 10 times.

Cultivation of microspores and pollen. Microspores and pollen were isolated and cultivated in a modified Lichter medium (NLN, Lichter, 1982), free of potato extract and growth regulators as described by Pechan and Keller (1988). After cultivation for 0, 2, 4, 6, 8, 10, 12, and 24 h at embryogenic conditions (32°C) or at non-embryogenic conditions (18°C; J.B.M. Custers, personal communication), samples were collected for further analysis. The embryogenicity of cultures was controlled at day 4 by the analysis of nuclei stained with 0,02 μg/ml 4,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co., St. Louis, Mo.). Embryos were counted after 3 weeks.

Treatment with cytochalasin B. At the onset of the cultivation 5 μg/ml cytochalasin B

33

(SERVA, Heidelberg, Germany) were added to the NLN-medium to disorganize actin filaments. After 8 h of cultivation either at 32°C or at 18°C the cells were washed twice with NLN-medium on sieves with 7 μm mesh, and then cultivated in NLN-medium for 3 weeks at 32°C and at 18°C, respectively.

Staining of microfilaments and nuclei. After harvesting, microspores were fixed in 3% (w/v) paraformaldehyde (PFA) and 0.1% (v/v) Triton X-100 in microtubule stabilizing buffer (MSB) consisting of 0.1 M 1,4-piperazinediethanesulfonic acid (Pipes), pH 6.9, 1 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM $MgCl_2$, and 0.4% (w/v) polyethylene glycol (PEG) 6000 at room temperature for 15 min. After two washing steps in MSB of 10 min each, the microspores were incubated in staining buffer for 4 h at 4°C in the dark. The buffer contained 0.1 M Pipes, pH 6.9, 10 mM EGTA, 5% (v/v) dimethyl sulfoxide, 0.5% (v/v) Nonidet P-40, 10% (w/v) sucrose, 0.25 units/ml rhodamine-phalloidin (Molecular Probes Inc., Eugene, Oreg.) and 0.02 μg/ml DAPI.

Preparation of microspores and pollen for immunolabelling of microtubules. Cultured microspores, pollen and embryogenic cell complexes were collected in sieves and fixed at room temperature for 1.5 h with 3% (w/v) PFA in MSB. Triton X-100 (0,05% [v/v]) was added to the fixative. After fixation samples were rinsed in MSB and immobilized in 3% (w/v) agar and 1% (w/v) gelatine in MSB. Pieces of agar were dehydrated in a graded series of ethanol, embedded in PEG (PEG 1500 : PEG 4000 = 2 : 1), cut in 2 μm sections and processed as described by Van Lammeren et al. (1985). Immunolabelling was carried out with a monoclonal anti α-tubulin-IgG1 (Sigma Chemical Co., St. Louis, Mo.) as the primary antibody, and a goat-anti-mouse-IgG-BODIPY conjugate (Molecular Probes) as secondary antibody. After immunolabelling, sections were stained with 0,1 μg/ml DAPI for 30 min and mounted in Citifluor-glycerol (Citifluor Ltd., London). Control experiments were performed by omitting the first antibody.

Fluorescence microscopy. The fluorescence of nuclei, MFs and MTs was visualized with a Nikon Microphot-FXA epifluorescence microscope supplied with a mercury lamp (HBO 100 W) using the following filters: (i) for DAPI: EX 365/DM 400/BA 420, (ii) for rhodamine: EX 510-560/DM 580/BA 590 and (iii) for BODIPY: EX 470-490/DM 510/BA 515 EF. Black and white images were recorded on Kodak TMY 135-film.

RESULTS

Embryogenicity of the culture. Microspores at the late unicellular stage and pollen grains at the early bicellular stage were optimal for the induction of embryogenesis. The culture was started with a mixture of microspores and pollen mainly in these stages. After 4 days of cultivation at 32°C up to 30% of the microspores and pollen exhibited embryogenic development. Symmetrically divided microspores and proembryos with 3 or more nuclei were formed. After 3 weeks of cultivation up to 2000 torpedo-shaped embryos were counted per Petri-dish containing 2 ml of suspension with approximately 2.10^4 cells/ml. Normal pollen

development and configurations intermediate between pollen and embryo development were also found at 32°C. Embryogenesis did not occur at 18°C but pollen were formed.

Microfilamental configurations during the first 24 h of cultivation. The MF patterns at the start of cultivation corresponded with the *in vivo* configurations, described by Hause et al. (1992). No changes, caused by the isolation procedure, were found.

Microfilaments in cultured microspores. From 6 h of cultivation onwards fine filamental networks appeared around the nucleus and in the cytoplasm of late microspores at both temperatures (Fig. 1). They were not observed at this stage *in vivo*.

Under non-embryogenic conditions mitosis proceeded as *in vivo* microspores (Fig. 2). Phragmoplast formation gave rise to unequal division and gametophytic development (Fig. 6). Under embryogenic conditions changed division planes were observed: During the first 12 h of cultivation, the nucleus maintained its marginal position as in normal microspore mitosis. The division plane, however, rotated up to 90° (Fig. 3). Colocalization with, or connections between MFs and the spindle apparatus were not observed (Figs. 2a, 3a), but MFs occurred in phragmoplasts at both temperatures (Figs. 4, 6). After 24 h of culture at 32°C phragmoplasts were also observed in the centre of the microspores (Fig. 4). They were formed in microspores in which the nucleus migrated to the centre of the cell before mitosis. Both types of microspore divisions at 32°C resulted in equally sized daughter cells. The daughter cells exhibited MFs in the peripheral cytoplasm (Fig. 5).

Microfilaments in early bicellular pollen. At 18°C culture bicellular pollen contained a generative cell which is initially attached to the intine (i.e. early bicellular stage I). Hereafter, the generative cell becomes round, separates simultaneously from the cell wall (the early bicellular stage II) and migrates to the centre of the vegetative cell (the mid-bicellular stage, cf. Fig. 21). Microfilaments of the vegetative cytoplasm enclosed the migrated generative cell which itself did not show MFs (Fig. 7).

Cultivation at 32°C induced differences in the MF-patterns in early bicellular pollen: The generative cell rounded up, but did not separate from the intine and exhibited MFs around its nucleus (Fig. 8). The vegetative cell exhibited a criss cross pattern of MFs and the characteristic banding pattern of *in vivo* grown bicellular pollen was lost. Hereafter the vegetative nucleus divided (Fig. 9) and a proembryo of 3 cells was formed (Fig. 10). The generative cell remained in its marginal position.
Pollen grains, which were in the mid-bicellular stage at the onset of cultivation, developed into mature, tricellular pollen grains at both temperatures. Their MF-patterns did not differ from those observed *in vivo* (data not shown).
To confirm the results about the embryogenic capacity of the bicellular pollen grains that have an arrested generative cell, a culture was started with only bicellular pollen (Table 1). After 96 h of cultivation the number of proembryos with 3 to 10 nuclei was comparable with the number of pollen grains which had an arrested generative cell after 24 h of cultivation.

Treatment with cytochalasin B during the induction phase of embryogenesis. In order to perturb MFs during the induction phase of embryogenesis, cytochalasin B was added at the start of cultivation. After 8 h of treatment the MF patterns in microspores and vegetative cells of bicellular pollen were disturbed. Only some MFs were detected within or around the generative cell (Figs. 11 a, b). Four hours after the removal of cytochalasin B the MF pattern was restored in all microspores and pollen (Figs. 11 c, d).

Further development of microspores and pollen was determined after 24 h and 96 h. The number of embryogenic complexes (bicellular pollen with arrested generative cell and symmetrically divided cells) at 24 h and the number of proembryos at 96 h was in the same range as in a culture at 32°C without cytochalasin B treatment (Table 2). In both cultures comparable numbers of embryos arose after 3 weeks of cultivation. Cytochalasin B did not influence the development of cultivated microspores and pollen at 18°C.

Plate I. Survey of changes in the microfilamentous (MF) cytoskeletons and nuclei observed in microspores of *Brassica napus* cultivated for 0 to 24 h under non-embryogenic (Figs. 1, 2, 6, 7) and under embryogenic (Figs. 3-5, 8-11) conditions. Figs. 1a - 11a and 11c show the MFs visualized by staining with rhodamine-phalloidin. Figs. 1b - 11b and 11d show the DAPI-stained nuclei of the same cells. Bar represents 10 μm for all figures.
Fig. 1: Late unicellular stage at 12 h of cultivation at 18°C. MFs are visible in the whole cytoplasm, the nucleus is surrounded by MFs.
Fig. 2: Microspore in anaphase at 24 h of cultivation at 18°C. MFs form a complex network through the whole cytoplasm, connections or colocalization with the spindle apparatus are not visible, the division plane (white line in b) corresponds with the division plane in vivo.
Fig. 3: Microspore in anaphase at 6 h of cultivation at 32°C. The division plane has rotated by 90° as indicated by the white line in (b). MFs do not show connections or colocalization with the spindle apparatus.
Fig. 4: Cytokinesis of microspore mitosis at 24 h of cultivation at 32°C. The MF-containing phragmoplast is located in the center of the cell, after cell wall formation two daughter cells of the same size will arise.
Fig. 5: Symmetrically divided microspore exhibits two cells of similar size. The daughter cells are separate and the MF-pattern is visible.
Fig. 6: Cytokinesis of microspore mitosis at 24 h of cultivation at 18°C. MFs are mainly observed in the region of the phragmoplast between the daughter nuclei. The position of the phragmoplast is typical for an asymmetric division of the microspore.
**Fig. 7:** Early bicellular stage II at 12 h of cultivation at 18°C. The generative cell (arrow) has rounded up and already moved to the center of the vegetative cell. The MF-network of the vegetative cell encloses the generative cell.
Fig. 8: Early bicellular stage II at 10 h of cultivation at 32°C. The generative cell (arrow) has rounded up, but remained at the intine. This cell is completely separated from the cytoplasm of the vegetative cell as visible by the MFs near the common wall (arrow-heads in a). Both cells exhibit a clearly distinct MF-network.
Fig. 9: Proembryo with vegetative nucleus in mitosis (big arrow in b). The previous generative cell (small arrow in a) is still arrested at the previous pollen wall. The hoop-like pattern in the vegetative cell, characteristic for this cell in vivo, is not observed.
Fig. 10: Proembryo with 3 nuclei. The previous generative cell (arrow) is distinguished from the daughter cells of the vegetative cell by its separate MF-network localized at the previous pollen wall.
Fig. 11: Bicellular pollen cultivated under embryogenic conditions in the presence (a, b) and after the removal of cytochalasin B (c, d). (a, b): Bicellular pollen after 8 h cultivation with cytochlasin B. The generative cell (small arrow) still shows intact MFs (small arrow-head in a), while the MFs within the vegetative cell are disrupted. There are only few short residues of the MF cytoskeleton visible (big arrow-heads in a). (c, d): Bicellular pollen 4 h after removing of cytochalasin B. The vegetative cell (big arrows) and the generative cell (small arrows) show a restored MF-network.
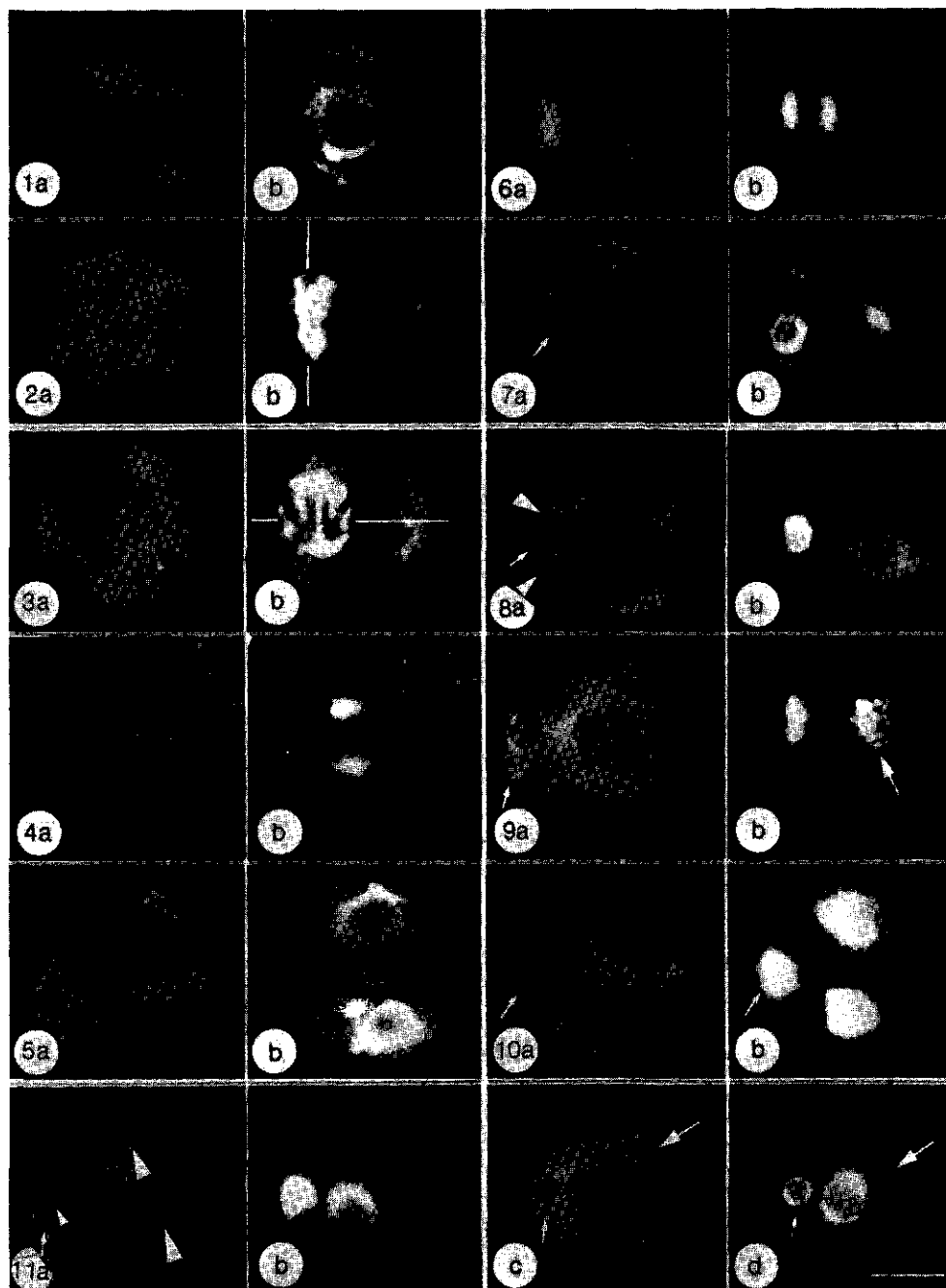
Developmental stage	Proportional distribution	
	after 24 h of culture	after 96 h of culture
broken	15.0	29.7
unicellular	0	0
bicellular: without MF	0	10.7
arrested generative cell	**18.3**	0
mid-bicellular	5.2	
late bicellular	21.6	21.5
two nuclei from the same size	3.3	0.8
symmetrical divided	0	0
mature pollen grain	33.3	20.4
3 nuclei (vegetative nucleus divided)	3.3	6.0
4 nuclei	0	4.7
5 nuclei	0	3.4
6 nuclei	0	0.8
7 - 10 nuclei	0	2.0
Σ 3 - 10 nuclei	3.3	**16.9**

Table 1: Survey of the development of bicellular pollen cultured for 24 h and 96 h at 32°C. The culture was started with about 35% early bicellular, 35% mid-bicellular and 15% late bicellular pollen grains. 15% of the pollen grains were broken. After 3 weeks of cultivation 2.3% of the cultivated pollen grains had formed embryos. Percentages are based on the counting of at least 300 cells per sample. Note the comparable percentages of the bicellular stages with arrested generative cell at 24 h and of the proembryos at 96 h.

Microtubule configurations at the start of culture. At the onset of culture three types of MT configurations were distinguished: (i) about 30% of the cells exhibited normal cytoskeletal arrangements as observed *in vivo*, (ii) about 40% of the cells showed abnormal MT configurations, and (iii) about 30% of the cells were without MTs (Fig. 12). Normal MT configurations like those found *in vivo* were mainly observed from late microspore stage

onward (Fig. 12a, arrows). Cells with abnormal configurations exhibited thick and short MT structures (Fig. 12a, small arrow).

Microtubule configurations during the first 24 h of cultivation. At 18°C numerous microspores and pollen showed no labelling or only short and thick labelled structures. However, MT configurations comparable to the *in vivo* situation were also observed, e.g. mitotic spindles (Fig. 13), phragmoplasts during microspore mitosis (Fig. 14) and MTs in early bicellular pollen (Fig. 16). Early bicellular pollen contained MTs parallel to the border between generative and vegetative cell, and in the vegetative cell also perpendicular to this border (Fig. 16a). Between 4 and 8 h of culture, microspores with prophase nuclei contained abundant arrays of MTs never seen during *in vivo* development (e.g. Fig. 15). These arrays showed a high diversity in shape, in orientation within the cell, and in spatial relation to the nucleus. Sometimes MTs appeared in dense arrays opposite the nucleus. Other times, spindle-like configurations were observed. DAPI-staining clearly revealed, however, that the prophase-nuclei or chromosomes were not located in such 'spindles' (Fig. 15).

The aberrant MT-configurations found at 18°C between 4 and 8 h (e.g. Fig. 15), were also formed at embryogenic conditions. Additionally, new MT-configurations occurred at 32°C.

At the start of culture at 32°C, microspores divided in a manner similar to those *in vivo*. The orientation of a normal fluorescent spindle is shown in figure 12a. After 2 h of culture, about half of the microspores maintained the normal division pattern. The other microspores, however, exhibited chromosome containing spindles which were still in acentric positions but with orientations which had shifted up to 90°. Between 4 and 8 h of culture, the majority of dividing microspores exhibited turned spindles and equatorial plates (Fig. 17). The phragmoplast remained in an acentric position and because of its rotated position (Fig. 18) division resulted in the formation of two symmetric cells. In intermediate cases the division plane turned less than 90° and two asymmetric cells were formed.

Microspore mitosis *in vivo* only occured when the nucleus is in an acentric position, but after 12 h of culture at 32°C, mitosis also occurred in microspores which had their nucleus in central position (Fig. 19). Karyokinesis and cytokinesis resulted in symmetrical division.

After 12 h of culture, bicellular stages were observed in which the generative cell remained arrested at the intine. There were two differences to the early bicellular pollen grain developed *in vivo*: (i) The generative cell contained a spherical instead of an elongated nucleus. (ii) The vegetative cell did not contain the MTs perpendicular to the common border (Fig. 20).

After 24 h of cultivation, some pollen exhibited vegetative nuclei in mitosis whereas the generative cells were still connected with the intine. The MT-patterns were comparable to those found after 12 h of cultivation.

developmental stage	distribution (in %) with cytochalasin B-pulse		distribution (in %) in control without cyto-chalasin B	
	24 h	96 h	24 h	96 h
broken	18.5	27.9	26.6	42.5
unicellular: without MFs	19.3	24.4	19.8	17.2
late	33.1		19.8	
mitosis	0.5	0	0.5	0
bicellular: early	2.5	0	3.1	0
mid-	8.8	6.4	8.9	8.3
arrested generative cell	**5.4**	4.1	**5.7**	0
symm. divided	**11.9**	*15.4*	**13.5**	*11.8*
late	0	1.0	0	0
mature pollen	0	2.6	0.5	2.9
proembryo with 3-10 nuclei	0	*18.2*	1.6	*17.3*

Table 2: Survey of the cultivation of microspores and pollen treated with cytochalasin B for 8 h and without cytochalasin B. Both cultures at 32 °C started from the same isolation and contained about 30 % mid-unicellular and 30 % late unicellular microspores, 3.5 % microspores in mitosis, 11 % early bicellular and 5.5 % mid-bicellular pollen grains. 20 % of the isolated cells were broken. In both cultures the yield of embryos was 3.1 % after 3 weeks; controls at 18 °C were without embryos. Percentages are based on the counting of at least 300 cells per sample. Note the comparable percentages of the bicellular stages with arrested generative cell as well as of the symmetrical divided microspores at 24 h in both cultures (fat arabics), and of the symmetrical divided microspores as well as of the proembryos at 96 h in both cultures (fat italic arabics).

DISCUSSION

Embryogenicity of the culture. Microspores and pollen were isolated from at least 20 buds per cultivation to analyze 10 samples from one culture at both cultivation temperatures. On the one side, such culture has the advantage to analyze all developmental stages of microspores and pollen simultaneously, on the other side, it was not possible to get such high percentages of embryos as reported by Pechan and Keller (1988) or Telmer et al. (1992) who used preparations from single buds. Moreover, it was not possible to follow the development

of one certain developmental stage in relation to time during the cultivation. This disadvantage was, however, overcome by the mathematical evaluation of culture development shown in Tables 1 and 2.

The results of the analysis of MFs and MTs are summarized in the schematic presentation of the development of microspores and pollen *in vivo* and under embryogenic conditions *in vitro* (Fig. 21). Three embryogenic pathways are presented. Approximately 30% of all microspores and pollen cultivated at 32°C followed these pathways. There were also cells which did not show clearly either embryogenic or pollen development. It was not possible to determine how these cells developed further. Based upon the quantitative analysis of the stages during the first 4 days of cultivation it is suggested that these cells degenerate and die.

Microfilamental cytoskeletons during induction of embryogenesis in microspores. In late microspores two pathways lead to symmetrical cell division (Fig. 21, I and II): (I) During the first 12 h of cultivation at 32°C the nucleus and mitotic spindle are still in a marginal position, but the division plane turns. (II) After 12 h of culture division is initiated, while the nucleus is in the centre of the cell. In both cases MFs did not colocalize with the mitotic spindle as was also found during microspore mitosis *in vivo* (Hause et al., 1992). Such colocalization was described for various plant cells (Seagull et al., 1986, Traas et al., 1987, Van Lammeren et al., 1989, Schmit and Lambert, 1990). Furthermore there were no differences between the MF-configurations in the embryogenic and the gametophytic development. These results hint at the independence of the embryogenic development from the MF-cytoskeleton. Although evidence has been accumulated, that MFs can influence the division plane (Cho and Wick, 1991) and the positioning of the nucleus (Katsuta and Shibaoka, 1988, Url et al., 1992) in plant cells, a more prominent influence on these processes is demonstrated for MTs (Mineyuki and Furuya, 1986, Venverloo and Libbenga, 1987, Katsuta et al., 1990, Zaki and Dickinson, 1991).
Comparable with the *in vivo*-situation in *B. napus* (Hause et al., 1992), MFs are located in the phragmoplasts of dividing cultured microspores. Although, MFs probably cause the movement of the phragmoplast from the initially central position to the periphery of the cell as assumed by Lloyd and Traas (1988), they are likely not responsible for the shift of the orientation of the phragmoplast. The MFs in the phragmoplast are newly assembled (Schmit and Lambert, 1990), but the orientation rather depends on the orientation of the spindle-apparatus and the existing division-plane.

Microfilamental cytoskeleton during the induction of embryogenesis in bicellular pollen. The third pathway which will initiate embryogensis starts at the early bicellular stage (Fig. 21 III). For *B. napus* it is demonstrated that the generative cell remains arrested near the intine during the first division of the vegetative nucleus. This arrest, visible after 12 h of cultivation at 32°C, appears to be a prerequisite for embryogenic development, because a divided vegetative nucleus was only observed when the generative cell was arrested. Additional proof was found after the evaluation of a culture, starting with bicellular pollen only. This culture showed 18.3% bicellular structures with an arrested generative cell after 24 h. Nearly all these complexes turned into proembryos after 96 h (16.9%, Table 1). The reason for the arrest is not detectable in the pattern of the MFs. Additionally, treatment with

cytochalasin B did not change the embryogenicity of cultures (Table 2). If there is a function for MFs during the induction of embryogenesis, the number of proembryogenic structures after 24 h should be lower in the treated culture because of the temporal difference in development of at least 8 h.

Microtubular configurations during cultivation. The MT configurations observed at the start of cultivation clearly differ from those observed *in vivo* (Hause et al., 1992). The differences are caused by the procedure i.e. mechanical stress, liquid nutrient medium and centrifugation forces. The absence of MTs in 30% of the cells might be the result of irreversible cell degeneration due to the isolation. The cells with thick and short bundles of MTs probably represent a group in which breakdown of MTs is followed by their renewed and less differentiated synthesis of MTs. However it cannot be excluded that some of these cells were dead at the start of fixation. Microspores and bicellular pollen with normal MT cytoskeletons and those having changed configurations most probably developed to normal pollen at 18°C or to normal pollen and embryos when cultured at 32°C.

Plate II. Survey of changes in microtubules (MTs) and nuclei visualized in sectioned microspores and pollen of *Brassica napus* at the start of culture and during cultivation under embryogenic and non-embryogenic conditions. Figs. 12a-20a show MTs visualized immunocytochemically with BODIPY. Figs. 12b-20b show the identical sections illuminated for the DAPI-stained nuclei.
Bars represents 10 μm (at Fig. 12b for 12a,b; at Fig. 20b for 13a,b - 20a,b)
Fig. 12: Microspores at the onset of cultivation containing MTs as during *in vivo* development (long arrow: late microspore; short arrow: microspore mitosis), short labelled structures (small arrow) and without labelling (arrowhead).
Figs. 13 - 16: Microspores and pollen cultivated at 18° C.
Fig. 13: Mitotic spindle during microspore mitosis in acentric position and division plane as in vivo (a, white lines) containing condensed chromosomes (b); 4h of cultivation.
Fig. 14: Cytokinesis during microspore mitosis at 8 h of cultivation. The phragmoplast is oriented as in vivo (a, white lines) and located between the generative (b, small arrow) and vegetative (b, arrow) nuclei.
Fig. 15: Prophase stage at 8 h of cultivation. The nucleus containing condensed chromosomes (b) is outside the spindle-like structure which is formed by MTs (a, arrow - position of the nucleus).
Fig. 16: Early bicellular stage at 12 h of culture. The generative cell is attached to the pollen wall (b, arrow - vegetative nucleus, small arrow - generative nucleus). MTs in the vegetative cell beside others perpendicular to the common border between both cells (a).
**Figs. 17 - 20: Microspores and pollen cultivated at 32°C.**
Fig. 17: Metaphase stage at 8 h of cultivation. The mitotic spindle (a) and the chromosomes in the metaphase plate (b) have a 90° turned orientation (white lines) in comparison to normal pollen development.
Fig. 18: Cytokinesis at 12 h of cultivation. Phragmoplast with an acentric starting point (a) between two nuclei (b) formed after a turn of 90° of the division plane (a, white lines).
Fig. 19: Prophase stage at 12 h of cultivation. The nucleus is located in the center of the cell, contains condensed chromosomes (b) and is surrounded by MTs (a).
Fig. 20: Bicellular stage at 12 h of cultivation. The vegetative nucleus (b - arrow) is near the generative cell (b - small arrow) which is rounded up. MTs were only observed parallel to the border between both cells (a).
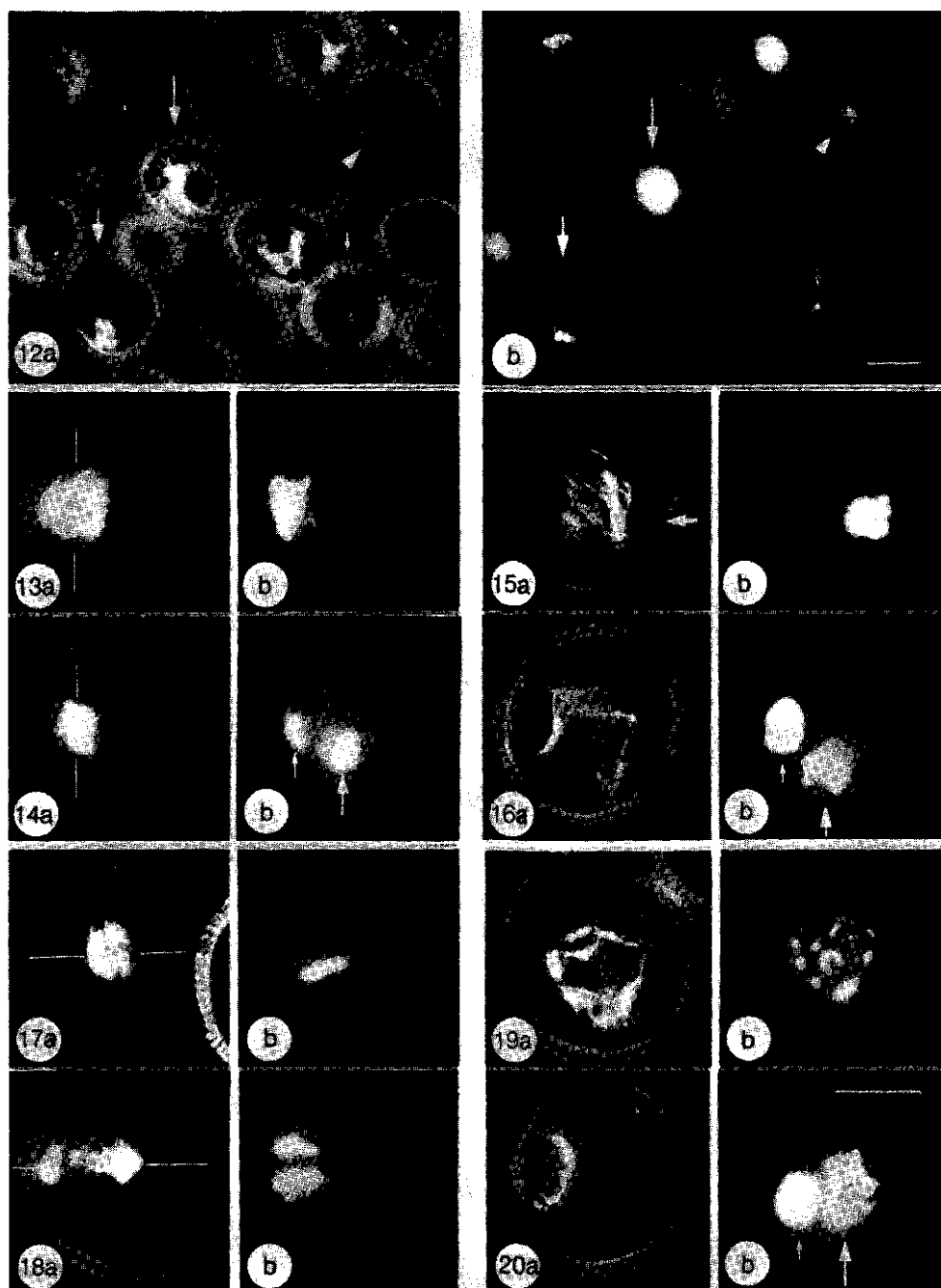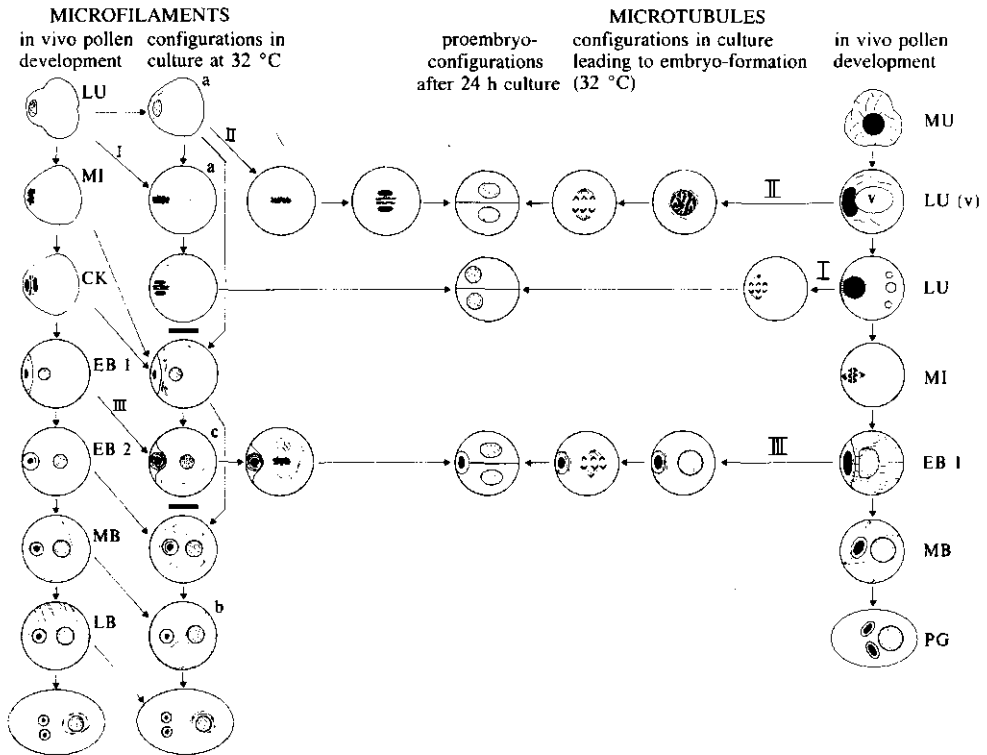
Fig. 21: Schematic representation of the development of *Brassica napus* microspores and pollen *in vivo*, and *in vitro* under embryogenic conditions. The diagram shows the characteristic changes of the microfilamental and microtubular cytoskeletons and the localization of nuclei during the development to tricellular pollen and proembryos. Abbreviations: MU - mid unicellular stage, LU(v) - vacuolate late unicellular stage, LU - late unicellular stage, MI - mitosis, CK - cytokinesis, EB 1 - early bicellular stage I, EB 2 - early bicellular stage II, MB - mid-bicellular stage, LB - late bicellular stage, PG - tricellular pollen grain.

Prominent MT arrays and non-functional spindles were observed 4 h after the isolation at embryogenic and at non-embryogenic conditions. Those structures are probably not relevant to further embryo development because (i) we did not find microspore nuclei which divided after the appearance of those structures and (ii) cultures at 18°C never formed embryos. It is possible that stress-induced enhanced synthesis of tubulin, as shown for microspores of *Zea mays* after heat shock (Hopf et al., 1992), and a spontaneous self reorganization of MTs (Bajer and Molè-Bajer, 1986) in the microspore are the reasons for those structures.

44

Microtubular cytoskeleton during induction of embryogenesis in microspores. In the process of microspore derived embryogenesis two developmental pathways can be distinguished. Firstly, the turn of the division plane in microspores cultured at 32°C is of outmost importance (Fig. 21 I). The rotated division plane, observed at first after 2 h and more frequently after 6 and 8 h of culture, depends on the *de novo* synthesis of MTs in a new position. Microspores with normal spindle orientations were mostly observed untill 2 h of culture. These microspores were probably isolated at metaphase and a rotation of the division plane is not to be expected. Because the turn of the spindle was only observed at 32°C, it is a strong indication that this process, leading to symmetrical cell division, is a stimulus for embryo formation.

Secondly, after 12 h culture at 32°C, mitosis was mainly initiated when the nucleus was located in the centre of the microspore (Fig. 21 II). This abnormal location of nuclei when in embryogenic condition was reported by Zaki and Dickinson, too (1990). Probably those microspores were in the late vacuolate stage at the beginning of culture. Late microspores *in vivo* have their nuclei in an acentric position because of the central vacuole, and the MTs that attach the nucleus to the cell membrane (Hause et al., 1992). The disappearance of the central vacuole (Telmer et al., 1992) and the apparently temperature induced disappearance or disturbed synthesis of MTs permitted the nucleus to migrate to the cell centre. Hence mitosis will give rise to symmetrical cells and embryogenesis. Because Zaki and Dickinson (1990) started the culture exclusively with late microspores having large vacuoles, they described pathway II as the only possibility for the formation of embryos from microspores.

Microtubular cytoskeleton during induction of embryogenesis in bicellular pollen. After 24 h culture we only observed dividing vegetative nuclei in pollen when the generative cell was still arrested at the intine (Fig. 21 III). At the start of culture in such bicellular pollen the generative cell was just formed and attached to the intine. The temperature treatment in culture caused changes in the normal development which mainly disturbed the detachment of the generative cell from the intine. The arrest of the generative cell is most likely caused by the disappearance or the disturbed synthesis of those MTs, which are normally located in the vegetative cell and perpendicular to the common wall. *In vivo* and at 18°C these MTs are probably involved in the translocation of the generative cell (Hause et al., 1992). The subsequent division of the vegetative nucleus is the most unusual phenomenon leading to embryogenesis.

CONCLUSIONS

The embryogenic pathways I, II and III (Fig. 21) are all clearly characterized. They deviate from the gametophytic pathway by the common occurrence of symmetrical or nearly symmetrical divisions.

The original polarization in the cells disappears and changes in various ways. Embryogenic pathway I should be considered to be the expression of changed polarization; there is a new

orientation of the mitotic spindle although the nucleus keeps its location in the cell. Embryogenic pathway II rather expresses a loss of polarization because the nucleus migrates in a central position before mitosis. In our opinion, the MF-configurations in late and dividing microspores are a response to the disappearance or the change of polarity. The newly formed MFs might be involved in the reorganization of the cytoplasm.

The division in the vegetative cell in embryogenic pathway III should be regarded as a result of dedifferentiation, because the vegetative cell restores its capacity to divide instead of forming a pollen tube. This is also concluded from the organization of the MF network in the embryogenic vegetative cell.

From the presented results it is suggested that MFs do not have a primary function in the induction of embryogenesis. Against that, the changes in the MT cytoskeleton clearly coincide with events which lead to embryogenesis. The role of MTs in the deviating pathways was determined in detail, the regulatory mechanisms, however, still need to be elucidated.

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47

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48

Chapter 4

Nuclear DNA synthesis during the induction of embryogenesis in cultured microspores and pollen of *Brassica napus* L.

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ABSTRACT

The dynamics of nuclear DNA synthesis were analyzed in isolated microspores and pollen of *Brassica napus* that were induced to form embryos. DNA synthesis was visualized by the immunocytochemical labelling of incorporated Bromodeoxyuridine (BrdU), applied continuously or as a pulse during the first 24 h of culture under embryogenic (32°C) and non-embryogenic (18°C) conditions. Total DNA content of nuclei was determined by microspectrophotometry.

At the moment of isolation, microspore nuclei and nuclei of generative cells were at G1, S or G2 phase. Vegetative nuclei of pollen were always in G1 at the onset of culture.

When microspores were cultured at 18°C, they followed the normal gametophytic development. When cultured at 32°C, they divided symmetrically and became embryogenic or continued gametophytic development. Because the two nuclei of the symmetrically divided microspores were either both labelled with BrdU or not labelled at all, it is concluded that microspores are inducible to form embryos from G1 till G2 phase.

When bicellular pollen were cultured at 18°C, they exclusively exhibited labelling in generative nuclei. This is comparable to the gametophytic development occurring *in vivo*. Early bicellular pollen cultured at 32°C, however, exhibited replication in vegetative nuclei, too. The majority of vegetative nuclei re-entered cell cycle after 12 h of culture. Replication in the vegetative cells preceded division of the vegetative cell, a prerequisite for pollen-derived embryogenesis.

Key words: *Brassica napus* - BrdU - embryogenesis - microspore and pollen culture - DNA synthesis

INTRODUCTION

In higher plants, microspores undergo an ordered sequence of mitotic cell divisions which lead to the formation of pollen grains consisting of a vegetative cell and two sperm cells that are committed to specialized functions. The formation of embryos from microspores and pollen represents a fundamental switch in this development. Several studies with microspores and pollen at various developmental stages have shown that there exist discrete developmental windows in which microspores and pollen become embryogenic in response to culture conditions. The re-entering of the cell cycles of the almost differentiated cells and the switch to embryogenic development were studied recently in tobacco pollen cultures. Using ^{3}H-thymidine incorporation under embryogenic conditions it was shown that replication in the vegetative nucleus led to embryo development (Zarsky et al. 1992)

Morphological studies in *Brassica napus* have indicated that embryogenesis was induced in late microspores and in early bicellular pollen (Fan et al. 1988; Pechan and Keller 1988; Telmer et al. 1992; Hause et al. 1993). The development of embryos from symmetrically divided microspores of *B. napus* was studied in detail (Zaki and Dickinson 1990, 1991). Less attention has been paid to the development of embryos from early bicellular pollen. From immunocytochemical studies on the cytoskeleton and the behaviour of nuclei as visualized by 4,6-diamidino-2-phenylindole (DAPI), it is well known that changes in the cytoskeletal patterning interact with deviating patterns of nuclear divisions in cultured late microspores and early bicellular pollen (Hause et al. 1992, 1993). In order to understand the early events of microspore and pollen derived embryogenesis in more detail we studied the nuclear DNA synthesis in microspores and pollen during the first 24 h of culture using the immunolabelling of incorporated Bromodeoxyuridine (BrdU). The method was successfully applied for plants to study nuclear DNA synthesis in tissues, cultured cells and protoplasts (Levi et al. 1987; Pfosser 1989; Wang et al. 1989; Stroobants et al. 1990; Wang et al. 1991). The application of short pulses of BrdU allowed us to analyze the dynamics of the replication in vegetative and generative nuclei.

MATERIAL AND METHODS

Plant material
Plants of *Brassica napus* L. cv. "Topas" were first grown for 4 weeks under greenhouse conditions at 18-23°C followed by a low temperature treatment at 10°C in the light (300 μmol photons . m^{-2}s^{-1} for 16 h) and at 5°C in the dark (8 h) until the onset of flowering. Flower buds, 3.2-3.8 mm long, were harvested from the terminal raceme.

Cultivation of microspores and pollen
Microspores and pollen were isolated as described by Pechan and Keller (1988). They were cultured in the dark at a density of 2×10^4 ml^{-1} in NLN medium (Lichter 1982) with 13% sucrose and free of potato extract, pH 6.0, at 18°C (non-embryogenic condition) and 32°C (embryogenic conditions). Two days later, the cultures incubated at 32°C were transferred to 25°C. The numbers of embryos were counted after 3 weeks.

Two types of cultures were used to determine the DNA synthesis during the first 24 h of culture under embryogenic and non-embryogenic conditions. The first culture was isolated from flower buds with sizes ranging from 3.2 to 3.4 mm and consisted of a mixture of microspores (Type A culture). The second culture (Type B culture) consisted mainly of late microspores, mitotic microspores and early bicellular pollen isolated from buds with sizes ranging from 3.6 to 3.8 mm.

BrdU labelling
Pulse labelling and continuous labelling with the thymidine substitute BrdU were applied to microspore and pollen cultures within the first 24 h of culture under embryogenic and non-embryogenic conditions. The BrdU labelling solution (supplied by Amersham) was added to the cultures at final concentrations of 1:500.
The viability of the cells was tested with fluorescein diacetate (FDA, Heslop-Harrison and Heslop-Harrison 1970) directly after the pulse labelling to determine the influence of increasing periods of BrdU incubation. The remaining cells were analyzed for BrdU incorporation. Control cultures without BrdU were also analyzed for viability.
The influence of BrdU on the embryogenicity of the culture was tested by adding BrdU to the cultures immediately after isolation, either for a period of 1 h or for 24 h. The numbers of embryos in the two cultures were counted 3 weeks after BrdU removal and compared with control cultures.
Two variants of BrdU labelling were used, (1) Cells were continuously labelled for 4, 8, 12, 16 and 24 h of culture, and (2) cells were pulse-labelled in the last hour of a culture period of 1, 4, 8, 12, 16 and 24 h. All of the experiments described were repeated at least twice.

Immunocytochemistry
Samples of microspores and pollen from BrdU labelled and control cultures were fixed immediately after the labelling for 1.5 h in 3.5% paraformaldehyde in phosphate buffered saline (PBS), supplemented with 0.1% Triton X-100. After fixation, the samples were rinsed, dehydrated, embedded in polyethylene glycol (PEG) and sectioned according to Van Lammeren et al. (1985). Sections were mounted on poly-L-lysine coated slides, treated with 0.1 M NH$_4$Cl and washed twice with PBS. The last washing was done with 0.1% BSA in PBS. The sections were then incubated for 1 h with anti-BrdU monoclonal antibody (Amersham) containing nuclease. After rinsing in PBS, the secondary antibody goat anti-mouse IgG BODIPY (Molecular Probes) was applied in dilution of 1:100, and the sections were incubated for 45 min. Both incubations were done in the dark at 26°C. For DNA staining, the same slides were incubated in DAPI solution (0.01 mg/l) for 10 min, then washed in PBS and covered with Citifluor-glycerol (Citifluor Ltd., London). The fluorescence of BrdU-labelled and DAPI-stained nuclei was visualized with a Nikon Microphot epifluorescence microscope using proper filters for DAPI and BODIPY. Black-and-white images were recorded on Kodak TMY 135-film.
The percentage of microspores and pollen grains having labelled nuclei was determined from a total number of 250-300 microspores and pollen grains for each sample in at least two independent experiments.

Microspectrophotometry

For the microspectrophotometric analysis control cells were collected immediately after isolation from the flower buds used for the Type A and the Type B experiments. From the Type B culture samples were also collected after 24 h of culture under embryogenic conditions. The cells were fixed in ethanol-acetic acid (3:1) and stored at -20°C. Feulgen staining was performed according to Dolezel (1989), with hydrolysis in 5 mol l^{-1} HCl at 25°C for 25 min. The slides were then stained for 60 min in Schiff reagent prepared according to Lilie (1951) using parafuchsin (Serva). Afterwards, the slides were washed by three changes of SO_2^- water, graded alcohols and xylene. Cover slips were mounted with Depex (Serva). The amount of DNA was measured by mirror scanning cytophotometry with a Leitz MPV-3 microspectrophotometer interfaced to a microcomputer with a Nucleiscan programme (Dolezel 1989), taken erythrocytes as an internal standard. The reference 1C value was given by the early microspores obtained from the Type A and B culture.

RESULTS

BrdU incorporation and effects on viability after short pulse labelling

The minimal pulse length needed to detect DNA synthesis was determined by adding BrdU to cultured microspores for 10, 20, 30, 45, 60 min at the end of a 12-h culture period of at 32 °C. The shortest pulse of BrdU which enabled the detection of the S-phase in microspores and pollen was 30 min (Fig. 1a, a'). A 1-h pulse labelling was sufficient to show replication in generative, vegetative and microspore nuclei. It was exceptional that progression through the cell cycle from S phase to mitosis was observed in microspores within a 1-h pulse of BrdU labelling (Fig. 1b, b'). Bromodeoxyuridine pulses of 1 h had no influence on the viability of the culture, but a continuous BrdU labelling during 24 h caused a 10-20 % decrease in viability. Embryogenicity was not changed after 1-h pulse labelling, but 24 h of continuous labelling reduced the number of embryos by 8 % to 17%.

Quantification of nuclear DNA by microspectrophotometry

Analysis of the Type A culture showed that predominantly microspores were present (Table 1). This mixture of microspores had DNA contents corresponding to values from 1C up to 2C at the onset of culture. Microspores with C values between 1 and 2 were in the S-phase (Fig. 2A). Analysis of the Type B culture revealed that it contained 30% microspores and 70% bicellular pollen (Table 2). The microspores had 1C to 2C DNA contents at the time of isolation (Fig. 2B). Late microspores were in G2. Bicellular pollen contained vegetative nuclei with DNA contents of about 1C at the time of isolation (Fig. 2D). Generative nuclei showed 1C levels and higher DNA contents, indicating further progress through the cell cycle up to G2 (Fig. 2C).

After 24 h of culture under embryogenic conditions, a portion of the vegetative nuclei of the bicellular pollen reached the G2 phase of the cell cycle (Fig. 2F). Some nuclei showed DNA contents higher than 2C at that time. The generative nuclei were predominantly in G1, but some did reach G2 within 24 h of culture (Fig. 2E). Microspores which had been cultured for 24 h exhibited a range of C values from about 1C up to about 3C (Fig. 2G). Daughter

cells formed by the symmetrical division of microspores were often at G1 phase. The DNA contents indicated that some daughter nuclei progressed through the S phase and reached the G2 phase (Fig. 2H), and others exhibited progression through the cell cycle simultaneously.

Differences in nuclear DNA synthesis in Type A and B cultures
Type A culture:
At the onset of culture the Type A culture consisted of a mixture of microspores at early and late developmental stages. Table 1 gives an overview of both, the developmental fate of these microspores from the onset of culture up to a period of 24 h and the incorporation of BrdU
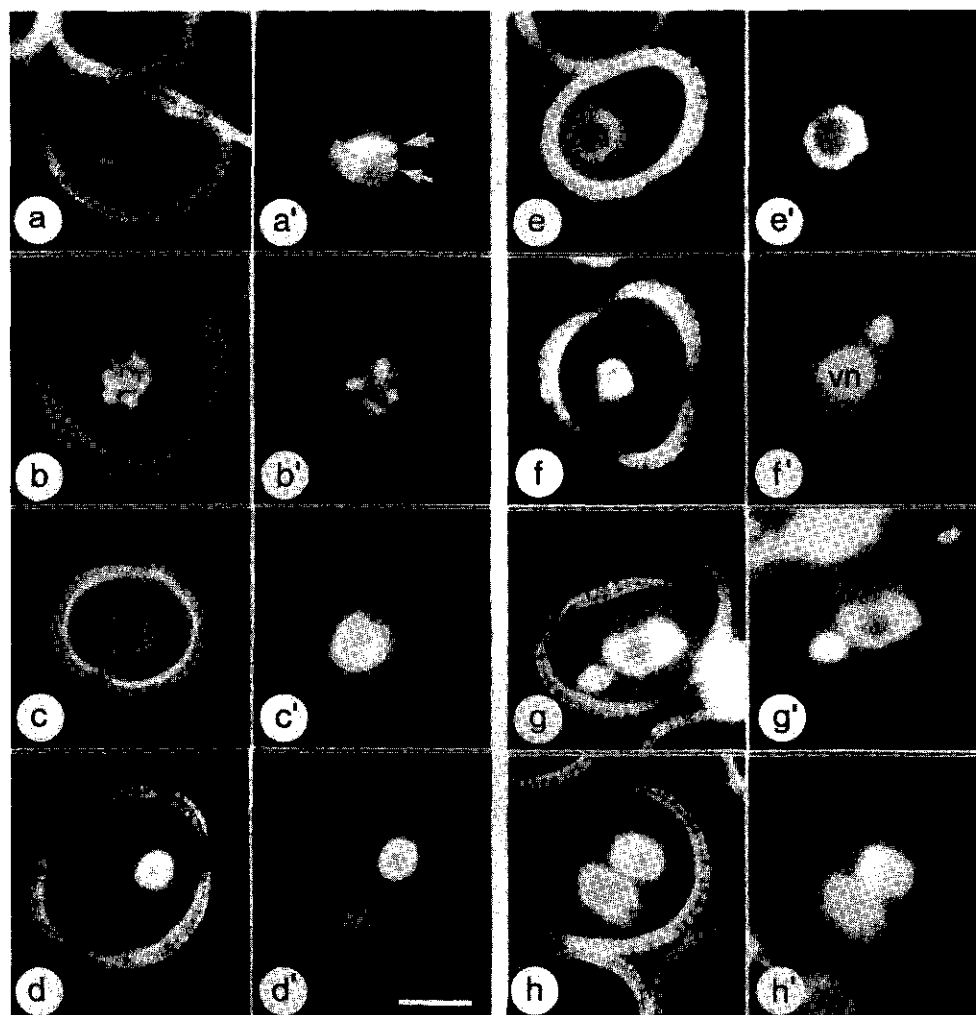
as a signal for replicative DNA synthesis during the first 24 h under embryogenic and non-embryogenic conditions. DAPI-stained nuclei exhibited a shift from the late microspore stage to the early bicellular pollen stage (up to 18% of the cells) within 24 h when cultured at 32°C. Embryo formation in Type A cultures was less than 0.5%.

At non-embryogenic conditions 0.8% of the microspores replicated DNA during an 8-h labelling period. Figure 1 c,c' is an example of such labelling after 4 h of incubation. After 24 h continuous labelling up to 1.8% of the microspores and newly formed pollen were labelled (Table 1). Incorporation only occurred in nuclei of late microspores and in generative nuclei at the bicellular pollen stage.

Under embryogenic conditions the percentage of microspores and pollen with BrdU-labelled DNA increased from 0.6% after 4 h of continuous labelling to 4.4% after 24 h of continuous BrdU labelling. Replicating nuclei were mostly observed in late microspores; only a few pollen with labelled vegetative nuclei were found (Table 1).

Type B culture:

The Type B culture consisted initially of a mixture of late microspores, mitotic microspores and early bicellular pollen (Table 2). Relative to the Type A culture, embryo formation in the Type B culture was much higher, up to 6.5%. Table 2 gives an overview of both, the developmental fate of the microspores and pollen from the onset of culture up to a period of 24 h and the incorporation of BrdU as a signal for nuclear DNA synthesis during the first 24 h under embryogenic and non-embryogenic conditions.

At non-embryogenic conditions (18°C), BrdU incorporation was always observed in the late microspore stage, but the majority of the replicating nuclei were the generative nuclei in middle or late bicellular pollen (Fig. 1d, d').

The DNA synthesis under embryogenic conditions (32°C) was much higher. The total number of labelled nuclei increased from 4% after continuous labelling for 4 h to about 15% after 24 h of BrdU labelling. An example of a labelled microspore nucleus is given in Fig. 1e, e'. The rate of entrance of microspores into the S-phase was constant during the 24-h period. The beginning of DNA synthesis in the vegetative nucleus of bicellular pollen was

Fig. 1. Fluorescence micrographs of semi-thin sectioned microspores and pollen of *Brassica napus* cultured under embryogenic (32°C) and non-embryogenic (18°C) conditions. a-h show the incorporation of BrdU, labelled with BODIPY; a'-h' depict the position of the nuclei in the same cells stained with DAPI. Bar represents 10 μm for all micrographs.

a-a'   Two-cellular structure from Type A culture, grown at 32 °C for 12 h, exhibits fluorescence in the two nuclei after a 30-min BrdU pulse;

b-b'   microspore grown at 32 °C for 8 h shows labelling in the prophase nucleus after a 1-h BrdU pulse;

c-c'   late microspore from Type A culture, grown at 18 °C for 4 h with BrdU, shows labelling in its nucleus;

d-d'   pollen from Type B culture, grown at 18 °C for 4 h with BrdU, exhibits labelling in the generative nucleus only;

e-e'   microspore from Type B culture, grown at 32 °C for 4 h with BrdU, shows labelling in the nucleus;

f-f'   pollen from Type B culture, grown at 32 °C for 1 h in the presence of BrdU, shows labelling in the vegetative nucleus (vn);

g-g'   pollen from Type B culture, grown at 32 °C for 12 h with a 1-h BrdU pulse, exhibits labelling in both the vegetative and the generative nucleus;

h-h'   symmetrical division in Type B culture, grown at 32 °C for 12 h, exhibits labelling in the two nuclei after 1-h BrdU pulse.

55
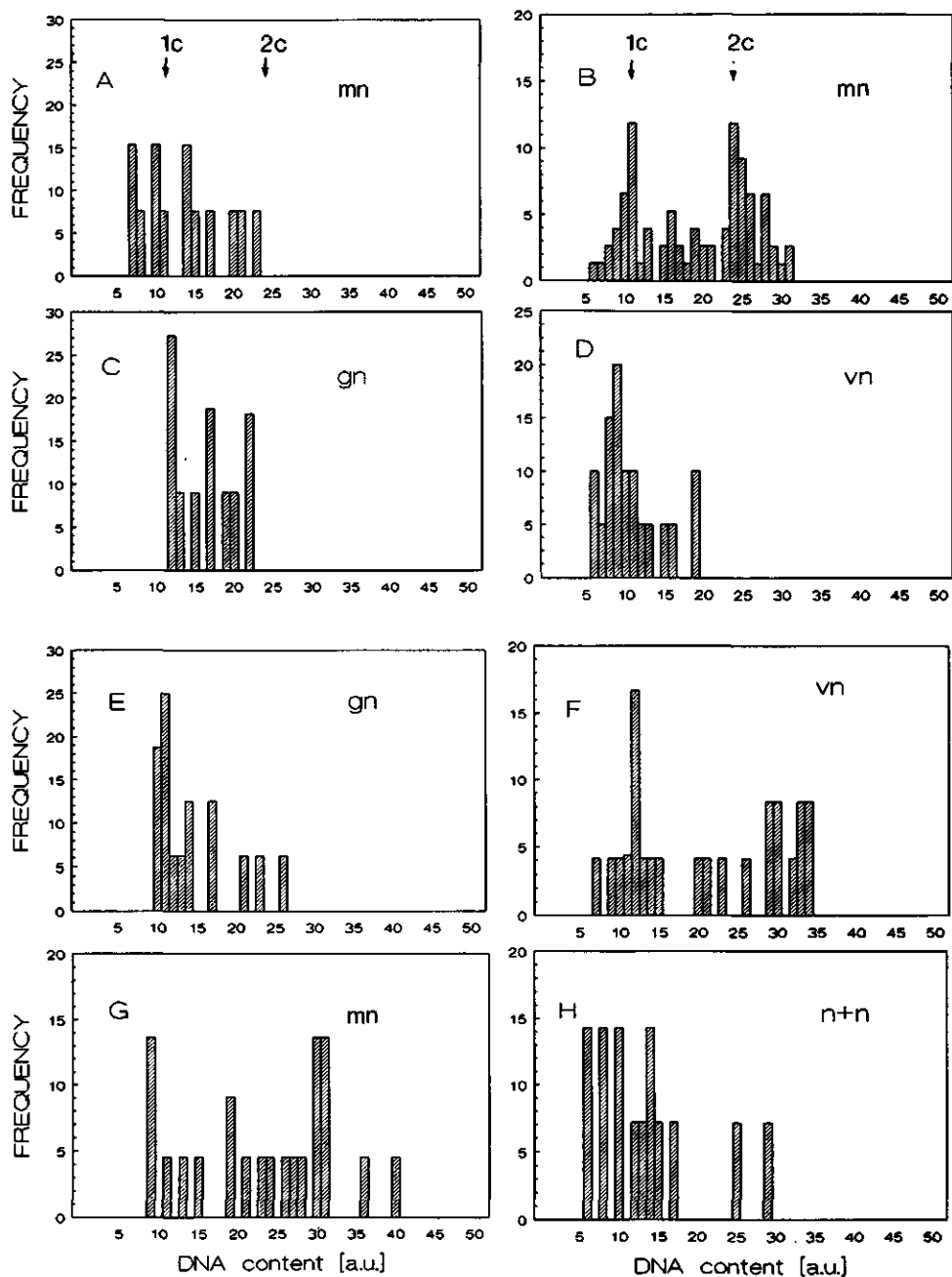
observed as early as 1 h of culture (Fig. 1f, f'). In this case no labelling was found in the generative nucleus. Pollen in which the generative and the vegetative nuclei were labelled were also observed (Fig. 1g, g' for 8 h of culture). Their number increased only twice after 24 h of culture. The highest number of labelled vegetative cells was found after 24 h of continuous BrdU labelling. Pollen with replicating vegetative nuclei often showed a configuration with the generative cell arrested near the intine and clearly separated from the vegetative cell. The incorporation patterns observed after 1 h of BrdU pulse often showed high heterogeneity in labelling in vegetative nuclei as compared to the homogenous labelling found in generative nuclei. Symmetrically or almost symmetrically divided microspores with labelled DNA in both nuclei were often observed from 12 h of culture onwards (Fig. 1h, h'; Table 2). Both nuclei were labelled after continuous incubation with BrdU and incidentally after 1 h of pulse labelling. On the other hand, we also found symmetrically divided microspores in which the daughter nuclei were not labelled after a continuous BrdU treatment for 8 h or 16 h.

DISCUSSION

The application of BrdU pulses enables the visualization of nuclear DNA replication (Lacy et al. 1991). Pulse labelling clearly provides information on the dynamics of DNA-synthesis, whereas continuous labelling visualizes total DNA-synthesis.
Type A and Type B cultures were analyzed because they consisted of two different populations of microspores and pollen. In the Type A culture, which contained mainly microspores, replication appeared only at low percentages and no symmetrical divisions were found although a portion of the microspores were in a late stage at the onset of the culture. This corresponds to results of Telmer et al. (1992). The Type B culture consisted of a mixture of late microspores, mitotic cells and up to 53% young bicellular pollen, and was appropriate to obtain high yields of embryos.
Microspores and pollen cultured for 24 h at non-embryogenic conditions exhibited DNA

Fig. 2A-H. Frequency histograms of Feulgen-stained nuclei of freshly isolated (A-D) and cultured (E-H) microspores and pollen of Brassica napus. DNA contents are measured by cytophotometry and expressed in arbitrary units (a.u.). Culture was in the embryogenic condition for 24 h. n = number of analyzed nuclei. In A and B the reference 1C and 2C values of DNA are indicated by arrows in the microspore populations from Type A and B cultures.

A: Microspore nuclei (mn) from Type A isolation (n=13);
B: Microspore nuclei (mn) from Type B isolation (n=76);
C: Generative nuclei (gn) of pollen from Type B isolation (n=11);
D: Vegetative nuclei (vn) of pollen from Type B isolation (n=20);
E: Generative nuclei (gn) of pollen from Type B culture (n=16);
F: Vegetative nuclei (vn) of pollen from Type B culture (n=24);
G: Microspore nuclei (mn) from Type B culture (n=22);
H: Nuclei (n+n) after symmetrical division (n=14).

Table 1. Nuclear DNA synthesis in isolated microspores of *B. napus* during the first 24 h of culture under non-embryogenic and embryogenic conditions. Cells of Type A culture were labelled with BrdU continuously (C) or for 1 h at the end of the cultivation period.

developmental stage of microspores and pollen (%)						
	EMs	MMs+LMs	M!	EB	MB+LB	Σ
after isolation	24	74	-	2	-	100
24 h in cult. at 32 °C	4.3	77.7	-	18	-	100

culture at non-embryogenic condition, 18 °C							
			% labelled cells				
period of labelling	LMs	M!		BC			Σ	T
			g	g+v	v	n+n		
4 h C	-	-	-	-	-	-	-	250
4 h 1 h	-	-	-	-	-	-	-	235
8 h C	0.8	-	-	-	-	-	0.8	285
8 h 1 h	-	-	-	-	-	-	-	218
16 h C	1.7	-	0.4	-	-	-	2.1	295
16 h 1 h	0.8	-	-	-	-	-	0.8	235
24 h C	1.4	-	0.4	-	-	-	1.8	284
24 h 1 h	0.4	-	-	-	-	-	0.4	211

culture at embryogenic condition, 32 °C							
			% labelled cells				
period of labelling	LMs	M!		BC			Σ	T
			g	g+v	v	n+n		
4 h C	0.6	-	-	-	-	-	0.6	305
4 h 1 h	0.4	-	-	-	-	-	0.4	251
8 h C	1.7	-	-	-	-	-	1.7	235
8 h 1 h	0.4	-	-	-	-	-	0.4	261
16 h C	2.0	-	-	-	0.6	-	2.6	291
16 h 1 h	0.4	-	-	-	-	-	0.4	245
24 h C	3.2	-	-	0.4	0.8	-	4.4	254
24 h 1 h	0.8	-	-	-	0.4	-	1.2	250

Abbreviations: BC, bicellular structure; EB, early bicellular structure; EMs, early microspores; g, generative nucleus; LB, late bicellular structure; LMs, late microspores; M!, mitosis; MB, mid-bicellular structure; MMs, mid microspores; n, nucleus of symmetrically divided microspore; T, total number of analyzed cells; Σ, sum of percentages; v, vegetative nucleus

Table 2. Nuclear DNA synthesis in isolated microspores and bicellular pollen of *B. napus* during the first 24 h of culture under non-embryogenic and embryogenic conditions. Cells of Type B culture were labelled with BrdU continuously (C) or for 1 h at the end of the cultivation period. For abbreviations see Table 1.

developmental stages of microspores and pollen (%)						
	EMs	MMs+LMs	M!	EB	MB+LB	Σ
after isolation	3.5	26	7.5	53	10	100
24 h in cult. 32 °C	1	17	0	24	58	100

culture at non-embryogenic condition, 18 °C								
			% labelled cells					
period of	LMs	M!		BC			Σ	T
labelling			g	g+v	v	n+n		
4 h C	0.4	-	0.8	-	-	-	1.2	250
4 h 1 h	0.4	-	-	-	-	-	0.4	231
8 h C	1.3	-	2.7	-	-	-	4.1	294
8 h 1 h	-	-	0.7	-	-	-	0.7	280
16 h C	1.4	-	5.9	-	-	-	7.3	287
16 h 1 h	-	-	0.7	-	-	-	0.7	290
24 h C	1.3	-	10.3	-	-	-	11.6	224
24 h 1 h	-	-	1.04	-	-	-	1.04	287

culture at embryogenic condition, 32 °C								
			% labelled cells					
period of	LMs	M!		BC			Σ	T
labelling			g	g+v	v	n+n		
4 h C	1.6	-	1.2	0.8	0.4	-	4.0	250
4 h 1 h	0.4	-	0.8	0.4	1.3	-	2.9	239
8 h C	1.9	-	0.4	2.9	1.9	-	7.9	208
8 h 1 h	0.4	0.4	0.8	0.4	0.4	-	2.4	237
16 h C	2.2	0.3	1.6	1.2	3.5	2.2	11.0	318
16 h 1 h	0.4	-	0.4	0.4	1.2	0.4	2.9	237
24 h C	1.3	-	2.3	1.7	5.0	4.9	15.2	297
24 h 1 h	0.3	-	0.3	-	1.2	-	1.8	287

replication in the nuclei of late microspores and in the nuclei of generative cells of middle and late bicellular pollen. Vegetative nuclei did not show DNA replication. These results are comparable with those expected during gametophytic development and are in agreement with the data of Aruga et al. (1982) and Zarski et al. (1992). Thus, 18°C is an acceptable control state at which embryogenesis does not occur.

Microspore-derived embryogenesis
Microspore-derived embryogenesis starts with symmetrical divisions induced in late microspores (Zaki and Dickinson 1991; Hause et al. 1993). In the present investigation the daughter nuclei were sometimes labelled and sometimes unlabelled. When they were labelled, either the BrdU was incorporated in the DNA during the S-phase of the microspore or the daughter nuclei progressed through the cell cycle simultaneously. The former explanation implies that microspores were at S-phase or still in G1. The latter explanation is possible because it was often observed that the two daughter nuclei were labelled after a 1-h BrdU pulse; one hour is too short for the progression of the cell cycle from the microspore S-phase, via mitosis, to G1 of the bicellular structure. Some symmetrically divided microspores had unlabelled nuclei in cultures even though BrdU was present continuously. This indicates that microspores which are isolated in G2 can also give rise to embryogenesis. So it can be concluded that microspores can be induced to enter the embryogenic pathway from G1, during S-phase, up to G2. We sometimes observed microspores with C-values larger than 2 (Fig.2G). This is most likely caused by endoreduplication within the microspore nucleus which has also been demonstrated in microspore cultures of *Zea mays* (Pretova et al. 1993).

Pollen-derived embryogenesis
When isolated bicellular pollen were cultured at 32°C, vegetative nuclei could enter the S-phase within 1 h. This observation shows that the re-entering of the cell cycle is an immediate response to the high temperature and might be of great importance to our understanding the initial changes that occur during development. Many vegetative nuclei were replicating DNA after 12 h of culture at 32°C. These data together with the quantitative determinations of DNA contents at the onset of culture and after incubation at 32°C indicate that the vegetative nucleus in *B. napus* is arrested in G1 phase *in vivo*. It re-enters the cell cycle within the induction period at 32°C. Similarly, Aruga et al. (1982) and Zarsky et al. (1992) confirmed that the vegetative nuclei of pollen of *Nicotiana tabacum* are arrested in G1. Contrary to these results, De Paepe et al. (1990) found that the DNA content of the vegetative nuclei of pollen from *Nicotiana sylvestris* corresponds to the G2 phase of the cell cycle.
Bicellular pollen with labelled generative nuclei appeared early in culture at 32°C. They were probably isolated in the middle or late bicellular stage of pollen development and are not competent to switch to the developmental pathway (Telmer et al. 1992).
Replication in the vegetative nucleus was sometimes preceded by DNA replication in the generative nucleus. Similarly, Zarsky et al. (1992) observed in tobacco that replication in the generative nucleus was first completed, and only then followed by DNA replication in the vegetative cell when induced to embryogenesis by starvation. As compared to tobacco pollen embryogenesis, the replicating vegetative nuclei of *B. napus* were more often found together with non-labelled nuclei of generative cells. Generative cells were often attached to the intine,

60

a characteristic of embryogenic development in bicellular pollen (Hause et al. 1993). Vegetative nuclei exhibiting DNA contents up to 3C most likely represent examples of endoreduplication. This pathway is not expected to give rise to embryogenesis because cell division is absent (see also Pretova et al. 1993).

High temperature treatment in Type B cultures resulted in up to 6.5% embryos after 3 weeks of culture, whereas up to 15.2% of the microspore and pollen population (Table 2) exhibited BrdU labelling within 24 h. It should be realized that the labelling of late microspores (1.3%) and generative cells (2.3%) not necessarily leads to embryo formation. So at least 11.6% of the cells changed DNA replication in the embryogenic direction. As it was observed that multicellular structures stopped further development regularly (B. Hause, unpublished), probably because of concurrence or disturbed endogenic regulation, it is understandable that the eventual percentage of embryos is lower than 11.6.

It can be concluded that our qualitative and quantitative analysis of nuclear DNA synthesis revealed the dynamics of the replication with respect to microspore- and pollen-derived embryogenesis. Embryogenic cultures can be started with microspores from late G1 to G2-phase. Vegetative cells of pollen always have to re-enter the cell cycle before embryogenesis can occur.

ACKNOWLEDGEMENTS

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Chapter 5

Cell cycle dependent distribution of phosphorylated proteins in microspores and pollen of *Brassica napus* L., detected by the monoclonal antibody MPM-2

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SUMMARY

The monoclonal antibody MPM-2, which interacts with a mitosis-specific phosphorylated epitope, has been used to study phosphorylation of proteins in microspores and pollen of *Brassica napus*. One- (1-D) and two-dimensional (2-D) immunoblots revealed that MPM-2 recognized a family of phosphorylated proteins in freshly isolated microspores and pollen. The same set of phosphorylated proteins was found after 8 h of culture at embryogenic (32°C) and non-embryogenic (18°C) conditions. Two major spots were observed on 2-D immunoblots, one of which ($M_r \approx 75$ kD, $pI \approx 5.1$) co-localized with the 70 kD heat shock protein. Immunolabelling of sectioned microspores and pollen showed that MPM-2 reactive epitopes were predominantly observed in the nucleoplasm from G_1 until G_2-phase, and in the cytoplasm during mitosis. This may be due to a cell cycle related translocation of phosphoproteins from the nucleus to the cytoplasm, or alternate phosphorylation and dephosphorylation in nucleus and cytoplasm. Detectability of epitopes on sections depended on the embedding procedure. Cryo processing revealed epitope reactivity in all stages of the cell cycle whereas polyethylene glycol embedded material showed no labelling in the cytoplasm during mitosis. Processing might reduce the antigenicity of cytoplasmic MPM-2 detectable proteins, probably due to dephosphorylation. The MPM-2 detectable epitope was observed in all cells investigated, irrespective of culture conditions, and its intracellular distribution depended on the cell cycle stage and was not related to the developmental fate of the microspores and pollen.

Keywords: Androgenesis, *Brassica napus*, Microspore culture, Pollen embryogenesis, Protein phosphorylation

INTRODUCTION

When placed in culture, isolated late microspores and early bicellular pollen from *Brassica napus* L. cv. Topas can be induced to switch their developmental fate from gametophytic development to sporophytic development, resulting in the formation of androgenic embryos in up to 70 % of the cells (Pechan and Keller 1988, Telmer et al. 1992). The early stages of this sporophytic development are characterized by an altered pattern of cell division: a symmetric cell division instead of an asymmetric first pollen mitosis, when starting from late microspores, or the participation of the vegetative cell instead of the generative cell in cell division, when starting from early bicellular pollen. The redirection of gametophytic development to embryogenesis is irreversibly induced by elevating the culture temperature to 32 °C (Hause et al. 1993, Clusters et al. 1994) for a period of at least 8 h. The 8 h inductive temperature treatment has been reported to coincide with (1) rearrangements of the microtubular cytoskeleton, such as changing orientations of mitotic spindles in late microspores and redistribution of cytoplasmic microtubules in young bicellular pollen (Hause et al. 1992, 1993), (2) re-entry into the cell cycle of the vegetative cell from 4 h of culture onwards (Binarova et al. 1993b), and (3) changes in the synthesis of 25 proteins detected on two-dimensional (2D) gels (Cordewener et al. 1994). Transition through different phases of the cell division cycle are controlled by cascades of protein phosphorylation and dephosphorylation (Murray and Kirschner 1989). As the nucleus of the vegetative cell re-enters the S-phase of the cell cycle, and divides only when pollen were cultured under embryogenic conditions (Binarova et al. 1993b), we aimed at comparing phosphorylation events occurring in vivo, and during culture under embryogenic and non-embryogenic conditions. To this end we used the monoclonal antibody MPM-2, which was raised against mitotic Hela cells (Davis et al. 1983). MPM-2 recognizes a set of phosphoproteins which exhibit a cell cycle dependent expression with maximum at the G_2/M transition both in mammalian (Davis et al. 1983) and plant cells (Traas et al. 1992, Binarova et al. 1993a). In mammalian cells it was shown that the MPM-2 epitope may be an important substrate of cdc2 kinase (Kuang et al. 1994). Taagepera et al. (1993) identified DNA-topoisomerase IIa as the major chromosomal protein detected by MPM-2. Western blot analysis of proteins, isolated from tobacco cell suspension cultures, showed that MPM-2 recognized a family of phosphorylated proteins (Traas et al. 1992).

During starvation of tobacco pollen under conditions yielding either embryogenic cells or mature pollen, Kyo and Harada (1990) observed differences in the degree of phosphorylation of four proteins (M_r range 15-30 kDa). Highest degrees of phosphorylation were found when pollen were cultured under embryogenic conditions (Kyo and Harada 1990, Kyo and Ohkawa 1991). Here, we show the intracellular distribution of MPM-2 reactive epitopes in microspores and pollen of *B. napus*, cultured under both embryogenic and non-embryogenic culture conditions.

MATERIAL AND METHODS

Plant material and in vitro culture
Plants of *Brassica napus* L. cv. Topas were first grown under greenhouse conditions and then at low temperature as described previously (Hause et al. 1993). Flower buds from 3.5 to 4.2 mm were harvested from the terminal raceme to obtain a range of developmental stages (see Pechan and Keller 1988). Isolation and in vitro culture of microspores and pollen was performed as reported by Pechan and Keller (1988). Microspores and pollen were sampled at the onset of culture, and after 8, 24 and 48 h of culture. A period of 8 h was chosen because then culture conditions had irreversibly changed the developmental pathway. At 4 h of culture first DNA synthesis was observed in vegetative nuclei and at 12 h first sporophytic divisions occurred (Binarova et al. 1993b).

2-D gel electrophoresis and silver-staining of proteins
2-D gel electrophoresis was performed as described by Cordewener et al. (1994). First dimension isoelectric focusing was performed on Immobiline DryStrips pH 3.0-10.5 (Pharmacia). Each strip was loaded with protein extract prepared from 4×10^5 microspores and pollen. Second dimension was run on precasted 8% - 18% gradient SDS ExelGels (Pharmacia). After electrophoresis the gels were either fixed in 10% acetic acid, 50% ethanol and silver-stained (Blum et al. 1987) or directly used for immunoblotting.

Immunoblotting
Proteins were transferred directly from the ExcelGels to nitrocellulose membranes using a semi-dry Western Blotting technique (MultiphorII Novablot). Nitrocellulose blots were incubated for 5 min in TBS (10 mM Tris-HCl, 140 mM NaCl, pH 7.5) and stained in Ponceau S solution (0.3% Ponceau S in 3% trichloro acetic acid). Some protein spots were marked by a needle and used as landmarks for matching the antibody reactive proteins on the nitrocellulose blot to a silver-stained 2-D gel.
Nitrocellulose blots were incubated in TTBS (0.5% Tween 20 in TBS) for 20 min and next for 2h in TTBS with a 1/500 dilution of the primary monoclonal antibody MPM-2 described by Davis et al. (1983) or a with a 1/750 dilution of the monoclonal anit-human HSP72/73 antibody (StressGen). After washing, the blots were incubated in TTBS with Goat anti-Mouse IgG alkaline phosphatase conjugate for 2 h. After washing in TBS the labelled proteins were visualized by reaction with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. All steps were performed at room temperature (RT).

Embedding and sectioning for immunocytochemistry
Cryoembedding Microspores and pollen were collected in sieves just after isolation or after 8 and 24 h of cultivation at either embryogenic (32°C) or non-embryogenic (18°C) conditions. The material was fixed in 3% paraformaldehyde (PFA) in phosphate buffered saline (PBS, 135 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.2) for 90 min, rinsed in PBS, and gradually infiltrated with 0.1 M sucrose (4h, RT), 1.0 M sucrose (overnight, 4°C) and 2.3 M sucrose in PBS (4h, RT). Droplets of $3 \mu l$ pollen suspension were placed onto holders and frozen in liquid propane. Sections ($3 \mu m$ thick) were cut with a

Reichert cryo ultra microtome at -20°C (knife temperature -60°C), picked up with a loop containing a droplet of 2.3 M sucrose and, after thawing, adhered onto slides coated with organosilane.

Polyethylene glycol (PEG) embedding Samples comparable to those taken for cryoembedding were fixed with 4% PFA in PBS containing 0.1% Triton X-100 , dehydrated in ethanol, embedded in PEG, sectioned and mounted on slides as described by Van Lammeren et al. (1985). Sections (2μm thick) were stuck on polylysine coated slides.

Immunocytochemistry
Slides were rinsed with PBS, blocked with 0.1 M NH$_4$Cl, rinsed again in PBS, and finally blocked with 0.1% acetylated bovine serum albumin in PBS (PBS/BSAac). MPM-2 monoclonal antibody (dilution 1:500 in PBS/BSAac) was applied overnight at 4°C. After rinsing in PBS, the second antibody Goat anti-Mouse IgG Bodipy (dilution 1:100 in PBS/BSAac) or Rat anti-Mouse IgG fluorescein isothiocyanate (RaM IgG FITC, dilution 1:25 in PBS/BSAac) was applied for 1 h at 37°C in the dark. Next, slides were washed in PBS and stained with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma Chemical Co., St.Louis, MO, USA) for 20 min at RT. After washing they were enclosed in Citifluor-glycerol (Citifluor Ltd., London) and observed by a Nikon FXA Microphot. The specificity of the MPM-2 antibody labelling was checked by omitting the first antibody as well as by treatment of sections with alkaline phosphatase followed by the complete immunolabelling procedure; bacterial alkaline phosphatase (*E. coli* type III-S; Sigma Chemical Co.; 20 units per ml) was prepared in buffer (50 mM Tris-HCl, 0.1mM EGTA, pH 8.0) supplemented with the protease inhibitors phenylmethylsulphonyl fluoride (PMSF, 1 mM) and leupeptin (0.3 mM). Sections were incubated overnight, washed three times with PBS and then incubated with MPM-2.

RESULTS

Identification of phosphoproteins recognized by MPM-2.
Protein extracts were prepared from a population of potentially embryogenic cells, containing a mixture of late unicellular microspores and early bicellular pollen. Figure 1a shows the MPM-2 reactive bands after SDS-polyacrylamide gelelectrophoretic separation of the proteins from a microspore and pollen population at the onset of culture (lane 1), and after an 8 h period of culture at either 18°C (lane 2; gametophytic development) or 32°C (lane 3; embryogenic development). All three lanes contained the same set of labelled protein bands with an M$_r$-range of 30 to 100 kDa. Slight differences in the level of labelling between the three lanes were observed (Fig. 1a). For a more detailed analysis of the MPM-2 staining patterns, protein extracts were run on 2-D gels before blotting and immunostaining. Figure 1b shows the 2-D immunoblot of an 8 h embryogenic culture, together with the corresponding silver-stained 2-D gel (Fig. 1c). By superimposing the silver-stained 2-D gel on the Western blot (see Material and Methods), the positions of immunoreactive spots were determined in the silver-stained gel. Two major spots (M$_r$ ≈ 75 kDa, pI ≈ 5.1 and M$_r$ ≈ 35 kDa, pI ≈ 6.6) and

67

some faint spots all at approximately 55 kDa but with pI values ranging from pI 6.5 to pI 7.0 were observed. A comparison of the 2-D labelling pattern of an embryogenic and a non-embryogenic culture showed no qualitative changes in MPM-2 reactive protein spots, and only minor changes in the labelling intensity (data not shown). The 75 kDa major MPM-2 reacting spot on the 2-D gel appeared to run at the same position as a string of proteins that were previously identified to be isoforms of heat shock protein 70 (HSP 70) (Cordewener et al. 1995). In order to conform this, two 2-D immunoblots were prepared from a 48 h embryogenic culture, in which we know there are high levels of HSP 70 present (unpubl. obs.). One blot was hybridized with MPM-2 (Fig. 2a), whereas the other was hybridized with a monoclonal antibody raised against human HSP 72/73; (anti-HSP 72/73; Fig. 2b). Anti-HSP 72/73 reacted with only one spot on the blot, at exactly the same position as the major MPM-2 reacting spot in Fig. 2a. Furthermore, the size of the immuno reacting 75 kDa MPM-2 spot increased concomitantly with the increase in size of the HSP 70 spot after 48 h in culture
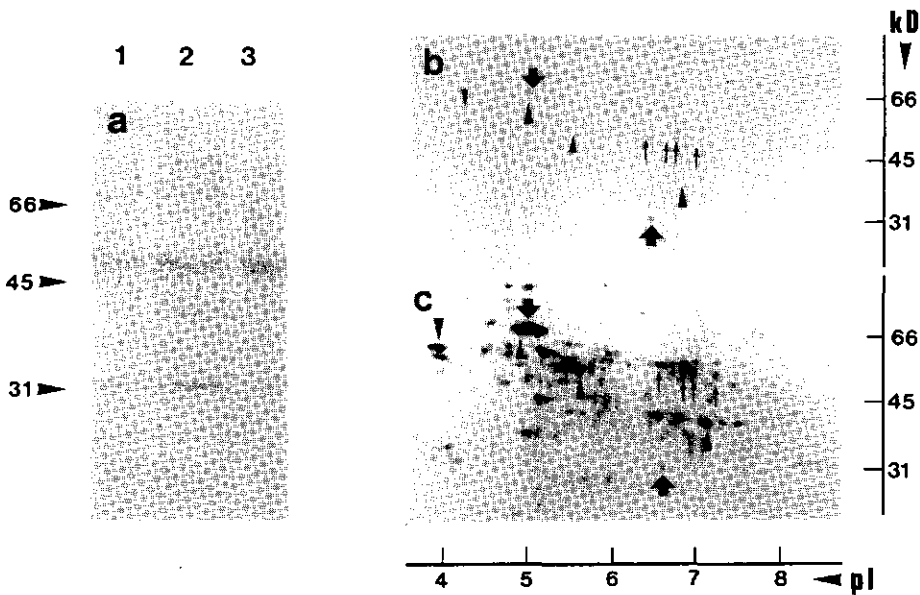

Fig. 1. Expression of MPM-2 antigens in microspores and pollen of *B. napus*.

a   SDS-PAGE and MPM-2 immunoblotting of protein extracts from microspores and pollen at the onset of culture (1) and after 8 h of culture under non-embryogenic (18°C; 2) and embryogenic (32°C; 3) conditions. Arrow heads indicate the position of protein markers and their M_r values in kDa.

b   2-D immunoblot showing MPM-2 reactive proteins of an 8 h embryogenic microspore culture. Large arrows indicate the strongly immuno-stained spots. Small arrows point to the site where faint spots were detected on the original blot, not visible on the final micrographs anymore. Arrow heads show the positions of the land marks used to match the immunoblot with the silver stained gel in c.

c   Silver-stained 2-D gel of the protein extract used for the 2-D immunoblot shown in b. Arrows and arrow heads point to identical positions as shown in b.
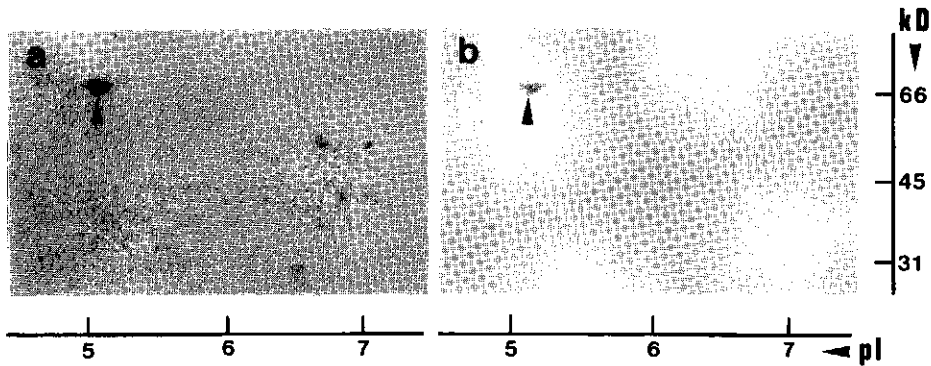
Fig. 2 Co-localization of the 75 kDa MPM-2 reacting phosphoprotein with HSP 70 on 2-D immunoblot. **a** 2-D immunoblot showing MPM-2 reactive proteins of an embryogenic microspore culture (48 h; 32 °C). **b** 2-D immunoblot showing HSP 70 reactive proteins of the same culture as used in **a**. Arrowheads mark the position of the major HSP 70 isoform.

(compare Fig. 1b to Fig. 2a). These results suggest that MPM-2 reacts with a phosphorylated epitope present on certain isoforms of HSP 70. HSP 70 and MPM-2 immunoblots were also made from a 48 h non-embryogenic culture (18 °C). The labelling pattern was comparable to that found under embryogenic culture conditions (blots not shown).

MPM-2 localization by immunofluorescence microscopy
MPM-2 epitopes were detected on cryosections and PEG sections. Controls, in which the first antibody was omitted or in which alkaline phosphatase treatment was done (Fig. 4a), always appeared negative. Only nucleoli exhibited a faint diffuse fluorescence at wavelengths not related to the fluorescence of FITC.
CRYOSECTIONING: In freshly isolated microspores MPM-2 labelled epitopes were observed in the nucleoplasm and to a lower extend in the nucleolus (Fig 3a). During early prophase, the nucleoplasm was intensively fluorescent and the cytoplasm became labelled (Fig. 3b). The labelling in the nucleoplasm persisted during late prophase but the sites occupied by condensing chromosomes were not labelled (Fig. 3c). The area surrounded by the condensing chromosomes was intensively labelled now. At metaphase the whole cytoplasm exhibited labelling but chromosomes appeared negative (Fig. 3d). Pollen at early bicellular stages showed MPM-2 labelling in the plasma of both the generative as well as the vegetative nucleus, comparable to the labelling found in microspore nuclei, and to a lower extend in the nucleolus and cytoplasm. Often the labelling of the vegetative nucleus was brighter than that of the generative nucleus, especially in early bicellular pollen (Fig. 3e). Late bicellular pollen still exhibited labelling in the outer region of the plasma of both nuclei. Labelling intensities in the two nuclei were comparable, and nucleoli showed faint labelling (Fig. 3f).
The labelling patterns of microspores and pollen, cultured at 18°C for 8 and 24 h, appeared comparable to those found for freshly isolated cells (data not shown). When cultured at 32°C for 8 and 24 h, again the same pattern of immunolabelling was observed in microspores and pollen which did not yet exhibit symmetrical divisions. Thus the localization of MPM-2
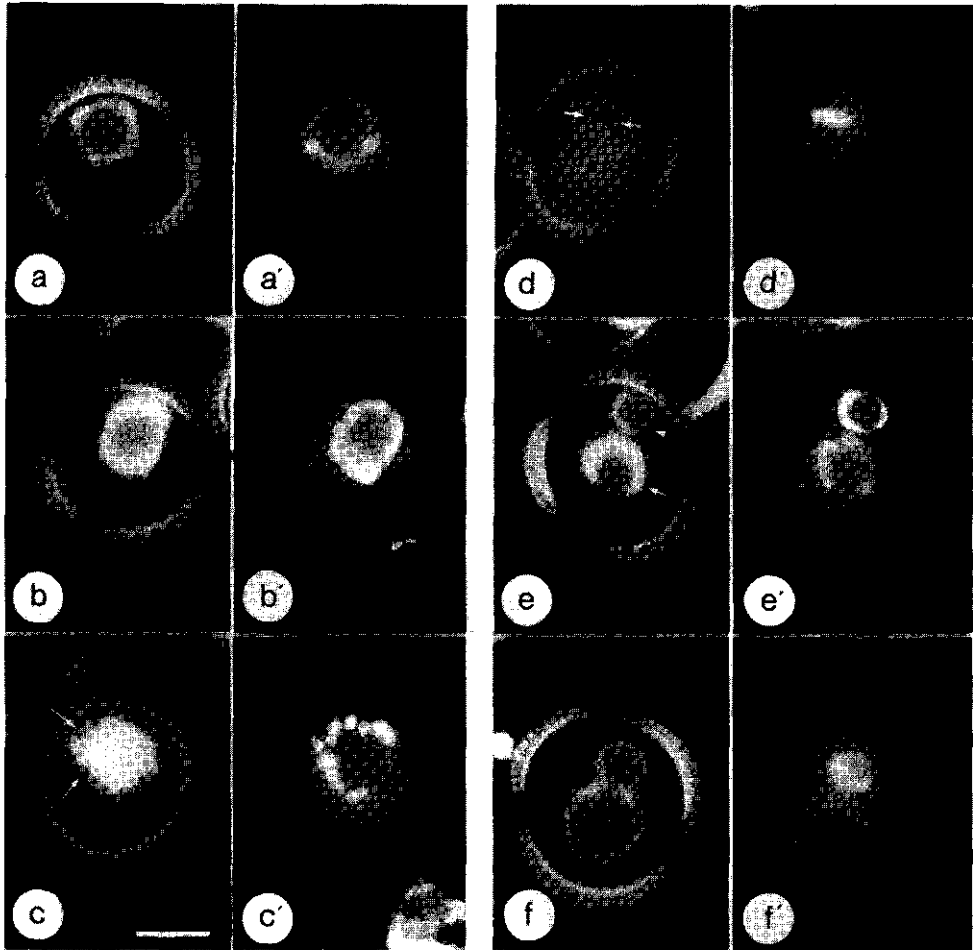
69

Fig.3 Survey of distribution of MPM-2-FITC labelling on cryo sectioned microspores and pollen of *B. napus* at the onset of culture from late unicellular microspores until late bicellular pollen. a-f Representative views of the MPM-2 distribution, a'-f' DAPI stained images of the same sections for the determination of the phase of the cell cycle. Bar: 5 μm, for all panels

a. Late microspore stage with accumulation of MPM-2 labelling in the nucleoplasm. Note that the nucleolus exhibits a weak labelling. The cytoplasm is not stained.

b. Early prophase of microspore mitosis with further accumulation of MPM-2 labelling in the nucleoplasm.

c. Late prophase stage in which the nucleolus had disappeared. The MPM-2 labelling is spread over the nucleus except for those regions where the chromosomes are localized (arrows). Note that the cytoplasm is now labelled weakly.

d. Metaphase of microspore mitosis. Note that the MPM-2 signal is present throughout the cytoplasm and not at the sites of the condensed chromosomes in the metaphase plate (arrows).

e. Early bicellular pollen with MPM-2 labelling in the nuclei. Note the differences in labelling intensity in generative (arrowhead) and vegetative (arrow) nucleus.

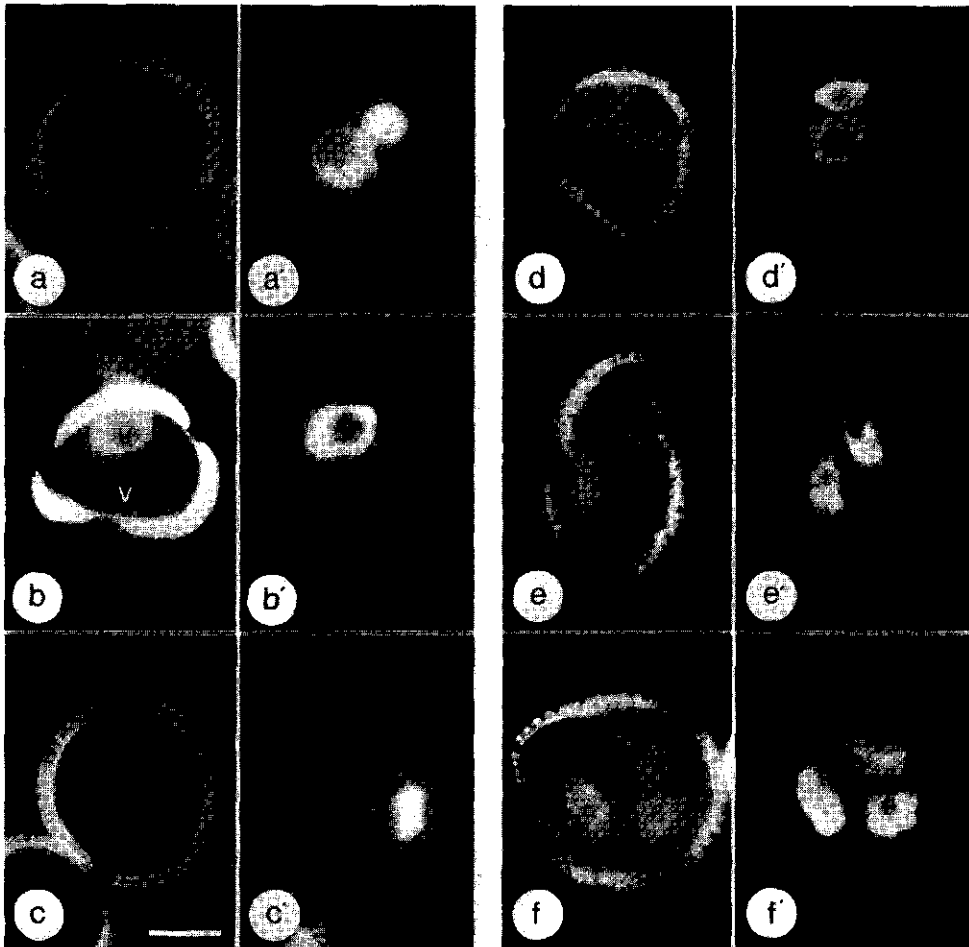f. Late bicellular pollen with MPM-2 labelling in the nucleoplasm of the generative and vegetative nuclei.

70

Fig. 4 Survey of distribution of MPM-2-FITC labelling on PEG sectioned microspores and pollen of *B. napus* at the onset of culture (a and b) and after cultivation (c-f). a'-f' DAPI stained images of the same sections. Bar: 5 µm, for all panels.

a. Control labelling after treatment of section with alkaline phosphatase. There is no specific fluorescence.
b. Late vacuolate microspore showing high labelling in nucleoplasm, low labelling in the cytoplasm, and no labelling in the vacuole (v).
c. Microspore mitosis. Cytoplasmic labelling is absent.
d. Early bicellular pollen cultured for 1 day at 18°C showing faint labelling in both nuclei and a very weak labelling in the cytoplasm.
e. Bicellular structure formed by symmetrical division of a microspore cultured for 1 day at 32°C. Weak labelling was observed in the nuclei.
f. Tricellular structure formed by division of the vegetative cell after cultivation of bicellular pollen for 1 day at 32°C. Note the labelling in all nuclei and weak granular labelling in the cytoplasm. The upper nucleus belongs to the generative cell. It is grazed in the section and therefore less fluorescence after DAPI staining than expected (f´)
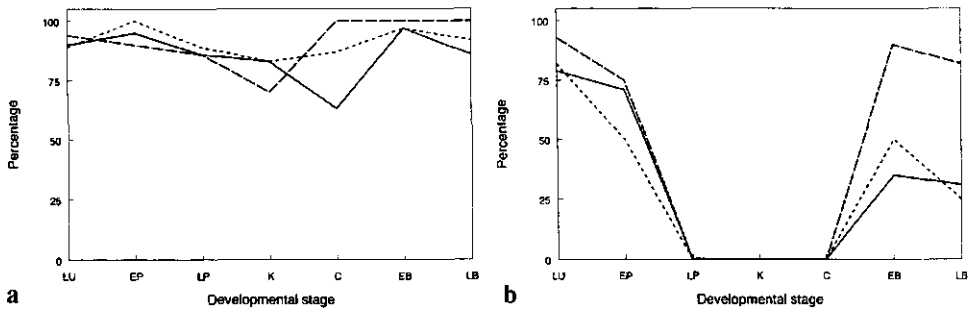
71

Fig. 5 Graphical representation of labelling frequencies at various developmental stages of microspores and pollen of *B. napus* after cryo embedding (a) and PEG embedding (b). The data represent percentages of cells that exhibited labelling. The solid lines represent the situation at the start of culture, the broken lines the situation after 8 h of culture at 18°C, and the dotted lines the situation after 8 h of culture at 32°C. Total number of pollen counted 3280. **LU** late microspore stage, **EP** early prophase, **LP** late prophase, **K** karyokinesis, **C** cytokinesis, **EB** early bicellular stage, **LB** late bicellular stage.

reactive epitopes exclusively depended on the developmental stage of the microspores and pollen. This was confirmed in up to six independent experiments.

PEG-SECTIONING: When microspores and pollen were embedded in PEG just after isolation, the labelling of microspores before mitosis, up to early prophase (Fig. 4b) and the labelling of bicellular pollen after cytokinesis was comparable to that found after cryosectioning but less intense. No labelling was observed during mitosis (Fig. 4c).

After culture at 18°C the labelling of microspores and pollen was comparable to that found in a freshly isolated population of cells. Fig. 4d shows the labelling of both nuclei of an early bicellular pollen cultured for 24 h at 18°C.

When cultured at 32°C, microspores and the vegetative cell of pollen divided symmetrically forming bi- and tricellular structures, respectively. MPM-2 labelling showed an equally intense fluorescence in both daughter nuclei of a bicellular structure (Fig. 4e). The tricellular structure of Fig. 4f' was formed by symmetrical division of the vegetative cell. After DAPI staining it showed two equally fluorescent nuclei and one less fluorescent nucleus, the original generative nucleus which was only grazed in this section. The nucleoplasm of all three nuclei was labelled by MPM-2 and some granular labelling was observed in the cytoplasm (Fig. 4f). Again, no labelling was found during mitosis.

Comparing the data obtained after cryosectioning and PEG sectioning it appeared that cryosections always revealed labelling whereas PEG sections showed a decrease of labelling intensity at interphase and the absence of labelling during mitosis, both at the onset of the culture as well as after 8 h and 24 h of culture. This phenomenon was independent of the culture temperature. Figure 5 presents the data on the MPM-2 labelling frequencies of fresh and cultured microspores and pollen after cryo and PEG embedding.

72

DISCUSSION

In a variety of systems, including plants, it has been demonstrated that mitosis specific protein phosphorylation can be followed with the monoclonal antibody MPM-2 (Davis et al. 1983, Osmani et al. 1991, Traas et al. 1992). Using HeLa cells, synchronized in S phase, Davis et al. (1983) showed strong MPM-2 labelling of three protein bands (70 kDa, 118 kDa and 182 kDa), only during metaphase and anaphase. Osmani et al. (1991) found specific labelling of a 58 kDa protein during mitosis in *Aspergillus nidulans*. Recently Traas et al. (1992) have shown that also in higher plants MPM-2 recognizes a phosphorylated epitope which is most abundant during mitosis. Using 2-D immunoblot analyses of tobacco whole cell extracts, these authors found that MPM-2 reacted with a group of phospho-proteins, one of which ($M_r \approx 65$ kDa) was associated with the cytoskeleton. In Fig. 1 we show on 2-D immunoblots that microspores and pollen of *B. napus* cultured for 8 h at 32 °C contain two major MPM-2 reactive proteins, a 75 kDa protein ($pI \approx 5.1$) and a 35 kDa protein ($pI \approx 6.6$). Whereas the 35 kDa protein forms a distinct spot in the silver-stained 2-D gel, the 75 kDa protein is part of a large, not well-resolved area. A better resolution of this area was obtained using a smaller pH-range for the first dimension iso-electric focussing (pH 4-7; Cordewener et al. 1994). Under these conditions the 75 kDa MPM-2 reactive spot co-localized with a set of spots, which had previously been identified as members of the 70 kDa heat shock protein family (HSP 70) (Cordewener et al. 1995). This suggests that certain isoforms of HSP 70 contain the MPM-2 epitope. *In situ* localization of HSP 70 with anti HSP 70 on sectioned microspores and pollen (Cordewener et al. 1995) did not reveal codistribution of HSP 70 and MPM-2 in all developmental stages. For example, the nucleus of the vegetative cell, which is arrested in the G_1 phase of the cell cycle, showed MPM-2 labelling but no HSP-70 labelling. This may be due to the other immunoreactive MPM-2 phosphoproteins detected on the immunoblot.

Phosphorylation of members of HSP 70 has been reported for several species, including plants (Miernyk et al. 1992, Sherman and Goldberg, 1993). Certain members of the HSP 70 family might be associated with the cytoskeleton (Tsang, 1993), and one of the major MPM-2 reactive protein spots in the tobacco extract represents a 68 kD protein which is enriched in the cytoskeleton fraction (Traas et al. 1992). Immunocytochemical MPM-2 labelling showed similar staining patterns in freshly isolated microspores and pollen and microspores and pollen under both embryogenic and non-embryogenic conditions. Because there are remarkable changes in the microtubular cytoskeleton of microspores and pollen after 8 h of culture under embryogenic conditions (Hause et al., 1993), but not in the MPM-2 patterning, we were not able to correlate changes in the MPM-2 protein patterns with the changes in the cytoskeletal network. This suggests that in contrast to the tobacco suspension cells, MPM-2 reactive proteins found in *B. napus* microspores and pollen are not structurally associated with the microtubular cytoskeleton.

The 2-D immunoblot analyses of embryogenic and non-embryogenic *B. napus* cultures revealed that the change from gametophytic to sporophytic development was not preceded by major changes in the labelling pattern of MPM-2 reactive phosphoproteins. Since we started with a mixed population of microspores and pollen, the switch in development from pollen maturation to embryogenesis was possibly not synchronous enough to detect minor changes

in the level of protein phosphorylation during the first 8 h of culture. Also after 48 h, when a large number of microspores and pollen have divided in culture, no qualitative changes in the MPM-2 reactive protein patterns were observed between both culture conditions (unpubl. obs.). Immunocytochemical techniques, used to monitor the distribution of the MPM-2 epitope in individual cells during pollen development and embryogenesis, did not reveal changes in the distribution of MPM-2 detectable proteins either. It is therefore concluded that the change in developmental fate is not correlated with changes in MPM-2 detectable phosphorylation. MPM-2 detectable proteins occur both in the nucleoplasm and cytoplasm of plant cells (Davies et al. 1989, Traas et al. 1992, Binarova et al 1993a, 1994, Young et al. 1994). Immunocytochemistry on sectioned microspores and pollen of *B. napus* showed that the distribution of MPM-2 labelled phosphoproteins depended on the developmental stage of the cells. Labelling was predominantly present in the nucleus during interphase and in the cytoplasm during mitosis. This is in agreement with the observations of Traas et al. (1992) and Binarova et al. (1993a), but differs from the mitosis specific expression found in *Aspergillus nidulans* (Osmani et al. 1991) and HeLa cells (Davis et al. 1993). It also implies that there is either a change in concentration and/or phosphorylation of certain proteins that have a fixed location in the nucleus and the cytoplasm, or the cell cycle dependent change of the MPM-2 labelling pattern is caused by translocation of phosphorylated proteins. During mitosis chromosomes were not labelled. The MPM-2 labelled proteins found in the nucleoplasm at prophase are therefore likely not bound to chromosomes.

Under embryogenic conditions, symmetrical cell divisions are the first morphological sign for the initiation of embryogenesis (Hause et al. 1993). This symmetry was also expressed in the MPM-2 labelling patterns, which showed an equal distribution of immunofluorescence between both daughter nuclei. This is in contrast to the large differences in MPM-2 labelling intensity which were often observed after asymmetric division of a microspore observed under non-embryogenic and *in vivo* conditions.

The PEG embedding procedure caused the total loss of the MPM-2 immuno reactivity which was found in the cytoplasm of cryosectioned mitotic cells. The immuno reactivity of nuclei in interphase cells was preserved after PEG embedding, although of lower intensity than in interphase cells after cryosectioning. Binarova et al. (1994) observed intense MPM-2 labelling of the kinetochore region of mitotic chromosomes in squashed material, but in embedded material, proceeded for immuno-gold electron microscopy, the kinetochore associated labelling was much less intense. The MPM-2 labelling associated with nuclei at interphase was the same, with or without embedding. It is not clear whether the selective loss of phosphoepitopes due to the embedding procedure is caused by the dephosphorylation of MPM-2 recognized phosphoproteins or by the total disappearance of the antigen.

In cultured tobacco cells and root meristem cells of *Vicia faba* and *Zea mays* a highly cell cycle specific MPM-2 labelling in the nucleus increased from almost no labelling at G1 to a maximum at G2/M phase (Traas et al. 1992, Binarova et al. 1993a, 1994). Most MPM-2 labelling was associated with the interchromatin region of nuclei in late G2 phase. This was confirmed by observations made at the ultrastructural level using immunogold MPM-2 labelling (Binarova et al., 1994). At the onset of the experiments described here, it was known that the non-dividing nucleus of the vegetative cell was arrested in G1 (Binarova et al., 1993b). From the investigations on the root meristem cells, it was hypothesized that vegetative nuclei of *B. napus* pollen would also be free of MPM-2 labelling. This was not the

case. It was frequently found that the vegetative nucleus exhibited even more intense labelling than the generative nuclei. This might be due to the larger volume of the vegetative nucleus than that of the generative nucleus, it contains more interchromatine material which may either contain more MPM-2 detectable proteins or in which the accessability of these phosphoproteins is higher than in a condensed generative nucleus.

In conclusion, MPM-2 recognizes a family of phosphoproteins in microspores and pollen. Most of the fluorescence observed after MPM-2 labelling of sections can be ascribed to the two most intensively labelled spots in the 2-D immunoblots. One of these proteins may be a phosphorylated form of HSP 70. Eventhough the MPM-2 staining pattern varies throughout the cell cycle, it appears that it does not report (only) mitosis-specific phosphoproteins in microspores and pollen of *B. napus.*

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Chapter 6

Changes in synthesis and localization of members of the 70-kDa class
of heat-shock proteins accompany the induction of embryogenesis in
Brassica napus L. microspores

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SUMMARY

Elevation of the culture temperature to 32°C for approximately 8 h can irreversibly change the developmental fate of isolated *Brassica napus* microspores from pollen development to embryogenesis. This stress treatment was accompanied by de-novo synthesis of a number of heat-shock proteins (HSPs) of the 70-kDa class: HSP68 and HSP70. A detailed biochemical and cytological analysis was performed of the HSP68 and HSP70 isoforms. Eight HSP68 isoforms, one of which was induced three fold by the stress treatment, were detected on two-dimensional immunoblots. Immunocytochemistry revealed a co-distribution of HSP68 with DNA-containing organelles, presumably mitochondria. Six HSP70 isoforms were detected, one of which was induced six fold under embryogenic culture conditions. During normal pollen development, HSP70 was localized in the nucleoplasm during the S phase of the cell cycle, and predominantly in the cytoplasm during the remainder. Induction of embryogenic development in late unicellular microspores was accompanied by an intense anti-HSP70 labeling of the nucleoplasm during an elongated S phase. In early bicellular pollen the nucleus of the vegetative cell, which normally does not divide and never expresses HSP70, showed intense labeling of the nucleoplasm with anti-HSP70 after 8 h of culture under embryogenic conditions. These results demonstrate a strong correlation between the phase of the cell cycle, the nuclear localization of HSP70 and the induction of embryogenesis. As temperature stress alone is responsible for the induction of embryogenic development, and causes an altered pattern of cell division, there might be a direct involvement of HSP70 in this process.

Key words:
Brassica - Embryogenesis - Heat shock - Microspore - Pollen - Rapeseed

Abbreviations:
HSP = heat-shock protein; 2-D = two-dimensional; DAPI = 4,6-diamidino-2-phenylindole; 1-D = one-dimensional; pI = isoelectric point

INTRODUCTION

During normal development, angiosperm microspores divide asymmetrically to form bicellular pollen grains with a small generative and a large vegetative cell. The large vegetative cell becomes arrested in the G_1 phase of the cell cycle, whereas the generative cell divides once more to form the two sperm cells that can take part in double fertilization (for review see: Mascarenhas 1989; Bedinger et al. 1994). When placed in culture at 18°C, *Brassica napus* microspores continue gametophytic development and form tricellular pollen (Custers et al. 1994). Gametophytic development can be disrupted by elevating the culture temperature to 32°C (Lichter 1982); late unicellular microspores, or the vegetative cell of early bicellular pollen grains initiate symmetric divisions and so enter a program of embryogenesis, ultimately giving rise to torpedo shaped embryos (Pechan and Keller 1988; Hause et al. 1993). About 8 h of culture at 32°C is sufficient to synchronously and irreversibly induce this embryogenic development (Pechan et al. 1991). These features make

B. napus microspore embryogenesis an excellent system for studying the mechanisms by which embryogenic development can be initiated. Microspore embryogenesis is also an important tool for plant breeders, as it yields haploid embryos from which (homozygous) double-haploid plants can be regenerated (Hu and Huang 1987; Morrison et al. 1991).

Disruption of normal development by high temperature treatments or "heat shock", have been observed in a multitude of experimental systems (Petersen 1990; Zimmerman and Cohill 1991). For example, application of heat shock at specific times during *Drosophila* development causes characteristic reproducible developmental defects that phenocopy existing mutations (Goldschmidt 1949), and heat shock treatment during a specific time window of globular-stage carrot embryos causes developmental arrest (Zimmerman et al. 1989). Heat shock induces a program of gene expression in which the synthesis of a family of proteins, the so-called heat-shock proteins (HSPs), is highly induced (Morimoto et al. 1990; Hendrick and Hartl 1993). Attention has focused mainly on certain members of the 70-kDa family of HSPs, as these proteins are highly conserved throughout evolution (Boorstein et al. 1994), are essential for progression through the cell cycle, are thought to be associated with the cytoskeleton (Tsang 1993), are specifically localized in the nucleus during the S phase of the cell cycle (Milarski and Morimoto 1986), and are associated with a number of cellular proteins in a cell-cycle-dependent fashion (Milarski et al. 1989).

Because of the observed parallels between the induction of microspore embryogenesis and the cell-cycle effects of members of the 70-kDa class of HSPs in other experimental systems (Pechan 1991), we have analyzed the expression and subcellular localization of a number of HSPs from this class during the inductive phase of microspore embryogenesis.

MATERIALS AND METHODS

Materials
Anti-tomato HSP68 and anti-tomato HSP70 polyclonal antibodies were a generous gift from Dr. D. Neumann (Neumann et al. 1987, 1993; Nover et al. 1989). Anti-human HSP72/73 monoclonal antibody (clone N27F3-4) was obtained from StressGen (Victoria, Canada). Protein A-Sepharose 4 Fast Flow was purchased from Pharmacia (Roosendaal, Netherlands).

Culture and in-situ labeling conditions
Growth of the double-haploid *Brassica napus* L. cv. Topas plants and isolation of microspores were exactly as described previously (Cytochemistry: Pechan and Keller 1988; Biochemistry: Custers et al. 1994). Microspores were analyzed just after the isolation procedure, or after 8 h of culture at 18°C or 32°C. For [^{35}S]methionine labeling experiments, microspores were incubated for 8 h in 3 ml of medium containing 0.74 MBq L-[^{35}S]methionine (in-vivo cell labeling grade from Amersham, 's-Hertogenbosch, Netherlands; >37 TBq·mmol^{-1}) as described previously (Cordewener et al. 1994).

Protein sample preparation and gel electrophoresis
For the analysis of proteins by one-dimensional (1-D) immunoblotting, cells were collected by centrifugation at 1000.g (5 min, 4°C) and the proteins were extracted by boiling in SDS-sample buffer (65 mM Tris-HCl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 1 μg/ml^{-1}

bromophenol blue, pH 6.8; 10 μl per ml of culture). For two-dimensional (2-D) immunoblotting, 0.3 ml ice-cold 80% trichloroacetic acid (TCA; w/v) was added to 3 ml of in-situ [^{35}S]methionine-labeled microspore culture. After centrifugation, 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 boiled in 30 μl SDS-sample buffer and clarified at 10000g. Aliquots (2 μl) were TCA precipitated on Whatman 3MM filter paper (Merck, Darmstadt, Germany) followed by scintillation counting to determine the total amount of ^{35}S incorporation. The proteins were then re-precipitated by the addition of 9 vol. of cold acetone, and the pellet was washed once with acetone before drying and resuspending in 50 μl of isoelectric-focusing sample buffer [8 M urea, 0.5% Triton X-100, 2% β-mercaptoethanol, 2% Pharmalyte 3-10 (Pharmacia), 1 μg.ml^{-1} bromophenol blue]. The samples were frozen and thawed once prior to application to the isoelectric-focusing gel strips.

For the immunoprecipitation experiments, the cells were harvested and boiled in SDS-sample buffer as described above, except that the concentration of SDS in the sample buffer was lowered to 0.5%.

One-dimensional polyacrylamide gel electrophoresis was performed either on pre-cast homogeneous 7.5% ExelGels (Pharmacia) or on 7.5% Midget gels (Pharmacia). Two-dimensional gel electrophoresis and data analysis were performed as described previously (Cordewener et al. 1994).

Immunoblotting
After electrophoretic separation on 1-D or 2-D gels, the proteins were blotted onto nitrocellulose (0.1 μm; Schleicher & Schuell, Dassel, Germany). Blots were rinsed in TBS (10 mM Tris-HCl, 0.83% NaCl, pH 7.5), incubated in TTBS (0.5% Tween 20 in TBS) for 20 min and next for 2 h in TTBS with a 1:750 dilution of the primary anti-HSP antibody. After washing several times in TBS for a total of 30 min, the blots were incubated in TTBS with goat anti-mouse or goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad, Veenendaal, Netherlands) for 2 h. After washing in TBS the labeled proteins were visualized by reaction with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Dried 2-D blots were exposed to a PhosphorImager plate for the desired time, after which the plates were scanned with the use of a 400B PhosphorImager (Molecular Dynamics; B & L Systems, Zoetermeer, Netherlands).

Immunoprecipitation
The lysate of [^{35}S]methionine-labeled microspores, obtained after boiling for 5 min in SDS-sample buffer containing 0.5% SDS, was clarified at 10000g (5 min, 4°C) and the supernatant was diluted in PBSTD (0.05 M phosphate buffer, 0.15 M NaCl, 2% Triton X-100, 1% sodium deoxycholate, pH 7.2) such that the final SDS concentration was 0.1%. After the addition of rabbit polyclonal antibody (1:75 dilution) the mixture was incubated on a rotary shaker for 2 h at 4°C. The immune complexes were captured by incubation with Protein A-Sepharose FF (1:20 dilution) for another 2 h at 4°C. Following centrifugation (2 min, 10000g), the immunoprecipitate was washed three times with PBSTD, resuspended in isoelectric-focusing sample buffer and stored at -70°C until analysis by 2-D gel electrophoresis.

Immunocytochemistry and microscopy
Microspores and pollen were collected in sieves and fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.2) for 90 min. After rinsing in PBS twice, samples were infiltrated step wise with first 0.1 M sucrose (4 h, at room temperature), then with 1.0 M sucrose (overnight, at 4°C), and finally with 2.3 M sucrose in PBS (4 h, at room temperature). Droplets of 3 μl pollen suspension were placed onto aluminium stubs and frozen in liquid propane. Semi-thin sections (3μm thickness) were obtained from a Reichert-Jung (Vienna, Austria) Kryo-ultramicrotome at -20°C with a knife temperature of -60°C. Sections were picked up with a loop containing a droplet of 2.3 M sucrose and, after thawing, were stuck onto slides coated with 2% organo-silane solution in absolute ethanol. After rinsing in PBS, blocking with 0.1 M NH$_4$Cl, and rinsing in PBS, sections were rinsed in PBS containing 0.1% acetylated bovine serum albumin (PBS/BSA). Sections were then incubated overnight at 4°C with a 1:250 dilution in PBS/BSA of anti-HSP68 or anti-HSP70, or a 1:125 dilution of anti-HSP72/73 antibody. After five rinsing steps in PBS for 15 min each, secondary antibodies were applied. For anti-HSP68 and anti-HSP70 goat-anti-rabbit-fluorescein isothiocyanate (FITC) (1:40 in PBS/BSA) and for anti-HSP72/73 rat-anti-mouse Ig-FITC (dilution 1:25 in PBS/BSA) were used, all for 1 h at 37°C in the dark. Slides were washed in PBS and stained with 1μg.ml^{-1} 4,6-diamidino-2-phenylindole (DAPI; Sigma, Bornem, Belgium) for 20 min at room temperature. Two washings preceded the enclosure of the sections in Citifluor-glycerol (Van Loenen, Leiden, Netherlands). The specificity of the labeling was checked by controls in which the first antibody was omitted.

Pictures were recorded on Kodak TMY 135 film using an FXA Microphot epifluorescence microscope (Nikon, Tokyo, Japan) equipped with FITC (EX 470-490/DM 510/BA 515 EF) and DAPI (EX 365/DM 400/BA 420) filters.

RESULTS

Cross-reactivity of anti-HSP antibodies with HSPs from B. napus. Three different antibodies were used to study the expression of HSPs of the 70-kDa class during the induction of embryogenesis in microspores of *B. napus*: (i) anti-HSP68, a polyclonal antibody raised against tomato HSP68 (Neumann et al. 1987, 1993), (ii) anti-HSP70, a polyclonal antibody raised against tomato HSP70 (Neumann et al. 1987), and (iii) anti-HSP72/73, a monoclonal antibody raised against human HSP72/73 (clone N27F3-4). Immunoblot analysis of a protein extract derived from an embryogenic *B. napus* microspore culture (8 h, 32°C) revealed that each of the three antibodies recognized at least one protein band in the 70-kDa M$_r$-range (Fig. 1a). When the immunoblot was first developed with anti-HSP68, and subsequently with anti-HSP72/73, one extra band appeared on the blot with a slightly higher M$_r$ than the anti-HSP68 reacting protein band (compare the first three lanes in Fig. 1a). This indicated that anti-HSP68 cross-reacted with protein of a slightly lower M$_r$ (designated HSP68) than the protein recognized by anti-HSP72/73. The anti-HSP72/73 and anti-HSP70 antibodies recognized proteins of the same size (Fig. 1a, see also below). The proteins recognized by both anti-HSP70 and anti-HSP72/73 antibodies are collectively referred to as HSP70.
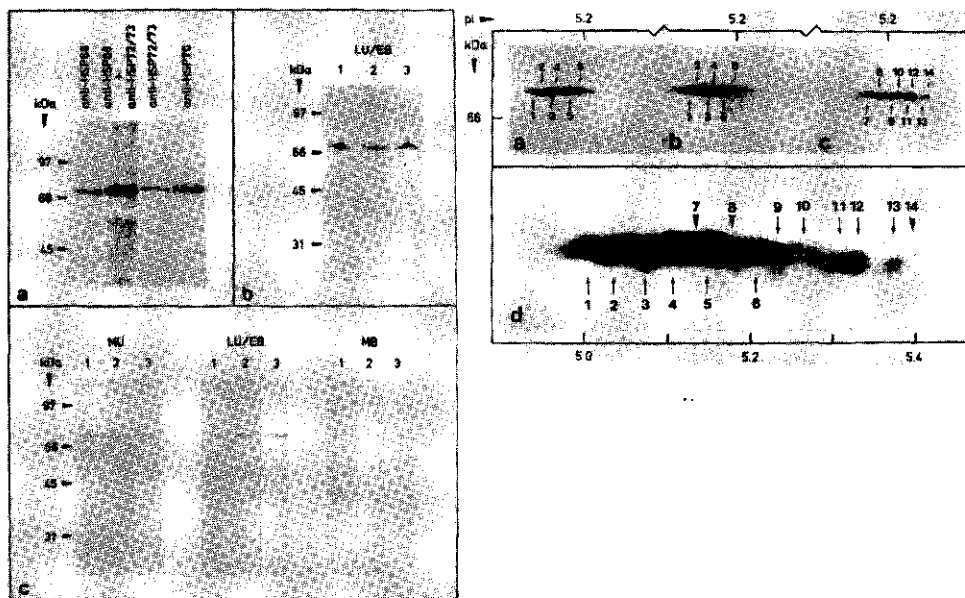
Fig. 1a-c. Expression of HSP68 and HSP70 in *B. napus* microspores isolated at different stages of development and cultured at different temperatures. **a** An embryogenic microspore population, containing both late unicellular (*LU*) microspores and early bicellular (*EB*) pollen, was cultured for 8 h at 32°C. Proteins were analyzed by SDS-PAGE (7.5% Midget gels) and immunoblotting using anti-tomato HSP68 polyclonal antibody (*lane 1*), anti-human HSP72/73 monoclonal antibody (*lane 3*) and anti-tomato HSP70 polyclonal antibody (*lane 4*). The immunoblot of *lane 2* was reacted in two consecutive steps with anti-HSP68 and anti-HSP72/73, respectively. **b** Proteins from an embryogenic microspore population (*LU/EB*) were extracted directly after isolation from the anthers (*lane 1*), or after 8 h of culture at 18°C (*lane 2*) or 32°C (*lane 3*). After SDS-PAGE (7.5% ExcelGel) and immunoblotting the blots were probed with anti-HSP68. **c** Proteins were extracted from microspores and pollen isolated at three different stages of development (*MU*, mid unicellular microspores; *LU/EB*, mixture of late unicellular microspores and early bicellular pollen; *MB*, mid bicellular pollen). Further analysis of the proteins and also the numbering of the lanes was the same as described in **b**, except that anti-HSP72/73 was used as a probe for hybridization.

Fig. 2a-d. Two-dimensional gel analysis of HSP68 and HSP70 isoforms expressed in an embryogenic microspore culture. *Brassica napus* microspores were labeled with [^{35}S]methionine for 8 h at 32°C. Total cell proteins were separated by 2-D gel electrophoresis (first dimension: pH 4-7; second dimension: 8-18% gradient ExcelGel) and blotted onto nitrocellulose. Blots were probed with anti-HSP70 (**a**), anti-HSP72/73 (**b**) and anti-HSP68 (**c**). The figures show only the immunoreactive region of the 2-D Western blots. The radiolabeled spots on each of the blots were visualized by PhosphorImager technology placing crosses on recognized spots. **d** A digitized image of the radioactivity present in the immunoreactive region of the blot shown in **c**. *Upward pointing arrows* in **d** indicate the various isoforms recognized by anti-HSP70 and anti-HSP72/73 antibody (1-6), while the *downward pointing arrows* indicate the isoforms recognized by anti-HSP68 (7-14). Spot numbers 7, 8 and 14 (*arrowheads*) were not radioactively labeled.
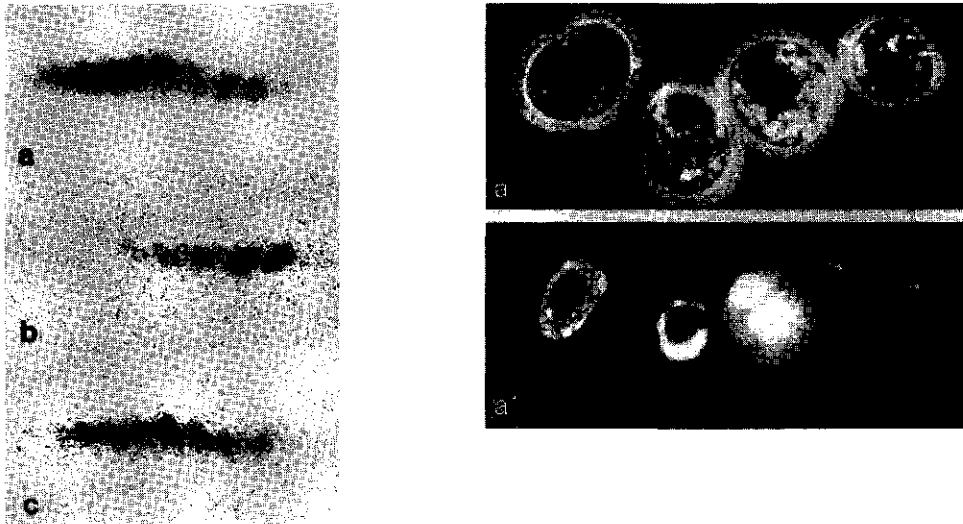
Fig. 3a–c. Two-dimensional gel analysis of [³⁵S]methionine-labeled proteins immunoprecipitated with anti-HSP68 and anti-HSP70. The *B. napus* microspores were labeled with [³⁵S]-methionine for 8 h at 32°C. Total cell extracts were used in immunoprecipitation studies with anti-HSP68 and anti-HSP70. After 2-D gel electrophoretic separation (first dimension: pH 4-7; second dimension: 7.5% homogeneous ExcelGel) of the resultant immunoprecipitates, the gels were scanned on a PhosphorImager. A grey-scale printout of the radioactive region of the gels is shown: a immunoprecipitate using anti-HSP70; b immunoprecipitate using anti-HSP68; c 1:1 mixture of the two immunoprecipitates, consisting of half the amount loaded in a plus half of that loaded in b.

Fig. 4a, a'. Intracellular localization of HSP68 in microspores and pollen as determined by indirect immunofluorescence using anti-HSP68. The presence and localization of HSP68 were visualized by immunocytochemical methods on cryo-sectioned microspores and pollen. a HSP68 labeling of microspores and pollen directly after isolation. A spot-like staining pattern of the cytoplasm was observed, whereas the nuclei were unlabeled. a' DAPI-staining of nuclei of the same section as shown in a. Note the DAPI stained organelles which match to immunolabeled spots (*arrows*). The bar represents 10 μm

Expression of HSP68 and HSP70 during microspore embryogenesis. We investigated whether the induction of embryogenesis in microspores was associated with changes in the total amount of HSP68 and HSP70. The levels of HSP68 and HSP70 were estimated by immunoblot analysis of protein extracts prepared immediately after isolation of the microspores from the anthers (lanes 1 in Fig. 1b, c), after an 8 h period of culture at 18°C (lanes 2 in Fig. 1b, c; conditions for pollen development), and after an 8 h period of culture at 32°C (lanes 3 in Fig. 1b, c; conditions for embryogenic development). Three different populations of microspores and/or pollen were compared in this analysis: (i) potentially embryogenic microspores, containing a mixture of late unicellular microspores (LU) and early bicellular pollen (EB), (ii) mid unicellular microspores (MU), too young to undergo embryogenesis, and (iii) mid bicellular pollen (MB), too old to undergo embryogenesis.
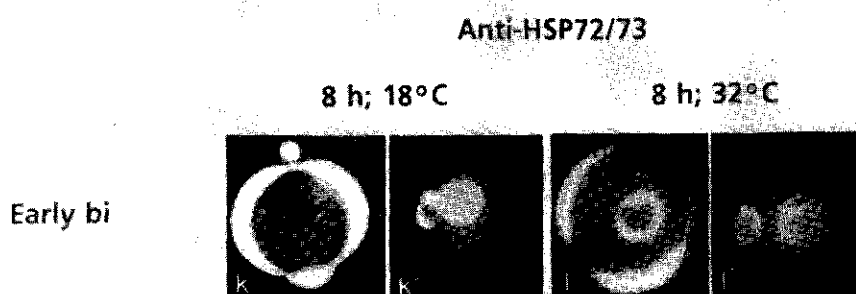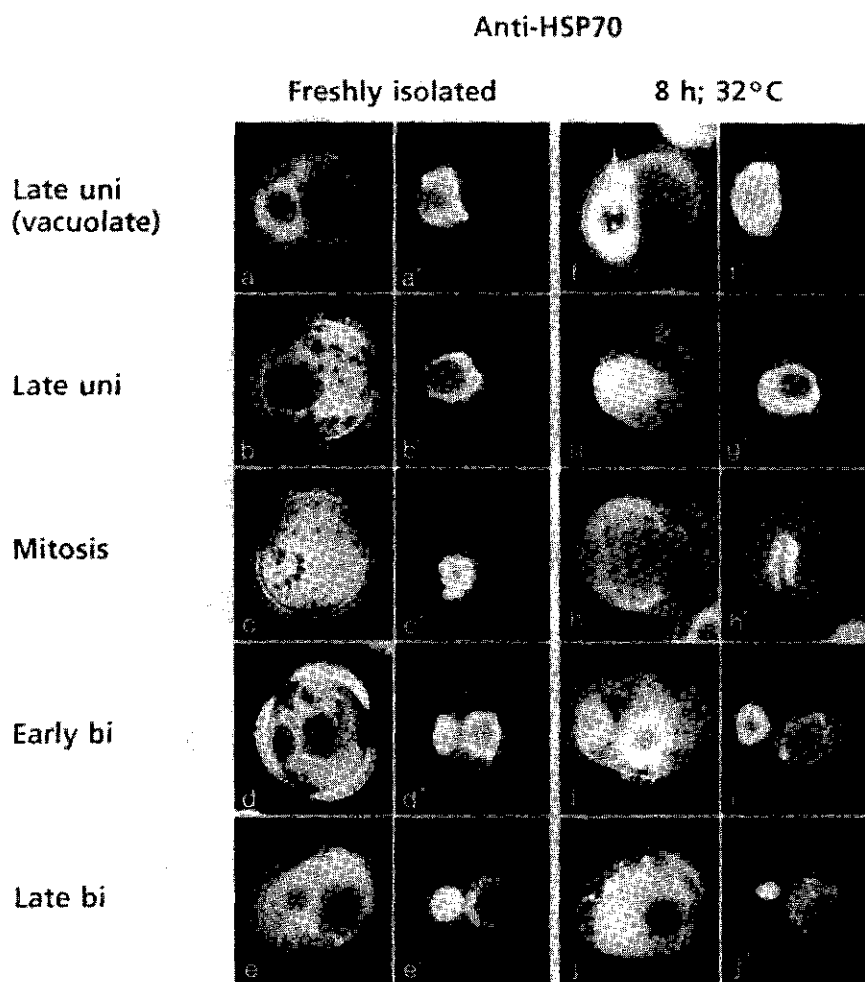
83

Protein extracts from all the samples probed with anti-HSP68 showed a band of approximately the same intensity, indicating that the induction of embryogenesis in microspores was not accompanied by visible changes in the total amount of HSP68. Only the results obtained by analyzing a potentially embryogenic microspore population (LU/EB) for HSP68 content are shown (Fig. 1b). All samples were also tested for the amount of HSP70 as detected by the anti-HSP72/73 monoclonal antibody (Fig. 1c). Whereas the initial amounts of HSP70 present in the three populations from different developmental stages were similar (compare the lanes 1 in Fig. 1c), clear differences in the level of HSP70 were observed after 8 h of culture at 32°C (compare the lanes 3 in Fig. 1c). When a population of mid unicellular (too young) microspores was cultured (Fig. 1c, left panel) the level of HSP70 did not change detectably after 8 h of culture at either 18°C or 32°C. In contrast to this, the potentially embryogenic microspore population showed an increase in HSP70 level after 8 h of culture at 32°C (Fig. 1c, middle panel), whereas the mid bicellular (too old) pollen cluttered under the same conditions contained little or no HSP70 (Fig. 1c, right panel). These results show that the total amount of HSP70 present after an 8 h 32°C treatment strongly depended on the developmental stage of the microspores and/or pollen at the onset of culture. Because of this, we studied the expression of members of the 70-kDa class of HSPs in relation to the induction of embryogenesis.

Identification of HSP68 and HSP70 isoforms in potentially embryogenic microspores. A more detailed analysis of the expression of HSP68 and HSP70 under the different culture conditions (18°C and 32°C) was performed by in-situ [^{35}S]methionine-labeling of potentially embryogenic microspores. Proteins from a [^{35}S]methionine-labeled embryogenic microspore extract (8 h, 32°C) were separated by 2-D gel electrophoresis, transferred to nitrocellulose, and probed with the various antibodies. Figure 2a-c shows the immuno-reactive area of three identical radioactive 2-D blots hybridised with the different antibodies, i.e. anti-HSP70 (Fig. 2a), anti-HSP72/73 (Fig. 2b) and anti-HSP68 (Fig. 2c). To facilitate alignment of the immunochemically stained spots with the corresponding [^{35}S]methionine-labeled spots (Fig. 2d), small dots of radioactive ink were applied to each blot as landmarks before exposure to the PhosphorImager plate. Accurate matching of the immuno-reactive spots with the PhosphorImager data was achieved by computerized image analysis (Cordewener et al. 1994). The anti-HSP70 and anti-HSP72/73 antibodies each reacted with the same set of six protein spots with isoelectric points (pIs) ranging from 5.0 to 5.2 (spot numbers 1-6 in Fig. 2a, b). Eight protein spots were recognized by anti-HSP68, with varying pI-values from 5.1 to 5.4 (spot numbers 7-14 in Fig. 2c). Figure 2d represents a digitized image of the radioactive spots present on the blot shown in Fig. 2c. An identical image was obtained for the two other blots (Figs. 2a and b), confirming the reproducibility of the applied 2-D technique. The assignment of the different immuno-reacting spots to [^{35}S]methionine-labeled spots is given in Fig. 2d. All HSP70 immuno-reacting spots (numbers 1-6) could also be detected as distinct spots on the autoradiograph, indicating that all HSP70 isoforms detectable were actively synthesized during the initial 8 h of culture of microspores at 32°C. Only five out of eight HSP68 isoforms visible on the blot matched spots which were detected as single radioactive spots after computer analysis of the autoradiograph (spot numbers 9-13). This indicates that the remaining three HSP68 isoforms were already present in the microspores before the radiolabel was added to the culture, and were not synthesized at a sufficient rate for detection

during the inductive phase of embryogenesis.

Increased synthesis of HSP68 and HSP70 isoforms during induction of embryogenesis.
Previously, we have constructed reference databases of [^{35}S]methionine-labeled proteins from
embryogenic (8 h, 32°C) and non-embryogenic (8 h, 18°C) microspore cultures (Cordewener
et al. 1994). Comparison of these two databases showed that 25 proteins were differentially
synthesized during the inductive phase of microspore embryogenesis. In order to see if any
of the HSP68 or HSP70 isoforms belonged to this subset of 25 proteins, we matched the 2-D
[^{35}S]methionine-labeled protein pattern of the immunoblots shown in Fig. 2 with the 8 h 32°C
reference database. This analysis revealed that one of the HSP70 isoforms (spot number 5 in
Fig. 2) and one of the HSP68 isoforms (spot number 12 in Fig. 2) belonged to this subset of
differentially synthesized proteins (SSP numbers 2709 and 3711 of the database; Cordewener
et al. 1994). After correction for the on average 2.3-fold higher overall incorporation of [^{35}S]-
methionine into proteins at 32°C than at 18°C, the induced HSP68 isoform showed a 3.0-fold
increase in synthesis at 32°C, and the HSP70 isoform was induced 6.0-fold at 32°C
(Cordewener et al. 1994). Apart from the very specific induction of these two HSP isoforms
caused by the temperature elevation, quantitative analysis (for details, see Cordewener et al.
1994) revealed that independent of the culture temperature, the overall rate of HSP70
synthesis (spots 1-6 from Fig. 2d) was at least two-fold higher than the rate of HSP68
synthesis (spots 7-14 from Fig. 2d).

Immunoprecipitation of anti-HSP68 and anti-HSP70 reacting proteins. To demonstrate the
validity of the matching of anti-HSP immuno-reacting proteins to the [^{35}S]methionine-labeled
proteins, immunoprecipitation experiments were performed. These experiments were carried
out with [^{35}S]methionine-labeled embryogenic cultures (8 h; 32°C) similar to those used for
the immunostaining experiments described previously. The 2-D gels of the various
immunoprecipitates were run analogously to those for the immunoblots of Fig. 2, except that
a 7.5% homogeneous gel was used instead of a 8-18% gradient gel in order to get a better
resolution in the second dimension. At least eight [^{35}S]methionine-labeled spots could be
visualized after 2-D gel electrophoretic separation of the anti-HSP70 immunoprecipitated
proteins (Fig. 3a). The most highly labeled radioactive spots of the anti-HSP70
immunoprecipitate focused in the same pH-range as the six immunoreactive spots of the 2-D
blots hybridized with anti-HSP70 and anti-HSP72/73 (Fig. 2a, b), whereas the more basic
radiolabeled proteins appear to run in the HSP68 area (Fig. 2c). The 2-D protein pattern of
the immunoprecipitate from anti-HSP68 was composed of at least four spots (Fig. 3b). Due
to the specificity of the immunoprecipitation, no protein landmarks were available for
accurate alignment of spots between the two 2-D gels. Therefore, a third 2-D gel was run
from a 1:1 (v/v) mixture of each of the two immunoprecipitates. A quantitative comparison
of the three protein patterns, using the ImageQuant software, revealed that in the 2-D gel of
the mixed immunoprecipitates (Fig. 3c) the anti-HSP68 reactive proteins were localized in
the same area as the more basic proteins from the anti-HSP70 immunoprecipitate. It therefore
appears that the anti-HSP70 antibody cross-reacts with some of the HSP68 isoforms under
the immunoprecipitation conditions employed here, whereas the anti-HSP68 antibody retains
its specificity.

Anti-HSP70

Freshly isolated   8 h; 32°C

Late uni
(vacuolate)

Late uni

Mitosis

Early bi

Late bi

Anti-HSP72/73

8 h; 18°C   8 h; 32°C

Early bi

Immunolocalization of HSP68 and HSP70. Sectioned microspores or pollen were subjected to immunocytochemical analysis to determine the cellular distribution of HSP68 and HSP70 proteins immediately after isolation and after an 8 h culture period at 18°C or 32°C. These experiments were carried out to determine whether the increased HSP68 and HSP70 synthesis during the induction of microspore embryogenesis, as revealed by [^{35}S]-methionine incorporation, was accompanied by changes in their localization.

The anti-HSP68 staining of freshly isolated microspores and bicellular pollen grains revealed a punctate cytoplasmic staining (Fig. 4a). Nuclei and vacuoles were never stained. Fluorescence of the microspore and pollen walls was due to autofluorescence. During the early stages of microspore development, no differences in staining were observed; after the mid bicellular pollen stage, the number of stained spots decreased. When placed in culture for 8 h at 18°C or 32°C, comparable HSP68 staining patterns were found at the various stages of microspore and pollen development (data not shown). Simultaneous staining with both anti-HSP68 and the DNA dye DAPI showed that the punctate HSP68 staining co-distributed with DNA-containing organelles (Fig. 4a, a').

In contrast to HSP68, a developmental stage specific intracellular localization of HSP70 was observed in freshly isolated microspores and pollen (Fig. 5a-e). Vacuolate late unicellular microspores showed labeling of the nucleoplasm and cytoplasm (Fig. 5a, a'). During the process of vacuole destruction, just preceding the first pollen mitosis, the intensity of HSP70 labeling increased in the cytoplasm while it gradually decreased in the nucleus (Fig. 5b, b'). During mitosis HSP70 was equally distributed in the cytoplasm; chromosomes and vacuoles were not labeled (Fig. 5c, c'). Early bicellular pollen also showed an intensive fluorescence

Fig. 5a-l. Intracellular localization of HSP70 in microspores and pollen as determined by indirect immunofluorescence using anti-HSP70 and anti-HSP72/73. The presence and localization of HSP70 (a-j) and HSP72/73 (k, l) were visualized by immunocytochemical methods on cryo-sectioned microspores and pollen, directly after isolation or after culture under embryogenic (8 h; 32°C) or non-embryogenic (8 h; 18°C) conditions. Every micrograph is accompanied by a UV micrograph (a'-l') in which DAPI staining visualizes the position of nuclei and chromosomes. The bar represents 10 μm. a-e HSP70 labeling of microspores and pollen directly after isolation. a Vacuolate late unicellular microspore shows labeling in the nucleoplasm, and weaker labeling in the cytoplasm. b Late unicellular microspore exhibits labeling in the cytoplasm, less in the nucleoplasm and none in the nucleolus. c Mitotic microspore shows labeling throughout the whole cytoplasm. Note negative staining of the chromosomes. d Early bicellular pollen shows labeling in the cytoplasm of both the generative and the vegetative cell. Note labeled spots near the wall between both cells. e Late bicellular pollen shows a stronger labeling of the cytoplasm of the generative cell compared to that of the vegetative cell. f-j HSP70 labeling of microspores and pollen after 8 h of culture at 32°C. f Vacuolate late unicellular microspore shows strong labeling in nucleoplasm, and much weaker labeling in the cytoplasm. Note the intensive labeled area (*arrow*) which frequently occurred during microspore stage. g Late unicellular microspore exhibits an intensively labeled nucleoplasm, and a weakly labeled cytoplasm. h Mitotic microspore with changed chromosome orientation characteristic for embryogenic development. The cytoplasm is labeled whereas the chromosomes are unlabeled. i Early bicellular pollen shows strong labeling of both the generative and vegetative nucleus, in contrast to the situation observed at 0 h (d). j Late bicellular pollen shows diffuse labeling of the cytoplasm and nucleoplasm of both the generative and vegetative cell. Note the intensively labeled spots in the cytoplasm of the vegetative cell. k-l The HSP72/73 labeling of microspores and pollen after 8 h of culture at 18°C or 32°C. k Early bicellular pollen shows no fluorescence in the cytoplasm and nucleus after labeling with

anti-HSP72/73. l Early bicellular pollen exhibits labeling in the nucleus of the vegetative cell.

of the cytoplasm, but no labeling of the generative and vegetative nuclei (Fig. 5d, d'). Intensively labeled spots were frequently observed near the cell wall between the generative and vegetative cell. In late bicellular pollen the staining intensity of the cytoplasm of the vegetative cell decreased, whereas the cytoplasm of the generative cell continued to exhibit a strong labeling (Fig. 5e, e'). In some instances intensively labeled spots appeared in the cytoplasm of the vegetative cell of late bicellular stages (data not shown).

Eight hours of culture at 18°C did not affect the staining pattern of anti-HSP70 at any stage of development, when compared to that of freshly isolated microspores or pollen (data not shown). In contrast, major differences were observed after cultivation at 32°C for 8 h (Fig. 5f-j). After this temperature treatment, vacuolate late unicellular microspores showed a much stronger labeling of the nucleoplasm than observed for freshly isolated microspores of the same developmental stage (Fig. 5a, f). Non-vacuolate late unicellular microspores showed a strong labeling of the nucleoplasm, which was never observed in freshly isolated microspores or after 8 h of culture at 18°C (Fig. 5b, g). During microspore mitosis, frequently characterized by changed spindle placement (Fig. 5h, h') or symmetrical divisions, the chromosomes were not labeled. The signal in the cytoplasm was comparable to that of freshly isolated microspores (Figure 5c, h).

The HSP70 labeling of the cytoplasm of early bicellular pollen was of the same intensity as in the late microspore stage. In contrast to freshly isolated early bicellular pollen, and to microspores cultured for 8 h at 18°C, the generative and vegetative nuclei of the early bicellular pollen, cultured for 8 h at 32°C, did contain HSP70 in the nucleoplasm (Fig. 5i, i'). Frequently, the intensity of labeling was comparable in both nuclei, but we also observed early bicellular pollen with more intensely labeled vegetative nuclei. Late bicellular pollen exhibited a decreased labeling in the nuclei but bright spots in the cytoplasm (Fig. 5j, j'). These spots did not co-localize with DAPI staining.

Despite the use of high concentrations of antibodies and the prolongation of labeling procedures it was only possible to observe a very faint labeling with anti-HSP72/73 in microspores and pollen, both directly after the isolation as well as after cultivation at 18°C. This indicates that, under these conditions, HSP70 could not be detected by the monoclonal anti-HSP72/73 antibody, but could be detected by the polyclonal anti-HSP70 antibody (Figure 5d, k). After cultivation for 8 h at 32°C, HSP70 proteins could be labeled with anti-HSP72/73 in a pattern similar to that of anti-HSP70, albeit less intensive (for example see Fig. 5i, l). The specific anti-HSP72/73 labeling disappeared after 24 h of culture. The general lower staining intensity with the monoclonal anti-HSP72/73 antibody, when compared to the polyclonal anti-HSP70 antibody, is probably caused by less efficient labeling of HSP70 by the monoclonal antibody than by the polyclonal antibody. Lack of staining by the anti-HSP72/73 monoclonal antibody during non-inductive culture conditions might be due to the inaccessibility of the epitope, caused by selective association with other proteins under non-heat-shock conditions. Selective association with specific epitopes under different conditions has been observed previously for HSP70 (Milarski et al. 1989).

DISCUSSION

Microspores and/or pollen of a number of plant species have the remarkable ability to switch their developmental fate from becoming a mature pollen grain to becoming an embryo. The most common procedures used to bring about this switch, are cold pre-treatment, heat treatment, or a period of starvation of sugar or nitrogen sources (Prakash and Giles 1987). In cultured *B. napus* microspores, embryogenesis is most efficiently induced by heat treatment, but ethanol or gamma rays are also effective (Pechan and Keller 1989). Throughout biology, severe stress is characterized by the shut down of normal protein synthesis, and the almost exclusive synthesis of the 'heat shock proteins' (Morimoto et al. 1990). The most abundant HSPs belong to the 70-kDa HSP family. These proteins act as molecular chaperones in the (re)folding, assembly, and transport of cellular proteins, and as such are essential for cell survival (Hendrick and Hartl 1993). As mild stress seems to be the common theme in the induction of microspore embryogenesis in general, we were interested to see whether the temperature elevation that causes the switch in developmental fate of *B. napus* microspores also causes changes in HSP synthesis and localization.

Irreversible commitment of *B. napus* microspores to embryogenic development can be accomplished within 8 h of culture at 32°C (Pechan et al. 1991). Only microspores around the first pollen mitosis can undergo this process. The overall level of HSP70 increased by this mild stress treatment during the commitment phase (Fig. 1). Microspores that were in a developmental stage too old to undergo embryogenesis, were unable to increase their total amount of HSP70 in response to the 8 h 32°C treatment. It has been reported that mature pollen can not mount a heat-shock response (Duck et al. 1989; Frova et al. 1989; Hopf et al. 1992). This ability is lost gradually after the first pollen mitosis, and may explain why the competence of pollen to initiate embryogenic development in culture decreases with developmental age.

Heat-shock proteins belong to the most conserved proteins known (Boorstein et al. 1994). However, it remains important to verify the validity of using antibodies from different species in a heterologous system. For the anti-HSP68 antibody this has been well documented in eight different plant species (Neumann et al. 1993). Tomato HSP68 was shown to belong to the constitutively expressed, but heat inducible, family of HSPs, and migrates as a 68 kDa set of isoforms, with a pI range of 5.5-5.7 (Nover and Scharf 1984; Neumann et al. 1987). The cDNAs encoding an HSP68 recognized by this antibody were isolated from tomato and potato, and were shown to be highly homologous to several mitochondrial and plastid HSP70s, as well as to *Escherichia coli* DnaK (Neumann et al. 1993). In *B. napus* a similar set of HSP68 isoforms was detected by 2-D gel electrophoresis, of which one was differentially synthesized under embryogenic culture conditions (spot number 12 in Fig. 2, SSP 3711 in Cordewener et al. 1994). The threefold-induced synthesis of this HSP68 isoform could not be detected on 1-D immunoblots. This is likely due to the large contribution of the remaining seven isoforms, whose synthesis was not induced, to the overall level of HSP68 as detected by 1-D immunoblotting. At least four out of five [^{35}S]methionine-labeled HSP68 isoforms could also be detected after immunoprecipitation, further validating the use and specificity of the anti-HSP68 antibody.

Recently, it was shown that the anti-HSP68 antibody stains mitochondria of all eight plant species investigated (Neumann et al. 1993). The results presented here indicate that this is also the case for *B. napus* microspores; HSP68 is localized in DNA containing organelles. Owing to its mitochondrial localization and its apparently unaltered distribution under all conditions investigated, it is not very likely that HSP68 is directly involved in the induction of embryogenic development.

The anti-HSP70 antibody used in this study has not been characterized to the same extent as the anti-HSP68 antibody. In tomato, the anti-HSP70 antibody recognizes a 70-kDa set of isoforms, with a pI range 5.25-5.5 (Neumann et al. 1987). Heat shock was shown to induce the HSP70 expression, and the antibody shows cross-reactivity on immunoblot with 70-kDa proteins of protein extracts from four other plant species (Nover et al. 1989). In *B. napus* microspores, we detected at least six HSP70 isoforms with the anti-(tomato) HSP70 antibody, with pI-values ranging from 5.0 to 5.2 (Fig. 2). Exactly the same isoforms also reacted with the anti-(human)HSP72/73 monoclonal antibody, highlighting the conserved nature of this family of proteins. A similar large number of isoforms of HSPs of the 70-kDa class has also been found in *Arabidopsis*, encoded by at least three different genes (Wu et al. 1988). All isoforms were synthesized during the 8-h inductive treatment at 32°C. One of the isoforms was synthesized at a sixfold-higher rate than in the non-embryogenic 18°C control (spot number 5 in Fig. 2; SSP 2709 in Cordewener et al. 1994). This induced synthesis was also reflected in the total amount of HSP70 detected on 1-D immunoblot (Fig. 1). Immunoprecipitation experiments revealed that apparently all HSP70 isoforms can be captured by the anti-HSP70 antibody (Fig. 3). In addition to the HSP70 isoforms, anti-HSP70 also precipitated HSP68 isoforms. This indicates that under the conditions used for the immunoprecipitation experiments, anti-HSP70 is less specific than under the conditions used for immunostaining on nitrocellulose. In the immunocytochemical studies, punctate staining of the cytoplasm was frequently observed with the anti-HSP70 antibody. This staining did not co-localize with DNA containing organelles, indicating that under the conditions employed here, anti-HSP70 did not cross-react with HSP68. It is likely that the punctate staining by anti-HSP70 observed in late bicellular pollen in a 32°C culture (Fig. 5j) represents the heat-shock granules as described previously (Nover et al. 1989). Further evidence that anti-HSP70 antibody does recognize bona-fide HSP70 was obtained by purifying HSP70 by ATP-agarose affinity chromatography, and demonstrating co-purification of anti-HSP70 and anti-HSP72/73 immuno-reacting proteins (unpublished observations).

Previously, it was shown that, under non-heat-shock conditions, HSP70 isoforms are mainly localized in the cytoplasm of tomato cells (Neumann et al. 1987). In *B. napus* microspores, however, we detected stage-specific changes in the intracellular distribution of HSP70 during normal development. For example, only vacuolate late unicellular microspores, competent for undergoing embryogenic development, showed a clear staining of the nucleoplasm (Fig. 5). This developmental stage coincides with DNA replication as determined by 5-bromo-deoxyuridine incorporation (Binarova et al. 1993). Under non-heat-shock conditions, human HSP70 was shown to be synthesized exclusively during the S phase of the cell cycle, and to be primarily localized in the nucleus during S phase, and in the cytoplasm during the remainder of the cell cycle (Milarski and Morimoto 1986). *Brassica napus* HSP70, therefore appears to behave similarly to the human HSP70. The staining pattern of anti-HSP70 in freshly isolated microspores was identical to that of microspores that were placed in culture

for 8 h at 18°C, conditions which allow gametophytic development to proceed.

Staining patterns of anti-HSP70 in microspores that had been induced to enter embryogenic development by shifting the culture temperature to 32°C for 8 h, showed an increase in the overall labeling intensity, and changes in the cellular distribution of HSP70. The HSP70 staining remained in the nucleus, even after vacuole disintegration in the late unicellular stage, when under non-embryogenic conditions HSP70 disappeared from the nucleus (Fig. 5b, g). This may be related to an elongation of the S phase of the cell cycle, which is frequently observed after heat shock (Walsh and Morris 1989; Roti Roti et al. 1992; Walsh et al. 1993). During mitosis, HSP70 distribution was similar to that of control microspores (Fig. 5c, h). The most dramatic change in HSP70 staining could be observed in early bicellular pollen cultured under embryogenic culture conditions: HSP70 staining could now be observed in the nuclei of both the vegetative and the generative cell (Fig. 5i). At times only the vegetative nucleus could be stained (Fig. 5l). None of the nuclei could be stained if the generative cell had already moved into the cytoplasm of the vegetative cell, a developmental stage too late for undergoing embryogenic development (Hause et al. 1993). This pattern strongly resembles the pattern of DNA synthesis, as revealed by 5-bromo-deoxyuridine incorporation, in microspores cultured under embryogenic culture conditions (Binarova et al. 1993). There is therefore a strong correlation between the phase of the cell cycle, the nuclear localization of HSP70 and the induction of embryogenesis.

Elevation of the temperature is the sole external factor that causes the switch in developmental fate from pollen development to embryogenic development. This change in development may be caused by an inappropriate regulation of cell cycle timing, therefore derailing normal gametophytic development. It may be that the induced synthesis and nuclear transfer of HSP70 might be involved in this timing of the cell cycle, affecting the S phase in particular. It might be envisioned that HSP70 acts either directly, functioning analogous to the bacterial HSP70 homologue DnaK, which recruits the DnaB helicase to the origin of DNA replication (for review, see Stillman 1994), or indirectly by allowing the transfer of transcription factors to the nucleus, analogous to the activation of the glucocorticoid receptor (Pratt 1993; Shen et al. 1993; Hutchison et al. 1994).

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Chapter 7

Subcellular localization of cell specific gene expression in *Brassica napus* L. and *Arabidopsis thaliana* L. microspores and pollen by non-radioactive *in situ* hybridization

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ABSTRACT

Sections of microspores and pollen of *Brassica napus* L. and *Arabidopsis thaliana* L. were hybridized with a digoxigenated ssDNA probe which was picked up from cultured microspores and pollen of *Brassica napus* L. Specific localization of the signal was found in both *A. thaliana* as well as in *B. napus*. There was neither expression in microspores nor in vegetative cells of pollen. However, cytoplasm of generative cells of both *A. thaliana* and *B. napus* exhibited strong hybridization signal as did the sperm cell cytoplasm after division. With this finding we demonstrated for the first time cell specific gene expression in developing pollen. Detailed molecular characterization of this probe or the use of well characterized probes specific for pollen development combined with the results of *in situ* hybridization should give the possibility to get new insights into pollen development.

INTRODUCTION

Pollen, the male gametophyte of the plant, plays a key role in the reproductive cycle of higher plants. Pollen is formed in the anther from sporophytic tissue that forms microspore mother cells. The microspore mother cells undergo meiosis and form 4 microspores each (microsporogenesis). Microspores turn into pollen by the first mitotic division and then consist of a vegetative and a generative cell (microgametogenesis). The generative cell divides once again in the pollen grain (tricellular pollen) or in the pollen tube, and forms two sperm cells needed for double fertilization. Extensive characterization of microspores of mature pollen grains have been conducted from cytological and biochemical point of view (Mascarenhas 1975, Vijayaraghavan and Bhatia 1985). In *Brassica napus* and *Arabidopsis thaliana* the sperm cells are formed within the pollen grain which thus belongs to the tricellular type. In contrast to microsporogenesis, microgametogenesis is characterised by polarization, highly specialized cell division, and cell differentiation processes (McCormick 1993). As already indicated by light microscopic investigations (Geitler 1934) the first sign of polarity during pollen development is the eccentric position of the microspore nucleus before mitosis. The arising unequally sized cells develop into the morphologically and physiologically different generative and vegetative cell. So, there is a difference in developmental fate of the vegetative and the generative cell in the two-cellular pollen. The generative cell mostly contains only a small amount of cytoplasm. The differences in the size of mitochondria of generative and vegetative cells hint at differences in the metabolism of both cells (for review see Vijayaraghavan and Bhatia 1985). Lower density of nuclear pores in the generative nucleus hint also at lower transcriptional and subsequent lower metabolic activity in comparison to the vegetative cell (Wagner et al. 1990).

The processes of cell differentiation in the developing pollen grain are described till now only at the basis of structural data. Anther and/or pollen specific genes were identified and characterized (e.g. Stinson et al. 1987, Weterings et al. 1992, Twell et al. 1993, and Roberts et al. 1993). Specific gene expression in either the generative or the vegetative cells during pollen differentiation was not observed until now (Mc Cormick 1993).

A cDNA library was prepared by PCR from *B. napus* microspores and pollen that were

induced to become embryogenic. A clone, designated #231, has been isolated and sequenced (Angelis and Pechan, in preparation). We investigated the expression and subcellular localization of #231 in developing microspores and pollen of *B. napus* and *A. thaliana* by non radioactive *in situ* hybridization to reveal the specific expression of the corresponding gene during androgenesis and microgametogenesis.

MATERIAL AND METHODS

Plant material
Plants of *Brassica napus* L. cv. Topas were cultured as reported by Hause et al. (1993). Plants of *Arabidopsis thaliana* L. were cultured under greenhouse conditions, and buds of various sizes were harvested. Microspores of *B. napus* L. were isolated from buds with a size of 3.6 - 3.8 mm as described by Pechan and Keller (1988) and cultivated under non-embryogenic (18°C) and embryogenic (32°C) conditions (Hause et al. 1993). Samples were fixed at the onset of culture, after 8h and after 24h of cultivation. Additionally, microspores and pollen of *B. napus* and *A. thaliana* were isolated from anthers and fixed directly.

Fixation, embedding and sectioning
Microspores and pollen were collected in sieves, fixed at room temperature for 1.5 h with 3% (w/v) paraformaldehyde and 0.05% (v/v) Triton X-100 in phosphate buffered saline (PBS), pH 7.2. For the embedment in paraplast samples were rinsed in PBS and immobilized in 3% (w/v) agar and 1% (w/v) gelatine in PBS. Pieces of agar-gelatine were dehydrated in a graded series of ethanol and infiltrated gradually by paraplast using xylol at 56°C. Sections of 5μm thickness were transferred onto glass slides coated with organo-silane.
For cryosectioning, fixed samples were rinsed in PBS and then gradually infiltrated in series solutions of saccharose (0.1-1.0-2.3 M saccharose) in PBS, at room temperature. Drops of 3μl were frozen in liquid propane and cut with glass knives on an ultracryotome (Reichert-Jung). Sections of 3 μm thickness were transferred onto organo-silane coated slides.
For electron microscopy glutaraldehyde/OsO₄ fixation, Spurr embedding and post staining was used as published previously (Hause et al. 1992).

Acridine orange staining
Sections of paraplast and cryo embedded microspores and pollen were stained with acridine orange according to Gahan (1984) to detect RNA and DNA simultaneously. For control, RNAs were removed from the tissue by incubating the slides with 0.5 μg.ml⁻¹ RNase A for 2 h at 37°C. The paraffine sections were stained just after deparaffinization and cryosections were stained just after sticking the material onto glass slides. Sections were incubated with 0.05% (w/v) acridine orange in 0.2 M acetate buffer (pH 2.1) for 30 min at room temperature. Slides were rinsed and finally mounted in veronal-acetate buffer pH 7.8. The observation was performed by fluorescence microscopy with UV excitation.

Preparation of DIG labelled ssDNA probes

A cDNA library was regenerated by PCR from mRNA of cultivated microspores and pollen of *B. napus*, the clone #231 was selected and cloned into pSPORT (Angelis and Pechan, in preparation). NotI and G-specific primers, used for the PCR amplification of cDNA prior cloning into pSPORT, were also used for the amplification of inserts for the preparation of ssDNA probes. PCR fragment was purified from primers and spurious products on NuSieve GTG low temperature melting agarose (FMC), and was cleaned on "Magic PCR Preps" minicolumn (Promega). The PCR fragment was used for a second round of linear amplification with one primer and DIG-dUTP to prepare DIG-ssDNA probes. Other reagents and conditions were according to the manufacturer (Boehringer-Mannheim).

In situ hybridization

The *in situ* hybridization procedure of Cox and Goldberg (1988) was followed with modifications. In the prehybridization treatment, paraffine sections were incubated for 30 min with 1 μg ml^{-1} proteinase K in 0.1 M Tris-buffer, pH 7.5, supplemented with 50 mM EDTA. Both paraffin and cryosections were treated with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine buffer, pH 8.0, for 10 min and incubated in prehybridization buffer consisting of 50 % (v/v) formamide in 2 x sodium chloride/sodium citrate buffer (1 x SSC: 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), supplemented with 0.15 mg.ml^{-1} tRNA and 0.05 mg.ml^{-1} poly(A)-DNA for 0.5 h at 37°C.

For hybridization 1 μl PCR mixture containing the DIG-ssDNA probe was diluted in prehybridization buffer and denaturated at 80°C for 5 min. Buffer with 40 units of RNasine (Promega) was added to a final volume of 100 μl. Hybridization was done for 20 h at 45°C. After hybridization, the slides were washed twice in 50 % (v/v) formamide 2 x SSC, and several times for 15 min in 2 x SSC at room temperature. Last washing step was at 48°C. Hybridized DIG-labelled probe was detected with anti DIG sheep polyclonal antibody F(ab) fragment, conjugated with alkaline phosphatase (Boehringer Mannheim). Detection of alkaline phosphatase with nitro blue tetrazolium chloride and X-phosphate was done according to the manufacturer's instruction. Thereafter, sections were stained with 1 μg.ml^{-1} 4,6-diamidino-2-phenylindol (DAPI) in PBS buffer for 30 min. Finally, slides were enclosed in glycergel (DAKO). Controls were performed by omitting the DIG labelled probe or by treatment of slides with RNase as described above, and appeared negative.

Bright field and fluorescence microscopy was done with a Nikon Microphot FXA using the filter combination EX 365/DM 480/BA 420 for DAPI. Colour images were recorded on Kodak EPY 64T film for bright field photography and on Kodak Ektachrome P800/1600 ASA film for DAPI fluorescence.

RESULTS AND DISCUSSION

After release of the tetrad, microspore development in *Brassica napus* is characterized by an early stage in which the nucleus is still in central position (Hause et al. 1992). Hereafter a huge vacuole developed, the nucleus attained an eccentric position and thus the microspore attained a polar organization (Fig. 1a, Hause et al. 1992). The vacuole persisted until the late microspore stage and disappeared before prophase when the chromatin condensed. Microspore

mitosis resulted in the formation of bicellular pollen in which the generative cell was still attached to the wall of the pollen (Fig. 1b). The division resulted in a large vegetative cell and a small generative cell, which contained cytoplasm and organelles such as endoplasmatic reticulum (ER), mitochondria and exceptionally plastids. In mid and late bicellular pollen generative cells were separated from the pollen wall (Fig. 1c). They consisted of a small nucleus surrounded by a thin layer of cytoplasm containing small-sized mitochondria, RER and few vacuoles. Late bicellular pollen turned into tricellular pollen by division of the generative cell (Fig. 1d, Murgia et al. 1991). When the generative cell divided to form two sperm cells, most of its cytoplasm was located between the two daughter nuclei (Fig. 1d). However, organelles such as mitochondria and small vacuoles were exclusively found in the tip regions of the cell and not in the central region between the two daughter nuclei.

The main purpose of the resulting sperm cells is fusion with the egg cell and central cell (Strasburger 1884). The sperm cells are very small and their nuclei occupy most of the cell volume. The cells have very small mitochondria, no plastids, low numbers of pores in the nuclear membrane and perform low metabolic activity (Shi et al. 1991). These facts show the highly specialized determination of the sperm cells.

In situ hybridization is the most direct way of examination the modulation of gene expression during development at the individual cell level (Cox and Goldberg 1988, Pardue 1988, Wilkinson 1992). Therefore, we used the probe #231, isolated from cultivated microspores and pollen. This cDNA clone contains a sequence of 305 bp and detected seven bands by Southern hybridization on *Brassica* genomic DNA (Angelis and Pechan, in preparation). Moreover, acridine orange staining was introduced as a control to determine the presence of RNA before starting the hybridization procedure. The intensity of the orange-red signal indicated the concentration of RNA. Fig. 2a gives an image of a section suitable for *in situ* hybridization, Fig 2b gives an image of a section treated with RNAse before staining. Here the orange stain in the nucleolus and cytoplasm disappeared and the green colour indicates that the DNA in the nuclei persisted.

The #231 mRNA was localized in developing microspores and pollen of *B. napus* (Fig. 2c-f) and *A. thaliana* (Fig. 2h-k). The developmental stages of microspores and pollen were determined by DAPI staining (Fig.2 c'-k'). The staining did not disturb the quality of the *in situ* hybridization signal. The DAPI-fluorescence signal could, however, be reduced by the alkaline phosphatase reaction product when the in situ signal was too strong (Fig. 2i-i').

Hybridization signal after probing with #231 was not observed during microspore development until mitosis in *B. napus* (Fig. 2c). After mitosis, the young two cellular pollen exhibited signal in the region of the generative cell (Fig. 2d). Signal was not observed in the nucleus and nucleolus but in the cytoplasm. Sometimes the labelling was spread over the whole cytoplasm surrounding the nucleus, other times it was concentrated in the region directed towards the vegetative cell. In a later stage of pollen development, in which the generative cell is completely surrounded by the cytoplasm of the vegetative cell, signal was found exclusively in the cytoplasm of the generative cell (Fig. 2e). In a stage just after division of the generative nucleus the signal was only observed in the region between the two daughter nuclei (Fig. 2f). In tricellular pollen in which sperms were formed already, no signal was found any more (data not shown). Controls in which the probe was not applied, appeared negative independently of the developmental stage of the microspores and pollen (Fig. 2g). The signal with clone #231 was found in or near the generative cells in all developmental

99

stages of bicellular pollen. The signal was located in a thin layer around the generative nucleus. The EM studies (Fig. 1) showed that the generative cell had only thin a layer of cytoplasm surrounding the nucleus. Thus, it can be concluded that the signal is probably
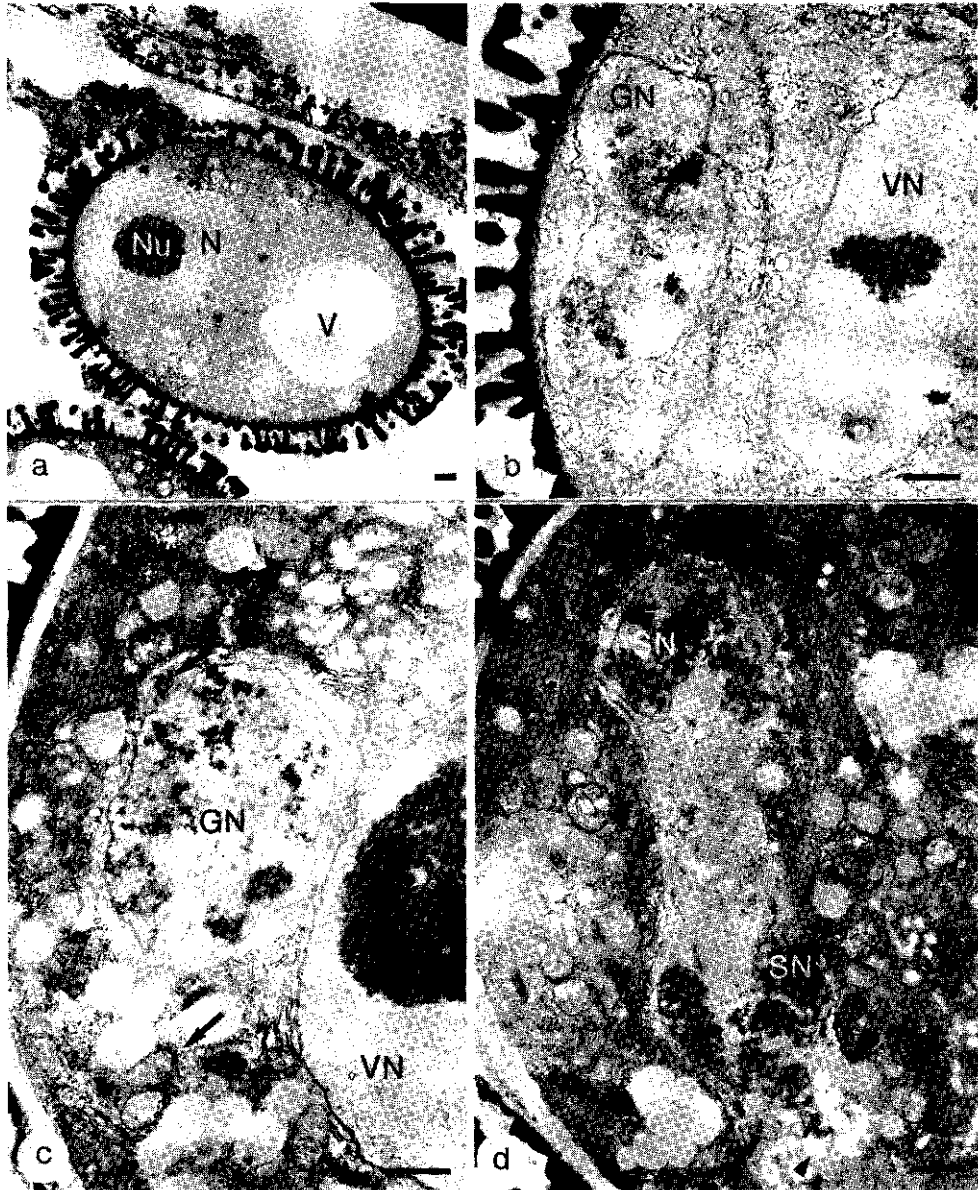
restricted to the cytoplasm of the generative cells. The generative cell, however, is an elongated structure with a length of 15 μm and a with of 2 μm. The precise localization of the mRNA enabled by the combination of cryosectioning and alkaline phosphatase reaction results in the subcellular localization in an area of about 4 μm^2 showing the high resolution of the method.

When sperm nuclei were formed by division of the generative nucleus, signal was mostly found between these nuclei (Fig. 2f). EM pictures showed that at this moment the greater part of the cytoplasm of the generative cell was located in the space between the newly formed nuclei (Fig. 1d). This finding again shows that the location of the strong *in situ* signal is just on the place of accumulation of cytoplasm originating from the generative cell.

When #231 was hybridized on sections of *A. thaliana* the distribution of signal was again restricted to phases running from microspore mitosis until the formation of the sperm cells (Fig. 2i-k) and again the distribution was restricted to the cytoplasm of either the generative cell (Fig. 2i, j) or the developing sperm cells (Fig. 2k) in a way comparable to that found in *B. napus*. Signal was absent in sectioned microspores (Fig. 2h) and mature pollen (data not shown). Summarizing, the same localization of signal as in *B. napus* was observed in the microspores and pollen of *A. thaliana*, probably because the *B. napus* is closely related to this model plant and the cDNA used is enough homologous.

Brassica napus microspores and pollen, which were cultured, were tested for the presence of signal after 8 and 24 h of culture. When cultured under non-embryogenic conditions (18°C) we observed #231 signal at stages like found in freshly isolated microspores and pollen, i.e. after microspore and pollen mitosis. Under embryogenic conditions (32°C) signal was again observed after microspore and pollen mitosis, both at 8 h and 24 h. Symmetrical divisions were observed after 24 h, however, these structures did not show signal after hybridization with probe #231. It is remarkable that cell division in microspores and pollen is accompanied by #231 signal expression, whereas the symmetrical divided structures formed by microspores and pollen cultured under embryogenic conditions for 24 h did not show such expression. This finding and the fact that we did not observe any differences between freshly isolated material and microspores cultured under embryogenic conditions suggest that the gene is not related to the induction of embryogenesis.

Because of its restriction to the cytoplasm of the developing and dividing generative cell, it

Fig. 1. Electron micrographs of ultra thin sections of Spurr embedded microspores and pollen of *Brassica napus* cv. Topas. Bars represent 1μm

a.   Vacuolate late microspore showing structural polarity by the eccentric position of its nucleus (N) and vacuole (V) (Nu=nucleolus).

b.   Cytokinesis of microspore mitosis with generative nucleus (GN) and vegetative nucleus (VN). Cell separation by cell plate (arrows) formation is about to finish.

c.   Bicellular pollen showing the generative cell surrounded by the cytoplasm of the vegetative cell. Note that in the generative cell only a thin layer of cytoplasm surrounds the nucleus (GN). Mitochondria in the generative cell (small arrow) are much smaller than those in the vegetative cell (large arrow) VN= vegetative nucleus).

d.   Pollen grain exhibiting the end of cytokinesis of the sperm cells. Note that most of the cytoplasm is between the two sperm nuclei (SN). Mitochondria (arrow) and most vacuoles (arrow head) were located at the outer ends of the elongated dividing generative cell.

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Chapter 8

Expression of polarity during early development of microspore-derived and zygotic embryos of *Brassica napus* L. cv. Topas

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ABSTRACT

Microspore derived (MS-)embryogenesis and zygotic embryogenesis of *Brassica napus* L. cv.
Topas were investigated by light and scanning electron microscopy to reveal the expression
of polarity during the transition phase from globular to heart and torpedo shape.
During the first 5 days of MS-embryo formation, the cell wall of the former microspores
remained intact and a globular mass of cells developed within. Pollen walls ruptured after 5
days of culture; embryos proceeded through heart-shape and a torpedo-shape stages within
15 days in a way comparable to, but faster than observed during zygotic embryogenesis.
Expression of polarity in globular and elongating MS-embryos was analyzed by detection of
the distribution of activated calmodulin as well as of free cytosolic calcium by using confocal
scanning laser microscopy, and by the detection of starch. Calmodulin was evenly distributed
in globular embryos and only exhibited clear polar distribution in elongated embryos. Free
cytosolic Ca^{2+} accumulated in the protoderm of globular embryos and in the central cylinder
of torpedo shaped embryos, but never showed polar distribution. Accumulation of starch
granules at the root poles of both sexual as well as MS-embryos, however, indicated polar
distribution before the transition from globular to heart shape stage. Since the local rupture
of the pollen wall of 6 day old MS-embryos was never preceded by the decrease of starch at
that site, it is likely that the rupture of the pollen wall plays an important role in the local
activation of the cell metabolism and thus in the determination of the polarity axis in MS-
embryos.

Key words:

Brassica napus - microspore culture - microspore embryogenesis - polarity - pollen culture

Abbreviations:

CLMS	confocal laser scanning microscopy
DAPI	4,6-diamidino-2-phenylindole
IPI	iodine/potassium iodide
MS	microspore
SEM	scanning electron microscopy

INTRODUCTION

The formation of haploid embryos from microspores (MSs) and pollen represents the main
tool for plant breeders to generate haploid and dihaploid plants which have considerable value
in plant breeding (for review see Evans et al., 1990). Microspore derived (Ms)embryogenesis
has been investigated extensively in rape (*Brassica napus*) because haploid embryogenesis was
obtained at high frequencies using anther cultures (Thomas and Wenzel, 1975) or cultures of
isolated late MSs and early bicellular pollen (Lichter, 1982) and because it served as a model

106

to study early events in zygotic embryogenesis (Taylor et al., 1990). Plant growth conditions, genotype variations, culture conditions, induction temperatures, all influence the ability of the isolated MSs to form embryos (Chuong and Beversdorf, 1985; Keller et al., 1987; Charne and Beversdorf, 1988; Chuong et al., 1988a, b; Gland et al., 1988; Huang et al., 1990; Telmer et al., 1992).

Morphological studies have shown that embryos are produced after a heat shock treatment of isolated late MSs and early bicellular pollen grains (Pechan and Keller, 1988). There are three embryogenic pathways deviating from the normal, gametophytic pathway: The occurrence of two types of symmetrical divisions in MSs, and the re-entry of vegetative cells in the cell cycle (Hause et al. 1992, 1993; Binarova et al., 1993). Beside these first processes of dedifferentiation, the formation of the embryo proper includes processes of ordered cell divisions as well as cell growth and differentiation. The establishment of polarity in the tissue of the fucoid zygotic embryo is essential for further development and differentiation (Kropf, 1992) and is expressed at the macroscopical level by the change from radial to axial symmetry (see also Tykarska, 1976, 1979). At the structural and biochemical level, polarity in the embryo is discernible in the distribution of organelles, and in the distribution of various compounds within the tissues, respectively (Raghavan, 1986). One of these compounds is Ca^{2+} which participates in the initiation and maintenance of plant processes which are important during plant embryogenesis (for review see Timmers, 1990). The distribution of Ca^{2+} and the calcium-binding protein calmodulin might express polarity during the development of MS-embryos as was described for other embryogenic systems (Timmers and Schel, 1990, 1991). Additionally, the distribution of starch in growing embryos might reflect differences in metabolic activity in the various cells of the embryo, and might thus reflect cell differentiation and an early expression of polarity in the developing embryo.

Therefore we analyzed the transition from the globular to the elongating phase in MS-embryogenesis from the morphological point of view and questioned whether this transition is preceded by a change in distribution of free cytosolic Ca^{2+}, calmodulin and a change in metabolic activity, expressed in the appearance or disappearance of starch.

MATERIALS AND METHODS

Plant material and cultivation of MSs and pollen
Plants of *Brassica napus* L. cv. Topas were first grown under greenhouse conditions and then submitted to a cold treatment as described by Hause et al. (1993). Flower buds with a size of 3.6 to 4.2 mm were used to isolate late MSs and early bicellular pollen. Isolation, and induction of embryogenesis in modified Lichter medium (NLN, Lichter, 1982) was according to procedures described by Pechan and Keller (1988). Embryos were analyzed after cultivation for 4, 5, 6, 7, 8, 9, 10 days, and 2 and 3 weeks.

Zygotic embryos were excised from ovules at developmental stages from 4 up to 12 days after anthesis. They were immediately stained by iodine/potassium iodide (IPI) to visualize the distribution of amyloplasts, or they were fixed with ethanol-acetic acid-formaldehyde (18:1:1) and further processed for scanning electron microscopy (SEM).

107

Immobilization of MS-proembryos
In order to monitor embryogenesis by confocal laser scanning microscopy (CLSM), embryos from 6-day-old liquid cultures were immobilized by transferring them to a double-layer culture system. Embryos were first collected on a nylon net (mesh-size 7 μm), next resuspended in 0.4 % (w/v) low melting point agarose in NLN at 30°C and then layered as a 1 mm thick sheet on top of solid 0.5 % (w/v) agar NLN medium. Cultures were incubated at 25°C in the dark and investigated daily by CLSM for 6 days.

Staining procedures

Staining of nuclei
Proembryos were harvested and incubated in 0.02 μg/ml 4,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co., St. Louis, Mo.) according to Hause et al. (1993).

Staining of activated calmodulin
Activated calmodulin was visualised according to Haußer et al. (1984) modified by Timmers et al. (1989): 2.10^{-5} M fluphenazine.2HCl (SERVA) was added to the culture medium and embryos were analyzed by fluorescence microscopy after 20 min.

Staining of free cytosolic calcium
Free cytosolic calcium was stained as described by Williams et al. (1990) and modified by Timmers et al. (1991). Embryos were harvested on sieves, incubated for 1 h in 20 μM FLUO-3 (Molecular Probes Inc, Eugene, Oregon, USA) in B5-medium (Gamborg et al., 1968) supplemented with 0.1% digitonin and rinsed in B5 for 1 min to remove the digitonin. Embryos were incubated again in 20 μM FLUO-3 in B5 for at least 1 h. After a final short wash in B5, the embryos were analyzed by CSLM.

Staining of starch
Embryos were harvested and incubated with IPI (2 g KI + 0.2 g I_2 in 100 ml H_2O) for at least 30 min.

Scanning electron microscopy
Microspore derived embryos were collected on nylon nets (mesh-size 7 μm) and fixed in 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0) for 2h at RT. After rinsing with buffer, samples were postfixed with 1 % (w/v) OsO_4 for 1h and rinsed again. These embryos as well as fixed zygotic embryos were dehydrated in a graded ethanol series. All samples were processed for critical point drying with liquid CO_2 using a Balzers union critical point dryer CPD 020, then coated with 3 nm platinum using an Oxford CT 1500 HF sputter coating unit, and finally observed in a JEOL JSM 6300 F scanning microscope.

Light microscopy
The fluorescence of DAPI and fluphenazine was visualized with a Nikon Microphot-FXA epifluorescence microscope supplied with a mercury lamp (HBO 100 W) using the filter EX 365/DM 400/BA 420. Embryos were observed in bright field mode and with differential interference contrast (DIC). The growth of immobilized embryos was monitored with a BIO-

RAD MRC-600 Laser Scanning Confocal Imaging System (CSLM) using transmission mode. FLUO-3 was visualized by CLSM using excitation wavelength 488 nm and long-pass filter 515 nm.

RESULTS

Morphogenesis of MS-embryos

Samples taken after four days of cultivation contained microspores, pollen and proembryos (Fig. 1a) with up to 12 nuclei. All nuclei belonged to individual cells which were still located within the former pollen wall (Fig. 1b). The scanning micrograph (Fig. 1c) shows a proembryo which had increased 1.6 times in diameter with respect to the original pollen. The exine had stretched and the colpi had enlarged.

Pollen walls ruptured after 5 days of cultivation. At 6 days, proembryos contained approximately 40 cells forming a slightly elongated cell mass (Fig. 2). Cell divisions were mainly observed in the peripheral cell layer of the embryo (Fig. 2a). When pollen walls ruptured at earlier stages of development, large cells arose. These cells had a callus-like appearance and did not divide further (results not shown).

After 7 days of culture embryos became globular again (Fig. 3a). They were no longer surrounded by the pollen wall and had formed a protoderm like cell layer (see e.g. Fig. 7a). Globular embryos exhibited exponential growth both in liquid medium and after immobilization as shown by the daily CLSM observations of living embryos (Fig. 3). During the first 3 days after immobilization, i.e. from the 6th till the 9th day of culture, the diameter of the globular embryo increased 3 times (Fig. 3a-d) but the shape did not change. At the 4th day, i.e. at the 10th day of culture, the symmetry of the embryo changed from radial to bilateral. Within 24 h the width of the embryo increased approximately 1.3 times and its length increased approximately 1.8 times resulting in a heart-shaped embryo (Fig. 3e). The next day the embryo elongated and formed cotyledons (Fig. 3f). Microspore-derived embryos sometimes had a well developed suspensor consisting of a hypophysis and a file of cells (Fig. 9a, b). Such embryos developed when the first divisions within the young globular embryo were regular and parallel cell walls were formed.

After 2 weeks of cultivation in liquid medium, torpedo-shaped embryos had developed. They consisted of small cotyledons, an elongated hypocotyledon and a root tip (Fig. 4). At the end of the cultivation, i.e. after 3 weeks, embryos exhibited cotyledons which were often fused (Fig. 5). The shoot apex did not form primary leaves yet. Up to 5% of the cultured microspores and pollen (4 x 10^4 ml^{-1} at the onset of culture) gave rise to such embryos.

Expression of polarity in MS-embryos

Globular embryos do not yet exhibit differentiation in shape. Therefore they were analyzed for the expression of internal polarity by visualizing the distribution of activated calmodulin, free cytosolic calcium, and starch.

Calmodulin staining with fluphenazine revealed that activated calmodulin was equally distributed throughout the globular embryos (Fig. 6a). When embryos exhibited a morphological polarity, the calmodulin distribution changed. In heart-shaped embryos calmodulin was predominantly found in the root apex and in the tips of the developing

109
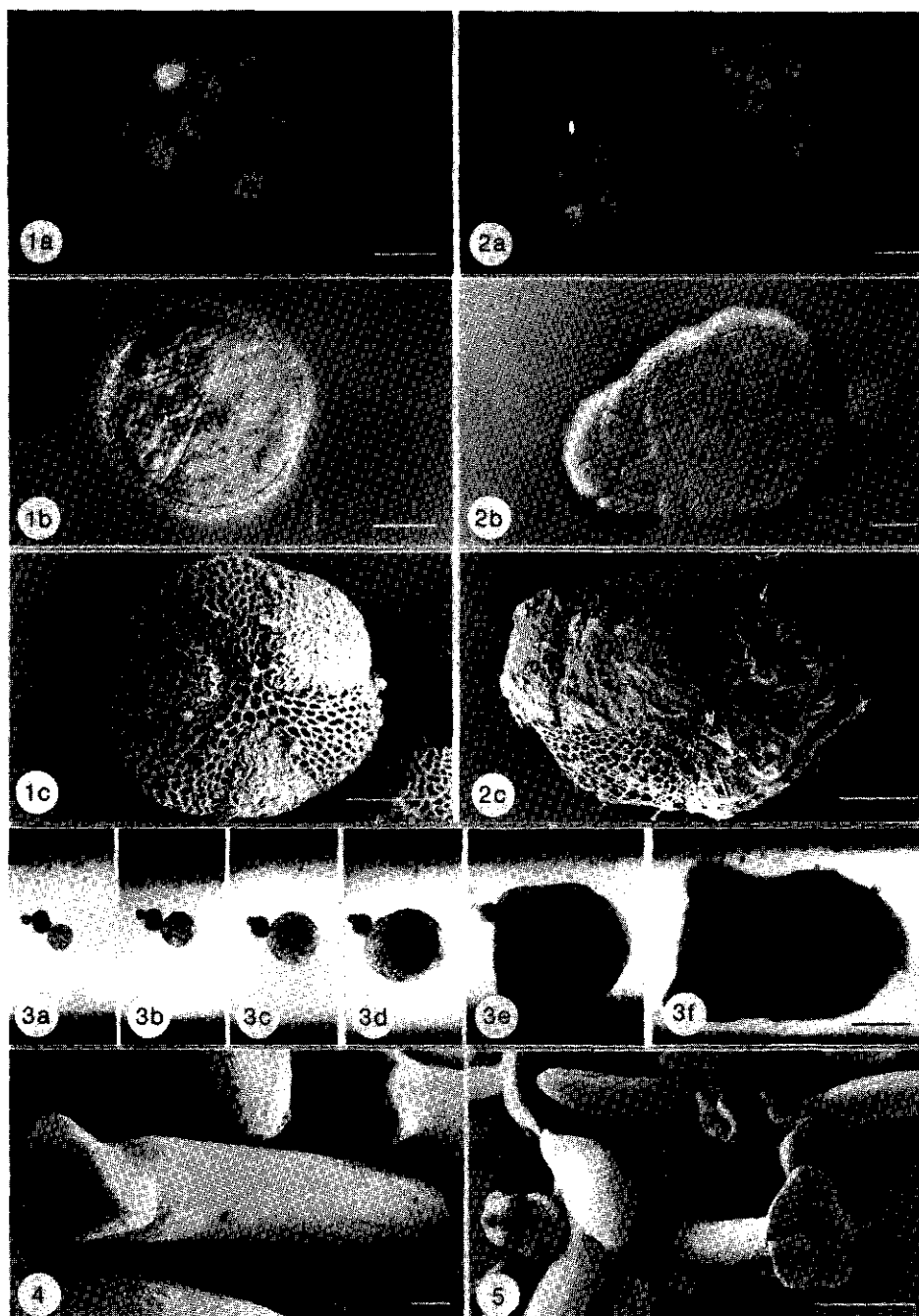
cotyledons (Fig. 6b). The distribution did not change in later developmental stages (results not shown).

The staining of free cytosolic Ca^{2+} in globular embryos with FLUO-3 showed that the calcium distribution in the embryo differed between the protoderm, and the sub-epidermal and central cells (Fig. 7a). There was, however, no polarity in the distribution. The staining in the epidermis persisted in the heart-shaped and young torpedo stages but the staining in the central area had disappeared. When older torpedo-shaped embryos were analyzed not only the epidermis was stained but the central cylinder exhibited prominent fluorescence as well (Fig. 7b). As a control, embryos were cut longitudinally and transversely and stained. The staining pattern did not change (data not shown).

The staining of starch with IPI revealed that starch granules were equally distributed in proembryos initially (Fig. 8a). From 6 days onward, however, the distribution changed. The bursting of the pollen wall preceded a change in the distribution of starch grains. Granules disappeared near the site where the pollen wall was broken and persisted at the opposite side (Fig. 8b). Globular embryos at 7 days of culture exhibited an accumulation of large starch grains at the side of the embryo that eventually formed the root apex (Fig. 8c). From 8 until 9 days of culture globular and heart-shaped embryos accumulated small starch grains in procambial strands, additionally (Fig. 8d, e). After 10 days of cultivation starch accumulation was found all over the embryo (Fig. 8f). When embryos had a suspensor, starch accumulated in the suspensor cells, and in cells of the embryo proper in the region adjacent to the hypophysis (Fig. 9c). When cell divisions in microspores had arrested in an early proembryonic stage, the accumulation of starch was found in huge amyloplasts in all the cells of such structure (Fig. 9d).

Morphogenesis of zygotic embryos

To compare MS-embryogenesis with zygotic embryogenesis morphologically, zygotic embryos of various developmental stages were dissected from the same plants as used for MS isolation, and analyzed by SEM (Fig. 10). The young globular stage (about 4 days after anthesis) showed an elongated suspensor, which was connected with the micropylar part of the ovule and consisted of at least 8 cells (Fig. 10a). The heart stage of the zygotic embryo (8 days after anthesis) exhibited the typical change from radial to bilateral symmetry (Fig.

Fig. 1 Proembryos after 4 days of cultivation. Fluorescence image of DAPI-staining (a) shows about equally sized nuclei. The differential interference contrast micrograph (DIC) (b) of the same proembryo as in (a) and the scanning electron micrograph of this stage (c) show the undisrupted pollen wall. Bars = 10 μm.

Fig. 2 Proembryos after 6 days of cultivation. Fluorescence image of DAPI-staining (a) focused on the peripheral cell layer, shows at least 30 nuclei, some in mitosis. The DIC-picture (b) of the same proembryo as in (a) and the scanning electron micrograph of this stage (c) show the broken pollen wall. Bars = 10 μm.

Fig. 3 Survey of growth of embryo immobilized after 6 days of cultivation in liquid medium. Pictures were taken daily by CLSM from 7 days of culture onward (a) till 12 days of culture (f). Bar = 100 μm for all micrographs.

Fig. 4 Scanning electron micrograph of young torpedo-shaped embryo cultured for 2 weeks in liquid medium. Bar = 100 μm.

Fig. 5 Scanning electron micrograph of torpedo-shaped embryo at the end of cultivation (3 weeks) in liquid medium. Bar = 1 mm.

10b), but contrary to the MS-embryos these embryos increased more in width than in length. The young torpedo-shaped embryo (Fig. 10c, 10-11 days after anthesis) and the maturing embryo (Fig. 10d, 11-12 days after anthesis) exhibited large and separate cotyledons.

Expression of polarity in zygotic embryos
Staining of dissected embryos with IPI revealed that globular-stage embryos of 3-4 days after anthesis only accumulated starch in the suspensor cells and mainly in the upper cells of the suspensor (Fig. 11a). When the globular embryo proper had enlarged, i.e. 5-6 days after anthesis, starch grains persisted in the suspensor and additional starch accumulated in embryo cells near the hypophysis, not at the opposite pole of the embryo proper (Fig. 11b). Heart-shaped embryos at 8-9 days after anthesis, still showed starch at the root pole and additional accumulation of starch grains in the procambial strands and other parts of the embryo proper (Fig. 11c, d).

DISCUSSION

Morphogenesis of MS-embryos
During the cultivation of MSs and pollen of *B. napus* under embryogenic conditions, proembryo formation starts either with the symmetrical division of the MSs, or with the division of the vegetative nuclei of bicellular pollen (see Hause et al., 1993). The microspore

Fig. 6 Fluorescence micrographs of the distribution of activated calmodulin in MS-embryos visualized by the fluorescence of fluphenazine. (a) The globular stage embryo shows an equal distribution of stained calmodulin. Bar = 10 μm. (b) Young heart-shaped embryo exhibits stained calmodulin in the region of cotyledons and root apex. Bar = 50 μm.
Fig. 7 CSLM images of the distribution of free cytosolic calcium in MS-embryos visualized by the fluorescence of FLUO-3. (a) The globular embryo shows an equal distribution of calcium within the inner cells, the protoderm exhibits a higher, but also equally distributed, fluorescence. Bar = 10 μm. (b) The mature torpedo-shaped embryo shows stained calcium within the epidermal cells and within the cells of the central cylinder. Bar = 100 μm.
Fig. 8 Survey of the distribution of starch during the development of MS-embryos visualized by staining with iodine/potassium iodide (IPI). (a) Proembryo after 5 days of cultivation. Note former pollen wall which is just broken at the top (arrows). (b) Proembryo after 6 days of cultivation (comparable to Fig. 2). The former pollen wall ruptured (arrows). Note the persistence of starch at the opposite site to that area. (c) Globular embryo after 7 days of cultivation. Big starch granules are localized at one side of this globular structure. (d) Late globular embryo after 8 days of cultivation. Starch granules are visible at the side of this embryo which is still in contact with the residues of the former pollen wall as well as in the procambial strands (arrows). (e) Young heart-shaped embryo after 9 days of cultivation. Starch is accumulated at the root apex and in the procambial strands. Note the different size of starch granules at the root apex (statoliths) and in the procambial strands. Bars for (a-e) = 10 μm. (f) Elongated heart-shaped embryo after 10 days of cultivation. The embryo accumulated starch in high amounts, only the region between the root apex and the hypocotyledon contains less starch. Bar = 50 μm.
Fig. 9 Suspensor-bearing embryos and incidentally observed structures from embryogenic microspore cultures after 9 days of cultivation. (a-c) Globular, MS-embryos with a suspensor-like structure: (a) DIC image and (b) fluorescence of the DAPI stained nuclei of the same embryo, (c) visualization of starch by staining with IPI. Note the accumulation of starch within the cells of the suspensor-like structure. (d) Staining of starch in an embryogenic structure, which stopped its development at an early proembryogenic stage. Bars = 10 μm.
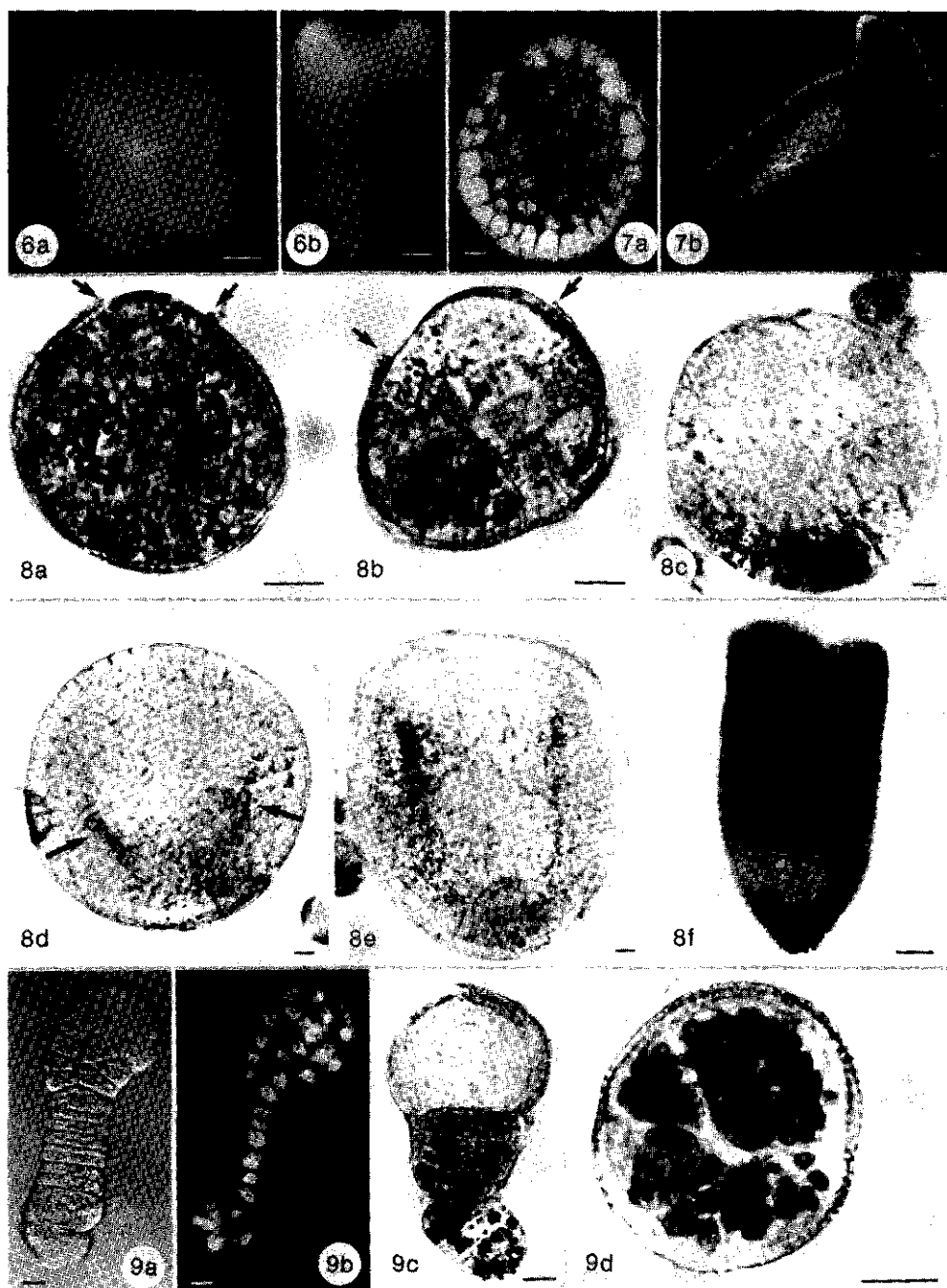
and pollen-derived pathways of embryo development are also described in the general scheme of embryo formation by Sunderland (1973) and Raghavan (1986). At the onset of culture, the generative cell sometimes divided once, too, but the shape of the resulting cells was different from that of the daughter cells of the vegetative cell (see also Fan et al., 1988). After four days, all cells of the proembryo of *B. napus* exhibited similar shapes. It is therefore concluded that only the daughter cells of the vegetative cell were involved in the further formation of the embryo, as was also described for pollen embryogenesis in other plants such as *Nicotiana* (Sunderland and Wicks, 1971), *Datura* (Sunderland and Dunwell, 1977), *Solanum* and *Luffa* (Sinha et al., 1978), and *Triticum* (Reynolds, 1993).

Based on the observation that the premature rupture of the pollen wall led to callus formation, it is concluded that it is essential for the formation of proembryos that the pollen wall remains intact during the first 5 days of cultivation. It is suggested that, by the limitation of volume, a mass of relatively small cells is produced. The stretching of the colpi and exine, however, enabled the proembryo to increase in volume about four times. Bursting of the pollen wall initially resulted in elongation of the embryo but it reformed its globular shape again. Thus the rupture itself does not cause elongation and the polarity is generated from within the globular embryo.

After the rupture of the pollen wall, the proembryo undergoes a zygote-like development by the formation of an embryo proper. This was demonstrated by the daily CLSM observations of living embryos as well as by scanning electron microscopy and comparison with zygotic embryos. Compared with the morphological development of zygotic embryos (Fig. 10, see also Tykarska 1976, 1979; Liu et al., 1993a), MS-embryos are, however, often characterized by the absence of a suspensor and by the occurrence of abnormalities in the shape of the cotyledons. These abnormalities were already described for *B. napus* by Thomas and Wenzel (1975). They could be caused by the more rapid plantlet development during culture (Swanson et al., 1987), although excised zygotic embryos grown *in vitro* showed faster development but the same shape of cotyledons as the *in planta* grown embryos (Liu et al., 1993a). Since it was found that different growth conditions of plants also influenced the shape of the developing embryos, it is more likely that the different culture conditions applied by the investigators have caused the different growth patterns. Although we observed that 60 % of the MS-embryos were haploid, 22 % were dihaploid or aneuploid and 18 % showed chimeric tissues (unpublished results), all MS-embryos exhibited small cotyledons. We therefore can exclude that there is a genetic background for the retarded growth of the cotyledons although changing ploidy levels influence further development of the plant shape (Lichter, 1982). The ratio of haploid and dihaploid embryos, was in the same range as described earlier for *B. napus* by other authors (Chuong et al., 1988b; Siebel and Pauls, 1989).
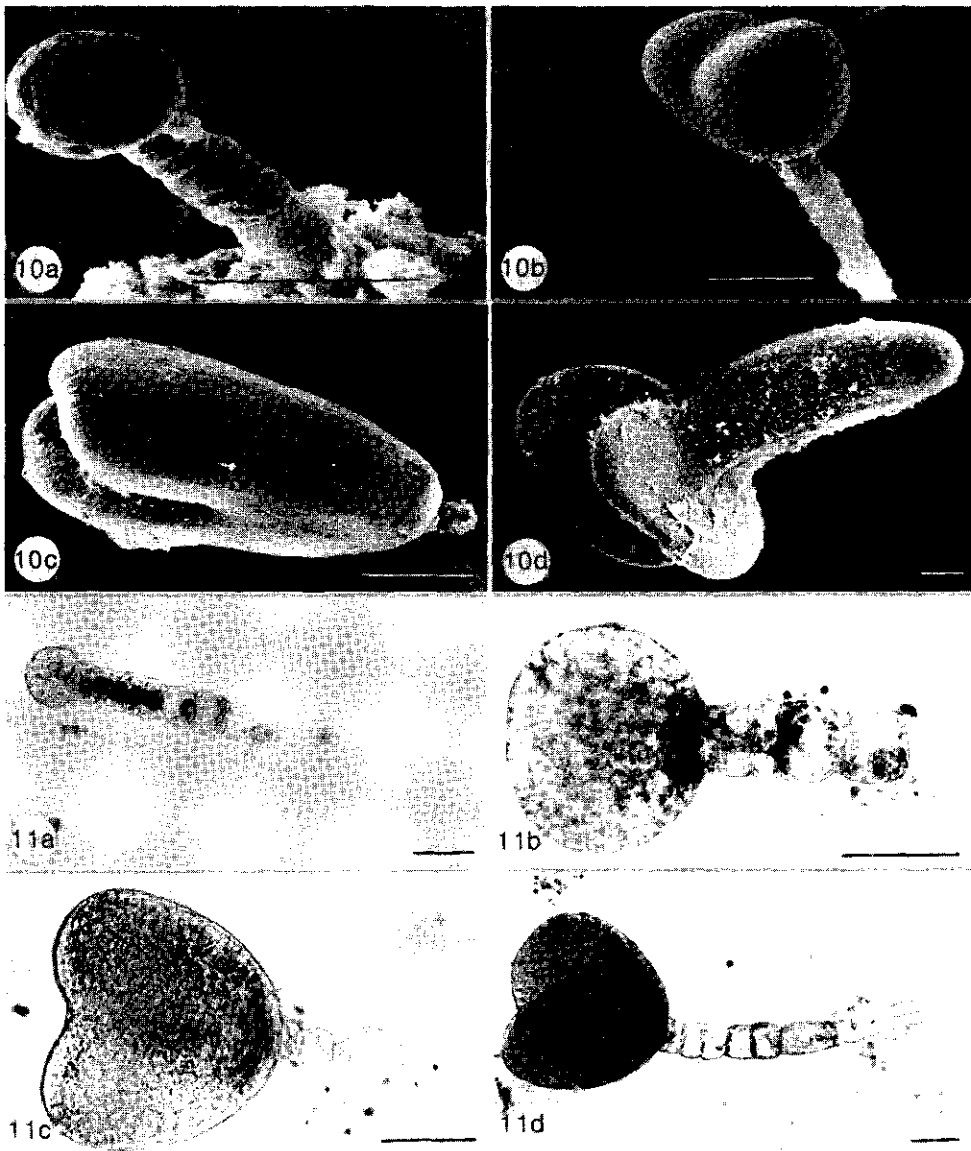
Fig. 10 Scanning electron micrographs of zygotic embryos. (a) Globular stage. (b) Heart-shaped stage. (c) Torpedo-shaped stage. (d) Nearly mature stage. Bars = 100 μm.

Fig. 11 Survey of the distribution of starch in zygotic embryos visualized by staining with IPI. (a) Young globular stage. Some starch granules are located within the suspensor. (b) Late globular stage. (c) Young heart shaped stage. (d) Heart shaped stage. (b-d) Starch is accumulated within the cells at the basal pole of the embryo proper. Bars = 50 μm.

Expression of polarity

The transition from the globular stage to the heart and torpedo-shape stages are expressions of changed polarities within the embryo proper. In zygotic embryos the first signs of polarity can already be observed in the egg cell and, later, in the unequal division of the zygote (see a.o. Van Lammeren, 1986). Establishment of polarity in zygotic embryos is reviewed by De Jong (1993). Hormones appear to play an important role during early zygotic embryogenesis in *B. juncea* (Liu et al., 1993b). Because the induction of polarity is related to cell division, cell differentiation and cell patterning, the role of calcium and the calcium binding calmodulin was analyzed in relation to cell growth and proliferation (Hepler, 1988), mitosis and cytokinesis (Hepler, 1989), organogenesis (Hush et al., 1991) and germination (Cocucci and Negrini, 1991). Based on the polar distribution of calcium and calmodulin found in carrot and maize embryos (Timmers and Schel, 1991; Timmers et al., 1989), calcium and calmodulin distributions were analyzed, but they did not express polarity in globular MS-embryos of rape. On the other hand the changed distribution of storage products, in this case starch, was an early sign of polarity expression, already before the globular embryo changed its shape. Based on the observation that MS-embryos, still encapsulated by the pollen wall, exhibited an equal distribution of starch grains in their cells, we suggest that the polarity is *induced* at this proembryo stage by the rupture of the pollen. The metabolic rate of the cells near the bursting place increased, witness the disappearance of starch at that site, whereas a retardation of metabolic activity at the opposite and future root pole resulted in the persistence of starch and even the enlargement of starch grains in that region. A comparable phenomenon was observed in the zygotic embryo proper. Here the accumulation of starch was observed near the hypophysis of the suspensor, an area without high metabolism where the root meristem including statoliths are formed (see also Tykarska, 1984).

In all, zygotic embryogenesis goes through two phases of expression of polarity. First at the zygote stage when unequal cell division occurs, and second at the globular embryo stage. MS-embryogenesis showed that suspensor formation is not a prerequisite for embryogenesis but the formation of a globular embryo is. The bursting of the pollen wall appeared the first sign of development of internal polarity within the globular embryo. Whether the bursting of the pollen wall is a random phenomenon or a strictly programmed and localized process remains to be elucidated.

ACKNOWLEDGEMENTS

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118

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Chapter 9

General Discussion

Bettina and Gerd Hause

GENERAL DISCUSSION

The cultivation of microspores and pollen represents a very efficient method for the production of haploid or dihaploid plants as a tool for plant breeding as well as for basic research. For several plant species quite efficient protocols were developed to produce a large number of embryos in culture. But the number of embryos depends upon the species and sometimes even on the cultivar. One model plant is *Brassica napus*. In this species a high temperature treatment for at least 8 hours is necessary to induce the formation of proembryo structures from isolated microspores or pollen (Pechan and Keller 1988). Some cultivars, such as the cultivar "Topas", form large numbers of embryos under these embryogenic culture conditions whereas other cultivars are low- or non-embryogenic. Because of significant differences in the rate of embryo induction between the cultivars it was of great interest to study the alterations in the microspores and pollen during the first 24 hours of cultivation. The aim of our investigations was to clarify processes in the induction of microspore embryogenesis from the cytological point of view. A general scheme of the pollen development and the pathways to induce embryogenic cultures is shown in Fig. 1.
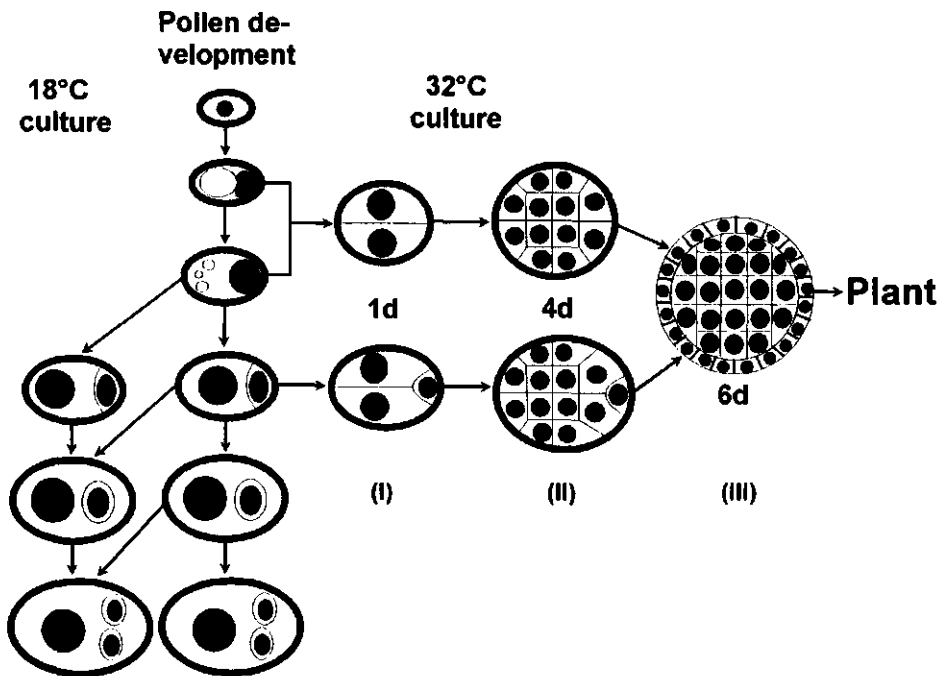

Fig. 1: Schematic representation of the variation of development of microspores and pollen of *Brassica napus* cultured under non-embryogenic (18°C) and embryogenic (32°C) culture conditions. Under embryogenic conditions three phases are distinguished: (I) induction of cell division, (II) multiplication of cells within the microspore wall, and (III) differentiation after rupture of the pollen wall once the embryogenic competence is established. d = days in culture

We claimed that the termination of microsporogenesis and following pollen development and the induction of embryogenesis should be accompanied by significant cytological changes. An important element which is a prerequisite essential for the cellular processes is the cytoskeleton. Investigations of the microtubular and microfilamental (F-actin) cytoskeleton during normal pollen development and in microspore cultures represented the start of our investigations.

In vivo F-actin configurations could be visualized by rhodamine-phalloidin only during microspore mitosis (phragmoplast) and in late bicellular pollen grains. The fact that we did not observe actin filaments during microspore development may be due to the methods which were used (Simmonds 1994). In embryogenic cultures the microfilamental pattern became more complex. After 6 hours in culture microfilaments could be detected in late microspores, in dividing microspores and in young bicellular pollen. The comparison of the actin pattern *in vivo* and *in vitro* (embryogenic and non-embryogenic culture conditions), as well as the results of experiments done with cytochalazin D, revealed that the alterations of the actin cytoskeleton were not related to the induction of microspore embryogenesis.

Immunocytochemical and electron microscopic investigations of the microtubular cytoskeleton showed prominent patterns during all stages of *in vivo* microspore and pollen development (**Chapter 2**). Cultivation of microspores and pollen under embryogenic conditions led to changes in the microtubular configurations (**Chapter 3**). These changes can be related to the induction of symmetrical divisions, a prerequisite to form proembryo structures. We identified three pathways for the formation of symmetrically divided structures (see also Fig. 2): (i) Migration of the peripherally located nucleus to the center of the microspore when the induced microspores were in the late vacuolated stage. The migration of the nucleus was caused by the disappearance or blocked formation of microtubules between the plasma membrane and the nucleus observed during normal pollen development. (ii) Isolation and cultivation of late microspores just before division led to a turn of the mitotic spindle of up to 90°. The nucleus stayed in the peripheral position, but the turn of the spindle caused the formation of symmetrically or nearly symmetrically divided structures. (iii) Very young bicellular pollen are the origin of the third pathway. The changes in the microtubular cytoskeleton of the vegetative cell led to an arrest of the generative cell at the pollen wall during further development. This arrest was always observed in pollen with a dividing vegetative nucleus, leading to the formation of proembryo-structures.

Microspore nuclei pass through the mitotic cell cycle both during pollen development as well as during embryo formation. The vegetative nucleus leaves the cell cycle and does not divide during pollen development but it does during embryo formation. The induction of division in the vegetative nucleus must be connected with changes in the cell cycle. For pollen of *Nicotiana tabacum* it has been described that the vegetative nucleus is arrested in G1 (Aruga *et al.* 1982, Zarsky *et al.* 1992) whereas De Pape *et al.* (1990) reported that the DNA content of the vegetative nucleus of *Nicotiana sylvestris* corresponds to the G2 phase of cell cycle. Thus the exact determination of the cell cycle in our system was very important. One possible way to analyse changes in the cell cycle is to investigate replicative DNA-synthesis. Our experiments with *B. napus* were performed using the immunocytochemical detection of bromodeoxyuridine which was given in pulses and incorporated into the DNA during replication (see **Chapter 4**). Just before microspore mitosis DNA-replication occurred in the

late microspore stage after the disappearance of the central vacuole, and in the generative cell replication occurs during the late bicellular stage of development. The vegetative nucleus was
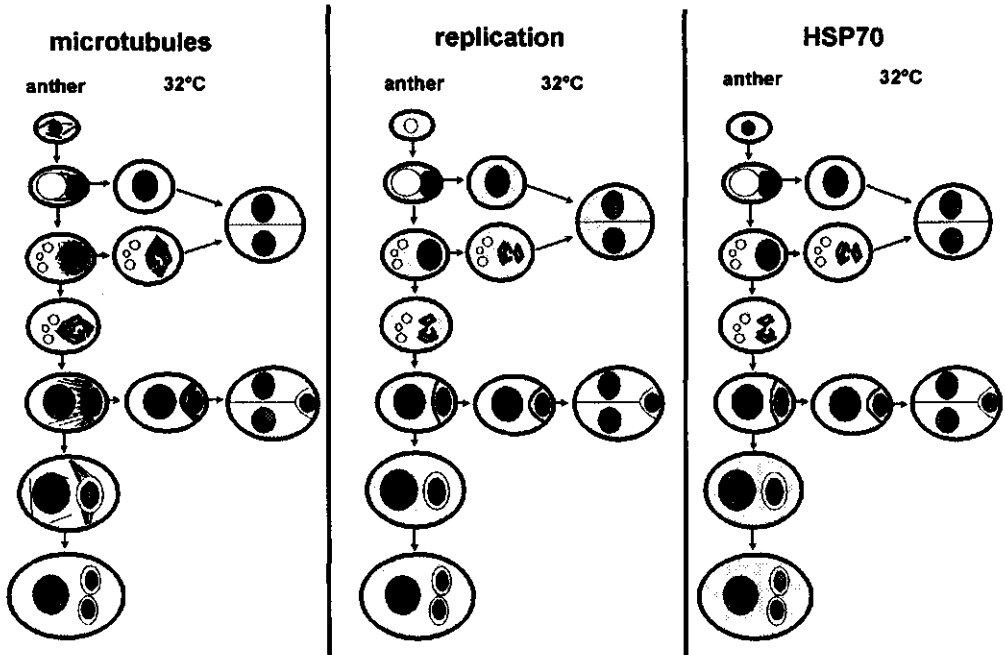

Fig. 2: Schematic representation of the onset of cell division in microspores and pollen of *Brassica napus*, *in planta* and after isolation and culture under embryogenic conditions. The first panel shows the changes in the microtubular cytoskeleton and the relation with the positions of nuclei and spindles. Nuclei are depicted in grey and microtubules as lines. The second panel shows DNA synthesis in nuclei as determined by the fluorescent labelling of incorporated bromodeoxyuridine. Black nuclei are labelled, grey nuclei are not labelled. Note the black nucleus of the vegetative cell of the two-cellular pollen grain indicating the re-entry into replication. The third panel shows the presence of HSP70 in microspores and pollen. Black nuclei are labelled with anti-HSP70, whereas the grey nuclei are not labelled. Note that HSP70 is located in the nuclei during or just before replication.

arrested in the G1- phase of the cell cycle and did not replicate DNA. In microspores cultured under embryogenic conditions, DNA synthesis occurred also in the late microspore stage. In early bicellular pollen embryogenic cultivation also caused replication in the vegetative nucleus. Thus the vegetative nucleus re-entered the cell cycle. On the other hand, the vegetative nuclei of mid- and late bicellular pollen remained in the G1 stage of the cell cycle even after cultivation under embryogenic conditions.

The cell cycle is accompanied by changes in the phosphorylation and dephosphorylation of certain proteins, substrates for specific kinases (for review see Pines 1993, Doerner 1994). We tried to find changes in the protein phosphorylation pattern using the antibody MPM-2 which is directed against a set of proteins phosphorylated before and during mitosis (Davies *et al.* 1983). Among the MPM-2 recognized phosphoproteins there are DNA topoisomerase IIα, activated mitogen-activated protein kinase p42 and cdc25 (Taagepera *et al.* 1993, 1994, Kuang et al 1994). Changes within the set of phosphorylated proteins recognized by MPM-2 can, however, not be distinguished. In *B. napus* MPM-2 labelling was detectable during all stages of microspore- and pollen development *in vivo* (see **Chapter 5**). Surprisingly, the vegetative nucleus of the pollen was also labelled, although it is known to be in G1. Moreover, alterations in the localization of the MPM-2 epitopes in the vegetative nuclei were not detectable after cultivation under embryogenic as well as non-embryogenic conditions. Taking into account that the MPM-2 antibody recognizes mitosis-specific events in plants (Traas *et al.* 1992, Binarova *et al.* 1993), little or no label within a nucleus arrested in G1 is to be expected. In the case of the vegetative nucleus of *B. napus* we could find no such correlation. The clear MPM-2 signal implicates that mitosis specific phosphorylations detected by this antibody occur, but they are probably not sufficient for cell cycle progression. Thus, in embryogenic cultures some additional stimulating or inhibiting regulators have to trigger the young vegetative nucleus to re-enter the cell cycle: (i) Cultivation under stress conditions could lead to the synthesis of one or more factors which are necessary for the G1-S transition. The synthesis of these factors may not occur during normal pollen development and during non-embryogenic cultivation. (ii) Stress might inactivate an unknown inhibitor or repressor which prevents the vegetative nucleus entering the S phase. For human cell cultures so-called CDEs (cell cycle dependent elements) have been described (Zwicker *et al.* 1995, Lubicello 1995) which can be regulated by a specific repressor. Repressor-binding to CDEs influences the expression of cell cycle-related genes such as cdc25, cdc2 and cyclin A. Zwicker *et al.* (1995) expected CDE mediated repression of activation by a specific set of transcriptional factors to be a common mechanism in the regulation of cell cycle-related genes. It is possible that in plants CDE-like sequences also exist and that they also play an important role during pollen development. In contrast to the young bicellular stage, in late bicellular pollen such a regulatory mechanism could be established with higher stability. There, it was not possible to disorganize the developmental program by the kind of stress used in the experiments: in late vegetative nuclei an inhibitor might be synthesized in sufficient amounts, might be very stable or could not be inactivated.

In *B. napus* heat shock treatment is the most efficient way to induce microspores and pollen to enter embryogenesis (Pechan and Keller 1988). This encouraged us to analyse the occurrence of heat shock proteins during cultivation at 32 °C in comparison to non-embryogenic cultivation at 18 °C (see **Chapter 6**). In Western blot analysis of proteins isolated from embryogenic and non-embryogenic cultures, only minor quantitative differences were visible. However, the immunocytochemical localization of one of the high molecular weight heat shock proteins, HSP70, exhibits significant changes following heat shock. HSP70, a member of the HSP 70 family is constitutively expressed in plant cells. The subcellular localization revealed that this protein was mainly located in the cytoplasm and in nuclei undergoing S-phase of the cell cycle, because nuclear localization was observed *in vivo* and

in vitro at 18°C only in the late microspore stage and in the generative cell during the late bicellular stage. When early bicellular pollen were cultivated at 32°C, however, HSP70 labelling was also regularly detected in the vegetative nuclei. The vegetative nucleus of late bicellular pollen, which can not be induced to divide, was not labelled. The HSP70 family is known as a class of so-called molecular chaperones (Georgopoulos and Welch 1993). HSP70 chaperones have been implicated in protein folding, the assembly and disassembly of oligomeric complexes, protein synthesis and degradation, and the translocation of polypeptides across cellular membranes (for review see Glick 1995). Moreover, a possible interaction of HSP70 with other cellular proteins in a cell cycle-dependent manner was reported by Milarski *et al.* (1989). Whether HSP70 also acts in plants as a carrier for cofactors of replication has not been clarified. On the one hand, results concerning activation of the glucocorticoid receptor hint at a carrier function of HSP70 during the activation of transcription (Hutchinson *et al.* 1994). On the other hand, HSP70 could act in a manner analogous to its bacterial homologue DnaK which influences replication (for review see Stillman 1994). Our results lead us to the conclusion that the nuclear location of HSP70 in microspores and pollen of *B. napus* is an unequivocal marker for replicative events.

Summarizing our results we have to distinguish between the induction of the embryogenic pathway in microspores and that in bicellular pollen. Isolated microspores are inducible by the disturbance of their microtubular cytoskeleton. The central position of the nucleus or the turn of the spindle apparatus leads to symmetrical divisions as a prerequisite of embryogenic development. The disturbance of microtubules can be caused by high temperature treatment, but also by chemicals such as colchicine (Iqbal *et al.* 1994, Zaki and Dickinson 1995, Zhao *et al.* 1996). Against this type of induction, the vegetative cell of bicellular pollen has to re-enter the cell cycle. Therefore, a stress seems to be necessary as a trigger for the progress through the cell cycle. Normally the cell cycle is blocked in the vegetative cell, but this block cannot remove the competence for cell division. With respect to the pteridophytes, the spermatophytes have a strongly specialized male gametophyte which is a tricellular unit consisting of two sperm cells and the vegetative cell. In comparison, the gametophyte (prothallium) of the pteridophytes (a preceding division of the plant kingdom) is a free, multicellular, autotrophic organism. This situation remains also in the microprothallia of the heterosporic fern producing only male gametes. In seed plants the development of the gametophyte takes place in the plant and the prothallium becomes heterotrophic. This makes it possible that during the phylogenesis of spermatophytes, the specialization of the male gametophyte of the angiosperms had to be connected with a reduction in the number of cell divisions. The overcoming of the blocked cell cycle during the induction phase of the embryogenic pollen cultures leads in its consequence to the formation of a haploid, multicellular structure similar to a prothallium. So the induction process could be analogous and comparable to a "re-induced" way of the vegetative reproduction of the gametophyte.

Our results concerning embryogenesis in microspore and pollen cultures implicate the occurrence of two fundamental processes: (i) stress-induced de-differentiation and (ii) the induction of embryo development in proembryo structures (see Fig. 3).

Firstly, the development to a male gametophyte has to be stopped in the late microspore, or in the early bicellular pollen stage, and symmetrical divisions must be initiated. Those

symmetrical divisions are the prerequisite for the development of a proembryo. The proembryo develops within the pollen wall by subsequent divisions. Up to the fifth day after induction no signs of polar embryonic development could be detected (**Chapter 8**). During this time a requirement for embryogenic development is the intactness of the pollen wall. If the pollen wall is ruptured too early, the cells grow without limitation in space resulting in a kind of callus. The expansion prevents that the embryo-specific ratio of the volume of the cytoplasm to the volume of the nucleus will be reached. If the pollen wall is ruptured on day 5 or 6 of culture, the cells of the proembryo did reach that ratio, the multicellular structure is conditioned to embryogenesis and can develop in a comparable manner to zygotic embryos. Thus, during the embryogenesis of microspores and pollen the second important step is the switch to embyogenesis at day 4 or 5 of cultivation, i.e. the induction of differentiation in the cytologically non-differentiated proembryogenic mass. Accordingly, it is very important to distinguish between the process of induction of symmetrical divisions, which in the literature is quite often referred to as the induction of embryogenesis, and the induction of embryo-specific differentiation after some days of cultivation.
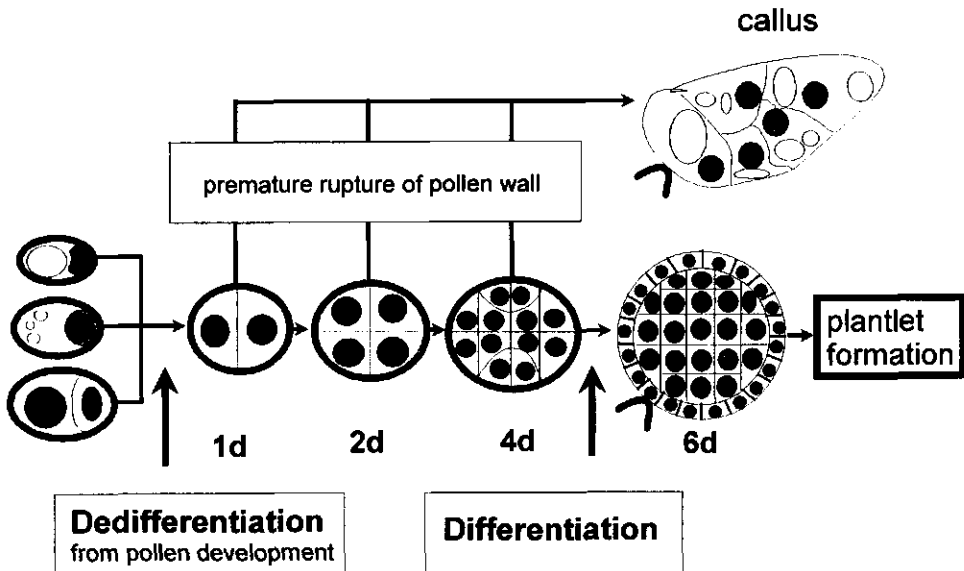

Fig. 3: Scheme of differentiation and dedifferentiation processes in embryogenic microspore and pollen cultures of *B. napus*. The initiation of symmetrical divisions in late microspores or young bicellular pollen represent a dedifferentiation from pollen development. Real embryogenic competence is reached after the 4th day of culture when embryo specific differentiation is possible without the growth limiting pollen wall. When the pollen wall ruptured premature (before day 4 of culture), a callus like structure is formed.

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128

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131

SAMENVATTING

De productie van kunstmatige plantenembryo's is een belangrijk instrument voor fundamenteel onderzoek en voor de plantenteelt. Het is mogelijk grote hoeveelheden embryo's te maken door methoden zoals somatische embryogenese en embryogene microsporecultures. Grote hoeveelheden embryo's zijn enerzijds een noodzaak voor het kunnen doen van biochemisch en moleculair genetisch onderzoek, maar anderzijds ook onontbeerlijk voor biotechnologische toepassingen. Bovendien zijn dergelijke embryo's makkelijker hanteerbaar dan zygotische embryo's en verschaffen embryo's die van microsporen en pollen afkomstig zijn, een geschikt uitgangsmateriaal voor de productie van hybriden: Vanwege hun haploïde oorsprong zijn de embryo's die van microsporen zijn afgeleid na diploïdisatie dihaploïd en dus homozygoot.

Embryogenese wordt bij microsporen en pollen van koolzaad (*Brassica napus*) geïnduceerd door ze onder "heat shock" condities in kweek te brengen, d.w.z. bij een verhoogde temperatuur (32°C) gedurende ten minste 24 h. Kweek van geïsoleerde microsporen en pollen bij 18°C leidt tot normale pollenontwikkeling. Dit proefschrift geeft de resultaten van ons onderzoek naar de inductiefase van de embryogenese. Cellulaire veranderingen die gedurende de eerste 24 h van de cultuur van microsporen en pollen optraden, werden geanalyseerd om een beeld te krijgen van de cytologische verschillen tijdens de inductiefase van de embryogenese en tijdens het normale (niet-embryogene) patroon van pollenontwikkeling *in planta*. Door licht- en elektronenmicroscopische technieken te combineren voor het onderzoek van het cytoskelet (microtubuli en microfilamenten) is nu aangetoond dat de ontwikkeling van microsporen en pollen tot een normale driecellige microgametofyt in *B. napus* vergelijkbaar is met die in andere bedektzadigen. Alleen het verdwijnen van de centrale vacuole vóór de deling van de microspore is afwijkend van het normale verloop (Hoofdstuk 2). Stadium-specifieke ontwikkelingspatronen van microtubuli en microfilamenten werden gevonden in de microsporen en pollen.

Symmetrische delingen zijn een voorwaarde voor de embryogene ontwikkeling van in cultuur gebrachte microsporen en pollen. Daarbij werden drie wegen gevonden waarlangs dergelijke delingen konden worden geïnduceerd (Hoofdstuk 3). (i) De kweek onder embryogene condities veroorzaakte in gevacuoliseerde microsporen een verschuiving van de kern naar het midden van de cel, waar dan de deling plaats vond. Dit verschijnsel wordt begeleid door afbraak of veranderde vorming van microtubuli. (ii) In "late" microsporen veroorzaakte kweek onder embryogene condities een draaiing van de mitotische spoel tot 90°. Beide verschijnselen (i en ii) leiden tot symmetrische deling en een bicellulair pro-embryo. (iii) De omschakeling van de ontwikkeling van jong bicellulair pollen naar de vorming van pro-embryo's werd veroorzaakt door een verstoorde pollenontwikkeling, m.n. het vast blijven zitten van de generatieve cel aan de wand van de pollenkorrel, gevolgd door deling van de vegetatieve cel. In dit geval zijn de microtubuli die normaal te vinden zijn in de vegetatieve cel en wel loodrecht op de generatieve cel, verdwenen. Samengevat kunnen alle drie de wegen leiden tot de vorming van pro-embryo's en het microtubulaire cytoskelet lijkt te zijn betrokken bij deze veranderingen in ontwikkeling. Hoewel er ook veranderingen zijn gezien in de organisatie van het microfilamentaire skelet onder embryogene condities kon de rol van de microfilamenten tijdens de inductie van embryogenese niet worden vastgesteld.

Omdat onder embryogene condities veranderingen in de celcyclus in het oog sprongen, is de

DNA synthese *in vivo* en *in vitro* onderzocht (Hoofdstuk 4). Inbouw en detectie van bromodeoxyuridine en het bepalen van het ploïdie-niveau van de kernen met behulp van microspectrofotometrie zijn daarbij als methoden toegepast. Replicatie van DNA werd *in vivo* aangetoond in de kernen van "late" microsporen en in de kern van de generatieve cel van late twee-cellige pollenkorrels. In de normale *in vivo* ontwikkeling blijft de vegetatieve kern in de G1-fase. Onder embryogene condities bleef het replicatiepatroon in microsporen hetzelfde maar de vegetatieve kern van de jonge twee-cellige pollenkorrel kwam opnieuw in de celcyclus en vertoonde DNA synthese.

Veranderingen in het patroon van fosforylering werden geanalyseerd door gebruik te maken van het monoclonale antilichaam MPM-2 (Hoofdstuk 5). Het antilichaam MPM-2 is opgewekt tegen mitose-eiwitten in HeLa-cellen en herkent gefosforyleerde, mitose specifieke eiwitten in dierlijke en plantaardige cellen. In ontwikkelende microsporen en pollen van *B. napus* bond MPM-2 aan eiwitten in alle ontwikkelingsstadia en speciaal aan de eiwitten in de kern. Bovendien waren er geen verschillen in gefosforyleerde epitopen tussen microsporen en pollenkorrels die gekweekt waren onder embryogene en niet-embryogene cultuur-omstandigheden. Dit zou veroorzaakt kunnen zijn doordat dit antilichaam gefosforyleerde epitopen in meerdere eiwitten herkent.

Omdat een warmtebehandeling gebruikt werd om embryogenese te induceren is ook de subcellulaire distributie van "heat shock proteins" (HSPs) nagegaan (Hoofdstuk 6). De analyse van Western blots verkregen na 2-D gel elektroforese gaf een sterk signaal op 70 kDa. Immunocytochemisch onderzoek met een antilichaam dat opgewekt was tegen HSP70 liet een duidelijke stadium-specifieke subcellulaire verdeling van HSP70 zien, zowel *in vivo* als *in vitro*. Kweek onder embryogene omstandigheden veroorzaakte een veranderde lokalisatie van HSP70: het werd nu detecteerbaar in de kern van de vegetatieve cel. De plaats van HSP70 kon dan ook gecorreleerd worden met de initiatie van de DNA-replicatie. Mogelijke relaties tussen HSP70 en de DNA-replicatie worden bediscussieerd.

Hoofdstuk 7 beschrijft de localisering van specifieke messenger RNAs in ontwikkelende microsporen en pollenkorrels van *B. napus* en *Arabidopsis thaliana*. Specifieke genexpressie werd aangetoond in de generatieve cel van beide soorten door gebruik te maken van vriescoupes en *in situ*-hybridisatie met een gedigoxygeneerde probe.

Tenslotte is de expressie van polariteit vergeleken in ontwikkelende zygotische embryo's en embryo's ontstaan uit microsporen en pollen (Hoofdstuk 8). Onderzoek met scanning electronenmicroscopische technieken liet zien dat de embryo-ontwikkeling vanaf het globulaire stadium hetzelfde is. De verdeling van calcium-ionen, calmoduline en zetmeel werd geanalyseerd om een vroeg signaal van polariteitsontwikkeling te herkennen. De ophoping van zetmeel en de plaats van de overgebleven pollenwand bleken de enige tekens die in een vroeg stadium aangaven hoe de radiale as van het zich ontwikkelende embryo zou komen te liggen.

In hoofdstuk 9 werd de embryogenese beschouwd als een proces met twee fasen. De inductie-fase van de embryogenese bestaat uit de dedifferentiatie van een zich ontwikkelend organisme. Deze fase wordt gevolgd door de differentiatie tot een echte plant. Onze resultaten zijn gecombineerd met resultaten van andere groepen om tot een algemeen schema te komen van de inductie van embryogenese in microsporen en pollen van *B. napus*.

133

SUMMARY

Artificial systems to produce plant embryos are important tools for basic research as well as for plant breeding. It is possible to produce large amounts of embryos by methods like somatic embryogenesis or embryogenic microspore cultures. Such high amounts of embryos, which are easier to handle than zygotic embryos, are the prerequisite for biochemical and molecular genetic investigations on the one hand, and for biotechnological use on the other hand. Moreover, embryos derived from microspores or pollen represent a very efficient basis for the production of plant hybrids: Because of their haploid origin, microspore-derived embryos are after diploidization dihaploid, and thus, homozygous.

The induction of embryogenesis in microspores and pollen of *Brassica napus* is realized by their cultivation under heat shock conditions (32 °C for at least 24 h). This dissertation presents results from our investigations on the induction phase of embryogenesis. Cellular changes during the first 24 h of cultivation of microspores and pollen were analysed to discern cytological differences between this induction phase of embryogenesis and the normal pattern of pollen development *in planta*. Combining light as well as electron-microscopy for the analysis of the cytoskeleton (microtubules and microfilaments), it was shown that the development of microspores and pollen in *B. napus* to a mature, tricellular microgametophyte is comparable to other angiosperms. Only the disappearance of the central vacuole before microspore mitosis is different from the common pathway (Chapter 2). Stage specific developmental patterns of microtubules and microfilaments could be detected in the microspores and pollen.

Symmetrical divisions are a prerequisite for the embryogenic development of cultivated microspores and pollen, and three pathways for the induction of such divisions were identified (Chapter 3). (i) In vacuolated microspores cultivation under embryogenic conditions causes a migration of the nucleus to the centre of the cell where the division takes place. This phenomenon is accompanied by the disruption or altered formation of the microtubules. (ii) In late microspores the embryogenic conditions cause a turn of the mitotic spindle up to 90°. Both events (i and ii) result in symmetrically divided cells forming a bicellular proembryo. (iii) The developmental switch from young, bicellular pollen to the formation of proembryos is caused by a disrupted pollen development (arrest of the generative cell at the pollen wall) followed by division of the vegetative cell. In this case, microtubules, normally detectable in the vegetative cell perpendicular to the generative cell, are disrupted. In summary, all three pathways can lead to the formation of proembryos, and the microtubular cytoskeleton seems to be involved in these developmental changes. Although changes were also visible concerning the microfilaments under embryogenic conditions, their role in the induction of embryogenesis could not be confirmed.

Because of the obvious changes in the cell cycle of microspores and pollen cultivated under embryogenic conditions, the synthesis of DNA was investigated *in vivo* and *in vitro* (Chapter 4). The incorporation and detection of bromodeoxyuridine as well as the determination of the ploidy level of the nuclei by microspectrophotometry were used for these investigations. DNA replication could be shown *in vivo* within the nucleus of the late microspore and also within the generative nucleus of the late bicellular pollen. In normal development the vegetative nucleus remains in the G1-phase. Under embryogenic conditions, the pattern of replication

in microspores remained the same, but the vegetative nucleus of the young bicellular pollen re-entered the cell cycle and exhibited DNA synthesis.

Changes in phosphorylation patterns were analysed using the monoclonal antibody MPM-2 (Chapter 5). The antibody MPM-2, raised against mitotic proteins of HeLa-cells, recognizes phosphorylated, mitosis-specific proteins in animal and plant cells. In developing microspores and pollen of *B. napus*, MPM-2 bound to proteins of all developmental stages, especially to proteins in the nuclei. Moreover, there were no differences in phosphorylated epitopes between microspores and pollen cultivated under embryogenic and non-embryogenic conditions. This might be caused by the fact that this antibody recognizes phosphorylated epitopes of various proteins.

Because of the heat shock conditions used for the induction of embryogenesis, the subcellular localisation of heat shock proteins (HSPs) was performed (Chapter 6). Western blot analysis of proteins separated by two-dimensional gel electrophoresis revealed a strong signal at 70 kDa. Immunocytochemical investigations using an antibody raised against HSP70 showed a distinct stage-specific subcellular localization of HSP70 *in vivo* as well as *in vitro*. The embryogenic cultivation caused an altered localisation of HSP70, which became detectable within the nucleus of the vegetative cell. Its localization could therefore be correlated with the initiation of DNA replication. Possible relations between HSP70 and replication were discussed.

Chapter 7 describes the localisation of a specific mRNA within developing microspores and pollen of *B. napus* and *Arabidopsis thaliana*. Using freeze sectioned material and *in situ*-hybridization with a digoxygenin labelled probe, specific gene expression was demonstrated for the generative cell of both species.

Finally, the expression of polarity during the development of microspore-derived and zygotic embryos was compared (Chapter 8). Investigations by scanning electron microscopic techniques showed that the embryo formation between them are similar from the globular stage onwards. The distribution of calcium ions, calmodulin and starch was used to find early signs of polarity. However, the accumulation of starch and the position of a residual pollen wall were the only hints for a predisposition of the radial axis of the developing embryo.

In chapter 9 the embryogenesis in isolated microspores and pollen is considered as a biphasic process. The induction phase of embryogenesis represents the dedifferentiation of a developing organism followed by differentiation to a real plant embryo. Our results are combined with results of other groups to create a general scheme on induction of embryogenesis in microspores and pollen of *B. napus*.

ZUSAMMENFASSUNG

Artifizielle Systeme zur Erzeugung pflanzlicher Embryonen stellen sowohl für die Grundlagenforschung als auch für die züchterische Praxis ein sehr wichtiges Hilfsmittel dar. Durch Methoden wie somatische Embryogenese oder embryogene Mikrosporenkultur ist man in der Lage, eine sehr große Zahl von Embryonen zu schaffen, die leicht zugänglich sind. Dies ist gegenüber zygotischen Embryonen ein sehr großer Vorteil, da einerseits biochemische und molekularbiologische Untersuchungen ermöglicht bzw. erleichtert werden, und andererseits wichtige biotechnologische Anwendungsmöglichkeiten bestehen. Außerdem weist ein solches System wie die embryogene Mikrosporenkultur eine weitere, pflanzenzüchterisch sehr relevante Eigenschaft auf. Da das Ausgangsmaterial für diese Kulturen aus haploiden Einzelzellen besteht, können spontan oder durch entsprechende Behandlung dihaploide, also homozygote Embryonen gebildet werden. Die hieraus entstehenden homozygoten Pflanzen sind für die Erzeugung von Hybriden hervorragend geeignet.

In der vorliegenden Dissertation werden die Ergebnisse der Arbeiten zur Induktion der Embryogenese in Mikrosporen und Pollen vom Raps (*Brassica napus* L.) dargelegt. Die Arbeiten konzentrierten sich vor allem auf zellbiologische Veränderungen während der Induktionsphase der embryogenen Entwicklung, d. h. während der ersten 24 Stunden der Kultivierung unter embryogenen Bedingungen (32 °C in NLN-Medium). Zytologische Untersuchungen der normalen Mikrosporen- und Pollenentwicklung (*in vivo*) stellten dabei eine wichtige Grundlage für die sich anschließenden Untersuchungen des Induktionsprozesses dar. Dazu wurden die licht- und elektronenmikroskopischen Arbeiten mit der Untersuchung des Zytoskeletts (F-Aktin und Mikrotubuli; Kapitel 2 und 3) kombiniert. In *B. napus* verläuft die Entwicklung der Mikrosporen und Pollen zum reifen, dreizelligen Mikrogametophyten ähnlich wie bei anderen Angiospermen, wobei der Abbau der Zentralvakuole kurz vor der Mikrosporenmitose eine Abweichung darstellt (Kapitel 2). Entsprechend der Entwicklungsstadien wurden *in vivo* spezifische Muster der Mikrotubuli und des F-Aktins gefunden. In embryogenen Kulturen konnten drei Möglichkeiten der Induktion von symmetrischen Teilungen, einer essentiellen Voraussetzung für die Bildung von Embryonen, nachgewiesen werden (Kapitel 3). Die erste Möglichkeit ist die Induktion vakuolisierter Mikrosporen. Werden diese Mikrosporen unter embryogenen Bedingungen kultiviert, wandert der Kern nach dem Abbau der Vakuole und vor der Mitose ins Zentrum der Zelle. Dies führt zu einer symmetrischen Zellteilung. Der Kern kann möglicherweise deshalb ins Innere der Zelle wandern, da Mikrotubuli, die *in vivo* den wandständigen Kern mit der Plasmamembran verbinden, in diesem Stadium nicht synthetisiert werden bzw. abgebaut werden. Der zweite Weg zur symmetrischen Zellteilung beginnt im späten Mikrosporenstadium, kurz vor der Mitose. In diesem Fall führt die Kultivierung unter embryogenen Bedingungen zu einer Drehung der Mitosespindel um bis zu 90°. Die damit verbundene Änderung der Teilungsrichtung führt zum Abbruch der normalen Pollenentwicklung und zur Bildung zweizelliger Proembryonen. Die dritte Möglichkeit zur Induktion der Embryogenese besteht in der Änderung der Entwicklung junger, zweizelliger Pollen. Hier bewirkt die Kultivierung bei hohen Temperaturen eine Unterbrechung der normalen Pollenentwicklung (Arretierung der generativen Zelle an der Wand) sowie die Teilung der vegetativen Zelle. Die

136

Untersuchung des mikrotubulären Zytoskeletts zeigte, daß dies einhergeht mit dem Abbau von Mikrotubuli, die von der Wand der generativen Zelle ins Innere der vegetativen Zelle hineinragen. Hinsichtlich des Aktin-Zytoskeletts wurden zwar Veränderungen unter embryogenen Kulturbedingungen im Vergleich zur *in vivo*-Situation gefunden, Beziehungen zur Induktion embryogener Teilungen konnten allerdings nicht nachgewiesen werden.

Die Kultivierung von Mikrosporen und Pollen unter embryogenen Bedingungen ruft offensichtlich Veränderungen im Zellzyklus hervor. Aus diesem Grund wurde die DNA-Synthese *in vivo* und *in vitro* untersucht (Kapitel 4). Hierzu wurde der Einbau von Bromdesoxyuridin in bestimmten Zeitintervallen sowie der Ploidiegrad der Kerne mittels Mikrospektrophotometrie bestimmt. Die Ergebnisse zeigten, daß *in vivo* die Replikation im späten Mikrosporenstadium sowie im generativen Kern des späten zweizelligen Pollenstadiums abläuft. Der vegetative Kern bleibt in der G1-Phase, es erfolgt keine DNA-Synthese. Unter embryogenen Kulturbedingungen wurde in den Mikrosporen das gleiche Replikationsmuster wie *in vivo* gefunden, während in zweizelligen Pollen Unterschiede auftraten. Hier tritt der vegetative Kern kurz nach der Zellteilung wieder in den Zellzyklus ein und synthetisiert DNA.

Die möglichen Veränderungen des Phosphorylierungsmusters in embryogenen Kulturen im Vergleich zu nicht-embryogenen Kulturen bzw. zur *in-vivo*-Situation wurden mit dem monoklonalen Antikörper MPM-2 untersucht (Kapitel 5). Dieser Antikörper reagiert in tierischen und auch in pflanzlichen Zellen spezifisch mit phosphorylierten Epitopen, die nur während der Mitose auftreten. In *B napus* erkennt der Antikörper MPM-2 jedoch während der gesamten Mikrosporen- und Pollenentwicklung spezifische Proteine, vor allem in den Kernen. Es wurden keine signifikanten Unterschiede zwischen den Kultivierungsvarianten gefunden, was möglicherweise mit der Vielzahl der Epitope zu erklären ist, die den Antikörper binden.

Da für die Induktion der Embryogenese in Mikrosporen und Pollen ein Hitzestress notwendig ist, wurden Untersuchungen zur subzellulären Lokalisierung von Hitzestressproteinen (HSPs) durchgeführt (Kapitel 6). Im Western-blot nach 2D-Gelelektrophorese wurde ein besonders starkes Signal bei Proteinen der 70 KD-Klasse detektiert. Immunzytochemische Untersuchungen mit einem Antikörper gegen ein HSP70 Protein zeigten *in vivo* und *in vitro* eine deutlich stadienabhängige Lokalisierung. Durch die veränderte Lokalisierung des HSP70 in der vegetativen Zelle unter embryogenen Bedingungen konnte die Lokalisierung von HSP70 im Kern mit der Initiation der Replikation korreliert werden. Mögliche Zusammenhänge werden diskutiert.

Im Kapitel 7 wird die Lokalisierung einer DNA-Sonde in sich entwickelnden Mikrosporen und Pollen von *B. napus* und *Arabidopsis thaliana* dargestellt. Mit der beschriebenen Methode der *in situ*-Hybridisierung mit Digoxygenin-markierten Sonden an Gefrierschnitten ist in beiden Spezies erstmals eine für generative Zellen spezifische Genexpression gezeigt worden. Den Abschluß der Arbeit bildet ein Abschnitt über die Analyse der Ausprägung der Polarisierung in Mikrosporenembryonen im Vergleich zur zygotischen Embryoentwicklung (Kapitel 8). Rasterelektronenmikroskopische Untersuchungen zeigten, daß in beiden Fällen die Embryoentwicklung ab dem globulären Stadium vergleichbar ist. Weiterhin wurden Calzium- und Calmodulin-Verteilung sowie die Stärkeakkumulation untersucht. Im Ergebnis liefern nur Stärkeverteilung und Lage der Pollenwandreste erste Hinweise auf die Anlage der

Längsachse des Embryos.

In Kapitel 9 wird noch einmal verdeutlicht, daß die Induktion der Embyogenese in Mikrosporen und Pollen ein Prozeß ist, der in zwei Phasen abläuft. Es ist einmal die stressinduzierte Dedifferenzierung eines sich entwickelnden Organismus, der nach einer Wachstumsphase embryospezifische Differenzierungsprozesse folgen. Unsere Ergebnisse werden in Zusammenhang mit den Resultaten anderer Gruppen diskutiert und ein allgemeines Schema der Induktion der Mikrosporenembryogenese in *B. napus* wird vorgestellt.

SAMENVATTING VAN HET WERK GEDAAN DOOR GERD HAUSE

Het proefschrift beschrijft onderzoek naar de inductie van microspore- en pollenembryogenese in koolzaad (*Brassica napus* L.). De voordelen van dit experimentele systeem zijn beschreven in de algemene samenvatting. Wij waren geïnteresseerd in de cytologische veranderingen die optraden in geïsoleerde microsporen en pollen die onder embryogene condities gekweekt werden (cultuur bij 32 °C). De veranderingen die optraden gedurende de eerste 24 h van de kweek werden vergeleken met cellulaire gebeurtenissen in niet-embryogene cultures (gekweekt bij 18 °C) en *in vivo*. De veronderstelling was dat de inductie van embryogenese gepaard zou gaan met belangrijke veranderingen op het cellulaire vlak aangezien de inductie een zeer ingrijpende verandering in het ontwikkelingspatroon veroorzaakt.

Het microtubulaire cytoskelet is een celstructuur die betrokken is bij diverse celfuncties zoals deling en morfogenese. Daarom is het onderzoek gestart met een gedetailleerde analyse van het microtubulaire celskelet gedurende de *in vivo* ontwikkeling van microsporen en pollen (een gedeelte van hoofdstuk 2) waarbij gebruik gemaakt werd van elektronemicroscopische en immunocytochemische technieken. Een stadiumspecifiek microtubulair patroon werd gevonden gedurende de vorming van de mannelijke gametofyt. Ultrastructureel onderzoek maakte duidelijk dat de kern van de microspore vóór de mitose met microtubuli vast zat aan het plasmamembraan. Waarschijnlijk zijn die microtubuli van groot belang om de kern in haar excentrische positie te houden zodra de centrale vacuole vlak voor de mitose verdwijnt. Preprofasebanden werden niet gevormd, noch voor de mitose van de microspore, noch voor de mitose van het pollen, maar mitotische spoelen en fragmoplastmicrotubuli werden wel gevonden en wel bij al de celdelingen die uiteindelijk leidden tot de vorming van het driecellig pollen.

Een deel van hoofdstuk 3 beschrijft de veranderingen van het microtubulaire cytoskelet die optreden tijdens de inductie van microspore- en pollenembryogenese. Deze worden vergeleken met de veranderingen die optreden gedurende de *in vivo* ontwikkeling. Na twee tot vier uur kweek werden vaak spoelvormige microtubulaire configuraties gevonden in microsporen die in profase verkeerden. De spoelen bleken echter op een andere plaats in de cel te liggen dan de kernen. Dit verschijnsel is waarschijnlijk een verstoring veroorzaakt door de isolatieprocedure. Celdelingen die tot embryogenese leidden, bleken op drie manieren tot stand te kunnen komen. De eerste mogelijkheid wordt veroorzaakt door het uiteenvallen of de verstoorde vorming van de microtubuli die de microspore-kern aan het plasmamembraan hechten. Als daarna de centrale vacuole verdwijnt, dat is vóór de mitose, verplaatst de kern naar het midden van de microspore. De kern deelt dan en er worden twee gelijkvormige cellen gevormd. De vorming van een dergelijk symmetrisch gedeeld pro-embryo is een voorwaarde voor embryogenese. De tweede weg waarlangs pro-embryo's gevormd kunnen worden begint in het late microspore stadium, net voor de celdeling. In dat geval wordt de kern nog door microtubuli vastgehouden in haar excentrische positie, maar de mitotische spoel draait tot 90°. De draaiing van de spoel veroorzaakt een draaiing in het delingsvlak en dat resulteert in de vorming van een symmetrisch gedeeld pro-embryo. Heel jong tweecellig pollen is ook instaat embryo's te vormen. Als dit pollen onder embryogene condities wordt gekweekt leidt dat tot een blokkering van de ontwikkeling van de generatieve cel en tot de aanzet tot een symmetrische deling van de vegetatieve cel. Dat is de derde weg. De aanzet

tot celdeling gaat gepaard met het verdwijnen van microtubuli in de vegetatieve cel. Die microtubuli hadden een loodrechte positie ten opzichte van de gemeenschappelijke wand van de generatieve en de vegetatieve cel.

Als geïsoleerde microsporen en pollen onder embryogene condities gekweekt worden, veroorzaakt dat veranderingen in de celcyclus. De vegetatieve cel komt daarbij opnieuw in de celcyclus hetgeen een uiterst interessant verschijnsel is (zie algemene samenvatting). Fosforylering en defosforylering van een aantal eiwitten is van groot belang voor de voortgang in de celcyclus. Daarom onderzochten we eiwitfosforylering in microsporen en pollen waarbij gebruik gemaakt werd van het monoclonale antilichaam MPM-2 (Hoofdstuk 5). Dit antilichaam herkent een mitose-specifiek gefosforyleerde epitoop. Eén- en tweedimensionale immunoblots lieten zien dat MPM-2 een "familie" van gefosforyleerde epitopen herkent in pas-geïsoleerde microsporen en pollen, maar ook in embryogene en niet-embryogene culturen. Als coupes van microsporen en pollen met immunolabelingtechnieken werden geanalyseerd, bleek dat de epitopen die met MPM-2 reageerden voornamelijk in het kernplasma werden gevonden vanaf de G1 tot de G2 fase, en in het cytoplasma tijdens de mitose. Herkenning was echter afhankelijk van de inbeddingsprocedure. In vriescoupes bleek het epitoop in alle celcyclusstadia reactief, maar als materiaal in polyethyleenglycol was ingebed werd geen labeling in het cytoplasma gevonden. Het epitoop dat door MPM-2 wordt herkend werd overigens in alle onderzochte cellen gevonden, onafhankelijk van de kweekomstandigheden. De intracellulaire verdeling was dus veeleer afhankelijk van het stadium waarin de celcyclus verkeerde dan van de ontwikkelingswijze van de microsporen en pollen.

Hoge temperatuurbehandeling (32 °C gedurende tenminste 8 h) is de aanzet tot de inductie van embryogenese in microsporen en pollen van *B. napus*. Deze "stress" behandeling ging gepaard met *de novo* synthese van een aantal "heat shock"-eiwitten (HSP) van de 70-kDa klasse (hoofdstuk 6). Acht HSP68 en zes HSP70 isovormen werden gevonden op twee-dimensionale immunoblots. Sommige van die isovormen waren meer abundant onder embryogene condities. Met immunocytochemie is aangetoond dat HSP68 in alle cellen voorkwam maar alleen in DNA-bevattende organellen. Gedurende de normale ontwikkeling werd HSP70 gevonden in het nucleoplasma als de cel in de S-fase verkeerde en voornamelijk in het cytoplasma als de cel in de andere fasen van de celcyclus was. De inductie van de embryogene ontwikkeling in late microsporen ging gepaard met een intensieve HSP70-labeling. Als vroeg tweecellig pollen 8 h onder embryogene omstandigheden was gekweekt, vertoonde de kern van de vegetatieve cel ook een intensieve labeling in het nucleoplasma, terwijl dat nooit voorkwam onder niet-embryogene kweekcondities en *in vivo*. Dit resultaat toont dat er een sterke correlatie is tussen de aanwezigheid van HSP70 in de kern, de fase van de celcyclus, en de inductie van embryogenese.

Om specifieke gen-expressie in microsporen en pollen te kunnen vaststellen is een protocol ontwikkeld voor een gevoelige niet-radioactieve *in situ* hybridisatie-procedure (Hoofdstuk 7). Hybridisaties werden uitgevoerd met gedigoxygeneerde probes op materiaal dat met cryotechnieken gefixeerd was. Coupes van microsporen en pollen van *B. napus* en *Arabidopsis thaliana* werden gehybridiseerd met een ssDNA probe die was geïsoleerd uit een kweek van microsporen en pollen van *B. napus*. Het signaal werd bij beide plantensoorten op specifieke plaatsen in de cellen gevonden. Er was geen expressie in microsporen en

vegetatieve cellen maar wel in het cytoplasma van de generatieve cel tijdens de deling en in het cytoplasma van de spermacellen vlak na de deling. Met deze waarneming is voor de eerste keer cel-specifieke gen-expressie aangetoond in ontwikkelend pollen.

In hoofdstuk 9 wordt de inductie tot celdeling en embryogenese bediscussieerd en wel in relatie tot de celcyclus. In het bijzonder wordt aandacht gegeven aan de distributie van "heat shock"-eiwitten in relatie tot veranderingen in de DNA-replicatie. Voor de aanzet tot sporofytische delingen in de vegetatieve cel worden twee mogelijke wegen voorgesteld en bediscussieerd. De resultaten van het onderzoek zijn samengevat in een algemeen schema.

SUMMARY FOCUSED ON THE WORK DONE BY GERD HAUSE

The dissertation describes investigations on the induction of microspore and pollen embryogenesis in rapeseed (*Brassica napus* L.). The advantages of this experimental system are described in the general summary. We were interested in the cytological changes that occurred in isolated microspores and pollen which were cultured under embryogenic conditions (cultivation at 32 °C). Changes found during the first 24 h of culture of isolated microspores and pollen were compared with non-embryogenic cultures (cultivation at 18°C) and the *in vivo* development.

It was assumed that the induction of embryogenesis in microspores and pollen of *B. napus,* being a dramatic change of the developmental fate, is accompanied by notable changes at the cellular level. One important cell structure, which is involved in several cellular processes such as cell division and morphogenesis, is the microtubular cytoskeleton. The investigations started with the detailed study of the microtubular cytoskeleton during microspore and pollen development *in vivo* (part of Chapter 2) using electron microscopy and immunocytochemical techniques. A stage specific pattern of microtubules was observed during the formation of the male gametophyte. The ultrastructural investigations revealed that before mitosis the nucleus of the microspore was attached to the plasma membrane by microtubules. Those microtubules are possibly very important to retain the eccentric position of the nucleus despite the disappearance of the central vacuole just before mitosis. A preprophase band was not formed neither before microspore mitosis nor before pollen mitosis. Mitotic spindles as well as the microtubules of phragmoplasts were detected during both mitotic divisions leading to the formation of the three-cellular pollen.

One part of chapter 3 describes the changes of the microtubular cytoskeleton during the induction of microspore and pollen embryogenesis in comparison with those during *in vivo* development. After 2 to 4 hours of cultivation spindle-like structures were frequently observed in microspores which were in prophase of mitosis. The nuclei were, however, located in an other region of the cell. This phenomenon probably represents a disturbance caused by the procedure of isolation. Again, preprophase bands were not observed.

Three pathways of the induction of embryogenic divisions were detected. The first is the disruption or disturbed formation of the microtubules connecting the microspore nucleus with the plasma membrane during the vacuolated stage of the microspore. This leads to the movement of the nucleus to the centre of the microspore as soon as the central vacuole disappears. The nucleus divides in this central position and two cells of the same size are formed. The formation of such a symmetrically divided proembryo is a prerequisite for embryogenesis. The second pathway to form proembryos starts from the late microspore stage, just before mitosis. In this case the nucleus is still fixed in its eccentric position by microtubules but the mitotic spindle turns up to 90°. This turn of the spindle causes a turn of the division plane, and thus, the formation of a symmetrically divided proembryo. Very young bicellular pollen are also inducible to form embryos (pathway three): Cultivation under embryogenic conditions leads to the arrest of the development of the generative cell and to the initiation of symmetrical division of the vegetative cell. This initiation of cell division is accompanied by the disappearance of microtubules in the vegetative cell. Those microtubules are located perpendicularly to the common wall with the generative cell *in vivo*.

142

It was shown, that the cultivation of isolated microspores and pollen under embryogenic conditions causes changes in the cell cycle. Especially the re-entry of the vegetative nucleus into the cell cycle (see general summary) is a very interesting phenomenon. Phosphorylation and dephosphorylation of certain proteins is very important for the progress of the cell cycle. We therefore investigated protein phosphorylation in microspores and pollen using the monoclonal antibody MPM-2 (Chapter 5). This antibody interacts with a mitosis-specific phosphorylated epitope. One- and two-dimensional immunoblots revealed that MPM-2 recognized a family of phosphorylated proteins in freshly isolated microspores and pollen as well as in embryogenic and non-embryogenic cultures. Immunolabelling of sectioned microspores and pollen showed that MPM-2 reactive epitopes were predominantly observed in the nucleoplasm from G1 until G2 and in the cytoplasm during mitosis. It was observed that the detectability of epitopes on sections depended on the embedding procedure. Cryo processing revealed epitope reactivity in all stages of the cell cycle whereas polyethylene glycol embedded material showed no labelling in the cytoplasm. The MPM-2 detectable epitope was observed in all cells investigated, irrespective of culture conditions, and its intracellular distribution depended on the cell cycle stage and was not related to the developmental fate of the microspores and pollen.

The trigger for the induction of embryogenesis in microspores and pollen of *B. napus* is high temperature treatment (32°C for at least 8 h). This stress treatment was accompanied by *de novo* synthesis of a number of heat shock proteins of the 70-kDa class (Chapter 6). Eight HSP68 and six HSP70 isoforms were detected on two-dimensional immunoblots. Some of these isoforms showed an increased synthesis under embryogenic conditions. Immunocytochemistry revealed a codistribution of HSP68 with DNA-containing organelles in all cells. During normal pollen development HSP70 was localized in the nucleoplasm during the S phase of the cell cycle, and predominantly in the cytoplasm during the remainder part of the cell cycle. Induction of embryogenic development in late microspores was accompanied by an intense HSP70 labelling. In early bicellular pollen the nucleus of the vegetative cell, which normally does not divide and never expresses HSP70, showed intense labelling of the nucleoplasm with anti-HSP70 after 8 h of culture under embryogenic conditions. These results demonstrate a strong correlation between the nuclear localization of HSP70, the phase of the cell cycle, and the induction of embryogenesis.

To identify specific gene expression in microspores and pollen, a sensitive protocol for non-radioactive *in situ* hybridization was developed (Chapter 7). The hybridizations were done with a digoxygenated probe on cryo-fixed material. Sections of microspores and pollen of *B. napus* L. and *Arabidopsis thaliana* L. were hybridized with a ssDNA probe which was picked up from cultured microspores and pollen of *B napus* L. Specific localization of the signal was found in both *A. thaliana* as well as in *B. napus*. There was neither expression in microspores nor in vegetative cells of pollen. However, the cytoplasm of generative cells of *A. thaliana* and *B. napus* exhibited strong hybridization signal as did the sperm cell cytoplasm after division. With this finding we demonstrated for the first time cell specific gene expression in developing pollen.

In chapter 9 the initiation of embryogenic divisions is discussed with respect to the changes of the cell cycle, especially the changes in the DNA-replication in relation to the localization of heat shock proteins. For the initiation of sporophytic divisions in the vegetative cell two

possible pathways are discussed. The results of the investigations are summarized in a general scheme.

SAMENVATTING VAN HET WERK GEDAAN DOOR BETTINA HAUSE

In dit proefschrift is het proces van embryo-inductie in microspore- en pollencultures van koolzaad *(Brassica napus* L.) onderzocht. De ontwikkeling van microsporen en pollen, gekweekt onder embryogene condities (32°C), werd daarbij vergeleken met microspore- en pollencultures gekweekt onder niet-embryogene condities (18°C) en met de *in vivo* ontwikkeling aan de plant. Daarbij werd vooral gelet op veranderingen in de organisatie van het cytoskelet, op de DNA-synthese in de kern, op fosforyleringspatronen en de lokalisatie van "heat shock"-eiwitten.

Het cytoskelet speelt een belangrijke rol bij de deling en vormgeving van cellen. Het was de verwachting dat microtubuli en microfilamenten betrokken zouden zijn bij de blokkering van de gametofytische ontwikkeling en de aanzet tot sporofytische delingen. Tijdens de ontwikkeling van de microsporen en pollen werden microfilamenten zichtbaar gemaakt door het filamentair actine (F-actine) te kleuren met phalloïdine waaraan een fluorochroom gekoppeld was (Hoofdstuk 2). Het phalloïdine-rhodamine-conjugaat werd toegevoegd aan gefixeerde en ongefixeerde cellen waarvan de celmembranen met detergentia waren gepermeabiliseerd. Bovendien werden vriescoupes van helmknoppen met microsporen en pollen in verschillende ontwikkelingsstadia gekleurd. In de anthere vertoonden jonge en late microsporen geen microfilamenten met de genoemde technieken maar microfilamenten waren wel zichtbaar tijdens de mitose: Er was colocalisatie met microtubuli in de fragmoplast maar niet in de spoelfiguur. Tweecellig pollen vertoonde een karakteristieke band met microfilamenten. De band met microfilamenten lag altijd loodrecht op de denkbeeldige as die getrokken kan worden door de generatieve en vegetatieve kern. Er werden slechts kleine verschillen in microfilamentpatronen gevonden tussen de *in vivo* ontwikkeling en de ontwikkeling onder cultuuromstandigheden (Hoofdstuk 3). Vanaf 6 uur kweek werden microfilamenten ook in een kris-kras patroon gevonden in microsporen. Vanaf dat moment lag de ruimtelijke positie van de band met microfilamenten in tweecellig pollen ook niet meer vast. Onder embryogene omstandigheden werden andere microfilament-configuraties gevonden. (i) Microsporen met een delende kern in het centrum van de cel en (ii) microsporen in mitose met een gedraaide spoel vertoonden microfilamenten in de fragmoplast die in beide gevallen een veranderde positie had. (iii) Vroeg tweecellig pollen met een vastliggende generatieve cel en een vegetatieve cel met een delende vegetatieve kern had in beide cellen een fijn netwerk van microfilamenten. Om na te gaan of microfilamenten al of niet betrokken zijn bij de inductie van embryogenese werden microsporen en pollen gedurende de eerste uren van de cultuur behandeld met cytochalasine B, een verbinding die microfilamenten afbreekt. Alhoewel microfilamenten na de behandeling, verdwenen waren, was het vermogen tot embryovorming in de cultuur niet aangetast. Op basis van dit resultaat is dan ook geconcludeerd dat de verandering in microfilamentpatronen eerder een uiting is van secundaire veranderingen die door het inductieproces in gang zijn gezet dan dat microfilamenten betrokken zijn bij de inductie van embryogenese. In tegenstelling hiermee lijken de microtubuli wel betrokken bij het inductieproces (zie het deel van G. Hause).

Om de vroege veranderingen in de celcyclus en de DNA-synthese in kernen van embryogene microsporen en pollen te begrijpen, werd gedurende de eerste 24 h van de cultuur de DNA-synthese bestudeerd. De DNA-synthese werd zichtbaar gemaakt door bromodeoxiuridine

145

(BrdU), dat in het kern-DNA geïncorporeerd was, immunocytochemisch te detecteren. De DNA-inhoud van microspore-kernen en van de kernen van generatieve en vegetatieve cellen van tweecellig pollen werd met microspectrofotometrie gemeten. Tijdens de *in vivo* ontwikkeling wordt kern-DNA gerepliceerd in late microsporen en in de generatieve cel van jong tweecellig pollen, terwijl de kern van de vegetatieve cel in de G1-fase blijft. Als microsporen en pollen onder niet-embryogene omstandigheden werden gekweekt, volgden ze de normale gametofytische ontwikkeling. Inductie van embryogenese in microsporen leidt tot symmetrisch gedeelde pro-embryo's. Vlak na de deling vertoonden de kernen soms wel en soms geen labeling door BrdU inbouw. Hieruit wordt geconcludeerd dat microsporen tot embryogenese kunnen worden geïnduceerd als ze in G1-, S- én in G2-fase zijn. Als vroeg tweecellig pollen gekweekt wordt onder embryogene condities vertoonde het pollen DNA-replicatie in de kern van de generatieve cel maar bovendien ook in de kern van de vegetatieve cel. De vegetatieve kern kon de S-fase ingaan binnen één uur kweek en na 24 h hadden veel kernen van vegetatieve cellen DNA gerepliceerd bij 32°C. Deze gegevens laten zien dat het opnieuw in de celcyclus komen een vroege respons is op de hoge-temperatuurbehandeling. Het kan tevens een belangrijke gebeurtenis zijn die de verandering van gametofytische naar sporofytische ontwikkeling induceert. Zoals in hoofdstuk 6 is beschreven kan de analyse van de distributie van de "heat shock"- eiwitten aanwijzingen geven over de relatie tussen de "heat shock" en de DNA-replicatie (zie het deel van G. Hause).

Naast de beschreven processen van inductie van symmetrische delingen in microsporen en pollen behelst de ontwikkeling van het eigenlijke embryo (embryo proper) een aantal differentiatieprocessen. Het tot stand komen van polariteit is noodzakelijk voor de verdere ontwikkeling en differentiatie. Op biochemisch en cytologisch niveau kan polariteit herkend worden aan de verdeling van verschillende verbindingen in de weefsels of in de verdeling van organellen. Hoofdstuk 8 beschrijft en vergelijkt de vorming van microspore-embryo's en zygotische embryo's en de expressie van polariteit in globulaire en langwerpige embryo's. Onderzoek met lichtmicroscopie en scanning-elektronenmicroscopie toonde aan dat gedurende de eerste vijf dagen van de embryo-vorming de celwand van de oorspronkelijke microspore of pollen intact bleef. Een globulair embryo ontwikkelde binnen deze wand en nadat de wand openbrak ontwikkelde het embryo verder naar het hartvormige en torpedovormige stadium zoals bij zygotische embryo's. De overgang van het globulaire naar het hartvormige en torpedovormige stadium is een uiting van veranderde polariteit binnen het eigenlijke embryo. Een eerste teken van polariteit in zygotische embryo's is de ongelijke deling van de zygote en in de vorming van de suspensor. In pro-embryo's die uit microsporen zijn ontstaan, zijn alle cellen gelijk in vorm en grootte. Omdat inductie van polariteit gerelateerd is met celdeling en groei, werd de verdeling van calmoduline, vrij calcium in de cytosol en de ophoping van zetmeel geanalyseerd. Calmoduline was gelijkmatig verdeeld in globulaire embryo's. Het vrije calcium in de cytosol accumuleerde in het protoderm van de globulaire embryo's maar vertoonde nooit een polaire verdeling. De ophoping van zetmeel bij de wortelpool van het microspore-embryo was het enige teken dat een radiale as in het globulaire embryo tot ontwikkeling kwam. Omdat zetmeel egaal verdeeld was in microspore-embryo's waarvan de pollenwand nog heel was, concluderen we dat het breken van de pollenwand de inducerende gebeurtenis is die leidt tot het vastleggen van de polariteit.

In hoofdstuk 9 worden de resultaten van het onderzoek naar de inductie van embryogenese

in microspore- en pollencultures bediscussieerd. Er is geconcludeerd dat het gehele proces van embryogenese in microsporen en pollen bij *B. napus* gezien moet worden als een twee-fasen proces: De inductie van symmetrische delingen enerzijds en anderzijds de inductie van embryo-specifieke differentiatie na enige dagen kweek.

SUMMARY FOCUSED ON THE WORK DONE BY BETTINA HAUSE

The aim of this thesis was the investigation of the process of embryo induction in microspore and pollen cultures of rapeseed (*Brassica napus* L.). Therefore, alterations in the occurrence of the cytoskeleton, DNA synthesis and phosphorylation pattern, as well as in the localization of heat-shock proteins were analyzed by comparing the development of microspores and pollen in culture under embryogenic (cultivation at 32 °C) and non-embryogenic (cultivation at 18 °C) conditions with the normal pollen development (*in vivo*-development).

The cytoskeleton plays an important role in cell division and cell shaping. It was expected that microtubules and microfilaments (MFs) are involved in the stop of the gametophytic development and in the initiation of sporophytic divisions. The pattern of MFs during the development of microspores and pollen was analyzed by staining F-actin with fluorochrome-labelled phalloidin (Chapter 2). Phalloidin-rhodamine was infiltrated into fixed as well as unfixed cells permeabilized by detergents. Moreover, cryo-sectioned anthers of different developmental stages were stained. *In vivo*, MFs could not be visualized in young till late microspores, whereas MFs were visible during microspore mitosis. A colocalization with microtubules was detectable within the phragmoplast, but not within the spindle apparatus. Bicellular pollen were characterized by a specific banding pattern of MFs. This banding pattern appeared always perpendicularly to the imaginary axis between the vegetative and generative nuclei. Under non-embryogenic culture conditions only minor differences in the MF-pattern could be observed in comparison to the *in vivo* development (Chapter 3). From 6 hours onwards, MFs exhibiting a criss-cross pattern were detectable in microspores, too. Moreover, in bicellular pollen the direction of the banding pattern was lost. Against this, under embryogenic conditions different MF patterns were found - (i) Microspores with dividing nucleus in the center of the cell and (ii) microspores in mitosis with turned spindle contained MFs within the phragmoplast at the changed position, and (iii) in early bicellular pollen with arrested generative cell and dividing vegetative nucleus MFs showed a fine, distinct network in both cells.

To check whether or not MFs are involved in the induction of embryogenesis, microspores and pollen were treated with cytochalasin B during the first hours of cultivation. Although MFs were disrupted completely, there was no influence of this treatment on the embryogenicity of the culture. Concluding from this results, the altered pattern of MFs are rather the expression of "secondary" alterations caused by the induction process, than that they are involved in the induction of embryogenesis. Against this, microtubules seemed to be involved in the induction process (see part of G. Hause).

In order to understand the early events of microspore and pollen derived embryogenesis with respect to the cell cycle, nuclear DNA synthesis in microspores and pollen during the first 24 h of culture was studied. DNA synthesis was visualized by immunocytochemical labelling of bromodeoxyuridin (BrdU) incorporated into nuclei. The DNA content of microspore nuclei as well as of the nuclei of generative and vegetative cells of the bicellular pollen was measured by microspectrophotometry. During *in vivo* development DNA replication occurred within the late microspore and within the generative cell in late bicellular pollen, whereas the vegetative nucleus remained in the G1 phase. When microspores and pollen were cultured under non-embryogenic conditions, they followed the normal gametophytic development. The

induction of embryogenesis in microspores leads to symmetrically divided proembryos. Just after division the nuclei of both daughter cells showed either a signal caused by the incorporated BrdU or they were free from any label. So, it was concluded that microspores were inducible to undergo embryogenic divisions starting from G1, S and G2 phase. When early bicellular pollen were cultured under embryogenic conditions they exhibited DNA replication within the generative nucleus and additionally within the vegetative nucleus. The vegetative nucleus could enter the S-phase within one hour of culture and many vegetative nuclei had replicated DNA after 24 h of cultivation at 32 °C. This observation showed that the re-entering of the cell cycle is an early response to the high temperature treatment and might be an important event to induce the shift from gametophytic to sporophytic development. As described in Chapter 6 the analyses of the localization of heat shock proteins could give indications for possible relations between the heat shock and replication (see part of G. Hause).

Beside the described processes of induction of symmetrical divisions in microspores and pollen, the formation of the embryo proper includes processes of differentiation. Such processes are ordered cell divisions, cell elongation's and changes of cell shape. The establishment of polarity in the embryo is essential for its further development and differentiation. At the biochemical and structural level, polarity can be discerned in the distribution of various compounds within the tissues or in the distribution of organelles. The formation of microspore-derived embryos in comparison to the zygotic embryos, and the analysis of the expression of polarity in globular and elongating embryos are described in chapter 8. Investigations by light and scanning electron microscopy showed that during the first 5 days of the formation of microspore-derived embryos, the cell wall of the former microspore of pollen remained intact. A globular proembryo developed within this wall, and after its rupture embryos proceeded through heart-shape and torpedo-shape stages in a way comparable to zygotic embryogenesis. The transition from the globular stage to the heart- and torpedo-shape stages is an expression of changed polarity within the embryo proper. In zygotic embryos the first sign of polarity can already be seen in the unequal division of the zygote as well as in the formation of a suspensor. In microspore-derived proembryos all cells were equal in shape and size. Because the induction of polarity is related to cell division and elongation, the distribution of free cytosolic calcium and calmodulin, and the accumulation of starch were analysed. Calmodulin was evenly distributed in globular embryos. Free cytosolic calcium accumulated in the protoderm of globular embryos, but never showed polar distribution. However, the accumulation of starch at the root pole of the microspore-derived embryo was the only hint at the predisposition of the radial axis of the developing embryo. Based on the observations that starch is evenly distributed in proembryos covered by the pollen wall, and that residues of the pollen wall are detectable at the root pole, it is concluded that the rupture of the pollen wall could be the inducing event for the establishment of polarity.

In chapter 9 the results concerning the induction of embryogenesis in microspore and pollen cultures are discussed. It is concluded that the whole process of embryogenesis in microspores and pollen of *B. napus* has to be seen as an biphasic process: The process of the induction of symmetrical divisions is followed by the process of induction of embryo-specific differentiation after some days of cultivation.

149

CURRICULA VITAE

Bettina Hause was born in Mühlhausen (Thuringia, Germany) on 13 February 1961. After 8 years basic education in elementary school she visited the secondary school in Mühlhausen (1975-1979). From 1979 to 1984 she studied biochemistry at the Martin-Luther University in Halle. She finished her studies in 1984 with the diploma work about "Isolation and molecular characterization of mitochondrial DNA from cell suspension cultures of tomato". Then she worked as an assistant at the Department of Plant Biochemistry of the University of Halle and was investigating problems of compartmentation of nucleotides during the synthesis of RNAs. In 1987 she was honored by the degree of Doctor rerum naturalium after defending her doctoral thesis: "Compartmentation of nucleotides during the synthesis of different RNA species from tomato - analysed after cloning of the mitochondrial rRNA genes" (Promoter: Prof. D. Schlee). In 1988 and 1989 she worked as scientific assistant at the Institute of Biochemistry of Plants in Halle, under the supervision of Prof. B. Parthier. There, she was involved in biochemical studies about the induction of proteins in barley leaves by jasmonic acid. From 1991 to 1993 she was selected for an EC-fellowship (BRIDGE-program) at the Department of Plant Cytology and Morphology of the Wageningen Agricultural University (The Netherlands) studying the "Induction phase of microspore embryogenesis in *Brassica napus* L." (Prof. M.T.M. Willemse, Dr. A.A.M. Van Lammeren). From January 1994 till present she has been working as a scientific assistant at the Institute of Plant Biochemistry in Halle, Department of Hormone Research, investigating cytological aspects of different processes related to the action of jasmonic acid as well as to the pathogenic interaction between powdery mildew and barley. She participates in the education of students giving practical courses.

Gerd Hause was born on 25 July 1957 in Kleinpaschleben (Region Halle, Germany). He visited the grammar school in Kleinpaschleben (1964 - 1972) and the secondary school in Köthen (1972-1976). From 1979 to 1984 he studied biology, specialization genetics, at the Martin-Luther University in Halle, Germany. In 1984 he defended the diploma work about "Organelle distribution during pollen development in *Pisum sativum*". From 1985 to 1990 he worked as an assistant at the Department of Genetics, University of Halle. He was investigating problems of organelle transmission during spore and gametophyte development as well as during fertilization of plants. In 1988 he finished the doctoral thesis: "Ultrastructure of megagametogenesis and fertilization in *Triticale*" (Doctor rerum naturalium, Promoter: Prof. R. Hagemann). From 1991 to 1993 he was selected for an EC-fellowship (BRIDGE-program) at the Department of Plant Cytology and Morphology of the Wageningen Agricultural University (The Netherlands) studying the "Induction phase of microspore embryogenesis in *Brassica napus* L." (Prof. M.T.M. Willemse, Dr. A.A.M. Van Lammeren). Since 1994 he is a scientific assistant at the Institute of Genetics, Martin-Luther-University Halle. There he is responsible for the electron microscopy-group investigating differentiation processes during plant embryogenesis. Further on he is involved in different research programs of the department.

The authors are married and have two sons.