Monitoring of nuclear activity during pollen development and androgenesis in *Brassica napus* L.

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Cover: Replica of the fractured surface of the nuclear envelope of a vegetative nucleus of *Brassica napus* L.

Contents

Abbreviations used in the text

ATP	adenosine triphosphate
BrdU	5-bromodeoxyuridine
BrU	bromouridine
BrUTP	5-bromouridine 5'-triphosphate
BSA	bovine serum albumine
BSAc	acetylated BSA
CSLM	confocal scanning laser microscope
CTP	cytidine triphosphate
DAPI	4,6-diamidino-2-phenylindole
DFC	dense fibrillar component
DIG	digoxigenin
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
	EGTA ethylene glycol-bis(ß-aminoethyl esther)-N,N,N',N' tetraacetic acid
FCs	fibrillar centers
FDA	fluorescein diacetate
FITC	fluorescein isothiocyanate
GC	granular component
GTP	guanosine triphosphate
hnRNA	heterogeneous nuclear RNA
IR	interchromatin region
mAb	monoclonal antibody
MES	morpholinoethane sulfonic acid
mRNA	messenger RNA
NOR	nucleolar organizer region
NPC	nuclear pore complex
PBS	phosphate buffered saline
PC	polycomplex
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
rDNA	ribosomal DNA
RNA	ribonucleic acid
RPI	RNA polymerase I
RPII	RNA polymerase II
RPIII	RNA polymerase III
rRNA	ribosomal RNA
SC	synaptonemal complex
snoRNP	small nucleolar ribonucleoprotein particle
snRNP	small nuclear ribonucleoprotein particle
TRITC	tetramethylrhodamine isothiocyanate
UTP	uridine triphosphate

Chapter 1

General introduction

K.R. Straatman

The development of a zygotic embryo in plants is a polar process. The first division of a zygote is asymmetrically, resulting in a larger cell that will form the suspensor and a smaller cell that will form the embryo proper. Cellular material is distributed unequally over these two cells. One of the main topics in biological research these days is the relation between genome activity and cellular organization and especially the question if polarity in development is mirrored by RNA transcription and transport.

The nucleus contains most of the genetic information necessary to control events in the eukaryotic cell. To understand nuclear processes like DNA synthesis, gene expression, gene regulation and RNA processing and to link these processes to cellular activities like cell division and cell differentiation more knowledge of the nuclear organization is crucial. The nucleus can easily be observed in the light microscope but when we want to obtain more information about its structure and function a higher resolution is needed, combined with cytological techniques. It is well known by now that the nucleus is a highly organized organelle where nuclear processes take place in distinct domains. How these domains are related to cellular functioning is, however, still rather unknown.

In this introduction I will explain the general nuclear organization and introduce the plant system used to study the relation between nuclear morphology and cellular functioning, with emphasis on transcriptional activity.

The nucleus

The nucleus is the main storage place for DNA molecules, which encode for the genetic information of an organism. DNA molecules are long chains of nucleotides and to store these long chains they are highly organized into several levels of folding by specific nuclear proteins (Getzenberg et al., 1991). The complex formed between DNA and these proteins is called chromatin. This DNA organization has to be flexible so that the DNA remains accessible for transcription and replication. During cell division the DNA molecules become highly condensed and are visible as chromosomes.

A piece of DNA containing a specific part of genetic information for the production of one single protein is called a gene. One group of genes, the ribosomal genes encoding large pre-ribosomal RNA (rRNA) which is split into 28S, 18S and 5.8S rRNA, is localized in the nucleolus. This is the only nuclear subdomain that is also visible in the light microscope. All other genes are transcribed in the nucleoplasm, the nuclear area surrounding the nucleolus. Two morphological types of chromatin can be found during interphase in the nucleoplasm: the condensed chromatin, called heterochromatin, representing the transcriptionally inactive genes, and the less condensed chromatin, called euchromatin, representing the transcriptionally active genes. Three types of RNA polymerases are involved in the transcription from DNA into RNA. The type of polymerase is depending on the type of gene to be transcribed. RNA polymerase I transcribes ribosomal genes in the nucleolus, whereas RNA polymerase II and III are located in the nucleoplasm. RNA polymerase II transcribes most of the genes active in the nucleoplasm; only some small RNAs, like 5S rRNA, transfer RNA and U6 small nuclear RNA are synthesized by RNA polymerase III.

The nucleus is separated from the cytoplasm by a double membrane. The outer nuclear membrane is continuous with the endoplasmic reticulum and the inner nuclear membrane is tightly associated with a filamentous network, the nuclear lamina. The major proteins of the nuclear lamina are called lamins (Moir et al., 1995; Moreno Días de la Espina, 1995). Both nuclear membranes are fused at the places where nuclear pore complexes (NPCs) are present. Transport of particles from and towards the nucleus, the nucleocytoplasmic transport, takes place via these NPCs (Fig. 1). The double nuclear membrane forms, together with the NPCs and the nuclear lamina, the nuclear envelope (reviewed by Goldberg and Allen, 1995).

The synthesis of mRNA, its processing and transport

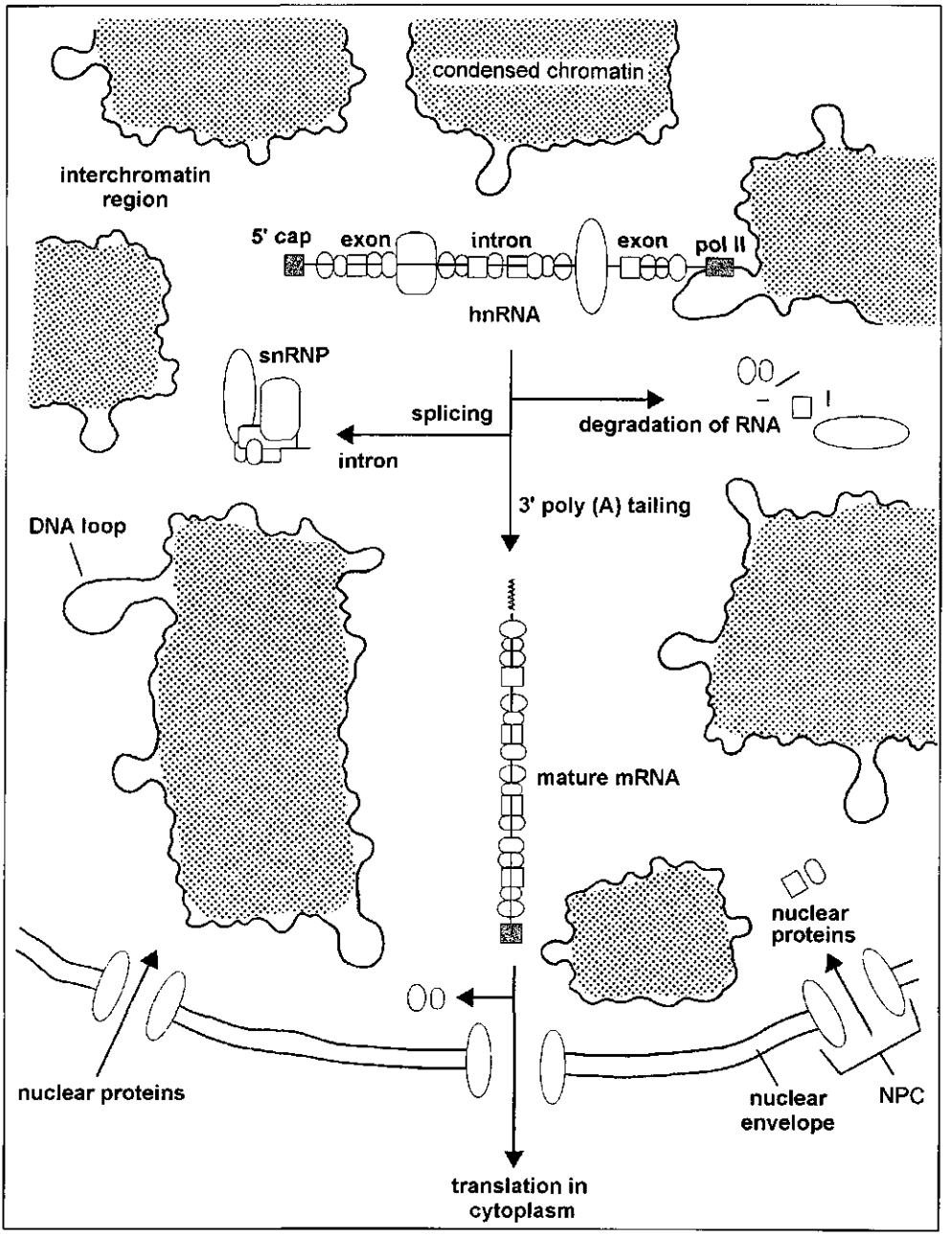
To regulate cellular activity, a part of the genetic information stored in the nucleus has to become available for translation in the cytoplasm, i.e. the production of the proteins the genes are coding for. Therefore, during transcription, copies are made of the genetic information. It is believed that transcription takes place on DNA loops at the

edge of more condensed DNA (Fig. 1). These copies are known as heterogeneous nuclear RNA (hnRNA). A part of this hnRNA is coding for messenger RNA (mRNA), the so called pre-mRNA, while the rest has unknown functions. There are several proteins that bind to the hnRNA, the ribonucleoproteins, which form together the ribonucleoprotein particles (RNPs). They protect the RNA molecule and are involved in several processes during its journey from the site of transcription to the site of translation in the cytoplasm.

Before pre-mRNA can be transported to the cytoplasm, it has to be further processed (Fig. 1). Processing of pre-mRNA includes 5'-end capping (Hamm and Mattaj, 1990), 3'-end cleavage and polyadenylation (Keller, 1995), and splicing (reviewed by Filipowicz et al., 1995). The 5'cap is probably important for transport of the mRNA into the cytoplasm and the poly(A) tail plays a role in the initiation of protein synthesis and the control of mRNA stability in the cytoplasm.

During splicing the introns are removed from the pre-mRNA and the remaining exons are bound together (Krämer, 1995). One group of proteins involved in this splicing process is the group of small nuclear ribonucleoprotein particles (snRNPs). The snRNPs are found in three nuclear domains: the interchromatin granules, the perichromatin fibrils and the coiled bodies. The interchromatin granules are thought to be storage places of snRNPs. These granules are difficult to observe in plant cells. The perichromatin fibrils are sites of transcription where snRNPs are available for splicing. The function of the coiled bodies is not clear but they are well visible and the number of coiled bodies increases when a cell becomes metabolically more active (Fakan et al., 1984; Ferreira et al., 1994; Beven et al., 1995). All these processes are linked to the nuclear matrix (see further). This implies that processes in the nucleus do not take place randomly, leading to an organized localization of proteins necessary for a nuclear process in a specific region (Spector, 1993; Strouboulis and Wolffe, 1996).

Fig. 1; Schematic drawing of a section through a cell nucleus according to the model of functional nuclear compartmentalization, adapted from Cremer et al. (1993), combined with the model of processing of mRNA, adapted from Dreyfuss et al. (1993). Loops of DNA containing active genes extend into the interchromatin region. Transcription takes place at the periphery of the condensed DNA by RNA polymerase (here RNA polymerase II (pol II)), at the base of these DNA loops. The transcript (hnRNA) is directly released into the interchromatin region. This contains all the necessary proteins for further processing of pre-mRNA into mature RNA nearby the site of transcription. Another part of the RNA will degrade in the nucleus. Mature mRNA is transported to the nuclear pore complexes (NPCs) by special proteins which stay in the nucleus. Via the NPCs the mature mRNA is released into the cytoplasm where translation of the mRNA into proteins takes place. Nuclear proteins are imported into the nucleus by the same NPCs. For further explanation see the text.


condensed chromatin
interchromatin region
5' cap
exon
intron
exon
pol II
hnRNA
snRNP
splicing
intron
degradation of RNA
3' poly (A) tailing
DNA loop
mature mRNA
nuclear proteins
nuclear proteins
nuclear envelope
NPC
translation in cytoplasm

The nucleolus

The nucleolus is the place of rRNA synthesis by RNA polymerase I and the processing of preribosomal particles. At least three series of small nucleolar RNPs (snoRNPs) can be found in the nucleolus, U3, U8 and U13, which are probably involved in rRNA processing.

At the ultrastructural level the nucleolus can be divided into four subdomains: the dense fibrillar component (DFC), the fibrillar centres (FCs), the granular component (GC) and the so called nucleolar vacuoles. The DFC is a dense nucleolar area containing at least DNA and 10 nm ribonucleoprotein particles (RNPs). Embedded in the DFC, lighter packed areas can be found, the FCs, which form the nucleolar organizing region (NOR) during interphase. The GC is more granular on micrographs and consists, at least partly, of pre-ribosomes. The nucleolar vacuoles are large electron translucent areas in the nucleolus. Highly active cells have often nucleoli with larger "vacuoles" than less active cells, but their function is not known. Where exactly in the nucleolus transcription takes place is still subject of discussion (reviewed by Hernandez-Verdun, 1991; Shaw and Jordan, 1995).

The nuclear matrix

The nuclear matrix has originally been defined as that part of the nucleus that remains after extraction of most of the proteins, DNA and RNA from the interphase nucleus (Berezney and Coffey, 1974). It is composed of the nuclear lamina with associated NPCs, remnants of the nucleolus (the nucleolar matrix) and an internal matrix. The nuclear matrix is the dynamic structural subdomain of the nucleus that directs the three-dimensional organization of the DNA into loop domains (Cook, 1995). Also, most key processes in the nucleus are associated with the nuclear matrix, like DNA synthesis, transcription and RNA processing (reviewed by Berezney et al., 1995).

The molecular composition of the internal matrix is still a matter of investigation. The composition and structure is depending on the isolation protocol used, often optimized for research on a particular part of it. It is clear by now that lamins are not only found at the nuclear periphery but are also part of the internal matrix and interact directly with the cytoplasmic intermediate filament network (Berezney et al., 1995).

The existence of a nucleolar matrix, as a part of the nuclear matrix, is still controversial. But, like the nucleoplasm, the nucleolus is also a place of synthesis and processing of RNA and therefore also needs to be highly organized (Moreno Díaz de la Espina, 1995).

The cell cycle

The functional stages of the nucleus during the cell cycle are divided into 4 phases, the G1, S, G2 and mitosis. The G1, S and G2 phase form the interphase. In this the S stands for the period of DNA synthesis whereas the G stands for a gap in DNA synthesis. Most non dividing cells are arrested in the G1 phase. During interphase the decondensed chromosomes occupy the so called "chromosome territories" in the cell nucleus. Nuclear processes take place at the periphery of these chromosome territories or in the interchromatin regions (Cremer et al., 1993). The interphase is followed by mitosis during sporophytic development. During mitosis, chromosomes become highly condensed and the nuclear membrane disassembles. In this stage, the chromosomes can easily be seen in a light microscope using DNA dyes. Each chromosome is doubled as a result of DNA replication and the two identical parts of the chromosomes are called chromatids. The two chromatids are separated and move to the opposite ends of the dividing cell providing both daughter cells with the same genetic information. Finally, they get decondensed while a nuclear membrane is formed around both groups of chromosomes.

An exception to this process is the meiosis, at the onset of gametophytic development. The meiosis actually consists of two divisions. After duplication of the chromosomes first the homologous chromosomes will pair. This means that during the first meiotic division the chromatids are not separated but that chromosomes are divided over the two newly formed cells. In the meiosis II the chromatids of each chromosome will be distributed over the two new cells like during mitosis. So after meiosis one cell has divided into four new cells which all contain half of the genetic information of the original cell. These cells will finally form the gametes, egg cells or sperm cells, which can fuse to form an embryo, containing the same amount of DNA as the parents.

One striking difference between the first meiotic division and the second meiotic division or the mitosis is that during the chromosome pairing in meiosis I the genes are transcriptionally active whereas in meiosis II and mitosis transcription is terminated (reviewed by Cook, 1997).

The plant system

The subject of this project has been to study nuclear morphology and activity during early plant embryogenesis. However, nuclear and cellular changes after fertilization are difficult to study *in vivo*. Several layers of sporophytic tissue cover the embryo and make it difficult to manipulate the embryo. This makes that the preparation of the material is laborious and the harvest of embryos is low. Rescue techniques to culture

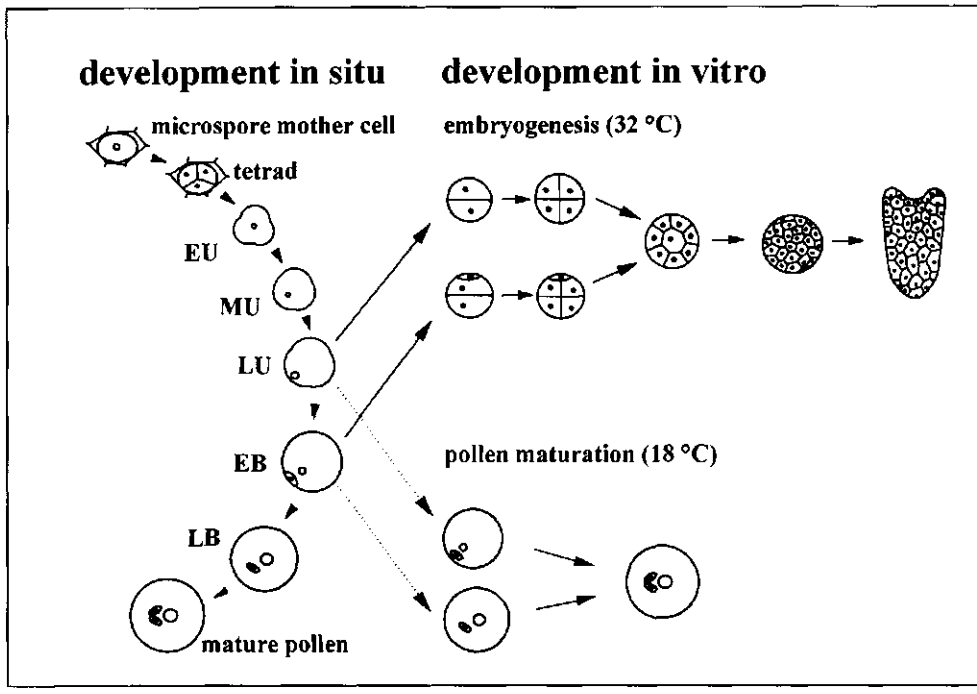


Fig. 2; The *in vivo* development of pollen of *Brassica napus* and the change in developmental pathway towards embryogenesis when late microspores and young pollen are cultured at 32 °C. When these stages are cultured at 18 °C normal pollen development takes place. Abbreviations: EB, young bicellular pollen; EU, young unicellular stage; LB, late bicellular pollen; LU, late unicellular stage; MU, middle unicellular stage (adapted from Cordewener et al., 1996).

zygotic embryos *in vitro* (Liu et al., 1993) are time consuming and early stages are difficult to culture *in vitro*. Therefore, an *in vitro* embryogenic system should be chosen. This system should be well known and embryogenesis should be easy to induce, in order to put most efforts in analysing the nuclei. The embryos should be easy to analyse, also in an early stage, and the system should give a large amount of embryos. All these conditions were found in the androgenic system of *Brassica napus*. Microspores and pollen can easily be isolated and when cultured for at least eight hours at 32 °C they will change their developmental pathway from pollen development towards embryogenesis (Fig. 2). Another advantage of this system is that when microspores and pollen are cultured at 18 °C normal pollen development will continue (Custers et al., 1994). This system allows to study two processes, pollen development *in vitro* and *in vivo* and, secondly, the developmental change from pollen development towards microspore and pollen embryogenesis. Both processes are studied in this thesis.

During sporogenesis in *Brassica napus* the microsporocyte undergoes meiosis, producing four haploid microspores which divide asymmetrically to give rise to a large vegetative cell and a smaller generative cell. The vegetative cell will not further divide whereas the generative cell gives rise to two sperm cells in the mature pollen. After pollination a pollen tube will grow into the style to transport the two sperm cells into one of the synergids of the embryo sac. One sperm cell will fuse with the central cell and form the endosperm and one sperm cell will fuse with the egg cell to form the zygote (Van Went and Willemse, 1984).

Microspore embryogenesis has several advantages over other *in vitro* embryogenesis systems: 1. Only a heat shock is necessary, together with a simple culture medium (Lichter, 1982), to induce the developmental change from gametophytic development to embryogenesis; no hormonal treatment is used; 2. Embryos develop directly from microspores, without a callus phase. This makes the system also interesting for transformation experiments; 3. Stages of microspores can be defined by the length of the buds (Kott et al., 1988); 4. High production of embryos can be reached (Fan et al., 1988; Pechan and Keller, 1988) 5. Besides the heat shock, also colchicine (Zaki and Dickinson, 1991; Iqbal et al., 1994; Zhao et al., 1996), gamma irradiation and ethanol (Pechan and Keller, 1989) can induce microspore embryogenesis in *Brassica napus*.

The culture system, however, has also disadvantages. 1. Until now it has not been possible to distinguish the microspores that change their developmental pathway directly after the induction treatment from those that do not become embryogenic; 2. The pollen wall prevents several chemicals and antibodies to penetrate into the microspore and hinders the detection of some signals inside the microspore; 3. A culture is always a mixture of dead and dying cells, embryogenic cells and non-embryogenic cells (microspores and pollen). This makes it more difficult to identify embryogenic cells in an early stage of culture.

Scope of the thesis

The main goal of this thesis has been to analyse nuclear morphology during pollen development and androgenesis of *Brassica napus*, and to relate structural changes with cellular functioning, focusing on transcriptional activity.

In Chapter 2 the nuclear changes during pollen development are analyzed at the ultrastructural level, using cytochemical staining techniques to visualize chromatin and snRNPs. In Chapter 3 the replication pattern of nuclear DNA is studied during pollen development and after the onset of the embryogenic culture. During *in vivo* pollen development the generative nucleus passes once more the S-phase and divides to form the two sperm nuclei. Concerning the vegetative nucleus of *Brassica napus*, it was not known whether it was arrested in the G1, and re-entered the cell cycle after

the embryo induction treatment, or was arrested in the G2.

Polarity in cellular development during embryogenesis might be reflected by polarity in transcriptional activity. Furthermore, cell regeneration and the re-entry of the cell cycle during microspore and pollen embryogenesis might have influence on the transcriptional activity as well. In an attempt to localize sites of transcription in the nuclei of plants, we adapted a run-on transcription system from mammalian origin for plant nuclei. This is presented in Chapter 4. Chapter 5 gives the results of experiments in which the transcription system was applied to whole plant cells using leaf and pollen protoplasts and growing pollen tubes. To study the relation between the sites of transcription and the proteins which are involved in splicing of pre-RNA, proteins of snRNPs and snoRNPs were localized as well. Chapter 6 presents labelling patterns at the light microscopical level of snRNPs during pollen development and androgenesis, paying special attention to the presence of coiled bodies. There are indications in literature that the appearance of coiled bodies might be related to the transcriptional activity.

When mature RNA has been produced it has to be transported to the cytoplasm via the NPCs. In 1985 Blobel stated in his "gene gating hypothesis" that the number and distribution of NPCs in the nuclear membrane might be indicative for nuclear activity, especially transcription. To test this hypothesis for the *Brassica* system the density of NPCs was studied on freeze-fractured material (Chapter 7) and the NPC densities were linked to the expected transcriptional activity. In Chapter 8 the results and conclusions of the foregoing chapters are generally discussed.

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Chapter 2

Ultrastructural cytochemistry of nuclei during *Brassica napus* pollen development

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Summary

Pollen development in *Brassica napus* was studied at the ultrastructural level using cytochemical approaches for staining of DNA and nuclear proteins, and *in situ* hybridization for the detection of ribosomal RNA. Shortly after meiosis, nuclear activity is low, as was indicated by the presence of polycomplexes in the nucleoplasm and a nucleolar organizer region attached to the nucleolus. In bicellular pollen the generative and vegetative nucleus and nucleolus were transcriptionally highly active. Both nuclei showed rRNA in the nucleolus and euchromatin in the nucleoplasm. After release of the generative cell from the intine wall, the chromatin of the generative nucleus became more condensed, indicating a decrease of transcriptional activity. In mature pollen the generative and vegetative nucleus were probably transcriptionally inactive, although their nuclear morphology was totally different. The sperm nuclei contained large areas of highly condensed chromatin whereas the condensed chromatin of the vegetative nucleus was distributed near the nuclear membrane, with some euchromatin in the nuclear interior.

Keywords: *Brassica napus*; cytochemistry; nucleus; pollen development; ultrastructure.

Introduction

The gametophytic development is an important process during plant sexual reproduction. The meiotic division of a diploid microspore mother cell leads to the formation of a tetrad, which releases four individual microspores. These haploid cells divide further to produce mature pollen, the male gametophytes in the life cycle of seed plants. After the microspore mitosis a small generative cell and a large vegetative cell are formed. In plants with bicellular pollen, the division of the generative cell into two sperm cells takes place during pollen tube growth. In tricellular pollen this mitosis takes place within the pollen grain, resulting in mature pollen consisting of two sperm cells surrounded by a large vegetative cell. *Brassica napus*, the plant of this study, belongs to the group which forms tricellular pollen.

Pollen development is controlled by the genome, and the nuclear morphology is thought to reflect cellular activity (Blobel, 1985; Testillano et al., 1995a). Inside the nucleus, processes are highly organized in separate nuclear domains (Spector, 1993). Some domains can easily be visualized by light microscopy (e.g. the nucleolus) or electron microscopy (e.g. nuclear bodies); however, most nuclear domains can only be observed using specific histochemical, immunocytochemical or *in situ* hybridization techniques.

The vegetative nucleus is responsible for most of the RNA synthesis after the microspore mitosis (reviewed by Tanaka, 1997). Several genes are known to be specifically expressed during pollen development (reviewed by Mascarenhas, 1992) whereas other mRNAs are synthesized during pollen development but have their function during germination or early pollen tube growth (reviewed by Mascarenhas, 1990).

An important domain, and source of information about the transcriptional activity, is the DNA itself. Highly condensed chromatin is considered to be transcriptionally inactive whereas decondensed DNA is transcriptionally more active. Using special cytological staining techniques, we can visualize the ultrastructure of the chromatin and correlate this with nuclear activity. The space between the condensed chromatin regions is called the interchromatin region (IR), the nuclear compartment that is identified by several groups as the region where many important nuclear processes take place, like transcription and pre-mRNA processing (Cremer et al., 1993; Visa et al., 1993; Testillano et al., 1993, 1995b).

Here we describe changes in nuclear morphology during pollen development of *B. napus* from the released microspores until mature pollen. Nuclear changes during pollen development are only partly documented at the ultrastructural level. Grant et al. (1986) and Telmer et al. (1993) have described pollen development of *B. napus* up to the microspore mitosis. Others have studied the formation of the sperm cells (Dumas et al., 1985; Charzynska et al., 1989; Murgia et al., 1991) but not with special attention to nuclear structure. To make a clear distinction between chromatin

structures and the IR, two cytochemical approaches were used, the NAMA method to stain specifically for DNA, (Testillano et al., 1991), and the EDTA regressive staining technique to accentuate ribonucleoprotein particles (RNPs) in the IR by bleaching the DNA, (Bernhard, 1969). To investigate nucleolar activity, high-resolution *in situ* hybridization was applied at some stages of pollen development using a ribosomal probe.

Materials and methods

Preparation of plant material

Plants from *Brassica napus* cv. Topas were grown from seeds in a phytotron at 18 °C with a photoperiod of 16 h light. Anthers containing young microspores, bicellular pollen and mature pollen were selected, cut into small pieces and fixed in 3% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4). Part of the material was treated as described below for cytochemistry. Afterwards, all the material was dehydrated in an ethanol series. The ethanol was slowly replaced by propylene oxide and the material was embedded in Epon. Ultrathin sections were stained for 30 minutes with 5% uranyl acetate at room temperature, in some cases followed by lead citrate staining according to Reynolds (1963). The sections were observed with a JEOL JEM-1200 transmission electron microscope.

Cytochemistry

For DNA staining the NAMA method was applied en bloc. The material was immersed overnight in 4% formaldehyde dissolved in 0.5 N NaOH, washed in water, treated 3 times one min with 1% acetic acid and then washed in water (NA-treatment). Afterwards the samples were dehydrated 15 min in 70 % methanol and 3 times 15 min in 100% methanol, immersed overnight in a freshly prepared methanol : acetic anhydride solution (5:1) followed by wash steps in water (MA-treatment). Some samples were only treated according the MA method, preferentially staining the nucleic acids and phosphorylated proteins.

The EDTA regressive staining was performed on ultrathin sections collected on copper grids. The sections were stained with 5% uranyl acetate for 30 min at room temperature, rinsed in water and immersed in 0.2 M NaEDTA for 3-5 min, rinsed in water and in some cases stained with lead citrate.

High resolution in situ hybridization

Material for *in situ* hybridization was fixed in 3% paraformaldehyde with 0.25% glutaraldehyde in PBS (140 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.4). After fixation the material was rinsed in PBS, dehydrated in an ethanol series, embedded in LR White hard grade (London Resin Company Ltd.,

Hampshire, UK), using 0.5% benzoinmethylether as a hardener, and polymerised under UV at -20 °C.

For *in situ* hybridization the entire 18S rDNA repeat (1000 bp) of *Pisum sativum* (Jorgensen et al., 1987; kindly provided by Dr. G. McFadden, The University of Melbourne, Australia) was subcloned in the pBluescript KS plasmid. An 18S DIG-rRNA labeled probe was synthesized using the DIG-RNA labelling kit (Boehringer, Mannheim).

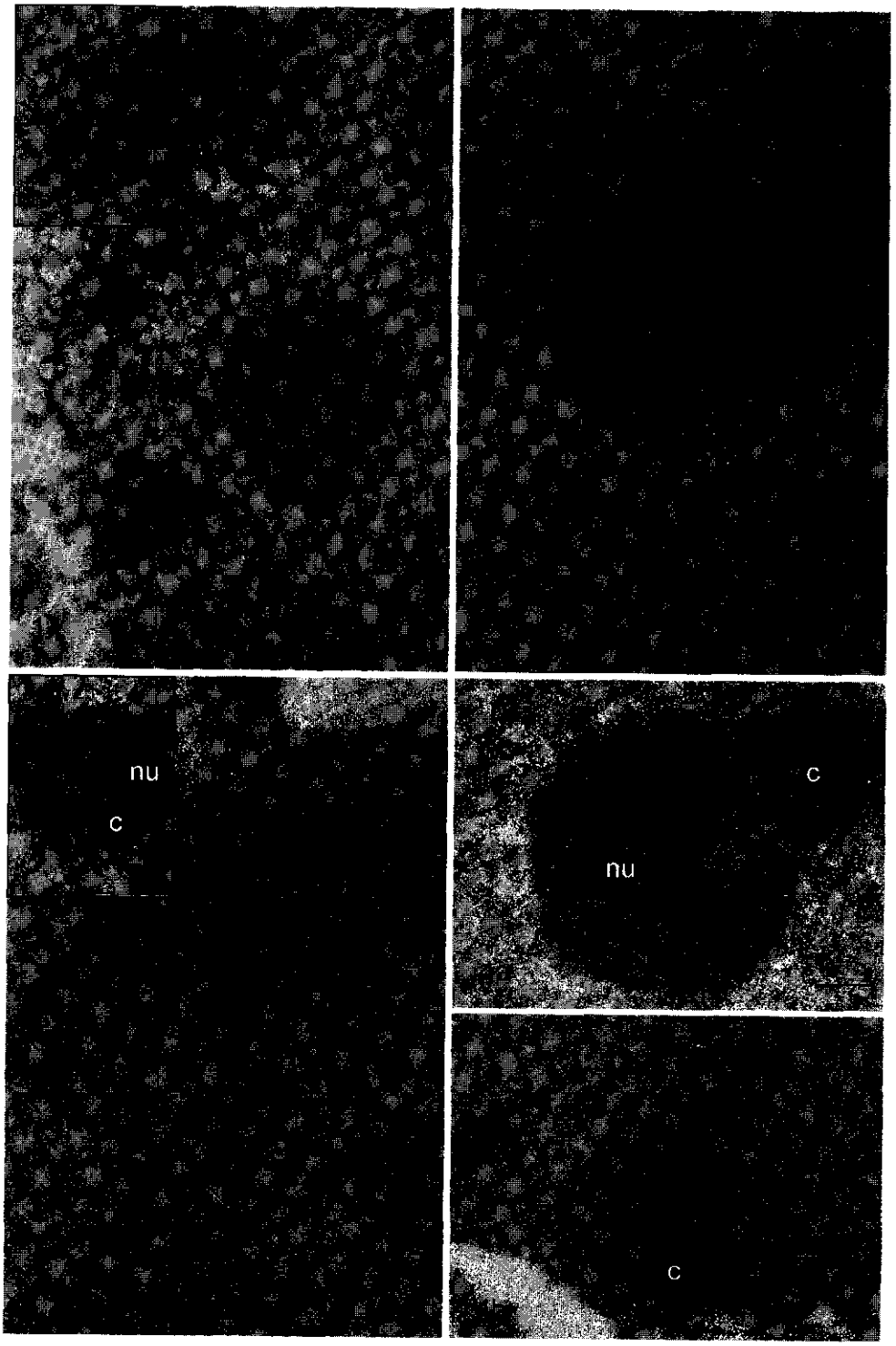
Ultrathin sections were treated for 2h with 2 µg/ml proteinase K at 37 °C, washed in water and hybridized overnight on 3 µl droplets of hybridization buffer containing the probe (50% formamide, 10 mM Tris, 200 mM NaCl, 5 mM $MgCl_2$, 5mM EDTA, 20 U/ml RNasine (Promega, Madison, WI, U.S.A), 5 µg/ml poly A, 5*Denhard) at 50 °C. Next morning the grids were washed 4xSSC, 2xSSC followed by 2h 1xSSC at 50 °C (1xSSC: 0.15 M sodium chloride, 15 mM sodium citrate, pH 7.0). Aspecific binding was blocked by 1% BSA + 0.2% Tween 20 (Sigma) in PBS, followed by detection of the hybridized DIG-labelled probe by anti-DIG 6nm gold (Aurion, Wageningen, The Netherlands), diluted 1:40 in 0.2% acetylated BSA (BSAc; Aurion) + 0.2 % Tween 20 in PBS, for 1h at 37 °C. The grids were washed 4 times in 0.2% BSAc + 0.2 % Tween 20 in PBS and 4 times in PBS. To stabilize the protein-gold complex the sections were fixed for 10 min in 2% glutaraldehyde and washed several times in water. The gold label was enhanced using the silver enhancement method developed by Danscher (1981).

Results

The nuclei of young microspores and the vegetative nuclei of young pollen showed an asymmetrical morphology with one nucleolus which was positioned laterally towards the nearby exine wall. Before condensation of the chromatin in the generative nucleus, the nucleolus was situated in the center. During pollen development only one nucleolus per nucleus was found.

Young microspores

In young microspores shortly after their release from the tetrads, the largest diameter found of the nuclei was about 5 µm and the largest found nucleoli measured 1 µm in diameter. Areas of heterochromatin were mostly found at the nuclear periphery. These clusters of DNA were in general smaller than 1 µm (Fig. 1). Most of the chromatin showed a decondensed pattern forming a dense network throughout the nucleoplasm. Using the EDTA-regressive staining technique, a fine distribution of RNPs was found in between the bleached chromatin (Fig. 2).


nu
c
c
nu
c

In several nuclei polycomplexes (PCs) were found. These structures are stacks of synaptonemal complex fragments consisting of an irregular formed region of lateral elements, a central element consisting of five parallel filamentous units, three in the center and at both sides a single one, and transverse filaments perpendicular to the lateral and central elements (Fuge, 1979). The distance between the lateral elements was about 150 nm (Fig. 3). In several cases the PCs were associated to the nuclear membrane and they were often found in close relation to small pieces of heterochromatin, the nucleolus or a nuclear body. Frequently, the central elements and transverse filaments continued from the PCs into the nucleoplasm.

The nucleoli consisted of a dense fibrillar component, showing a compact structure. After the NAMA treatment to stain specific the DNA, no dark coloring was found in the nucleolus (Fig. 1) indicating that there is no detectable amount of DNA in the nucleolus. In several microspores a nucleolar organizer region (NOR), often referred to as the fibrillar centers in interphase nuclei, is situated at the periphery of the nucleolus. The NOR was mostly positioned at the side of the nucleolus which was most close to the exine wall.

Attached to the NOR a piece of condensed chromatin could be seen (Fig. 4a). After MA and EDTA treatment the chromatin was bleached whereas the light stained region of the NOR remained unchanged (Fig. 2). After the NAMA-treatment, the chromatin was electron dense whereas the nucleolus was less electron dense. The lightly stained NOR seemed unaffected by the NAMA treatment (Fig. 4b).

Figs. 1-4; Nuclei of young microspores of *Brassica napus* post-stained with uranyl acetate

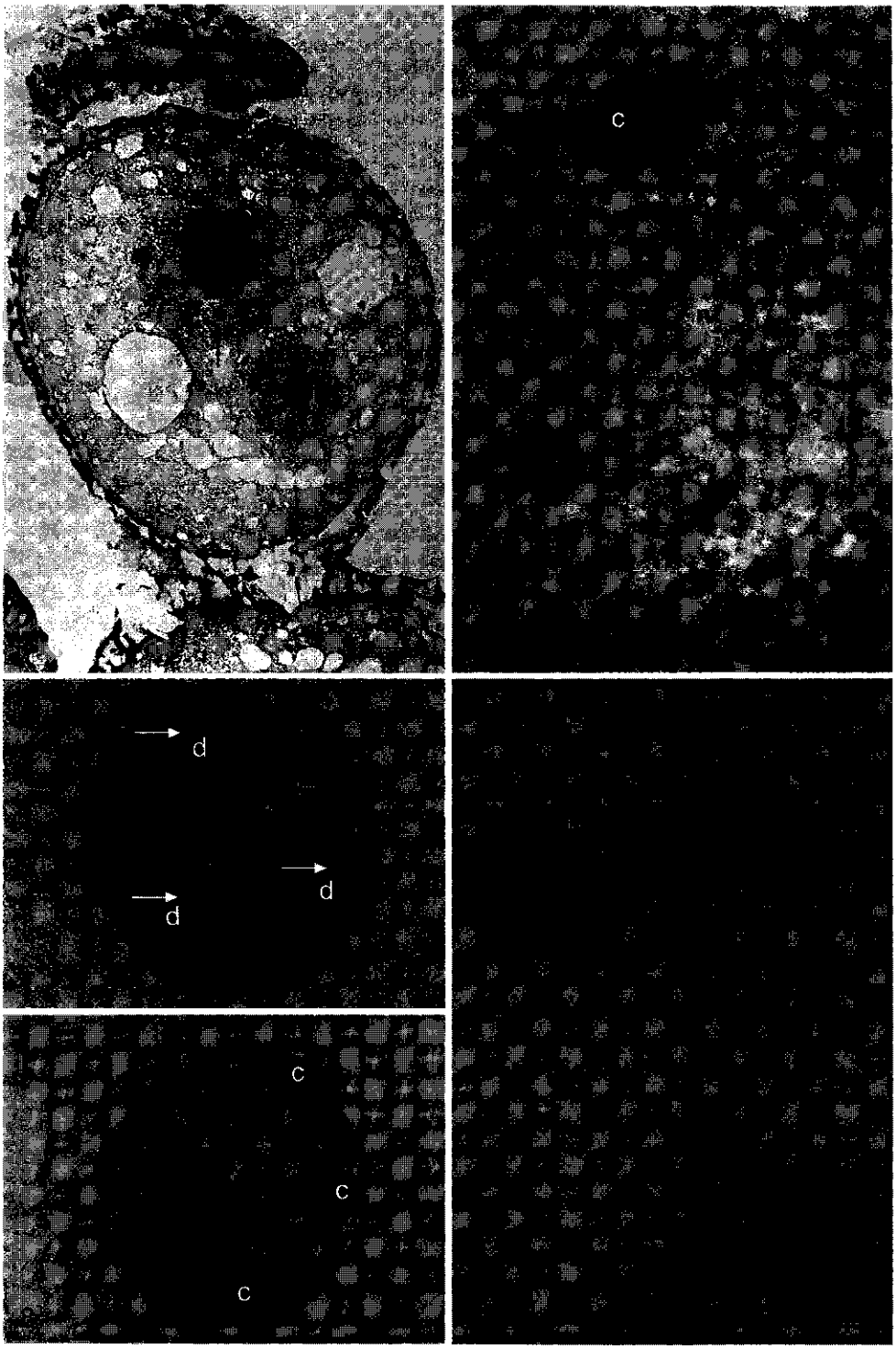
Fig. 1; The nucleus of a young microspore after the NAMA treatment to visualize the DNA. Some small clusters of condensed DNA can be seen near the nuclear periphery. The nucleolus (nu) consists of a dense fibrillar component. The inset shows an overview of this nucleus.

Fig. 2; The nucleus of a young microspore after MA treatment and EDTA regressive staining. RNPs (arrows) can be found in between bleached DNA areas. Attached to the nucleolus a NOR can be found (*), together with a piece of condensed chromatin (c).

Fig. 3; PC in the nucleus of a young microspore, not cytochemically treated. Note the close relation of the PC to the nuclear membrane and the heterochromatin (arrow). The substructure of the PC can clearly be seen. ce, central element; le, lateral element; arrowhead, transverse filament. The inset shows an overview of this nucleus with a nucleolus (nu), condensed chromatin (c) and the PC (arrow).

Fig. 4; A detail of the NOR (*) after MA-treatment (Fig. 4a) and after NAMA treatment (Fig. 4b) with attached chromatin (c). It is clear that the nucleolus (nu) only contains a dense fibrillar component.

Bars: 200 nm, 900 nm (insets) .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d
d
d
c
c
c

Bicellular pollen

After the microspore mitosis, an unequal cell division, a large vegetative cell with a smaller generative cell is formed. At first, the generative cell remains attached to the intine wall. However, most samples contained pollen in the middle bicellular stage, when the generative cell has migrated towards the center of the vegetative cell.

In the vegetative cell the largest diameter of the nucleus was 9 µm and of the nucleolus 5 µm. Small pieces of condensed DNA were mostly observed at the nuclear periphery (Fig. 5). The euchromatin had a more woolly appearance than in the young microspores (compare Fig. 6 with Fig. 1). The nucleolus showed a morphology of high activity with a large granular rim and granules around the dense fibrillar components, fibrillar centers in the dense fibrillar components and nucleolar vacuoles (Fig. 7).

In the generative nucleus the degree of condensation of the chromatin was higher than in the vegetative nucleus and also more areas of condensed chromatin were found. The heterochromatin was found not only at the nuclear periphery but also in the nuclear center (Fig. 8). The generative nucleus was smaller in diameter than the vegetative nucleus, 5 µm for the nucleus itself and maximal 2 µm for the nucleolus. The nucleolus of the generative nucleus was more compact than the nucleolus of the vegetative nucleus and hardly showed granular structures. In early bicellular pollen after *in situ* hybridization, using the rRNA-probe, labelling was found in both nucleoli (Fig. 9).

Figs. 5-9; Nuclei of bicellular pollen of *Brassica napus* treated with EDTA regressive staining or NAMA treatment and after *in situ* hybridization using a ribosomal probe.

Fig. 5; A bicellular pollen after NAMA-treatment, stained with uranyl acetate and lead citrate. The DNA in the vegetative nucleus (v) is highly decondensed whereas the DNA in the smaller generative nucleus (g) shows a more condensed structure. The nucleolus of the vegetative nucleus shows a large granular rim. In both nuclei a coiled body is visible (arrows).

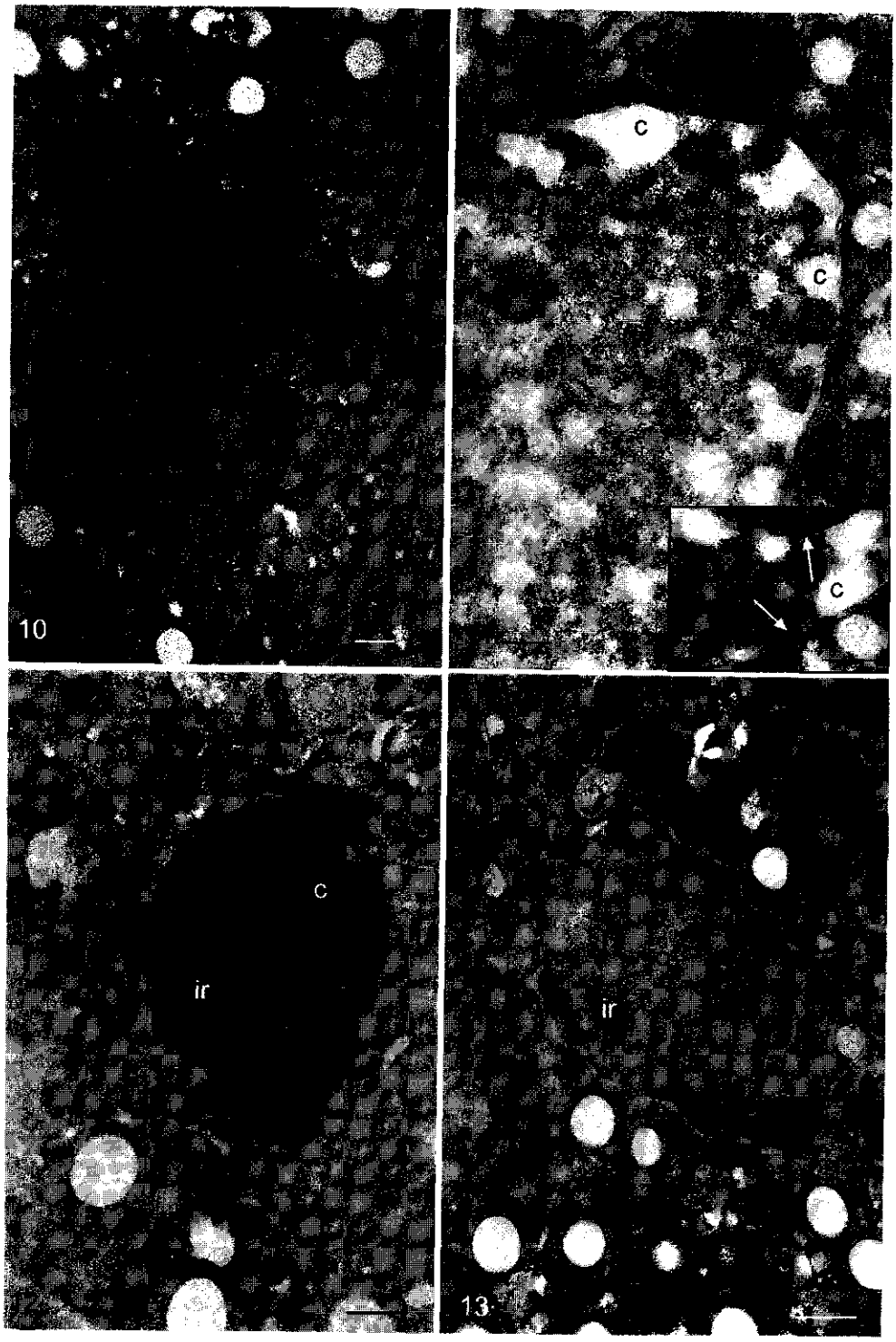
Fig. 6; The nucleoplasm of the vegetative nucleus of bicellular pollen after NAMA-treatment followed by uranyl acetate. cyt, cytoplasm; n, nucleus; c, condensed chromatin.

Fig. 7; The nucleolus of a vegetative nucleus of bicellular pollen with the appearance of high activity after EDTA treatment. Large areas of granular components, a nucleolar vacuole (v) and dense fibrillar components (d) with fibrillar centers (arrows) can be seen. The nucleoplasm shows a fine distribution of RNPs.

Fig. 8; The generative nucleus after NAMA treatment showed a higher degree of chromatin condensation, heterochromatin (c) and a more compact nucleolus (nu) as compared with the vegetative nucleolus. A coiled body (*) is attached to the nucleolar surface.

Fig. 9; *In situ* hybridization using a ribosomal probe clearly showed gold labelling in both nucleoli of the generative (g) and vegetative (v) nucleus of early bicellular pollen. The nucleoplasm remained almost negative but label was found in the cytoplasm.

Bars: 2 µm (5), 200 nm (6), 500 nm (7, 8), 1 µm (9).


10
c
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c
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ir
ir
13

In both the vegetative and the generative nucleus often a nuclear body was found. This body was mostly linked to the nucleolus, although in some cases it was found free in the nucleoplasm. This nuclear body was identified by its structure, appearance and bleaching after EDTA-treatment as a coiled body (Figs. 5 and 8).

Mature pollen

The vegetative nucleus of mature pollen showed a multi-lobed appearance. Therefore, no maximal diameter size could be given. A nucleolus was not found in this stage. Small condensed areas of chromatin were observed in close relation to the nuclear membrane. Often nuclear pore complexes were seen in between these heterochromatin areas (Fig. 11, inset). The center of the nucleus only contained a very small amount of euchromatin (Fig. 10, 11). In some vegetative nuclei condensed DNA was found more inside the nucleus. This is probably the result of heterochromatin near the nuclear periphery of the lobed nuclear membrane, which was tangentially cut. The EDTA treatment revealed that there were still RNPs in the IR of the vegetative nucleus, but the IR was only partly filled with it (Fig. 11).

In mature pollen the shape of the sperm nuclei had become elongated and the diameter in transverse section was small, about 2.5 µm. The DNA was highly condensed and a nucleolus was not found. Using the NAMA-method, large areas of IR became visible in between the condensed chromatin (Fig. 12). A difference in DNA staining was found between different sperm nuclei on the same grid using only uranyl acetate staining. In some nuclei DNA stained with uranyl acetate whereas in other nuclei the DNA remained almost unstained (Fig. 13). In nuclei with unstained DNA, the IR regions seemed to be smaller.

Discussion

Three stages of pollen development of *B. napus* were analyzed to characterize nuclear developmental changes. The NAMA method has been reported to be specific for DNA (Testillano et al., 1991, 1995b) and in this study we used this

Figs. 10-13; The vegetative and sperm nuclei of mature pollen of *Brassica napus*.
Figs. 10, 11; The highly lobed vegetative nucleus of mature pollen. After NAMA treatment (Fig. 10) condensed DNA is found near the nuclear envelope. The nuclear center hardly showed labelling for DNA. EDTA treatment (Fig. 11) clearly reveals condensed chromatin at the nuclear periphery (c) and the presence of RNPs (arrows). The inset shows nuclear pore complexes (arrows) in between areas with condensed chromatin.
Figs. 12, 13; The highly condensed sperm nucleus of mature pollen after NAMA treatment (Fig. 12), showing large areas of condensed chromatin (c) and interchromatin regions (ir), and after staining with uranyl acetate and lead citrate without a cytochemical pre-treatment (Fig. 13). In this case, the condensed DNA is much less stained by uranyl acetate.
Bars: 500 nm (10, 12, 13), 200 nm (11).

method as an indicator for nuclear transcriptional activity considering the presence of euchromatin and heterochromatin. The EDTA regressive staining was used to stain preferentially the RNPs to distinguish between the IR and chromatin (Testillano et al., 1993). Besides this, the RNP staining can also provide more information about the nucleolar structure (Risueño and Testillano, 1994; Testillano et al., 1995a).

The NAMA-method showed in young microspores and bicellular pollen DNA with relatively large areas of IR. Using the EDTA staining a finer distribution of RNPs was found, which does not match with the IR seen after the DNA staining. This phenomenon was also described by Testillano et al. (1993). They concluded that the major part of the RNPs was found in the IR not close to the condensed chromatin. However, our results give the impression that RNPs were present in the IR close as well as not close to the periphery of condensed chromatin.

Nuclei of young microspores showed a lot of euchromatin what could indicate a high level of transcription. However, there were still PCs and NORs found in the nucleoplasm. PCs are thought to be proteins of the synaptonemal complexes (SC) during meiosis, which are not bound to DNA anymore but are now bound together in a repetitive structure. In the literature, these structures are often described to appear prior to zygotene or after pachytene in the nucleus or cytoplasm of meiotic and germ-line derived tissues (Fuge, 1979; Goldstein, 1987; Grandi et al., 1994). Sometimes, they were found in the cytoplasm before the breakdown of the nuclear membrane or after reforming of the nuclear membrane (Bogdanov, 1977; Grandi et al., 1994). We found them in interphase nuclei of microspores, after the meiosis had been fully completed, as was also reported for *Orthoptera* species (Wolf and Mesa, 1993). This would imply that the nucleus is not yet completely rebuilt after meiosis. Furthermore, lateral PC elements could be observed in the nucleoplasm outside the PCs, but connected with the lateral PC elements in the PCs. This might indicate that the PCs play a role in the formation of the nuclear matrix. There has been reported some evidence for this. In some interphase nuclei without PC or SC, overexpression of a protein involved in the formation of SC and PC showed a nuclear network (Sym and Roeder, 1995). On the other hand, the finding of PCs in the cytoplasm, after formation of new nuclei (Grandi et al., 1994) makes it very difficult to give an interpretation of this phenomenon.

Another indication for a low transcriptional activity in these nuclei after meiosis is the observation that the nucleolus only consisted of a dense fibrillar component and still had an attached NOR. In general this is a sign for a quiescent nucleolus and it is often found during microspore maturation (Risueño and Medina, 1986). Above results, together with the relative small size of the nucleus and the nucleolus, indicate that after meiosis the transcription of mRNA and rRNA in nuclei of *B. napus* microspores is low. This suggests that a part of the RNAs, necessary for early microspore development, is produced before the tetrad stage.

After the microspore mitosis the vegetative nucleus had the appearance of a highly active nucleus. Both the nucleus and nucleolus were very large and the nucleoplasm hardly contained heterochromatin. In the nucleolus a high amount of granular components was found, the pre-ribosomal particles, indicating a high nucleolar activity. Also the appearance of a nucleolar vacuole is in general regarded as a sign for a high nucleolar activity (Risueño and Testillano, 1994). In this stage, the vegetative cell prepares itself for its future function. The vegetative cytoplasm contains a high density of organelles and membranes. After pollen maturation the vegetative cell is thought to be totally prepared for pollen germination. This process requires a high nuclear activity.

Although in middle bicellular pollen stages the generative nuclei gave the impression of low transcriptional activity, in earlier bicellular stages evidence was found for normal nucleolar activity. By use of *in situ* hybridization rRNA transcripts were found in the nucleolus of the generative cell, indicating that at least shortly after the microspore mitosis the generative nucleolus was transcriptionally active. Others have concluded that the generative nucleus of *Brassica napus* has to be considered as inactive (Murgia et al., 1991), but we found that, until the generative cell is released from the intine wall, the generative nucleus has a highly active appearance, containing a lot of euchromatin and an active nucleolus. Furthermore, in bicellular pollen many generative nuclei with a coiled body in the nucleoplasm were observed. These coiled bodies are generally found in transcriptionally active nuclei (Carmo-Fonseca et al., 1992; Andrade et al., 1993). Also the presence of a high amount of RNPs indicates that the generative nucleus is highly transcriptionally active. During migration of the generative cell, the chromatin in the nucleoplasm becomes more condensed, probably in preparation for the pollen mitosis.

The samples of mature pollen were taken just after anthesis. Pollen of this stage is dehydrated and the cytoplasm contains stacks of RER, lipids, ribosomes, mitochondria and many other small vesicles. The vegetative nucleus had a very large nuclear surface as a result of a lobed nuclear membrane. Often a large surface membrane is seen when structures need a high exchange with their surrounding. However, most of the DNA is found as condensed chromatin near the membrane and no nucleolus was found, indicative for a low nuclear activity. Furthermore, dehydrated mature pollen is thought to be transcriptionally inactive (Mascarenhas, 1975). The large nuclear surface of the lobed nucleus could therefore be explained as the remainder of previous high nuclear activity. Alternatively, it could also be the preparation for a required high nuclear activity during pollen germination but the general idea is that the vegetative cell is "ready" for pollen tube growth (Mascarenhas, 1975; Capkova et al., 1988; Frova, 1990), indicating that the first explanation has to be preferred.

The size, morphology and heterochromatin pattern completely differs between the sperm nucleus and the vegetative nucleus, although both nuclei are considered

to be transcriptionally inactive. The sperm nucleus has highly condensed chromatin in very large clusters, indicating transcriptionally inactive DNA. This is in agreement with the idea that sperm cell nuclei are arrested in late telophase (Gerassimova-Navashina, 1961; Palevitz and Cresti, 1989; Murgia et al., 1991). During mitosis there is no RNA synthesis, resulting in transcriptionally inactive sperm cell nuclei. The vegetative nucleus is silenced as a result of pollen maturation and shows condensed chromatin at the nuclear membrane, probably at places where chromatin was attached to this membrane as euchromatin in earlier stages.

The chromatin of the sperm cell nuclei was not always stained with uranyl acetate. After the NAMA treatment, the chromatin was always heavily stained, indicating that the poorly stained chromatin after only uranyl acetate treatment, is caused by penetration problems, due to a high grade of condensation of the DNA or changes in proteins involved in package of DNA during maturation of the pollen. NAMA treatment probably changes these proteins too, so that the binding sites for uranyl acetate are more attainable (Hayat, 1993)

In conclusion, the structural organization of microspore and pollen nuclei could be related to their activity and the developmental stage. This approach, giving information about the location of nuclear components, can be useful to study developmental processes in more detail, also in other plant systems.

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Chapter 3

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

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Summary

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, we concluded that microspores are inducible to form embryos from G1 till G2 phase. When bicellular pollen were cultured at 18 °C, they exhibited labelling exclusively 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.

Keywords: *Brassica napus*; bromodeoxyuridine; 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 on microspores and pollen at various developmental stages have shown that discrete developmental windows exist 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 can be 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* has been studied in detail (Zaki and Dickinson, 1990, 1991), but 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). This method has been successfully applied in 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.

Materials 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 $2x10^4$ 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_4Cl 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 37 °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.

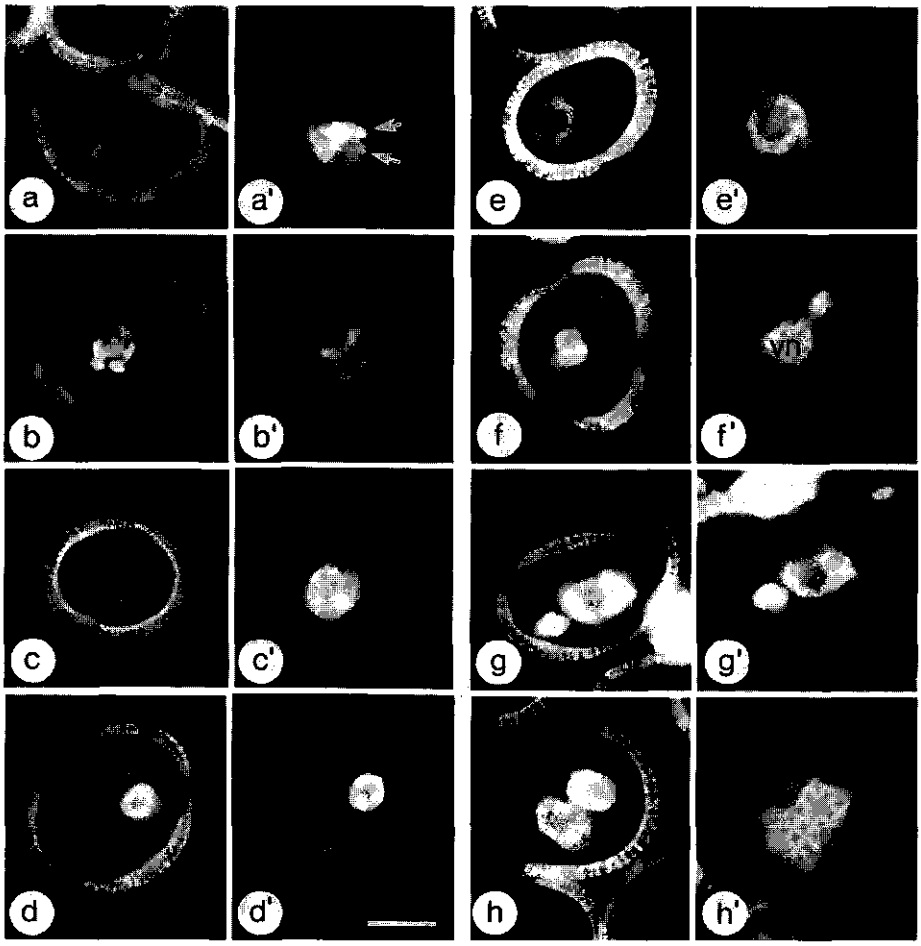
Microspectrophotometry

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 Lillie (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 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 and embryogenicity of the culture, but a continuous BrdU labelling during 24h caused a 10-20% decrease in viability and 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%.

Under 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.

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;

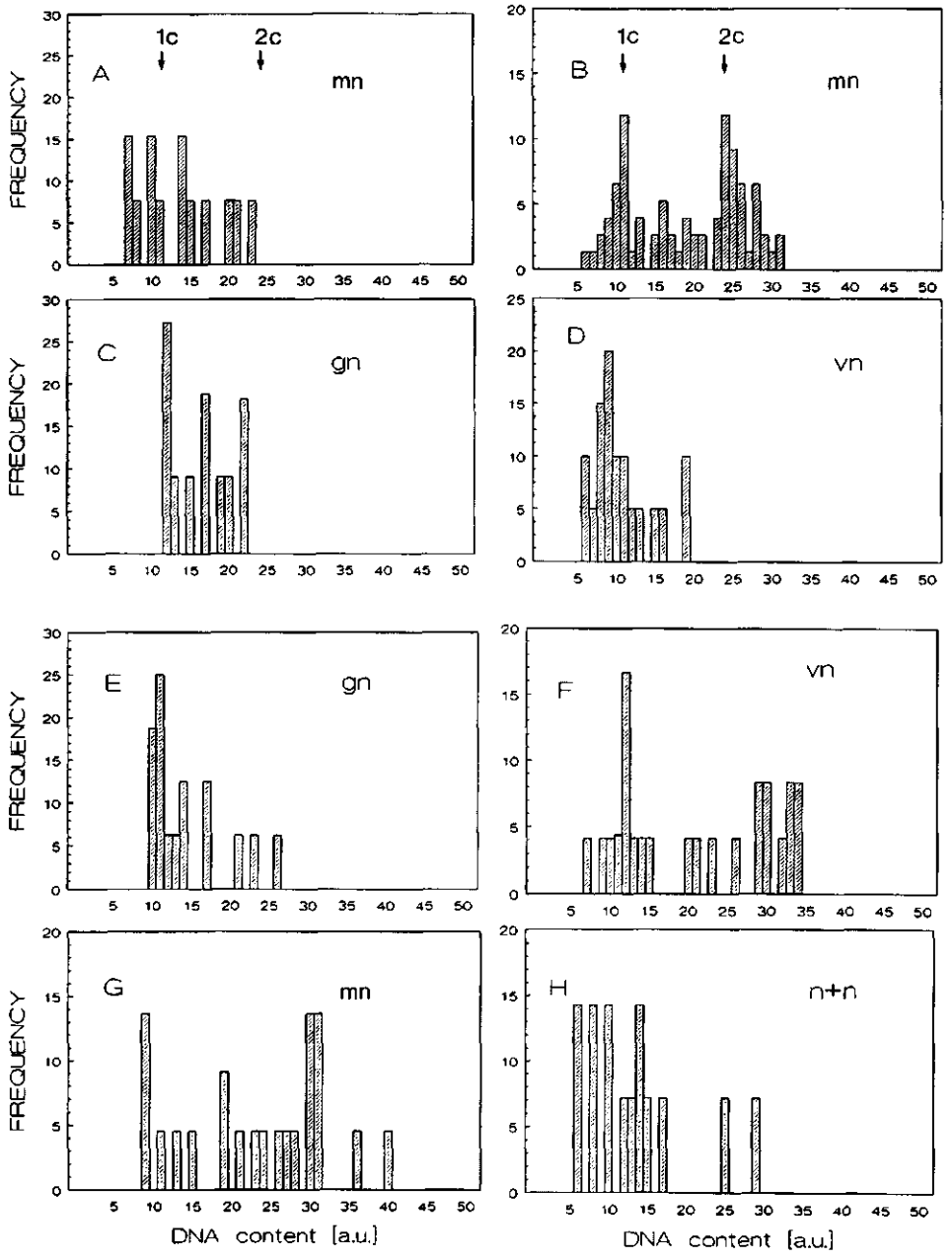
d-d' pollen from Type B culture, grown at 18 °C for 4 h with BrdU, exhibits labelling in the generative nucleus only;

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 3 °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 a 1-h BrdU pulse.


A
1c
2c
mn
B
1c
2c
mn
C
gn
D
vn
E
gn
F
vn
G
mn
H
n+n
FREQUENCY
DNA content [a.u.]

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.

Under 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 observed as early as after 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

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 *Brassica 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	MI	EB	MB+LB	Σ
after isolation	24	74	-	2	-	100
24 h in cult. at 32 °C	4.3	77.7	-	18	-	100

culture under non-embryogenic conditions (18 °C)								
	% labelled cells							T
period of labelling	LMs	MI	BC				Σ	
			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 under embryogenic conditions (32 °C)								
	% labelled cells							T
period of labelling	LMs	MI	BC				Σ	
			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; MI, 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 *Brassica 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	MI	EB	MB+LB	Σ
after isolation	3.5	26	7.5	53	10	100
24 h in cult. At 32 °C	1	17	0	24	58	100

culture under non-embryogenic conditions (18 °C)

	% labelled cells							T
period of labelling	LMs	MI	BC				Σ	
			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	-	-	1.0	-	-	-	1.0	287

culture under embryogenic conditions (32 °C)

	% labelled cells							T
period of labelling	LMs	MI	BC				Σ	
			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

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 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, 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.

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Chapter 4

Fluorescent labelling of nascent RNA reveals nuclear transcription domains throughout plant cell nuclei

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Summary

Transcription and RNA processing are main functions of the nucleus. These processes are found localized in specific nuclear domains. We have investigated the presence of nuclear transcription domains in plants by incorporating 5-bromouridine 5'-triphosphate in nascent RNA in isolated nuclei of *Brassica napus*. Bromo-uridine labelled RNA was visualized by a FITC-labelled biotin-avidin system in combination with confocal laser scanning microscopy. Labelled domains were found throughout the nucleus, in some cases including the nucleolus. This shows that the distribution of transcription sites in plant nuclei is similar to that of mammalian nuclei and that the same labelling procedures can be used.

Keywords: *Brassica napus*; 5-bromouridine 5'-triphosphate incorporation; confocal scanning laser microscopy; immunolabelling; isolated nuclei; transcription sites.

Introduction

In the interphase nucleus RNA synthesis, RNA processing and RNA transport are important processes. Three RNA polymerases are involved in the transcription process, termed RNA polymerase I (RPI), RNA polymerase II (RPII) and RNA polymerase III (RPIII). RPI synthesizes the 45S ribosomal pre-RNA in the nucleolus. RPII and RPIII are active in the nucleoplasm. RPII synthesizes the heterogeneous nuclear RNA (hnRNA) and most of the small nuclear RNAs (snRNA). RPIII synthesizes several small RNAs, like 5S ribosomal RNA and transfer RNA (Sentenac, 1985).

In recent years it has become clear that the nucleus is a highly organized organelle and that several steps in the metabolism of RNA are localized in specific nuclear domains (Stuurman et al., 1992; Testillano et al., 1993; reviewed by Spector, 1993). Also, tracks and foci of specific RNAs have been reported (Lawrence et al., 1989; Huang and Spector, 1991; Raap et al., 1991; Dirks et al., 1995). The most conspicuous nuclear compartment is the nucleolus. Other nuclear domains can be visualized only after, e.g., immunolabelling (see Testillano et al., 1993; Risueño and Testillano, 1994; Beven et al., 1995).

One step towards understanding the functional organization of the interphase nucleus is the *in situ* visualization of the sites of active chromatin, i.e., sites at which nascent RNA is found. In mammalian cells, these sites of transcription were labelled with fluorescent probes after incorporation of the UTP-analogue 5-bromouridine 5'-triphosphate (BrUTP) into nascent RNA in run-on experiments. This was done with permeabilized and microinjected human bladder carcinoma cells and human skin fibroblasts (Wansink et al., 1993), with permeabilized HeLa cells (Jackson et al., 1993) and with nuclear spreads of monkey kidney cells (Garcia-Blanco et al., 1995). After permeabilization of the cells, these *in vitro* systems continue the transcription processes that already had been initiated *in vivo*.

In plants, less is known about the structural organization of RNA processing. Recently, Sjödahl et al. (1993) have incorporated ^{32}P-UTP into nascent RNA of isolated nuclei of *Brassica napus* seeds in order to identify the synthesis of transcripts belonging to three cruciferin gene families. This, however, does not allow a precise localization of the sites of transcription because of the long path-length of the ß-radiation. Therefore, we decided to use the BrUTP incorporation to visualize nascent RNA in plant cells.

This study is part of a larger project on nuclear activity during plant embryogenesis, in which embryogenic microspore cultures of *B. napus* are used (see, e.g., B. Hause et al., 1993; G. Hause et al., 1995). In earlier experiments we were able to detect DNA synthesis in cells from microspore cultures using bromodeoxyuridine (BrdU) (Binarova et al., 1993), but we did not succeed to detect incorporated BrU in nascent RNA in the nucleoplasm, using permeabilized cells from both microspore

cultures and root tips of *B. napus*. In all mammalian cells studied so far for BrUTP incorporation, permeabilization of the membrane or microinjection of BrUTP proved to be necessary, because nucleotide triphosphates cannot cross the cell membrane. For plant cells, the cell wall forms an extra barrier. To investigate whether the BrUTP incorporation method can be used in plant systems, we decided to use isolated nuclei.

Materials and methods

Isolation of nuclei

Plants of *Brassica napus* cv. Topas were grown in the greenhouse at 18-23 °C. Young leaves were harvested and nuclei were isolated at 4 °C according to the protocol of Gustavsson et al. (1991) with some minor changes. The leaves were homogenized in 30 ml of MG-buffer (50% glycerol (Merck, Darmstadt, Federal Republic of Germany), 25 mM morpholinoethane sulfonic acid (MES, pH 6.0), 250 mM sucrose, 5.0 mM $MgCl_2$, 1 mM 1,4 dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM EDTA) in a mortar. The homogenate was filtered through an 80 HD filter and centrifuged in 15 ml M-buffer (as MG buffer, but without glycerol and with 750 mM sucrose) for 30 minutes in a HB4 swing-out rotor at 6000 g. The pellet was resuspended in 5.0 ml M-buffer containing 0.4% Triton X-100 (BDH, Poole, England), layered on a 40%/80% Percoll gradient and centrifuged for 30 min at 6000 g. The nuclei were harvested from the gradient and washed twice in HG-buffer (as MG-buffer, but with 10 mM HEPES instead of 25 mM MES). During the first wash, 0.2% Triton X-100 was added. The nuclei were stored at -20 °C in HG-buffer.

BrUTP incorporation

The protocol for BrUTP incorporation was according to Wansink et al. (1993), adapted for plant nuclei with the transcription buffer according to Sjödahl et al. (1993). A drop of HG-buffer containing isolated nuclei was put on a aminopropyl-triethoxy-silane-coated slide (Sigma, Chemical Co., St Louis, MO, USA). The nuclei were incubated for 3 min in glycerol buffer (25% glycerol, 20 mM Tris(hydroxymethyl)-aminomethane (Tris)/HCl, pH 7.4, 5 mM $MgCl_2$, 0.5 mM EGTA) containing 1 mM PMSF followed by a 3 min incubation in glycerol buffer containing 0.05% Triton X-100, 1 mM PMSF and 20 U/ml RNasin (Promega, Madison, WI, U.S.A).

Run-on transcription was performed in transcription buffer (20 mM N-[2-hydroxyethyl]piperazine-N'-[2 ethanesulfonic acid] HEPES, pH 8.0, 12 mM $MgCl_2$, 200 mM $(NH_4)_2SO_4$, 50 mM KCL, 100 µg/ml tRNA, 100 U RNasin/100 µl, 0.5 mM ATP, CTP, BrUTP (Sigma) and GTP (Boehringer, Mannheim, Federal Republic of Germany) each, for 45 min at 37 °C. Subsequently, the nuclei were washed 3 min in TBS (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$) containing 0.05% Triton X-100, 1 mM PMSF and 6.6 U/ml RNasin, followed by a 3 min wash in TBS containing 1

mM PMSF and 6.6 U/ml RNasin. In control experiments ∝-amanitin (10 µg/ml; Sigma) or actinomycin-D (0.2 µg/ml; Sigma) was included until the wash steps. Also, BrUTP was replaced by UTP (Boehringer) to test the specificity of the first antibody.

Fixation and immunocytochemistry

Nuclei were fixed in 2% freshly prepared formaldehyde in PBS (140 mM NaCl, 2.7 mM KCl, 6.5 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4; pH 7.4) for 15 min at room temperature followed by 2 x 5 min PBS wash steps. After this, the nuclei were treated with 0.5% Triton X-100 in PBS and rinsed in PBS. The slides were blocked as follows: 10 min 100 mM glycine in PBS, 20 min 2% acetylated BSA (Aurion, Wageningen, The Netherlands) in PBS and 5 min PBG (0.5% BSA, 0.1% gelatin from cold water fish skin in PBS). The nuclei were incubated overnight at 4 °C with a mouse monoclonal antibody against BrdU (1:500 in PBG; Sigma), washed four times 5 min in PBG, incubated with a biotin conjugated goat anti-mouse IgG (H+L) (1:3000 in PBG; Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.), washed four times 5 min in PBG, incubated for 45 min at 37 °C with ExtrAvidin-FITC (1:1500 in PBG; Sigma) and washed twice 5 min in PBS. Nuclei were incubated for 10 min in 0.1 µg/ml propidium iodide in PBS to stain the DNA. Finally the slides were mounted in Citifluor in glycerol (Citifluor UKC, Chem. Lab., Canterbury, U.K.).

In a control experiment the slides were treated after fixation with 1 mg/ml RNase A (Boehringer) in STE buffer (10 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA) for 2 h at 37 °C, washed in STE and blocked as written above.

Confocal scanning laser microscopy (CSLM)

Images of double labelled nuclei were recorded with an MRC 600 Bio-Rad using an argon-krypton ion laser attached to a Nikon Diaphot inverted microscope equipped with a 100x/1.3 N.A. oil immersion lens. The images were collected with a pinhole setting at 6 units (about 3 mm), a K1/K2 filter combination and a Kalman filter (about 64 scans per image), either simultaneously in one scan or separately in two scans. In the last case the FITC scan was made first.

Processing of images

Images were processed with the Comos software package (Bio-Rad, Veenendaal, The Netherlands). The contrast was modified by contrast stretch and the 3 x 3 smooth filter. The background was measured in the images and subtracted (mean - 2 x S.D.) from the original signal.

To keep the signals of the scans of the controls in the same value as the positive labelled nuclei, they were scaled 16 times before the background was subtracted. This is the average factor with which the BrUTP incorporated nuclei were stretched. Prints were made on a Kodak XLS 8600 printer.

Results and discussion

To study the spatial distribution of transcription domains in the interphase nuclei of *Brassica napus*, an immunocytochemical technique was used to visualize newly synthesized RNA after incorporation of BrUTP. To detect incorporated BrU, an antibody that specifically recognizes BrU and BrdU was used (Jackson et al., 1993; Wansink et al., 1993). The sites of BrU incorporation were visualized by using a secondary antibody that was conjugated with FITC. In preliminary experiments only a faint positive labelling was found in the nucleolus of a few microspores in culture after incorporation of BrU but there was never a positive signal in the nucleoplasm. The labelling with antibodies was done on 2 µm polyethylene glycol coupes (not shown). This gave the indication that there were penetration problems, either from the BrUTP or from the antibodies or that BrUTP or BrU-labelled RNA was broken down by enzymes. Therefore BrUTP incorporation experiments were done on isolated nuclei from leaves of *B. napus*.

After incorporation and immunolabelling of BrU in nascent RNA of these isolated nuclei, a typical punctate labelling pattern was observed throughout the nucleus (Fig. 1a). In some nuclei the nucleolus was also labelled (Fig. 1b). Labelling in the nucleolus gave always a brighter signal than the domains in the nucleoplasm. These brighter labelled domains in the nucleolus might be caused by the fact that ribosomal genes belong to the most actively transcribed genes in the nucleus and because they are organized in tandemly repeated units. The fact that most nucleoli were not labelled might be due to penetration problems of the antibodies into the nucleoli. The results are in agreement with observations by Wansink et al. (1993) on human skin fibroblasts and Jackson et al. (1993) on HeLa cells. These authors have also shown that only a limited amount of BrU was incorporated in nascent RNA before the transcription stopped. Most of the RNA, in that case, was still connected with RNA polymerase or the underlying nuclear matrix structure. Wansink et al. (1993) further showed that the punctated pattern distribution remained unchanged after a longer period of BrUTP incorporation. There was only an increase in labelling intensity. Therefore, nuclear domains that are rich in BrU labelled nascent RNA are in majority sites of transcription. However, the possibility that some Br-labelled RNA is diffused or transported from the sites of transcription, producing a faint label, can not be excluded. A striking difference in the intensity of labelling of the various spots in a nucleus was often found. This difference in labelling intensity may have several reasons. The spatial resolution of this light microscopical study is not sufficiently sensitive to distinguish between single gene products when several active genes are situated close to each other or when a gene is transcribed by several RNA polymerases, as was shown in electron micrographs by Hozák et al. (1994). It is possible that one domain reflects the activity of a single gene or of a cluster of genes, which can result in a brighter spot. Secondly, the transcription of some genes may almost be finished *in vivo* when the nuclei were isolated, so only a

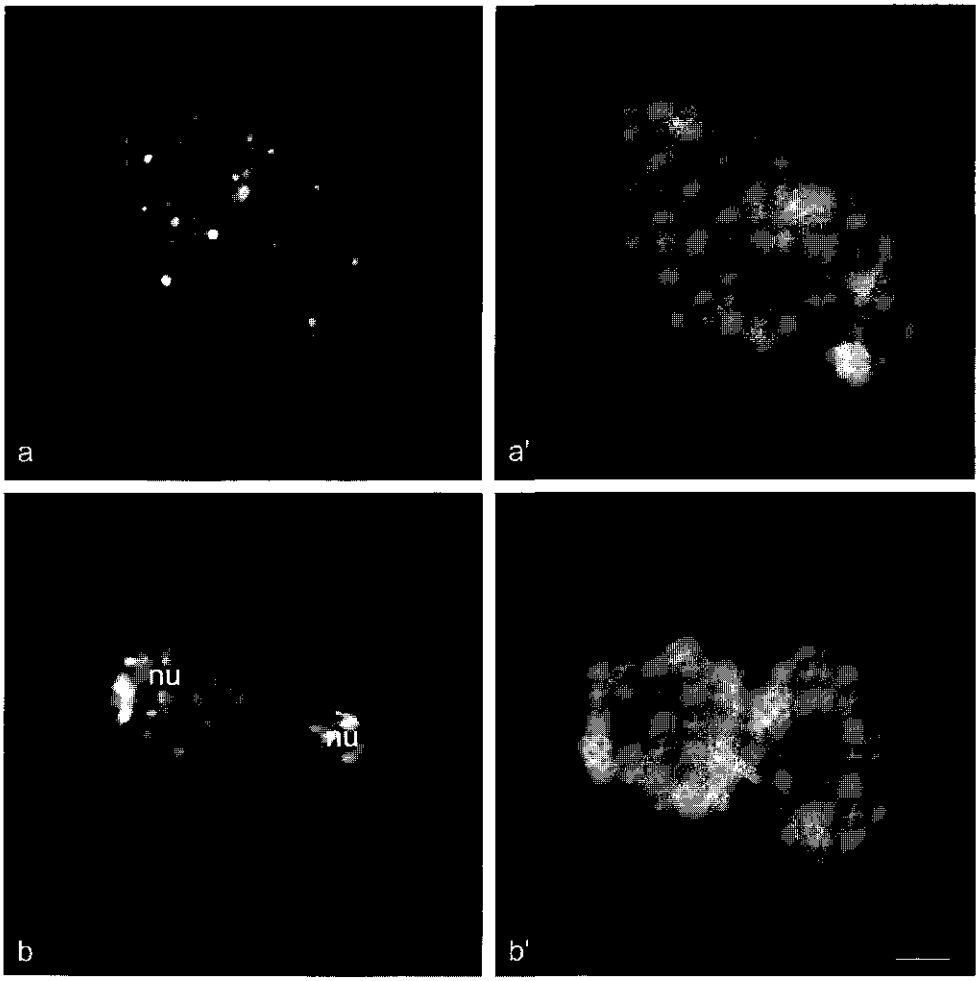

Fig. 1. Distribution of transcription domains in nuclei of *Brassica napus*. After isolation of the nuclei, BrU was incorporated into nascent RNA in a run-on experiment. Incorporated BrU was detected by indirect immunofluorescence. One optical section is shown. **a** Transcription domains are found throughout the nucleoplasm and **b** occasionally also the nucleolus was positive. **a'** and **b'** DNA staining with propidium iodide of the same nucleus as in **a** and **b** respectively. nu : nucleolus. Bar = 1 µm.

small amount of Br-labelled RNA is produced *in vitro*, resulting in a faint label. Furthermore, there will be a difference in BrUTP incorporation between AT or GC-rich genes. AT-rich genes produce BrU-rich RNA, which might give a brighter signal. On the other hand, it is unknown whether there is a difference in incorporation efficiency between genes. It is known that the efficiency of incorporation of BrUTP is about 40% lower than RNA synthesis in the presence of UTP in different cell systems (Nakayama

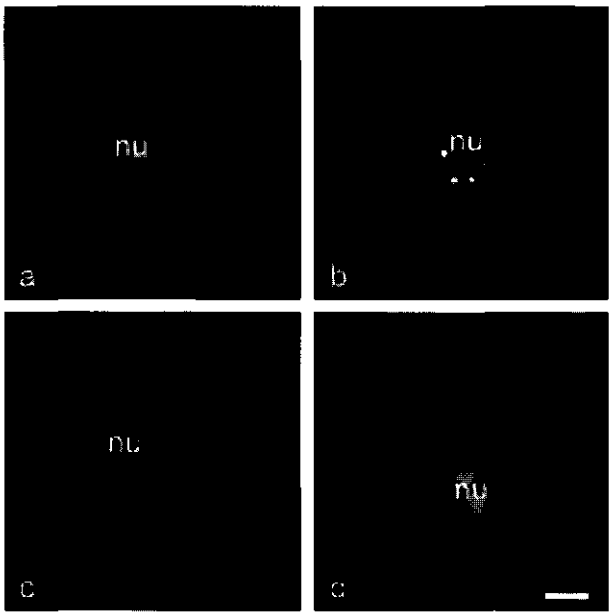

Figure 2. Controls. **a** BrUTP was substituted by UTP to examine the specificity of the anti BrU-antibody. Almost no label was found. **b** 10 mg/ml α-amanitin was present during run-on transcription to inhibit RPII and RPIII. Only transcription by RPI in the nucleolus was sometimes observed. **c** 0.2 mg/ml Actinomicyn D blocks all RP activity, so no label was found. **d** Also, no label was found when after BrU incorporation nuclei were treated with 1 mg/ml RNase A for 2 hours. nu: nucleolus; Bar = 1 μm.

and Saneyoshi, 1984; Wansink et al., 1993; Hozák et al., 1994). This can mean that BrUTP decreases the incorporation efficiency of BrU in RNA from GC-rich genes.

Figure 2 a-d shows control experiments. When the nuclei were treated with UTP instead of BrUTP no label was found (Fig. 2a). This indicates that the antibodies are specific for BrU-labelled RNA. Low concentrations of α-amanitin inhibit RPII. At higher concentrations RPIII is also inhibited (Roeder, 1976). The α-amanitin treated nuclei showed little or no label in the nucleus, except in some cases, where label was found in the nucleolus (Fig. 2b). This is due to the fact that RPI is not inhibited by α-amanitin and therefore transcription of ribosomal genes can take place. All RNA polymerases are blocked by actinomycin-D, which binds to DNA and in this way inhibits all RNA synthesis. In nuclei treated with 0.2 μg actinomycin-D/ml only background labelling was observed (Fig. 2c). Also, when cells were treated with RNase A after BrU incorporation, only background label was found (Fig. 2d). These controls show that the labelling seen in the BrU positive nuclei (Fig. 1) is due to the incorporation of BrU into RNA.

We conclude that it is possible to visualize sites of RNA synthesis after incorporation of BrUTP in nascent RNA in isolated nuclei of *B. napus*. The results are in good agreement with those obtained in animal and human cells (Wansink et al., 1993; Jackson et al., 1993; Garcia-Blanco et al., 1995) and are promising for further studies on various plant systems.

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Chapter 5

Immunocytochemical detection of transcription sites, fibrillarin and snRNPs in protoplasts and pollen tubes of *Brassica napus* L.

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Summary

Nuclear transcription sites were immunocytochemically identified after incorporation of the UTP-analogue 5-bromouridine 5'-triphosphate (BrUTP) into nascent RNA in cotyledon protoplasts, pollen protoplasts and whole plant cells of *Brassica napus*. In cotyledon protoplasts RNA synthesis was found at the periphery of condensed chromatin. In pollen tubes and pollen protoplasts only the vegetative nucleus showed transcription sites, whereas the sperm nuclei remained negative. This indicates that there is only RNA synthesis in the vegetative nucleus of mature pollen and during early germination in *B. napus*.

An antiserum against fibrillarin from mammalian origin, P89, was tested for the detection of small nucleolar ribonucleoprotein particles (snoRNPs) in plant cells using root tip cells of *B. napus* and *Allium cepa*. The labelling pattern was found to be similar to the distribution pattern of anti-fibrillarin in mammalian nuclei and therefore this antibody was used for further experiments. Depending on the pre-treatment, the labelling pattern of antibodies against snoRNPs was uniform, more speckled or absent. The same results were found in the nucleoplasm when antibodies directed against U1, U2, U4/U6 and U5 small nuclear ribonucleoprotein particles (snRNPs) were used. These results show that a speckled labelling pattern of snRNPs and fibrillarin in plant cells is a result of pre-treatment. Double labelling experiments with antibodies directed against Br-labelled RNA and snRNPs showed transcription sites in weakly labelled snRNP areas and no colocalization of Br-labelled RNA with the brightly labelled interchromatin granules and coiled bodies. In the nucleolus the Br-rRNA signal partly overlapped with the signal of anti-fibrillarin, indicating transcription of ribosomal genes in both the dense fibrillar component and the fibrillar centres.

Keywords: *Brassica napus*; 5-bromouridine 5'-triphosphate incorporation; fibrillarin; pollen; protoplasts; small nuclear ribonucleoprotein particles.

Introduction

One of the important processes in the nucleus is the synthesis of RNA. To visualize the sites of RNA synthesis the UTP-analogue 5-bromouridine 5'-triphosphate (BrUTP) can be incorporated into nascent RNA and detected by immunocytochemistry. This has proofed to be a powerful technique to localize sites of transcription in both nucleoplasm and nucleolus and to study the relation between these sites and nuclear and nucleolar proteins when applied to mammalian cells (Wansink et al., 1993; Hozák et al., 1994; Raška et al., 1995; Huang and Spector, 1996) and recently also to plant cells (Melcák et al., 1996; Straatman et al., 1996; Thompson et al., 1997).

In the nucleoplasm transcription results in the production of pre-mRNA. This pre-mRNA has to be processed to become mature before it can leave the nucleus. One of the steps during maturation is the removal of introns by spliceosomes in a process called splicing. These spliceosomes consist of various small nuclear ribonucleoprotein particles (snRNPs) and a group of proteins called the non-snRNP protein factors, like splicing factors. The snRNPs consist of a set of common proteins (or Sm-proteins) found in all snRNPs, a set of proteins characteristic for every group of snRNPs, and a specific small nuclear RNA (snRNA) (Krämer, 1995). The best known snRNPs in mammalian nuclei are U1, U2, U4, U5 and U6 snRNPs, so called because the snRNA they contain is U-rich. These snRNPs are distributed in a "speckled" distribution pattern in the nuclei of mammalian cells (Lamond and Carmo-Fonseca, 1993a). Several groups found a direct relation between the localization of proteins involved in splicing and the transcription activity (Sahlas et al., 1993; Spector et al., 1993; Baurén et al., 1996; Huang and Spector, 1996; Dirks et al., 1997).

Many studies have analysed the distribution of snRNPs using antibodies. Spliceosomal components were found in three different sub-nuclear compartments: perichromatin fibrils, which can be found in the weakly labelled areas; clusters of interchromatin granules, which are labelled more intense and coiled bodies, which are brightly labelled (Fakan et al., 1984; Spector et al., 1991; Ferreira et al., 1994). Perichromatin fibrils are fibrils in close relation to chromatin and they are visible in the electron microscope using cryofixed and cryosubstituted material. They are believed to be the sites of RNA splicing (Fakan, 1994). The clusters of interchromatin granules consist of aggregates of granules with a diameter of about 20-25 nm. In mammalian cells, the splicing factor SC-35 is also found in these granules. Finally, coiled bodies are nuclear bodies in which several nuclear and nucleolar proteins, including snRNPs and snoRNPs, and snRNAs are concentrated (reviewed by Lamond and Carmo-Fonseca, 1993b; Frey and Matera, 1995). In mammalian cells, transcriptionally active cells have more coiled bodies and less labelled clusters of interchromatin granules (Carmo-Fonseca et al., 1992). When RNA synthesis is blocked snRNPs no longer concentrate in coiled bodies but accumulate in clusters of interchromatin granules

(Carmo-Fonseca et al., 1992; O'Keefe et al., 1994). This indicates that the clusters of interchromatin granules might be the places of storage of snRNPs, but the function of coiled bodies in the splicing process is not clear yet.

The nucleolus, the most conspicuous nuclear compartment, is the place for rRNA processing. It is mainly divided in the fibrillar centres (FCs), the dense fibrillar component (DFC) and the granular component (GC) (see e.g. Testillano et al., 1993; Shaw and Jordan, 1995) and it contains its own snRNPs, the so called small nucleolar RNPs (snoRNPs), which are involved in the splicing of pre-rRNA. The best known is U3 snoRNP (reviewed by Mattaj, 1984; Kiss et al., 1985). One of the proteins of these snoRNPs is fibrillarin. Antibodies against fibrillarin immunoprecipitate with several snoRNPs, including U3 (Tyc and Steitz, 1989) and anti-fibrillarin is therefore often used for localization experiments. We used for the localization of snoRNPs a new antibody against fibrillarin from human origin. Therefore, the labelling pattern in plant cells was first studied using root tip cells of *Brassica napus* and *Allium cepa*.

We are interested in the nuclear organization in plant cells and the question if changes in developmental pathways are mirrored by changes in the nuclear architecture. A lot of effort is going on to identify the various domains in mammalian cells, but there are several functional differences between plant and animal genomes which might affect chromatin structure and gene expression (Shaw and Jordan, 1995) and consequently also the nuclear organization. Here we report on the localization of transcription sites in several types of plant cells and the relationship between these sites and snRNPs and snoRNPs. Recently, the technique of BrUTP incorporation to label the sites of transcription was shown on isolated nuclei of *B. napus* (Straatman et al., 1996). To extend these observations to *in vivo* situations and to obtain a better structural conservation of the nuclei for localization experiments, this system was now applied to cotyledon protoplasts of *B. napus*. We compared this labelling pattern with the distribution of snRNPs and snoRNPs. Protoplasts are required since BrUTP can not pass the cell wall. To visualize the difference in RNA synthesis between an active and a less active nucleus the incorporation experiments were also applied to protoplasts derived from mature pollen; the vegetative nucleus is considered to have a high nuclear activity and the sperm nuclei are considered to have a low nuclear activity. In an attempt to label nascent RNA in whole plant cells instead of protoplasts, BrUTP incorporation experiments were carried out on germinating pollen.

Materials and methods

Pollen protoplasts

Plants from *Brassica napus* cv. Topas were grown from seeds in a phytotron at 18 °C with a photoperiod of 16 h light. Flower buds at mature pollen stage were harvested, and pollen were isolated in NLN medium (Lichter, 1982), which was free from potato

extract and growth regulators (Pechan and Keller, 1988). Pollen were precultured overnight at 18 °C or they were cultured 3-6 h in BK medium (Brewbaker and Kwack, 1963) at 25 °C. After preculture, they were concentrated by centrifugation and resuspended in pollen protoplast isolation medium containing 1% pectinase (Merck, Darmstadt, Germany), 0.2% macerozyme R10 (Serva,Heidelberg, Germany) and 1% cellulase "Onozuka" R10 (Serva) dissolved in CPW medium with 0.8 M mannitol and 0.4 M sorbitol (Power and Chapman, 1985). The pollen were incubated in this solution for 2-4 h in the dark at 25 °C. Before use the pollen protoplasts were washed in CPW medium without enzymes.

Cotyledon protoplasts

Seeds of *B. napus* cv. Topas were germinated on ½MS medium with 3% sucrose and kept in the dark for 5 days followed by 3-4 days dim light at 25 °C. Cotyledons of 20-30 seedlings were harvested and cut into small strips before incubation in 10 ml enzyme solution containing 1% pectinase, 0.2 % macerozyme and 1% cellulase in CPW9 (Power and Chapman, 1985) supplemented with 5 mM MES, 5 mM arginine and 0.5% PVP, pH 5.6. After 4-6 h of incubation the protoplasts were filtered through a 100 μm nylon filter and centrifuged at 50g for 4 min. The pellet was resuspended in 3.5 ml CPW9 and layered on top of 4 ml 0.5 M sucrose solution in a centrifuge tube followed by 4 min centrifugation at 45g. The band with protoplasts was harvested and the protoplasts were washed in CPW9. The pellet was resuspended in KM8P culture medium (Kao and Michayluk, 1975).

Preparation of pollen tubes

Pollen of *B. napus* cv. Topas were germinated *in vitro* on coverslips in pollen tube growth medium according to Brewbaker and Kwack (1963; BK medium, optimized for *Brassica* by Taylor et al., 1991) containing 100 mg/l H_3BO_3, 300 mg/l $Ca(NO_3)_2.4H_2O$, 200 mg/l $MgSO_4.7H_2O$, 100 mg/l KNO_3, 20 % sucrose and 1% low gelling temperature agarose (Electran, BDH, UK). After 15 and 30 minutes of pollen tube growth the sites of RNA synthesis were labelled as described below.

Isolation of nuclei

Young leaves of *B. napus* were harvested from plants in the phytotron and nuclei were isolated as published before (Straatman et al., 1996). The isolated nuclei were stored in glycerol buffer (25% glycerol, 20 mM Tris (hydroxymethyl)-aminomethane /HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM EGTA) at -20 °C.

Labelling of transcription sites

Protoplasts were mounted on aminopropyl-triethoxy-silane-coated slides (Serva) and immobilized in 2% low melting point agarose (BDH, Poole, UK). Run-on transcription was performed as described before (Straatman et al., 1996) with some minor changes. Protoplasts were incubated in glycerol buffer containing 3 mM PMSF for 10 min. The protoplasts were permeabilized in glycerol buffer containing 0.1% Triton X-100, 3 mM PMSF, 40 U/ml RNasin (Promega, Madison, WI, USA.) and 2 mM 1,4-dithiothreitol (DDT) for 3 min followed by incubation in transcription buffer (20 mM N-[2-hydroxyethyl]piperazine-N'-[2 ethanesulfonic acid] Hepes, pH 8.0, 12 mM $MgCl_2$, 200 mM $(NH_4)_2SO_4$, 50 mM KCl, 100 µg/ml tRNA, 1000 U/ml RNasin, 2 mM DTT, 0.5 mM ATP, GTP, CTP and BrUTP each) for 15 min at room temperature. The protoplasts were washed 5 min (sometimes 10 min) in TBS (10 mM Tris/HCl pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM PMSF, 40 U/ml RNasin, 2 mM DDT) containing 0.1 % Triton X-100 followed by 5 min wash in TBS. Material was fixed in 2% paraformaldehyde in PBS (140 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2PO_4, 1.5 mM KH_2PO_4; pH 7.4) and immunocytochemistry was performed as described before (Straatman et al., 1996).

In control experiments α-amanitin (10 µg/ml; Sigma) which inhibits RNA polymerase II activity (Roeder, 1976), was included until the wash steps.

Immunolabelling

The following antibodies were used: Mouse mAb 72B9 detecting mammalian fibrillarin (Reimer et al., 1987) and P89, a human antiserum that recognizes *in vitro* translated human fibrillarin and immunoprecipitates U3RNPs (H. Pluk and W.J. van Venrooij, unpublished; both 72B9 and P89 were gifts from H. Pluk, Dept. of Biochemistry, Catholic University Nijmegen, The Netherlands); human antiserum B16, which recognizes all the proteins of the U1, U2, U4/U6 and U5 snRNPs (Lerner and Steitz, 1979; Habets et al., 1985); and the mouse monoclonal antibody 7.13 recognizing protein D of the U1, U2, U4, U5 and U6 snRNPs (Billings et al., 1982, 1985). The human antibodies were visualized with a TRITC conjugated goat-anti-human IgG (Sigma Chemical Co., St Louis, USA), diluted 1:20. The mouse antibodies were visualized with goat-anti-mouse Bodipy (Molecular Probes, Leiden, The Netherlands), diluted 1:100.

To analyse the specificity of the antibodies, they were studied using root tips of *B. napus* seedlings germinated at 25 °C and root tips of *A. cepa* grown in hydroculture in the greenhouse. Root tips were collected and fixed in 3% paraformaldehyde in PBS for 1 h at room temperature. Root tips were washed after fixation in PBS, treated with 2% cellulase "Onozuka" R10 (Sigma) for 1 h at room temperature, rinsed again in PBS and squashed on organosilane coated slides. The slides were air dried and washed in PBS, blocked with 1% BSA in PBS for 30 min and rinsed in 0.1% acetylated BSA

(BSAc; Aurion, Wageningen, The Netherlands), followed by incubation with the first antibody overnight. Next day the slides were washed in 0.1% BSAc, followed by incubation with the secondary antibody for 1 h at 37 °C. After washing in PBS the slides were mounted in Citifluor-glycerol (Citifluor Ltd., Canterbury, UK) and they were analysed with a Nikon Labophot epifluorescence microscope.

For the detection of Br-labelled RNA a mouse monoclonal antibody against BrdU (1:300; Sigma) was used followed by a biotin conjugated goat-anti-mouse IgG(H+L) (1:500; Jackson Immunoresearch Laboratories, West Grove, PA, USA). This complex was visualized using ExtrAvidin-FITC (1:1000; Sigma). DNA was stained with 0.1 µg/ml propidium iodide in single labelling experiments or with 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma) in double labelling experiments. The slides were analysed with a MRC600 Bio-Rad (Veenendaal, The Netherlands) confocal scanning laser microscope (CSLM) using an argon-krypton ion laser attached to a Nikon Diaphot inverted microscope equipped with a 100 x /1.3 N.A. oil immersion lens and the K1/K2 filter combination.

Results

Labelling patterns of anti-fibrillarin and anti-Sm

The antiserum P89, directed against mammalian fibrillarin, was used to label isolated root tip cells of *A. cepa* and *B. napus* to analyse the distribution pattern by immunofluorescence microscopy (Fig. 1). Cells of both species gave the same results, but root tip cells of *A. cepa* were mostly used because they are larger and therefore easier to study.

At interphase, a homogeneous label was observed in the nucleoli and, in some cases, also small foci were stained in the nucleoplasm (Fig. 1a). During late prophase and metaphase, a large spot was detected at the chromosome periphery, which is thought to correspond to the cytoplasmic nucleolar remnant (Fig. 1b). At late anaphase and telophase, several labelled spots were seen in close contact with the condensed chromosomes (Fig. 1c). The same results were obtained in root cells of *B. napus* using the anti-fibrillarin 72B9 (results not shown).

The antibody 7.13, directed against protein D of the splicing complexes U1, U2, U4/U6 and U5, showed labelling in the nucleoplasm of interphase nuclei of root tip cells, but no label was found in the nucleolus. In some cases the nucleoplasm was uniformly stained and in other cases a lightly speckled pattern was observed (Fig. 1d). Often a brighter labelled body was visible in the nucleoplasm. During mitosis a faint labelling was found dispersed through the cytoplasm. The same results were obtained with the antibody B16 on root cells of *B. napus* (not shown).

Isolated nuclei of *B. napus* leaves, fixed in 3% paraformaldehyde and labelled with anti-fibrillarin (P89 or 72B9), showed the same labelling pattern as the interphase

nuclei of root tip cells. However, the anti-snRNP (7.13 or B16) gave a heterogeneous label pattern in the nucleoplasm. In several nuclei even a speckled distribution pattern could be found (Figs. 1e, f). Such a clear speckled pattern was never found when whole cells or protoplasts were used for labelling experiments. When the isolated nuclei were first used for BrUTP incorporation experiments and then fixed and labelled for snRNPs or fibrillarin no label was found at all.

BrU-labelled RNA in nuclei of cotyledon protoplasts

The incorporation of BrUTP into nascent RNA in interphase nuclei of protoplasts derived from cotyledons of *B. napus* was investigated during run-on transcription experiments. Incorporated BrU was visualized using indirect immunolabelling and optical sections were made with a confocal laser scanning microscope. A typical punctate labelling pattern was observed throughout the nucleus (Fig. 2), sometimes including the nucleolus. The structure of the nuclei of cotyledon protoplasts was much better preserved after BrUTP incorporation (Figs. 3a, b) than in earlier experiments using isolated nuclei (Straatman et al., 1996). In most cases, labelling was found at the edges of the condensed chromatin and in weakly DNA stained areas. Often the transcription sites seem to be arranged in rows throughout the whole nucleus, oriented in different directions (Fig. 3d). Some of these rows were very long and were running from one side of the nucleus to the other side. The orientation of these rows was not interrupted by areas of condensed chromatin.

When α-amanitin was added during the experiments, only labelling in the nucleolus was observed (Fig. 3c) and when BrUTP was replaced by UTP no label was found (not shown) indicating that the observed labelling after BrUTP incorporation was derived from nascent RNA.

Transcription and splicing in cotyledon protoplasts

To study the relation between the sites of transcription and proteins involved in splicing, double labelling experiments were carried out after the run-on experiments to localize the sites of Br-labelled RNA and the distribution of the snRNPs or fibrillarin. Experiments carried out using cotyledon protoplasts often showed a totally labelled nucleoplasm or nucleolus when labelled with anti-Sm or anti-fibrillarin respectively, as was seen in the root tip cells. When, however, an extra 5 min 0.1% Triton X-100 treatment was given before fixation, a more speckled pattern was observed in the nucleus of some cells when anti-Sm was used (Fig. 4b). In the nucleoplasm a intensively labelled body was found, besides less intensively labelled areas and weakly labelled areas. However, the longer TritonX-100 treatment also resulted in a weaker signal of Br-labelled RNA and a worse preservation of the nuclear structure. The Br-labelled RNA was mostly found in the weakly labelled snRNP areas.

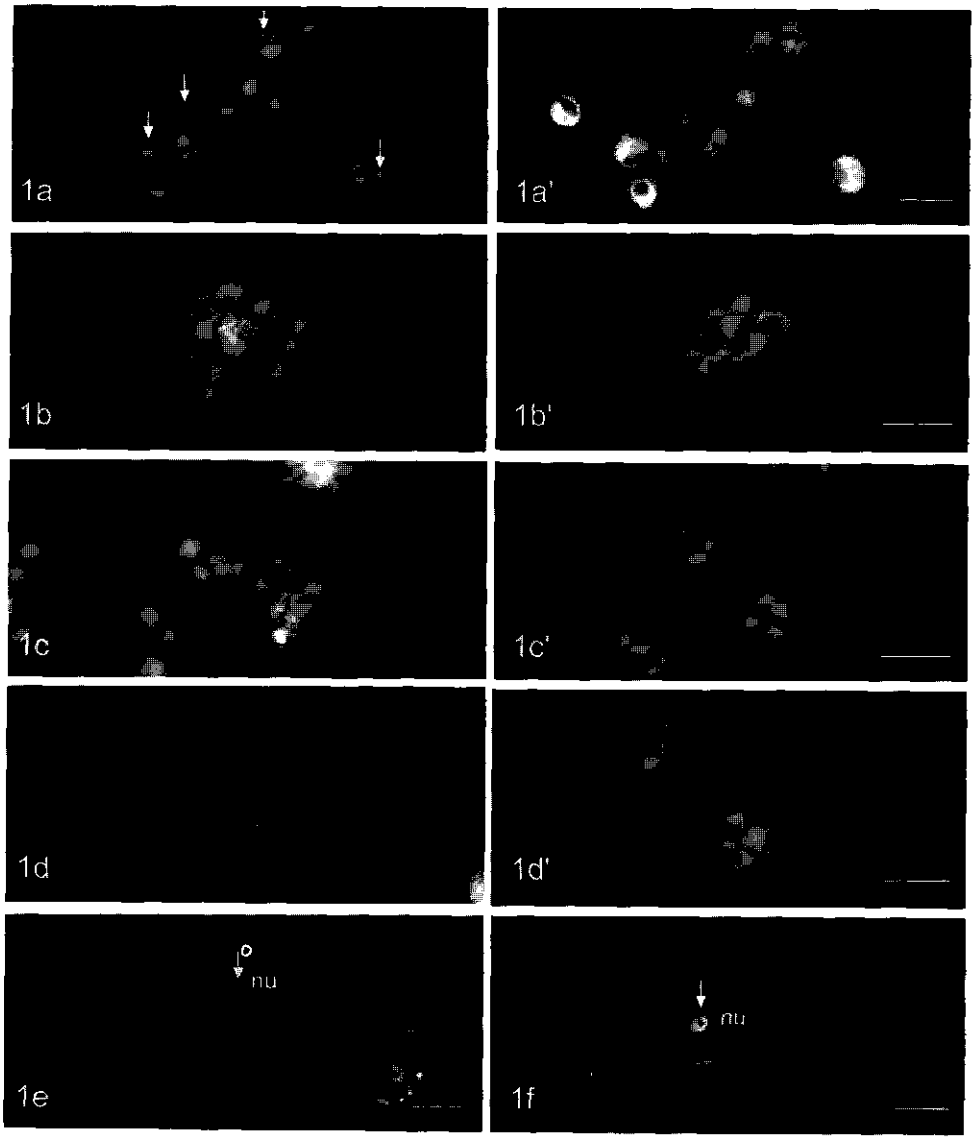


Fig. 1; Labelling patterns of anti-fibrillarin (P89) and anti-snRNP (7.13) in root tip cells of *Allium cepa*, and the labelling patterns of 7.13 in isolated nuclei of *Brassica napus*. **a**; P89 in interphase nuclei of root tip cells; both nucleoli and coiled bodies (arrows) are labelled. **b**; P89 in late prophase with labelling of the nucleolar remnant. **c**; P89 in telophase showing some larger and several smaller dots. **d**; anti-snRNP gave a signal in the nucleoplasm at interphase. During mitosis the cytoplasm was labelled. **a'**, **b'**, **c'** and **d'** show the matching DAPI pictures. **e** and **f**; optical sections of the speckled pattern of anti-snRNP in isolated nuclei of *B. napus*. The more intensely labelled loci are coiled bodies (arrows). nu; nucleolus. Bars: 2.5 µm (**1a**, **1a'**) 1 µm (**1b**, **1b'**, **1c**, **1c'**, **1d**, **1d'**), 3 µm (**1e**, **1f**)

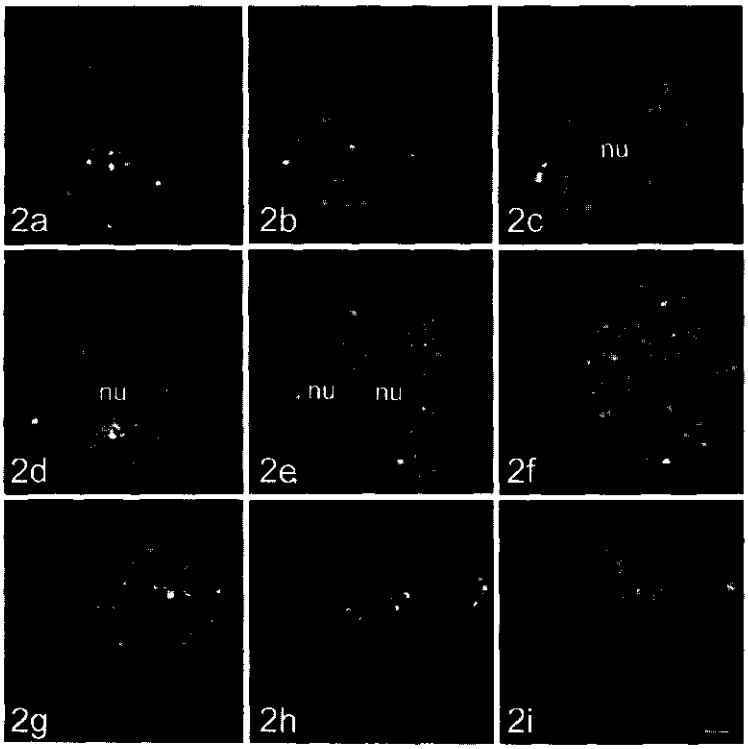


Fig. 2; Series of optical sections through a nucleus of a cotyledon protoplast of *Brassica napus* in steps of 1 µm after incorporation of BrUTP and detection of the Br-labelled RNA with antibodies. Labelling of nascent RNA was found throughout the whole nucleus but, in this case, not in the nucleolus (nu). bar: 2 µm.

After the longer TritonX-100 treatment and labelling with anti-fibrillarin, the nucleoli of some cells showed a more speckled staining pattern, too (Fig. 5b), besides a labelled body in the nucleoplasm. When this pattern was compared with the labelling of Br-rRNA synthesis (Fig. 5a), the bright fibrillarin signal and the Br-rRNA signal only partly colocalized; most Br-rRNA signal was found in the unlabelled or weakly labelled fibrillarin areas. When this picture was enlarged, sometimes a weak Br-RNA signal was found in the nucleolus besides the intensive rRNA labelling. This signal had almost the same intensity as the RNA labelling in the nucleoplasm.

BrU-labelled RNA in pollen protoplasts and pollen tubes

Incorporation of BrUTP into nascent RNA in nuclei of protoplasts derived from mature pollen (Fig. 6a) and in nuclei of pollen tubes (Figs. 6b and 6c) resulted in a punctate labelling pattern in the vegetative nucleus. Most sperm nuclei did not show label except in one case where label was found in the sperm nuclei of a pollen tube (not

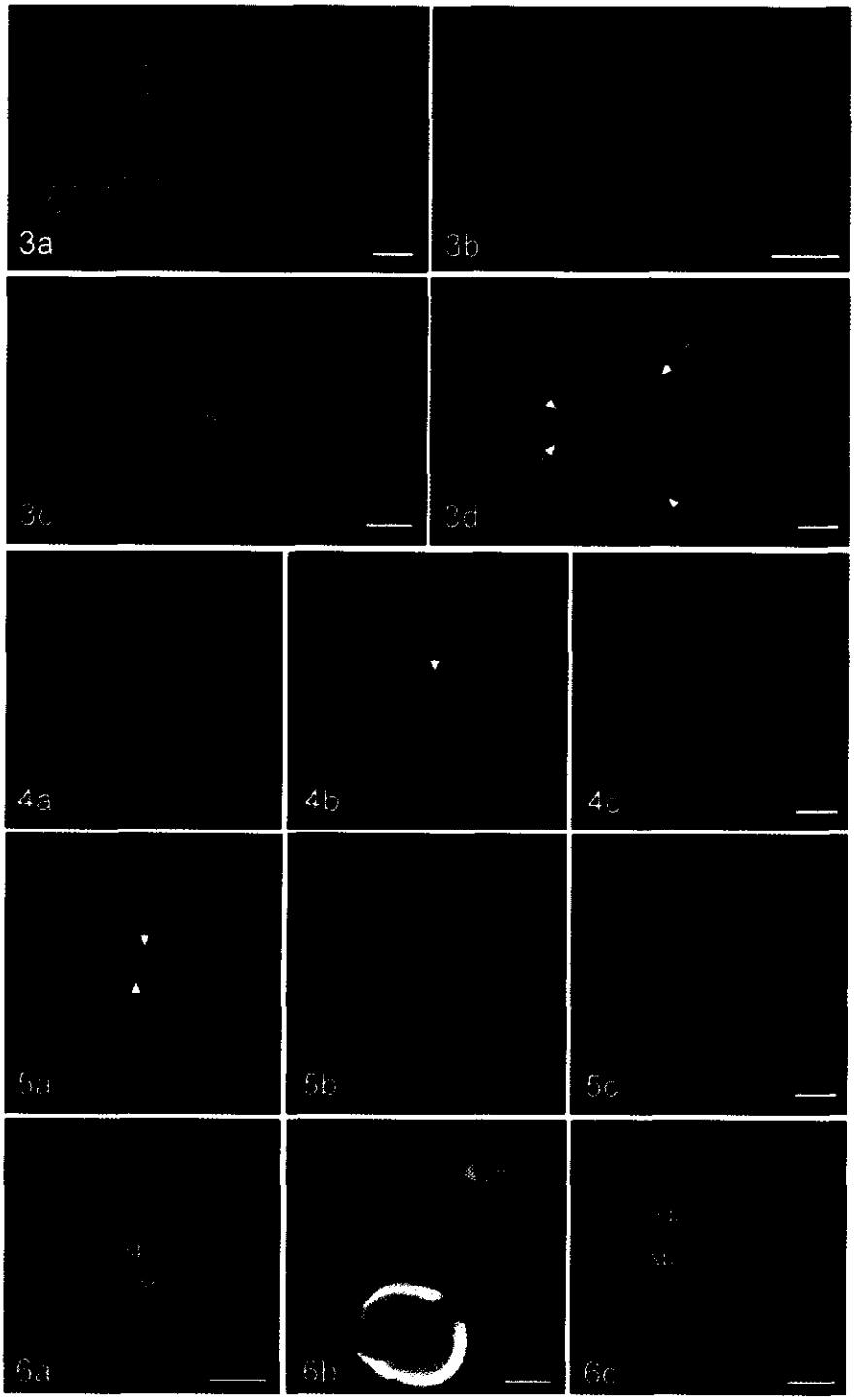
shown). In pollen protoplasts no label was detected in sperm nuclei at all. The label intensity in the vegetative nucleus of pollen protoplasts was high and uniformly spread over the nucleus, in contrast to the vegetative nucleus of the pollen tube where areas with and without label were found. Although most pollen germinated on slides, labelling of nascent RNA was only detected in a few cases. Double labelling experiments, using anti-fibrillarin or anti-snRNP together with BrU incorporation, gave a regular staining in the nucleolus and nucleoplasm respectively, so none of the known nucleolar or nuclear compartments of the vegetative nucleus could be identified (not shown).

Discussion

BrUTP incorporation in nuclei of cotyledon protoplasts

Recently we showed labelling of nascent RNA after run-on experiments in which the UTP-analogue BrUTP was incorporated in isolated nuclei of leaves of *B. napus* (Straatman et al., 1996). We optimized this technique to label *in vivo* situations and to allow a simultaneous localization of nascent RNA and nuclear proteins. Therefore, we adapted this technique to label nascent RNA in protoplasts, an approach recently also published by Melcák et al. (1996) for onion. We used protoplasts derived from cotyledons and from mature pollen, as well as germinating pollen of *B. napus*. It proved still necessary to remove the cell wall or exine to get BrUTP inside the cells. Until now the only exception found, where BrUTP could enter a plant cell only using Triton, was during pollen tube growth, although the number of nuclei showing BrUTP-incorporation was low, probably also due to these penetration problems. All results showed sites of transcription throughout the whole nucleus, sometimes including the nucleolus.

The transcription sites were often localized at the edges of condensed chromatin. This is in agreement with earlier observations (Fakan, 1994; Kurz et al., 1996; Strouboulis and Wolffe, 1996) and with published models where chromatin loops with active genes are located at or close to the surface area of chromosome territories (Getzenberg et al., 1991; Cremer et al.,1993). Sometimes these sites of transcription were found in rows, in some cases running from one side to the other side of a nucleus. The rows were not disturbed by areas with condensed DNA, suggesting a higher organizational structure which orients the transcription machinery. Thompson et al. (1997) recently showed labelling of rDNA transcription in *Pisum sativum* but they did not detect BrU-labelled RNA in the nucleoplasm. They suggest that this may reflect a greater sensitivity of RNA polymerases in the nucleoplasm to Triton, although they only used a one min 0.05% Triton treatment. In our hands, using *Brassica*, we always have labelling of transcription sites in the nucleoplasm and in less than 10% of the nuclei also RNA synthesis in the nucleolus is visualized. Different


3a
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5c
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duration of Triton treatments, as used in these experiments, did not seem to influence the presence of BrU-labelled RNA in the nucleoplasm or nucleolus. From earlier experiments (unpublished) we know that a shorter Triton treatment resulted in poor labelling of transcription sites. Raška et al. (1995) showed that depending on the pre-treatment only nucleolar or nuclear and nucleolar labelling was found in the same cell type. This shows that besides different cell types, also the way the material is prepared has a great influence on the final labelling found.

Labelling patterns of fibrillarin and nucleolar RNA synthesis

To see if P89, which labels mammalian fibrillarin, also labels fibrillarin in plant nuclei, the labelling pattern of P89 was compared with the labelling pattern of 72B9. This is a known antibody which labels fibrillarin in plant nuclei (Beven et al., 1996). In root cells of *B. napus* and *A. cepa* the labelling patterns of both antibodies were identical and comparable with the distribution of fibrillarin in Chinese hamster ovary cells (Azum-Gélade et al., 1994). This indicates that P89 can be used to label fibrillarin in plants.

The labelling of fibrillarin in interphase nuclei was influenced by the pre-treatment. Protoplasts of cotyledons, directly fixed, showed an almost homogeneous label in the nucleolus, whereas isolated nuclei often showed a speckle-like labelling in the nucleoli.

Fig. 3 a; Isolated cotyledon protoplast of *Brassica napus* after BrUTP incorporation experiment (left) with a nucleus labelled for Br-RNA (right). **b**; enlargement of the labelled nucleus of figure 1a showing Br-labelled RNA (yellow/green) on the edges of condensed DNA, stained with propidium iodide (red). **c**; control experiment using α-amanitin during the run-on experiments to block the transcription of RNA-polymerase II and III. Only transcription of RNA polymerase I was found in the nucleolus (nu). DNA is stained with propidium iodide (red) **d**; optical section through a nucleus of a cotyledon protoplast showing nascent RNA (green/yellow) organized in rows through the nucleus (see e.g. arrows). Red is DNA. Bars: 10 µm (**3a**), 5 µ (**3b**), 2 µm (**3c, 3d**).

Fig. 4; An optical section through a nucleus of a cotyledon protoplast with Br-labelled RNA in green (**a**) and labelling of snRNPs in red (**b**). The arrow in b points to a coiled body. In **c** the merge of a and b is shown. Bar: 2 µm.

Fig. 5; An optical section through a nucleus of a cotyledon protoplast. **a**; shows Br-labelled RNA in the nucleolus. A weak signal of Br-labelled RNA is visible in the nucleoplasm. Besides the more intensive Br-labelled RNA foci in the nucleolus, also weak labelled areas were found (arrows). **b**; labelling of fibrillarin in the nucleolus. In **c** the signals of a and b are merged showing that the Br-labelled RNA partly co-localized with intensive labelled areas of fibrillarin. Bar: 1 µm.

Fig. 6 a; Detection of transcription sites in nuclei of pollen protoplasts of *B. napus*. Both the sperm nuclei (sp) are negative whereas the vegetative nucleus shows a strong signal. **b**; a growing pollen tube with two sperm nuclei and the vegetative nucleus in the framework. At the place where the pollen tube emerges from the pollen, a part of it not in the focus plain of this optical section. Detection of sites of transcription in these nuclei (**c**) showed labelling in the vegetative nucleus but both sperm nuclei were negative. Bars: 5 µm (**6a**), 10 µm (**6b**), 3 µm (**6c**).

When, before fixation, BrUTP was incorporated, a more speckled nucleolar label was found in cotyledon protoplasts and no label at all in the nucleoli of isolated nuclei.

Fibrillarin is found to be associated with the DFC (Beven et al., 1996; Shaw et al., 1998) or with both the DFC and the GC (Azum-Gélade et al., 1994). Our results show that signal is lost from one or both of these compartments. This is in agreement with the results of Melcák et al. (1996) who found an influence of the Triton treatment on the nucleolar structure in onion protoplasts; after a short 0.05% Triton treatment the GC had been affected, while after a 12 minutes treatment also the transcriptionally inactive DFC subdomains had disappeared. Our results on isolated nuclei, after transcription experiments, suggest that even the transcriptionally active DFC subdomain can disappear; no label at all was found in these nucleoli when P89 was used.

Although the antibodies used do not allow to distinguish between the different nucleolar compartments, we can follow the nomenclature of Melcák and co-workers (1996) and assume that nuclei of *Brassica* react in the same way on a Triton treatment. This would mean that the GC, and maybe a part of the transcriptionally inactive DFC, was washed away. After incorporation of BrUTP in cotyledon protoplasts, we still find a large part of fibrillarin labelled in transcriptionally inactive parts, so this might indicate that the transcriptionally inactive DFC is still partly present. Sites of transcription were often found in the areas that did not label for fibrillarin. It implicates that part of the Br-labelled RNA is found in the FCs (the GC does not contain DNA and therefore also no nascent RNA, Risueño and Testillano, 1994; Raška et al., 1995; Melcák et al., 1996). Only a small part of the transcription sites was found in the DFC, on the border with the FCs. The localization of active rDNA in the nucleolus is controversial. In the literature evidence is presented for sites of rRNA synthesis in only the FCs (Thiry et al., 1988), only the DFC (Wachtler et al., 1992; Hozák et al., 1994; thompson et al., 1997) or in both the FCs and DFC (Raška et al., 1995; Melcák et al., 1996). Ribosomal DNA is found in both the FCs and DFC (Hozák et al., 1993; Risueño and Testillano, 1994; Raška et al., 1995). But, like Melcák et al. (1996) argue, different cell types and different stages of cell maturation or different metabolic states can result in different sites of rRNA synthesis.

In some of the nucleoli of cotyledon protoplasts a weaker signal was found besides the intensive label of transcription of the rDNA genes. It is not clear if this is an aspecific labelling or labelling of transcription of rDNA genes which were just switched on or off. Processing of rRNA is not plausible since BrUTP incorporation experiments have shown to label only the transcription sites; no further processing of RNA took place (Dundr and Raška, 1993; Wansink et al., 1994). Another possibility would be that transcription of some other nucleolar DNA is visualized, although ribosomal genes are the only active genes known in the nucleus.

Labelling patterns of snRNPs and RNA synthesis

Localization of the snRNPs U1, U2, U4/U6 and U5 in nuclei of cotyledon and pollen protoplasts showed label in the nucleoplasm, uniformly stained or as an interchromatin network. Also an intensively labelled nuclear bodies was found. When cells were labelled with anti-fibrillarin and anti-snRNP, the merged picture showed that the brightly labelled structure in the nucleoplasm contained both fibrillarin and snRNPs. This identified such structure as a coiled body (Ochs et al., 1994; Bell and Scheer, 1996). These results are in agreement with other reports on plant systems (Sanchez-Pina et al., 1989; Beven et al., 1995; Carmo-Fonseca and Ferreira, 1995; Glyn and Leith, 1995).

The labelling pattern of the snRNPs using B11 or 7.13 was influenced by pre-treatment as well. In directly fixed cotyledon protoplasts the nucleoplasm was homogeneously stained. When, before fixation, BrUTP was incorporated, a more speckled pattern was found in these protoplasts. In this case the labelling of the snRNPs showed, besides coiled bodies which appear as intensively labelled bodies, less intensively labelled areas, which probably are the clusters of interchromatin granules. The weakly labelled regions represent then the perichromatin fibrils. When the images of Br-labelled RNA were merged with such images, most of the transcription sites colocalized with the weakly labelled snRNP areas. Only in a few cases a colocalization with the interchromatin granule clusters was found. This is in agreement with the idea that splicing takes place at the site of transcription, i.e. the perichromatin fibrils, and not in the more intensively stained clusters of interchromatin granules or coiled bodies (Wansink et al., 1993; Fakan, 1994).

In isolated nuclei, fixed directly after isolation, a more speckled pattern could be found, even more speckled than in the leaf protoplasts after BrUTP incorporation. This pattern looked like the speckled pattern sometimes found in mammalian cells (Carter et al., 1993; Zhang et al., 1994). When transcription experiments were performed on isolated nuclei before fixation, no snRNPs could be detected in these nuclei. These results give the impression that a more speckled labelling pattern of snRNPs in plant nuclei is due to pre-treatment and is probably not the distribution of these snRNPs in living plant cells.

BrU-labelled RNA in pollen protoplasts and germinating pollen

Not much is known about transcription of sperm nuclei in pollen but all cloned genes investigated until now were active in the vegetative nucleus (for a recent review: Twell, 1994). In pollen protoplasts and pollen tubes of *B. napus*, Br-labelled RNA was only found in the vegetative nucleus. The signal obtained in vegetative nucleus in the pollen protoplast was much higher than that found in the vegetative nucleus in the pollen tube, indicating that there is a difference in transcription activity between the two stages. The sperm nuclei did not show BrU-labelled RNA, except for one case where

some label was found in a sperm nucleus. Therefore, we considered that the sperm nuclei do not synthesize RNA. However, we have to keep in mind that in order to let BrUTP penetrate the pollen, they were cultured in a protoplast deriving medium. Culturing of pollen in this isolation medium might have influenced the RNA synthesis. The fact, however, that also the sperm cells in pollen tubes did not show RNA synthesis is in agreement with the results obtained with the pollen protoplasts. From incubations with fluoresceindiacetate it is known that both the sperm cells and the vegetative cell were still viable after the isolation procedure (not shown). Moreover, microspores cultured at 18 °C showed normal pollen development (Custers et al., 1994). However, it does not prove that the RNA synthesis in the sperm nuclei of the pollen protoplasts was not turned off or the RNA synthesis in the vegetative nucleus turned on because of the isolation treatments. For example, in mature pollen no snRNPs could be detected indicating no RNA synthesis in *in vivo* (Chapter 6).

There are some technical reasons that can obstruct the detection of RNA synthesis in the sperm cells. The presence of the sperm cell membrane can still cause BrUTP penetration problems, although longer Triton treatments and the addition of 10% DMSO to the pre-incorporation treatments did not result in RNA labelling in the sperm cells. Secondly, the condensed chromatin can cause penetration problems for the antibodies. Finally, if only a few genes are transcribed, spread throughout the sperm nuclei, the obtained signal will be below the detection limit of the CSLM, because the transcription sites found are the visualization of clusters of active genes (Wansink et al., 1993; Iborra et al., 1996).

The scarcely published results of transcription in pollen are not congruent. Using [^{3}H]-uridine, Reynolds and Raghavan (1982) found silver grains in the vegetative nucleus and in the generative nucleus of the bicellular pollen of *Hyoscyamus niger*, but not in the sperm cells after germination. Surprisingly, also the vegetative nucleus was found to be negative in the pollen tube. However, Haskell and Rogers (1985) found RNA synthesis in both the vegetative nucleus and the sperm nuclei of *Secale cereale* in an early stage of germination, but they did not look for RNA synthesis in the pollen tube. In sea urchin spermatozoa the RNA synthesis is turned off in the sperm cells (Poccia, 1989).

Some structural evidence suits the obtained results indicating a silent sperm cell. In mature pollen grains of *Brassica* the chromatin of the sperm cells is highly condensed, whereas in the vegetative nucleus the chromatin is dispersed (Dumas et al., 1985). The condensation of the chromatin in the sperms cells is an indication for a low nuclear activity. It is thought that the condensation of the sperm nuclei is the result of a not completed mitotic cycle resulting in nuclei arrested in telophase (Gerassimova-Navashina, 1961). During mitosis also transcription is silenced. In general, the results suggest that there can be RNA synthesis in the vegetative nucleus but that RNA synthesis in the sperm cells is extremely low, if any.

Acknowledgements

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Chapter 6

Distribution of splicing proteins and coiled bodies in nuclei of *Brassica napus* L.

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Summary

We have analyzed the labelling pattern of small nuclear ribonucleoprotein particles (snRNPs) during pollen development and microspore and pollen embryogenesis in *Brassica napus* using an antibody which recognizes protein D of U1, U2, U4, U5 and U6 snRNPs. It was found that nuclei were labelled uniformly, with some more intensively labelled nuclear bodies. During pollen development no snRNPs were detected in the generative nuclei of late bicellular pollen or in the vegetative nuclei and sperm nuclei of mature pollen, both *in vitro* and *in vivo*. Using a second antibody against the nucleolar protein fibrillarin, which is also found in coiled bodies, the nuclear bodies could be identified as coiled bodies. Most nuclei contained one coiled body closely related to the nucleolus. These coiled bodies increased in size from 0.5 µm in young microspores to 2 µm in late microspores and young pollen, whereas the number of coiled bodies was rather stable. This increase in size of the coiled bodies coincides with a general increase in nuclear activity. After induction of embryogenesis the size of the coiled bodies decreased to around 0.5 µm whereas also the size of the nucleolus decreased. The place and dynamics of coiled bodies suggest that they might be involved in processes related to the nucleolus. Microspores and pollen were cultured at 18, 25 or 32 °C but we did not find any differences in labelling pattern caused by different temperature treatments.

Keywords: *Brassica napus*; coiled bodies; microspore embryogenesis; pollen development; spliceosomes.

Introduction

The nucleus is the major place for RNA synthesis. After transcription, pre-RNA is processed and the mature RNA is transported into the cytoplasm via nuclear pore complexes. Processing of pre-mRNA includes 5'-end capping (Hamm and Mattaj, 1990), splicing (reviewed by Filipowicz et al., 1995; Simpson and Filipowicz, 1996) and 3'-end cleavage and polyadenylation (Keller, 1995). During splicing, introns are removed from the pre-mRNA and the remaining exons are bound together (Krämer, 1995). Splicing takes place in the presence of small nuclear ribonucleoprotein particles (snRNPs), consisting of a set of proteins characteristic for every group of snRNPs, a set of common proteins found in all snRNPs (the so called Sm-proteins), and small nuclear RNA (snRNA). The Sm-proteins are recognized by auto-antibodies from patients with rheumatic diseases. The best known snRNP complexes in the nucleoplasm are U1, U2, U4, U5 and U6 snRNPs.

Several processes in the nucleus take place in distinct structural domains (reviewed by Spector, 1993; Strouboulis and Wolffe, 1996) and snRNPs can be detected in three different sub-nuclear compartments: perichromatin fibrils, clusters of interchromatin granules and coiled bodies (Fakan et al., 1984; Spector et al., 1991; Ferreira et al., 1994).

Perichromatin fibrils are fibrils closely related to chromatin and they are believed to be the sites where RNA splicing takes place directly after transcription (Fakan, 1994). The clusters of interchromatin granules are composed of 20-25 nm particles and linked together by thin fibrils. These clusters gave a weak positive signal after *in situ* hybridization with a poly(dT) probe (Visa et al., 1993) indicating that they contain polyadenylated mRNA. In mammalian cells, the splicing factor SC-35 has also been found in these granules (Xing et al., 1993; O'Keefe et al., 1994). The function of the clusters of interchromatin granules is not clear; however, snRNPs and several other proteins involved in splicing accumulate in interchromatin granules when RNA synthesis is blocked (O'Keefe et al., 1994; Huang et al., 1994).

A coiled body is a nuclear body, first identified by Cajal (1903) after nucleolar silver staining. They are visible in the electron microscope in both animal and plant cells. In mammalian cells they are roughly spherical and vary in size between 0.1-1 μm (Monneron and Bernhard, 1969; Lamond and Carmo-Fonseca, 1993) up to 2 μm in plant cells (Beven et al., 1995). Besides snRNPs, coiled bodies also contain the specific protein coilin P80 (Andrade et al., 1991), the splicing factor U2AF and several snRNAs (Carmo-Fonseca et al., 1991, 1992; Zhang et al., 1992) as well as several nucleolar proteins such as fibrillarin, a protein associated with U3, U8, U13, U14, U16, U20, U21 and Y snRNPs in the nucleolus (Lischwe et al., 1985; Ochs et al., 1985; Solymosy and Pollak, 1993). However, they do not contain rRNA (Carmo-Fonseca et al., 1993), nor the splicing factor SC-35 (Spector et al., 1991). Additionally, other

researchers did not find any accumulation of nascent pre-mRNA (Fakan and Bernhard, 1971; Fakan et al., 1976; Straatman et al., Chapter 5), or DNA (Raska et al., 1991) in coiled bodies. However, Frey and Matera (1995) and Smith and co-workers (1995) found an association of coiled bodies in mammalian cells with several genes, including histon genes, U1 and U2 genes. As a result of these data, coiled bodies are not believed to be sites of pre-mRNA splicing. On the other hand, in metabolically active cells coiled bodies are more numerous (Brasch and Ochs, 1992; Carmo-Fonseca et al., 1992: Andrade et al., 1993) and when transcription is inhibited by α-amanitin or actinomycin-D, snRNPs no longer concentrate in coiled bodies but aggregate in large clusters of interchromatin granules (Carmo-Fonseca et al., 1992; Huang et al. 1994). Also after mitosis, inhibition of transcription blocks the formation of new coiled bodies (Ferreira et al., 1994).

In plant systems, there are only a few reports on the processing of snRNPs. Sanchez-Pina et al. (1989) showed the presence of snRNPs as a speckled labelling pattern during somatic embryogenesis in *Daucus carota* but they did not register changes during development. They also found brightly labelled nuclear bodies. These labelled spots were sometimes localized in the nucleolus. Beven et al. (1995) analysed the distribution of U1, U2 and U6 snRNAs in the radicle of pea and identified the labelled nuclear bodies as coiled bodies using an anti-p80 coilin antibody. The snRNP label showed a fibrous network but the speckled pattern known from mammalian cells was not found. Concha et al. (1995) showed the appearance of snRNPs and U1 snRNA in the vegetative and generative nuclei of *Pinus radiata* and *Viburnum tinus*. In *Capsicum annuum* pollen snRNPs were found (Testillano et al., 1993, 1995; González-Melendi et al., 1995, 1996) but none of these authors reported the presence of nuclear bodies in the pollen. In *Triticum aestivum* and *Hordeum vulgare* the distribution of snRNPs has been studied during the cell cycle but it has not become clear which of the foci are coiled bodies (Glyn and Leitch, 1995).

In this study we report on the dynamics of snRNPs and coiled bodies in particular, during *in vivo* and *in vitro* pollen development and after induction of microspore and pollen embryogenesis in *Brassica napus*. Coiled bodies were identified using two antibodies, anti-Sm, to label the snRNPs, and anti-fibrillarin. The only nuclear body known to contain these two proteins is the coiled body. Besides microspores and pollen, also isolated nuclei derived from young leaves of *Brassica napus* and nuclei of leaf protoplasts were used to identify the coiled bodies and to study the different appearances of these nuclear bodies in plant cells.

When late microspores and early bicellular pollen of *Brassica napus* cv. Topas are cultured for at least 8 hours at 32 °C, their developmental pathway can change from pollen development into embryo development (Pechan and Keller, 1988). If cultured at 18 °C, normal pollen develop (Custers et al., 1994). Early events in microspore embryogenesis in *B. napus* have intensively been studied. Several studies

have focused on changes during the microspore-embryo induction period in relation to DNA synthesis (Binarova et al., 1993), RNA synthesis (Pechan et al., 1991), protein synthesis (Pechan et al., 1991; Cordewener et al., 1994, 1995; Hause et al., 1995) or cytoskeleton organization (Hause et al., 1992, 1993). We used this system also to study other nuclear changes during the switch from pollen development towards embryo development, e.g., rRNA distribution (Straatman and Schel, 1995), nuclear pore frequencies (Straatman et al., 1996b) and the expression of the putative embryo specific gene AGL15 (Straatman et al., 1996a).

Materials and methods

Plant material

Plants of *Brassica napus* L. cv. Topas were obtained from seeds in a phytotron at 18 °C with a 18h photoperiod. Anthers in different developmental stages were collected, ranging from early microspores to mature pollen.

Late microspores and early bicellular pollen were isolated and cultivated in NLN medium (Lichter, 1982), free from potato extract and growth regulators as described by Pechan and Keller (1988). They were either cultured for two days at 32 °C and thereafter transferred to 25 °C (embryogenic conditions) or they were cultured continuously at 18 °C (non-embryogenic conditions). Samples were collected after 0, 8 and 24 hours and 3 days of culture. Anthers, cultured microspores, cultured pollen and embryogenic cells were collected and fixed in 3% (w/v) paraformaldehyde in PBS (35 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4) for 3 hours at room temperature. Samples were rinsed in PBS and the cultured cells were immobilized in 2% low melting point agarose (BDH, Poole, England). Samples were merged in a 13% sucrose solution, frozen in droplets in liquid propane and stored at -70 °C.

Nuclei were isolated from young leaves of *B. napus* as described previously (Straatman et al., 1996c) and stored at -20 °C in glycerol buffer (50% glycerol (Merck, Darmstadt, Germany), 10 mM HEPES (pH 6.0), 250 mM sucrose, 5.0 mM $MgCl_2$, 1 mM 1,4 dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM EDTA).

Protoplasts were freshly derived from cotyledons of seedlings. The cotyledons were harvested and small strips were incubated in 10 ml enzyme solution containing 1% pectinase (Merck, Darmstadt, Germany), 0.2 % macerozyme R10 (Serva, Heidelberg, Germany), 1% cellulase Onozuka R10 (Serva), 5 mM MES, 5 mM arginine and 0.5% PVP, pH 5.6 in CPW9 (Power and Chapman, 1985) for 4-6 hours at room temperature. After incubation the protoplasts were filtered through a 100 µm nylon filter and centrifuged at 50 x g for 4 min. The pellet was resuspended in 3.5 ml CPW9 and layered on top of 4 ml 0.5 M sucrose solution in a centrifuge tube followed by 4 min centrifugation at 45 x g. The protoplasts were harvested and washed in

CPW9. The pellet was resuspended in KM8P culture medium (Kao and Michayluk, 1975).

Specimen preparation

Serial sections (5 µm) from frozen material, attached to a holder with M-1 embedding matrix (Lipshaw, Pittsburgh, USA), were prepared on a cryo-microtome (Micron, Biomed HM500) and mounted on aminopropyl-triethoxy-silane-coated slides (Serva, Heidelberg, Germany). The slides were dried on air and washed in PBS.

A drop of glycerol buffer containing isolated nuclei was put on a coated slide and the nuclei were allowed to stick on the slides for 5 min, followed by a short wash in PBS and a 15 min fixation in 2% paraformaldehyde. Protoplasts were mounted on coated slides and immobilized in 2% low melting point agarose (BDH, Poole, England) before fixation in 3% paraformaldehyde for 30 min.

Immunofluorescence labelling

All material was blocked in 1% BSA in PBS for 30 minutes and washed in 0.1% acetylated BSA (BSAc; Aurion, Wageningen, The Netherlands) followed by the first antibody incubation overnight at 4 °C. In single labelling experiments this first antibody was 7.13 (gift from F. Ramaekers, Dept. of Pathology, University of Nijmegen), a mouse monoclonal antibody which recognizes protein D of U1, U2, U4, U5 and U6 snRNPs (Billings et al., 1982, 1985; Sanchez-Pina et al., 1989) and was used undiluted. In double labelling experiments anti-fibrillarin P89 (kindly provided by W. Pluk, Dept. of Biochemistry, Nijmegen University, The Netherlands) was diluted 1:50 in antibody 7.13.

The next day the slides were washed in 0.1% BSAc followed by incubation in the second antibody for 1 hour at 37 °C. This second antibody was goat-anti-mouse-Bodipy (Molecular Probes, Leiden, The Netherlands) diluted 1:100 in 0.1% BSAc and goat-anti-human-TRITC (Sigma Chemical Co., St Louis, MO, USA) diluted 1:20. After washing in PBS, the DNA was stained with 0.1 µg/ml propidium iodide in single labelling experiments or with 1 µg/ml 4,6-diamidino-2-phenylidole (DAPI, Sigma) in double labelling experiments. The slides were mounted in Citifluor-glycerol (Citifluor Ltd., Canterbury, U.K.) and analysed with a MRC600 Bio-Rad confocal scanning laser microscope using an argon-krypton ion laser and a K1/K2 filter combination attached to a Nikon Diaphot inverted microscope equipped with a x 100/1.3 N.A. oil immersion lens.

Results

Labelling pattern of anti-Sm and anti-fibrillarin and the identification of coiled bodies

To examine the appearance of snRNPs in nuclei of *Brassica napus*, the mouse monoclonal antibody 7.13 raised against protein D of U1, U2, U4, U5 and U6 snRNPs was used on several cell types. In isolated nuclei a punctate labelling was seen in the nucleoplasm, besides intensively labelled foci. In some isolated nuclei of young leaves as well as in some nuclei of leaf protoplasts, these intensively labelled foci were also detected in the nucleolus (Figs. 1a, 2). In nuclei of leaf protoplasts and on cryosections of microspores and pollen, the labelling of snRNPs was uniform in the nucleoplasm (Figs. 2, 3) and, if present, also in the nucleolar vacuole. During pollen development and after induction of embryogenesis often one or more brightly labelled foci were found in the nucleoplasm (Fig. 3a). Labelled nuclear bodies in the nucleolus were never seen in these stages. When the signal was less intense, a fine network could be seen in the nucleoplasm, resembling labelling of interchromatin regions as shown by Testillano et al. (1993, 1995).

To characterize the intensively labelled foci, we tested several antibodies against the protein coilin p80, reported to be specific for coiled bodies, but no labelling was detected using *Brassica* cells. Therefore, double labelling experiments with the anti-snRNP 7.13 and the anti-fibrillarin P89 (see Chapter 5) were carried out to identify these nuclear bodies. Labelling with anti-fibrillarin resulted in a signal that showed some variety in intensity throughout the nucleolus of all tested material (isolated nuclei, nuclei of leaf protoplasts and nuclei of microspores, pollen and embryo-induced material) and labelling of one or more nuclear bodies in the nucleolus (Fig. 1b) or nucleoplasm (Fig. 3b). The labelling of nucleolar bodies was only found in nuclei originating from leaf material. In nuclei from protoplasts sometimes an intensively labelled nucleolar ring was found when labelled for fibrillarin (Figs. 4d, 4e).

When the signals obtained with anti-snRNP and anti-fibrillarin were merged, it became clear that in most cases the nuclear bodies labelled for snRNPs were also labelled for fibrillarin. This was found for the foci in the nucleolus as well as in the nucleoplasm (Figs. 1, 3). Only in nuclei of leaf protoplasts, nuclear bodies that only labelled for snRNPs and not for fibrillarin were found. In Fig. 4 a nucleus of a leaf protoplast of *B. napus* is shown. In this nucleus six nuclear bodies were found, three snRNP positive nuclear bodies, which were not labelled for fibrillarin, and three nuclear bodies that were positive for both antibodies. This was found in rare cases and only in nuclei of leaf protoplasts.

Pollen development

During *in vivo* pollen development, from early microspore stage towards late microspore stage, the nucleus and nucleolus enlarged and a nucleolar vacuole

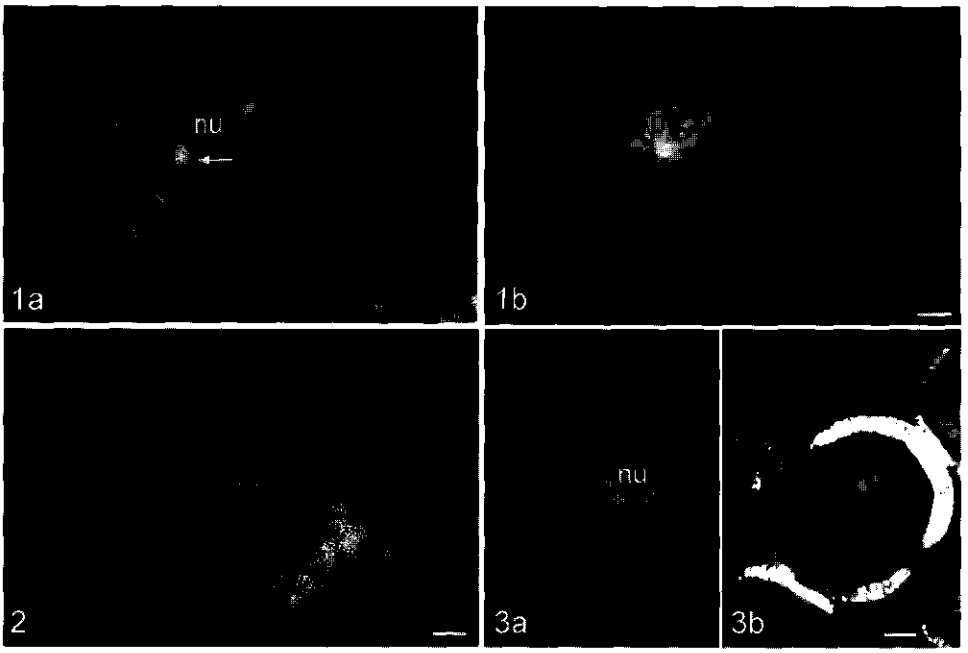


Fig. 1; Labelling of snRNPs in isolated nuclei of *Brassica napus* showed in optical sections a punctate labelling pattern in the nucleoplasm. **a**; in some cases nucleolar foci were found (arrow). **b**; the same nucleus as shown in **a**, but now labelled against the nucleolar protein fibrillarin. In the nucleolus (nu) the brighter labelled nuclear body was also found (arrow), identifying this body as a coiled body.
Fig. 2; Optical section through a nucleus of a leaf protoplast of *Brassica napus*. A nucleolus is visible with a large nucleolar vacuole (v). Both the nucleoplasm and the nucleolar vacuole are labelled for snRNPs as well as a nuclear body in the nucleolus (arrow).
Fig. 3; An optical section of a cryosection of a late microspore from *in vivo* material. **a**; a coiled body (arrow) is identified by labelling with an antibody against snRNPs and **b**; the same coiled body identified using an antibody against fibrillarin. Merging of **a** and **b** resulted in a perfect match of both signals. The labelling of snRNPs is more intense at the periphery of the coiled body whereas the labelling of fibrillarin is homogeneous.
Bars 1 µm (**1, 2**), 4 µm (**3**).

appeared. The nucleoplasm of young microspores showed a relatively weak signal when labelled for snRNPs, with little or no cytoplasmic labelling (Figs. 5, 6). In late microspores a stronger signal was found in both the nucleoplasm and the nucleolar vacuole (Fig. 7).

After the microspore mitosis the vegetative and generative nucleus were situated closely to the pollen wall and both nuclei showed a strong nucleoplasmic signal for snRNPs (Fig. 8). In the late bicellular pollen stage, the DNA of the generative nucleus became more condensed and the nucleolus disappeared coinciding with a loss of

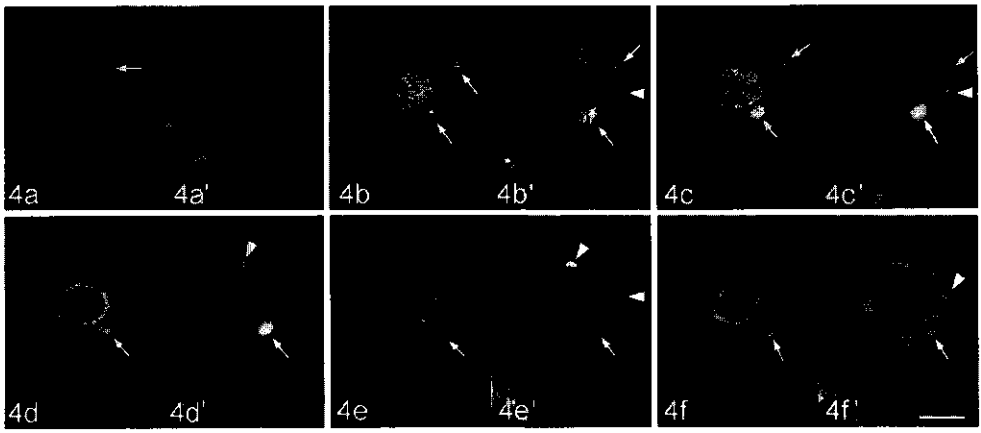
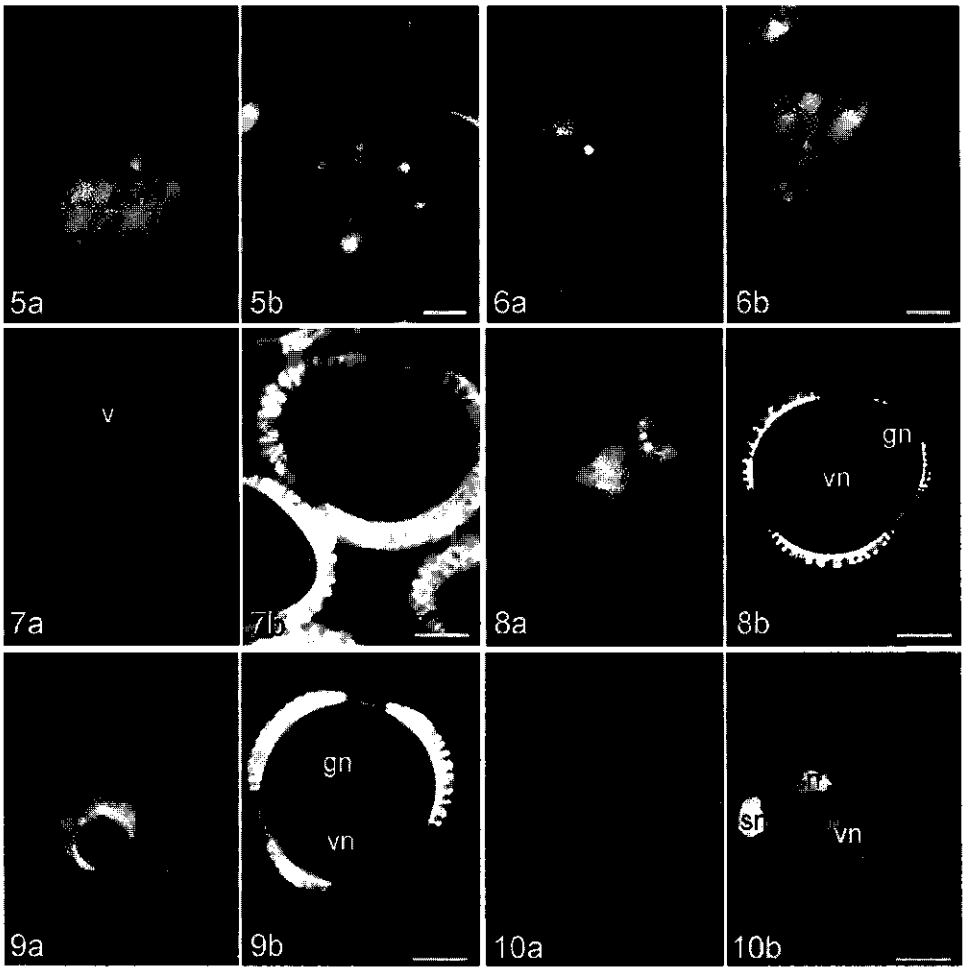


Fig. 4; Six optical sections through a nucleus of a leaf protoplast of *Brassica napus*; **Figs. 4 a-f** show the signal obtained with anti-fibrillarin and the **Figs. 4 a'-f'** show the signal obtained with anti-Sm to label the snRNPs. In this nucleus three nuclear bodies have been found that only labelled for snRNPs and not for fibrillarin (arrowheads) and three nuclear bodies that labelled with both antibodies (arrows).
Bar 5 µm.

signal for snRNPs. The vegetative nucleus was still large and contained a large nucleolus. It remained positive for snRNPs in this stage (Fig. 9).

After the pollen mitosis, the nuclei of the tricellular pollen did not show any signal when labelled for snRNPs (Fig. 10). When microspores and young pollen were cultured at 18 °C, resulting in *in vitro* pollen development, the same results were obtained. Only the background signal in the cytoplasm was stronger (not shown).

During the development from young microspores towards young pollen the number of coiled bodies per nucleus increased slowly (Table 1). In pollen stages the coiled bodies were only counted in the vegetative nucleus, not in the generative nucleus because most of these nuclei did not show labelling for snRNPs. A dramatic increase in size of the coiled bodies was observed during this development; from less than 0.5 µm in young microspores (Figs. 5a, 6a) towards more than 2.0 µm in late microspores and young pollen (Figs. 3a, 7a). The small coiled bodies showed a spherical morphology whereas the large coiled bodies mostly were more oval. In large coiled bodies the labelling of snRNPs was often observed at the periphery with less or not any label in the centre, whereas fibrillarin was found in the whole coiled body (see e.g. Fig. 3). After the microspore mitosis, coiled bodies were found in both the



Figs. 5-10; The labelling of snRNPs during *in vivo* pollen development. Optical sections through nuclei showing in **a** panels the labelling with anti-Sm and in **b** panels the DNA stained with propidium iodide.

Figs. 5, 6; In young microspores the nuclei are relatively weakly labelled in the nucleoplasm, with a small coiled body (arrow) closely related to a small nucleolus (arrowheads).

Fig. 7; In late microspores the nucleus has moved to the exine wall near the central vacuole (v). Both the nucleolus and the coiled body have increased in size.

Fig. 8; After the microspore mitosis both the generative (gn) and vegetative (vn) nuclei are positively labelled for snRNPs.

Fig. 9; In late bicellular pollen the generative nucleus (gn) is losing its signal for snRNPs whereas the vegetative nucleus (vn) remained positively labelled and still contained a coiled body (arrow).

Fig. 10; After the pollen mitosis in the vegetative nucleus (vn) and in both sperm nuclei (sn) not any label for snRNPs could be detected. Bars 2 µm (**5, 6**), 5 µm (**7, 9, 10**), 7 µm (**8**).

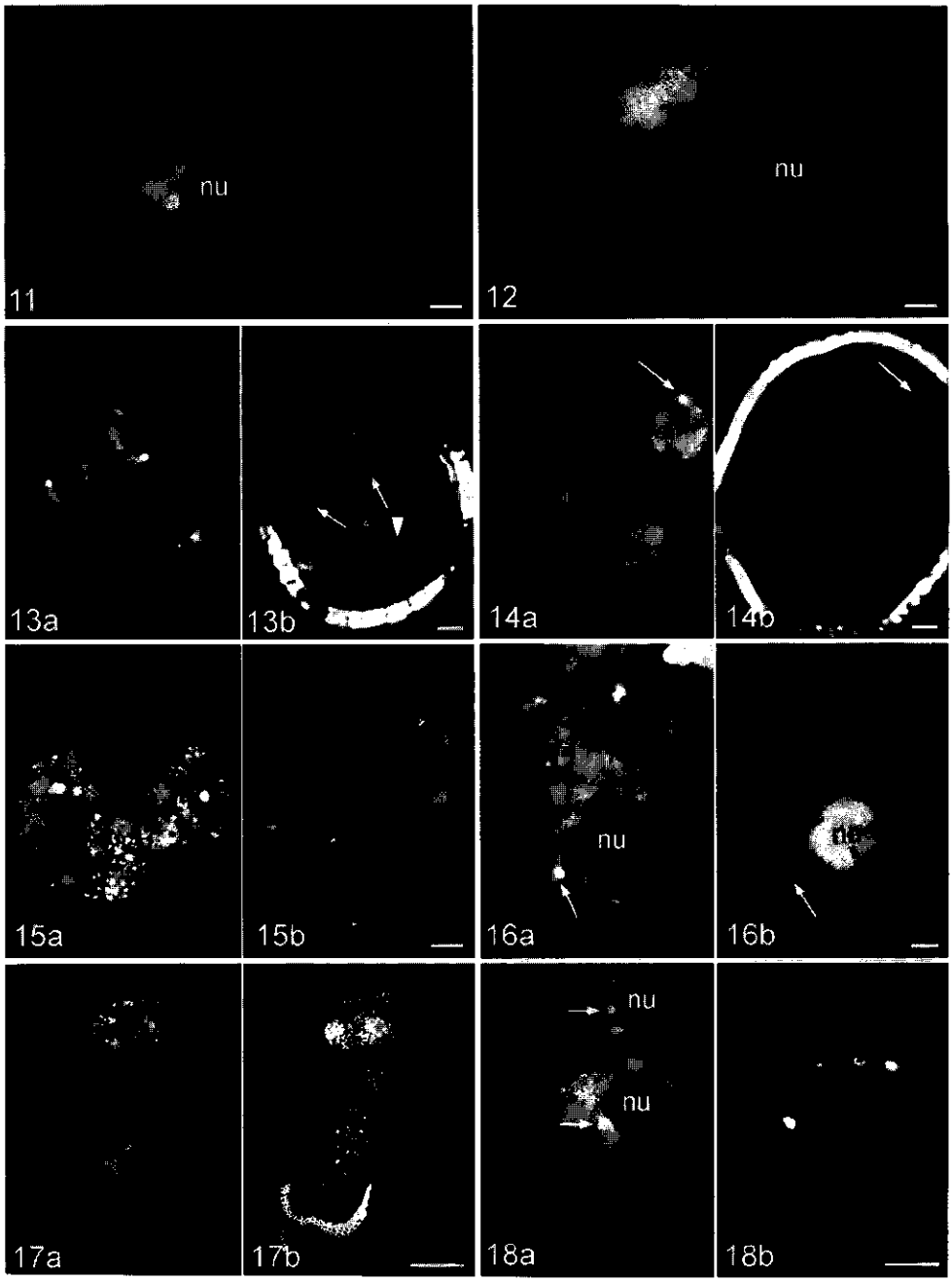
Table 1; The percentage of sectioned nuclei of different stages of pollen development *in vivo* and *in vitro* and after induction of microspore and pollen embryogenesis of *Brassica napus* with zero, one, two or three coiled bodies found in cryosections; N = the number of sectioned nuclei counted. In pollen the coiled bodies were only counted in the vegetative nucleus.

	number of coiled bodies				N
stage	0	1	2	3	
young microspores	65	33	2	-	101
late microspores	51	48	1	-	132
early bicellular pollen	44	41	14	1	71
late bicellular pollen	38	58	4	-	146
1 day 18 °C	41	56	2	-	94
3 days 18 °C	63	37	-	-	41
1 day 32 °C	59	41	-	-	46
7 days embryos	44	50	4	1	93

generative and vegetative nucleus (Figs. 8a, 9a). In a few cases, three to four small nuclear bodies were present in the generative nucleus shortly after this division, when both the vegetative and generative nuclei were still situated closely to the pollen wall (Fig. 8a). Unfortunately, we were not able to find these stages in double labelling experiments using both anti-snRNP and anti-fibrillarin. Between the early and late bicellular pollen stage, the generative nucleus lost it signal of snRNPs. The number of coiled bodies in the generative nucleus which did label with anti-snRNP decreased to approximately one coiled body per nucleus. The number of coiled bodies in the vegetative nucleus had slightly increased after the microspore mitosis, although most nuclei still contained one coiled body. The size of the coiled bodies had not changed after this division but from the early to the late bicellular stage it decreased to 1.5 µm (Fig. 9a) and the number of coiled bodies became deminished (Table 1). After the pollen mitosis all label of snRNPs disappeared, so no labelled coiled bodies were found (Fig. 10).

Microspore and pollen embryogenesis

When late microspores and young pollen are cultured for two days at 32 °C, they can change their developmental pathway from pollen development towards embryo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development. This change means that, instead of a asymmetrical pollen division which gives rise to a large vegetative cell and a small generative cell, the cells divide symmetrically. After the first symmetrical division both nuclei were positive when labelled for snRNPs (Figs. 11, 12). After 3 and 7 days of culturing under embryogenic conditions several nuclei showed a faint label or no label at all with this antibody. However, in an embryogenic structure, labelled and unlabelled nuclei could be found (Figs. 13, 14, 15).

From the moment of isolation until the first symmetrical division the size of the coiled bodies decreased from approximately 2 µm to 1.5 µm. In several nuclei the coiled body was no longer related to the nucleolus, but was found more to the periphery of the nucleus (Figs. 12, 13a). After the second embryogenic division only a few coiled bodies, with a size of 0.5 µm, could be visualized (Fig. 14a). After 7 days of culture, when the embryo is globular, the coiled bodies ranged in size from 0.5 to 0.8 µm (Fig. 16). The number of coiled bodies did not change during culturing.

In some cultures suspensor-like structures with a globular embryo can be found, as in the zygotic embryos (Fig. 17). The nuclei in these structures, often with a nucleolar vacuole, were positively labelled for snRNPs, both in the suspensor-like structure as well as in the globular part of the embryo. They contained coiled bodies with a size between 0.5 and 1 µm (Fig. 18), situated closely to the nucleolus. In general one coiled body per nucleus was found.

Figs. 11-18; The labelling of snRNPs after induction of microspore and pollen embryogenesis.
Figs. 11, 12; After embryo induction microspores divide symmetrically instead of asymmetrically. In both nuclei snRNP labelling is found in the nucleoplasm. Coiled bodies were found closely related to the nucleolus (nu) (**Fig. 11**) as well as free in the nucleoplasm (**Fig. 12**).
Fig. 13; An embryogenic structure is shown, probably after the second embryogenic division, three days in culture; **b** shows three nuclei labelled with propidium iodide (arrows and arrowhead), whereas the snRNP labelling in **a** only clearly showed two nuclei; one nucleus of **b** (arrowhead) is hardly labelled for snRNPs. Coiled bodies were still found in close relation to the nucleolus and free in the nucleoplasm.
Fig. 14 shows that also in early embryogenic structures the coiled bodies were labelled with both the antibodies against snRNPs (arrow) (**a**) and fibrillarin (arrow) (**b**).
Fig. 15 shows an overview of a globular embryo after 7 days of culture, **a** shows the snRNP labelling and **b** the fibrillarin labelling.
Fig. 16 is an enlargement of the embryo seen in **Fig. 15**, showing a coiled body free in the nucleoplasm (arrows) labelled against snRNPs (**a**) and fibrillarin (**b**).
Figs. 17, 18; In some cultures, embryos with a suspensor-like structure can be found. The nuclei of these structures label with antibodies against snRNPs (**Fig. 17a**) and often contain one coiled body in close relation to the nucleolus (nu) (**Fig. 18a**, arrows). **Figs. 17b** and **18b** show the DNA staining with propidium iodide.
Bars 2 µm (**11, 12, 16**), 3 µm (**13, 14**), 5 µm (**15, 18**), 25 µm (**17**).

Embryogenic cultures also contained cells which did not change into embryos but continued the regular path of pollen development. These cells showed a labelling pattern that was comparable to that found during *in vivo* pollen development and in pollen cultures at 18 °C.

Discussion

Labelling pattern of anti-Sm and anti-fibrillarin and identification of coiled bodies

Beven et al. (1995) used the mAb 4G3, raised against the human U2B" protein to label the U2 snRNPs in *Pisum sativum*. We did not get any signal using this antibody on root tip cells of *B. napus* or *Allium cepa*, although the controls, using cells of *P. sativum* were positive (not shown). This forced us to use another antibody raised against snRNPs and to label a larger group of the spliceosomes. In the past the mAb 7.13 had proven to give a good labelling of snRNPs in plant nuclei (Sanchez-Pina et al., 1989; Testillano et al., 1993) and was therefore used in these experiments.

To identify the coiled bodies, we tried several antibodies against coilin p80, but no signal was obtained in our cells. However, because coiled bodies contain fibrillarin and snRNPs, antibodies against both epitopes can be used to identify them. To our knowledge, there is no other subnuclear structure known that contains both proteins. All stages were analyzed with both antibodies. However, it was difficult to make good images from fibrillarin labelling in the coiled bodies of microspores and pollen material because both the exine and nucleolus gave a more intense labelling. As a result the coiled bodies were often difficult to see in the images (see Figs. 1b and 3b).

In double labelling experiments with material obtained from young leaves a nuclear body was found that only showed a signal when labelled against snRNPs and no signal with anti-fibrillarin. This might be a phase of the formation or deformation of coiled bodies. This was a rare phenomenon, which was only observed in leaf protoplasts and never during double labelling experiments using microspores or pollen nuclei. In microspore and pollen material all double labelling experiments showed labelling of the nuclear bodies with both anti-bodies. Only the high number of nuclear bodies in the generative nucleus of early pollen could not be positively identified as coiled bodies. In mouse 3T3 cells Raska et al. (1991) showed, using anti-fibrillarin and anti-coilin p80, some fibrillarin positive nuclear bodies that did not label with anti-coilin p80. However, they did not found any nuclear bodies labelled against snRNPs and not labelled with anti-coilin p80. Beven et al. (1995) showed in *P. sativum* that the nuclear bodies labelled with anti-coilin p80 always were labelled with an antibody against U2 snRNPs. In *Xenopus* egg extracts, Bauer and Gall (1997) found interdependence between the presence of coilin and snRNPs in coiled bodies, using immunodepletion experiments. Based on these data, we think that in *Brassica* nuclei it is plausible to identify the snRNP containing nuclear bodies as coiled bodies.

In nuclei of leaf material coiled bodies have also been found in the nucleolus. Such a nucleolar coiled body has been reported in both mammalian nuclei (Raska, 1991; Ochs et al., 1994) and plant nuclei (Sanchez-Pina et al., 1989; Olmedilla et al., 1997). Olmedilla et al. (1997) reported nucleolar coiled bodies to be present in microsporocytes during meiotic prophase in *Olea europaea*, a developmental stage not analysed in our experiment. However, we never found nucleolar coiled bodies during the pollen development as was also reported for pollen of *Capsicum anuum* (Testillano et al., 1993).

Pollen development

During pollen development, the labelling intensity for snRNPs increased from young microspores towards late microspores and young pollen. During this period, the size of the nucleus and the nucleolus increased and a nucleolar vacuole appeared, which also contained snRNPs. These changes indicate an increased nuclear activity. During this period, also the size of the coiled bodies increased from less than 0.5 µm to 2 µm. Most of these coiled bodies were found in close relation to the nucleolus. This could mean that coiled bodies are somehow involved in the maturation of ribosomal RNA.

From the sections it is difficult to say if the number of coiled bodies increased during this period. Because sections of 5 µm were cut, parts of a small nucleus, as in the early microspore stage, will be distributed over two or three sections and parts of a large nucleus, as in the late microspore stage, will be distributed over three or four sections. As a result of the increased size of the coiled bodies the chance that one coiled body is cut into two pieces and therefore can be found in two sections has increased as well. This makes it difficult to estimate the number of coiled bodies per nucleus. However, the number of nuclei with two coiled bodies in one section can also give an indication about the changes in numbers of coiled bodies. Table 1 shows that in the early bicellular pollen stages the number of sectioned nuclei with two or three coiled bodies has increased, indicating that in this stage the average number of coiled bodies per nucleus is higher than in the microspores and in the late bicellular stage. So, young microspores probably have zero to one coiled body per nucleus. This number increased to one or two coiled bodies per nucleus in early bicellular pollen. Raska et al. (1991) found a maximum from one to eight coiled bodies per nucleus depending on the species examined. It is not yet clear if this difference in number, size or place depends on the species, cell type, cell cycle or nuclear activity or on the development of the coiled bodies itself, or a combination of these factors. Our results suggest that during pollen development of *B. napus* increase of the size of the coiled bodies coincides with an increase of nucleolar activity.

It was found that in early bicellular pollen both the generative and vegetative nucleus were labelled for snRNPs, indicating that both nuclei are transcriptionally active. Additionally both nuclei showed coiled bodies. Shortly after the microspore

mitosis in a few cases three to four coiled bodies were found in a generative nucleus. This high number can be an indication for a change in activity of the generative nucleus before and after mitosis, because in the highly active vegetative nucleus this high number of coiled bodies was observed only once. In later stages of the generative nucleus only one small coiled body was found before all signal was lost.

In mature pollen both the vegetative nucleus and sperm nuclei lacked snRNP-labelling. This is not surprising because pollen is resting and viable for several days without RNA synthesis (Mascarenhas, 1975). However, it is also possible that the epitope for binding the antibodies was blocked as a result of survival strategies.

Microspore and pollen embryogenesis

In our experiments, inducing embryogenesis at 32 °C, we saw a slightly decrease in the number and size of coiled bodies at the onset of culture, which was not seen in non-embryogenic cultures at 18 °C. In higher plants, many heat shock protein genes contain introns. This implies that under heat shock conditions splicing of heat shock RNA still takes place (Christensen et al., 1992; Osteryoung et al., 1993). In wheat cells from germinating seeds Glyn and Leitch (1995) found that the number of coiled bodies increased when the temperature was increased from 4 to 37 °C. However, heat shock experiments with root tip cells from pea at 42 °C for one hour did not show any label of coiled bodies with both an U2 anti-sense probe and an antibody against U2 (Beven et al., 1995). So, the decrease found in our experiments might be due to the culture temperature.

After induction of microspore and pollen embryogenesis, several nuclei in multicellular structures did not show labelling of snRNPs. In an embryogenic structure some nuclei were labelled for snRNPs where some others were not. We think that this is caused by a bad penetration of the antibody or that binding sites for the antibody were not accessible. In non-embryogenic structures in these cultures, labelling of snRNPs was no problem, indicating that this is a specific problem for the embryogenic strucutres and not a result from the higher culture temperature.

After the first symmetrical division of the induced microspores and pollen large snRNP positive nuclei were observed with relatively small nucleoli. Also the size of the coiled bodies was reduced and several coiled bodies were found in the nucleoplasm, no longer proximity to the nucleolus. The smaller size of the nucleolus indicates a reduced transcriptional activity of ribosomale genes, which might be reflected by the reduced size of the coiled bodies. The fact that more coiled bodies were found at some distance from the nucleolus might also be an indication that they were transported away from the nucleolus and disintegrated resulting in one small remaining coiled body.

We have shown that during pollen development the generative nucleus in bicellular pollen and all nuclei in tricellular pollen lost their signal for snRNPs, probably

as a result of the lack of RNA synthesis in these stages. During pollen development not the number of coiled bodies is much influenced, but the size of the coiled bodies increased and this change coincided with a change in size of the nucleolus. After embryo induction the size of the coiled bodies decreased again as was the size of the nucleolus. However, the function of these coiled bodies remains unknown.

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Chapter 7

Nuclear pore dynamics during pollen development and androgenesis in *Brassica napus* L. cv. Topas

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Summary

This chapter describes changes in nuclear pore complex (NPC) densities, NPCs/nucleus and NPCs/μm^3 of freeze-fractured *Brassica napus* microspores and pollen, both *in vivo* and *in vitro*, and of early stages of microspore and pollen derived embryogenic cells. Changes found are discussed in terms of transcriptional activity. An increase in NPC density from 13 NPCs/μm^2 in young microspores up to almost 19 NPCs/μm^2 in the vegetative nucleus of tricellular pollen was found during *in vivo* pollen development. The total number of NPCs/nucleus increased from 979 to 3250. The NPC density of the vegetative nucleus of tricellular pollen *in vitro* increased to around 17 NPCs/μm^2 with a total number of NPCs/nucleus of 4300 after three and seven days of culture under non-embryogenic conditions. Both the results of *in vivo* and *in vitro* pollen development indicate an increase in transcriptional activity in the vegetative nucleus during maturation of the pollen. At the onset of microspores and pollen cultures the NPC density decreased from 15 NPCs/μm^2 at the stage of isolation down to 9 NPCs/μm^2, both under embryogenic and non-embryogenic conditions. This implies that the drop in NPC density is a result of culturing the microspores and pollen rather than an indication for microspore and pollen embryogenesis in *B. napus*. However, after one day in culture under embryogenic conditions, the NPC density had increased again and stabilised around 13 NPCs/μm^2 whereas under non-embryogenic conditions the NPC density was still around 9 NPCs/μm^2. The number of NPCs/nucleus in embryogenic cultures had increased from 3901 to 5136 within this first day of culture at 32 °C, whereas in non-embryogenic cultures the total number of NPCs/nucleus had decreased to 2563. The results of the NPC density were in general in agreement with changes found in the total number of NPCs/nucleus during these developmental stages. A low NPC density of 9 NPCs/μm^2 was found in the nuclei of sperm cells. This means that, although both the vegetative and sperm nuclei are believed to be transcriptionally inactive in mature pollen, the NPC density of the vegetative nucleus is twice as high as the NPC density of the sperm nuclei. In a few cases, the embryos formed suspensor-like structures which also had a low NPC density of 9 NPCs/μm^2, indicating a lower transcriptional activity in the nuclei of the suspensor cells in comparison with the nuclei in the embryo proper. In addition, also observations on NPCs and organelle organization, obtained by high resolution cryo-scanning microscopy, are presented.

Keywords: androgenesis; *Brassica napus*; freeze fracturing; nuclear pore complex; pollen development.

Introduction

In angiosperms, a meiotic division of the microsporocyte results in a tetrad which will release four microspores. These microspores divide asymmetrically to form bicellular pollen grains. In species with mature bicellular pollen the pollen mitosis will take place in the pollen tube whereas in tricellular pollen this pollen mitosis takes place inside the pollen grain.

In some species, microspores and young pollen can change their developmental pathway from gametogenesis to embryogenesis if cultured under special stress conditions. The resulting embryo develops from a single cell without an intervening callus phase. So, very early events involved in the induction of embryogenesis can be studied in such a system. Also for plant breeding, the production of homozygous plants after spontaneous duplication of the chromosomes or after a colchicine treatment (Zhao et al., 1996) and the possibility to produce transgenic plants (Dormann et al., 1996) makes microspore and pollen culture a powerful system. It has been developed and used for research in several plants, e.g. *Nicotiana* (Kyo and Harada, 1985; Garrido et al., 1993, 1995), *Triticum aestivum* (Datta and Wenzel, 1987; Touraev et al., 1996), *Hordeum vulgare* (Hoekstra et al., 1992), *Brassica* (Pechan and Keller, 1988) and *Capsicum* (González-Melendi et al., 1995).

Microspores and young bicellular pollen of *Brassica napus* cv. Topas can change their developmental pathway from pollen development towards embryogenesis if cultured for at least 8 hours at 32 °C (Pechan and Keller, 1988; Custers et al., 1994). Recently, Binarova et al. (1997) showed that also late bicellular pollen can be triggered to embryogenesis by a short heat shock of 41 °C followed by cultivation at 32 °C for 2 days. If microspores and young pollen are cultured at 18 °C normal gametophytic development continues (Custers et al. 1994). The change in developmental pathway after the heat shock coincides with changes in DNA synthesis (Binarova et al. 1993), cell division patterns (Hause et al., 1992, 1993), mRNA synthesis (Pechan et al., 1991) and protein synthesis (Pechan et al., 1991; Cordewener et al., 1994).

Changes in RNA and DNA synthesis are often reflected by changes in the total number of nuclear pore complexes (NPCs) per nucleus as well as the frequency of the NPCs (Maul et al., 1972; LaFountain and LaFountain, 1973; Jordan and Chapman, 1973; Wagner et al., 1990). Transfer of molecules between the nucleus and the cytoplasm occurs through these NPCs (Goldfarb and Michaud, 1991; Boelens et al., 1995; Hicks and Raikhel, 1995). Feldherr et al. (1984) showed that gold particles were entering the nucleus through all NPCs suggesting that all pores are functionally equal. Furthermore, Dworetzky and Feldherr (1988) showed that transport across the NPCs is bidirectional. NPCs are embedded in the nuclear envelope; they are composed of up to 100 different proteins (Reichelt et al., 1990) and cylindrically shaped with a diameter of approximately 100 nm. The NPCs are thought to be formed by three rings

of proteins, one at the nucleoplasmic surface, one at the cytoplasmic surface and the central transporter and 'spoke' ring complex. These rings show an eightfold symmetry. The rings are interconnected by eight vertical supports forming a so called 'basket' structure. An NPC has a resting channel size of 9-11 nm which allows passive transport and can open to 25-28 nm in diameter for active transport (Feldherr et al., 1984; Silver, 1991; Hinshaw et al, 1992; Goldberg and Allen, 1993; Panté and Aebi, 1993).

We have studied the NPC dynamics during *in vivo* pollen development and during the culture of microspores and young pollen under embryonic and non-embryonic conditions using freeze-fractured material. Using this technique, more information can be collected about nuclear activity during pollen development and about nuclear changes after the induction of embryogenesis. Besides information concerning the NPCs this technique also allows to get some information about other organelles during the different developmental stages.

Materials and methods

Plant material

Plants from *Brassica napus* cv. Topas were grown from seeds in a phytotron room at 18 °C with a photoperiod of 16 h light. Flower buds with various lengths, containing microspores, bicellular pollen and mature pollen, and free pollen from open flowers were collected to analyse the *in vivo* NPC density (Pechan and Keller, 1988).

Microspore cultures were obtained from flower buds between 3.2 and 3.6 mm length containing late microspores and early bicellular pollen. After rinsing the cells in B5 medium containing 13% sucrose, they were cultured for 1 day at 32 °C in modified Lichter medium (NLN, Lichter, 1982), free of potato extract and growth regulators as described by Pechan and Keller (1988), and further cultured at 25 °C (embryogenic condition). Other cultures were continuously maintained at 18 °C (non-embryogenic condition). After zero, two and eight hours and one, three and seven days of culture samples were taken.

Freeze-fracture

A high concentration of cells in a 13% sucrose solution was prepared by collecting the cells on a 25 µm filter. A droplet of this solution was transferred between two copper specimen holders and frozen in liquid propane. The sandwiched samples were fractured under high vacuum at -130 °C in a freeze-etching device (BAF 400, Balzers, Liechtenstein). The surfaces were shadowed with 2 nm platinum under an angle of 45°, followed by a deposition of 30 nm carbon under an angle of 90° to strengthen the replica.

To remove the replicas from the holders, they were drifted off on 13% sucrose and rinsed several times in distilled water. The water was replaced by 5% chromic acid and the replicas were cleaned overnight, rinsed in distilled water and etched for 2 h in 4% hypochlorite. After several wash steps in distilled water, the replicas were collected on glowed 200 mesh copper grids and examined with a JEM-1200 EXII transmission electron microscope operating at 80 kV. The nomenclature of the fractured nuclear membranes is according to Branton et al. (1975).

Quantitative study of nuclear pores

To calculate nuclear pore densities, calibrated micrograph prints were made from exposed nuclear fracture faces and analysed using the TIM image processing program (Difa Measuring Systems, Breda, The Netherlands). NPCs were counted from nuclear membrane surfaces of the different pollen stages and of the embryo induced cells and the nuclear area where the NPCs were counted was measured. After three and seven days of culture under embryogenic conditions only the embryogenic structures were analysed.

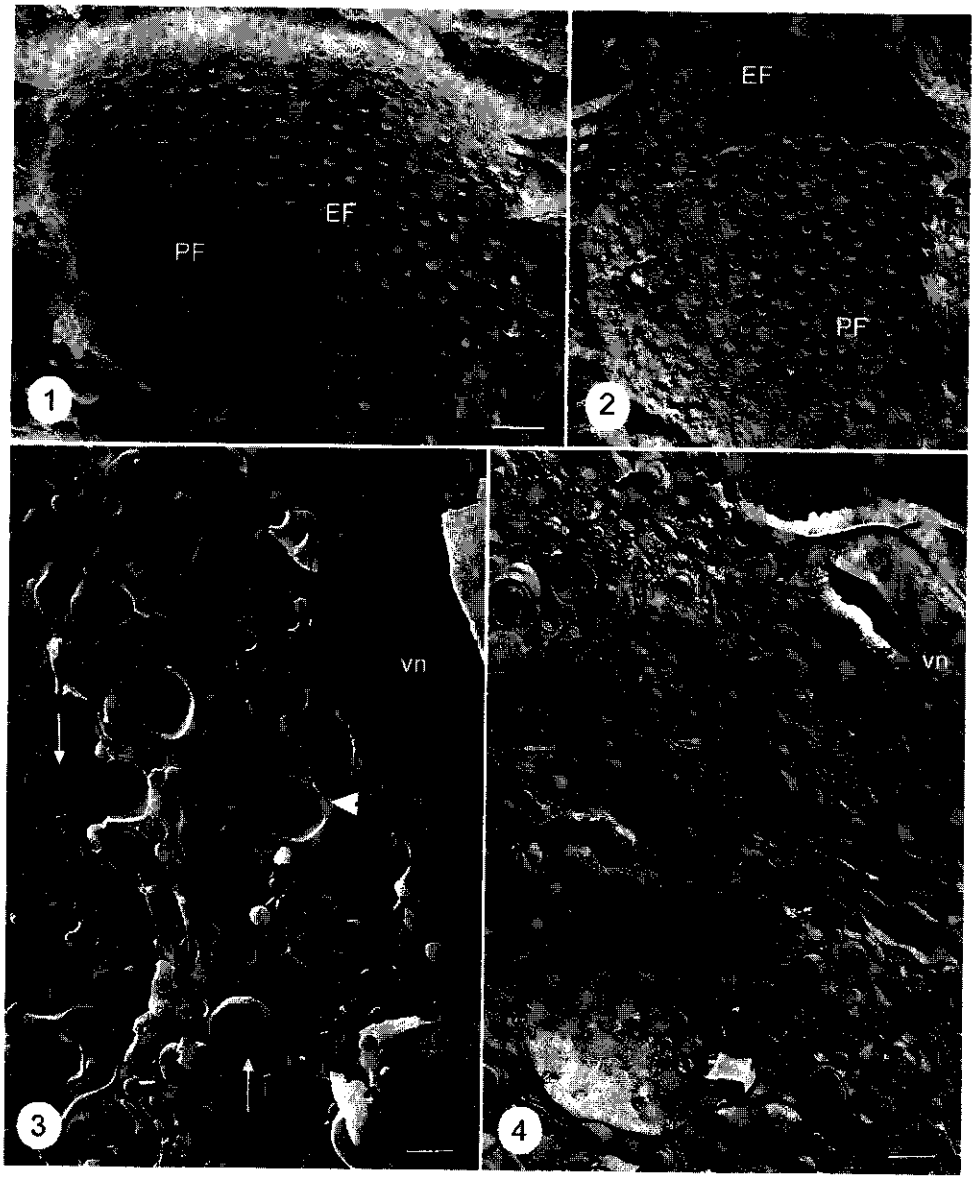
To measure the total nuclear surface and the nuclear volume, microspores and pollen were fixed in 3% paraformaldehyde + 0.25% glutaraldehyde and the DNA was stained with 4,6-diamidino-2-phenylindole (DAPI). Pictures of 100 nuclei of every sample were made with a digital camera (Panasonic WV-E550E) and the nuclear diameter was measured in two directions from the screen to calculate a mean diameter. The nuclear surface and volume was then calculated with this mean diameter assuming the nuclei were spherical.

CryoSEM

As a control of the replica images and to get additional information, fresh microspores and pollen were frozen as before and the sandwiches were broken in a low temperature field emission scanning electron microscope (LT-FESEM; JEOL JSM-6300F) equipped with an Oxford cryochamber. After sputter coating with 3 nm platinum the surfaces were analysed without further treatment.

Results

The youngest stage of microspores found in the samples of *in vivo* material were microspores which showed small vacuoles. This indicates that this stage was not harvested directly after release of the tetrads but a bit further in development. The second sample mostly contained young bicellular pollen besides late microspores. The third sample was taken just before flowering, containing tricellular pollen. Microspores and pollen cultured at 25 °C for six days, after a heat shock of 32 °C for one day, showed embryogenic multicellular structures, large swollen structures with one or two



Figs. 1-4; Comparison of fractured surfaces of pollen material of *Brassica napus* analysed directly in the LT-FESEM and as a replica in the TEM. Bars: 500 nm.
Figs. 1-2; A fracture showing the nuclear surface of a vegetative nucleus in the SEM (Fig. 1) and TEM (Fig. 2) showing in both cases the EF and PF faces and the NPCs.
Figs. 3-4; A comparable overview of the cytoplasm in mature *Brassica* pollen obtained using the LTFE SEM (Fig. 3) and TEM (Fig. 4). Clearly visible are multilamellate bodies (arrows) and the hole in a membrane structure (arrowhead) Abbreviations: gn, generative nucleus; vn, vegetative nucleus.

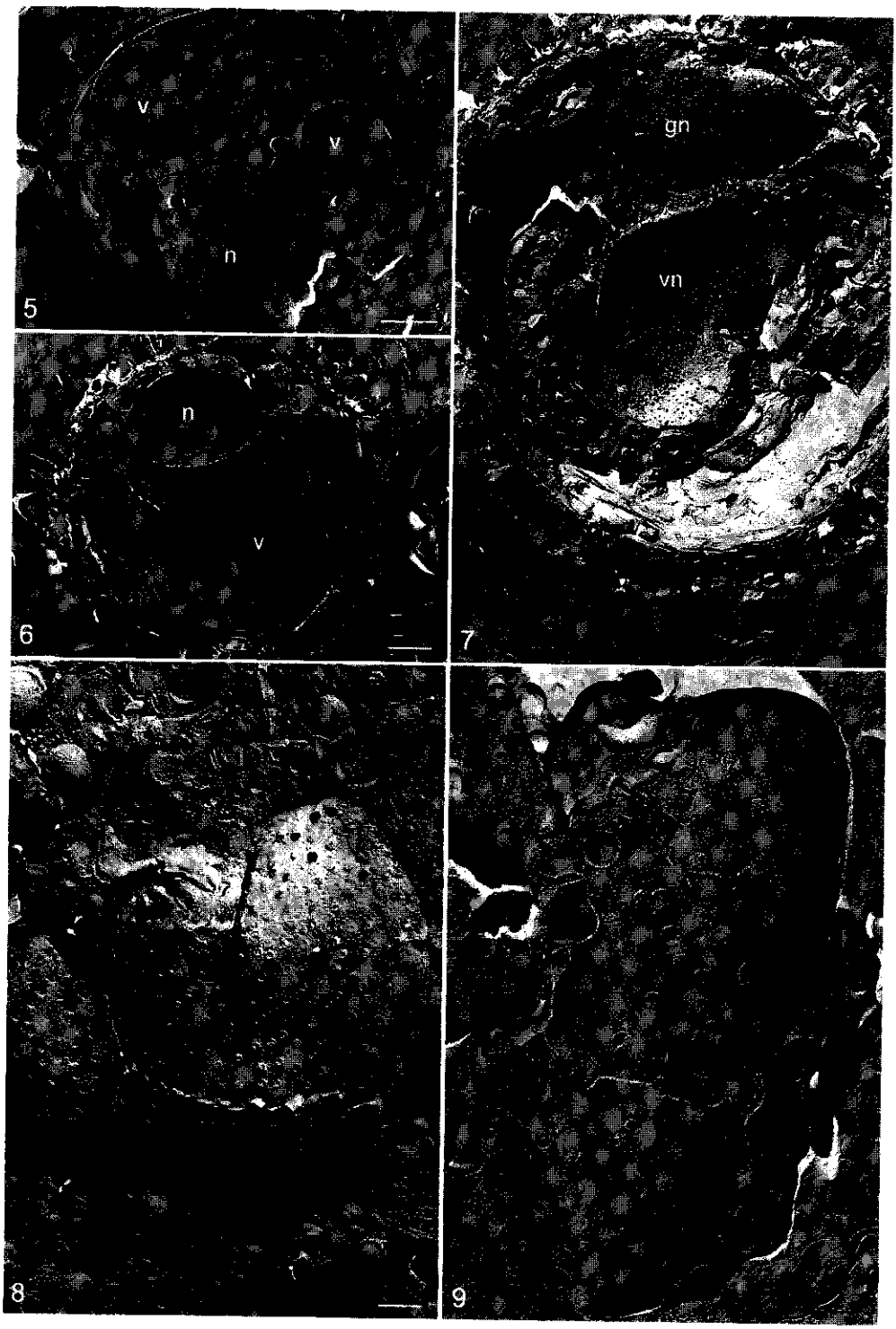
nuclei and pollen-like structures. In some cases embryos were found with a suspensor-like structure. Cells cultured for seven days at 18 °C developed into pollen.

In several replicas fractures of nuclear surfaces were found showing NPCs. In some cases a substructure of the NPCs was seen but the eight fold symmetry was not visible. Organelles like dictyosomes, ER, vesicles, often with multilamellate membranes, and vacuoles were well visible. The material fractured and sputter coated in the cryo-SEM showed similar nuclear surfaces (Figs. 1 and 2), indicating that the obtained replicas are a good representation of the native material. Figure 3, obtained in the cryoSEM, gives a view inside a vesicle and shows a hole in the membranes of this vesicle (arrowhead), suggesting tube-like extensions. Other vesicles seem not totally surrounded by membrane structures (Figs. 3 and 4, arrows).

Development of pollen in vivo and in vitro

The cytoplasm of young microspores *in vivo* showed a low density of organelles and a relatively small and round nucleus was present (Fig. 5). In late microspore stages the nucleus became larger and was flattened by a large vacuole, which pushed the nucleus and organelles towards the exine wall (Fig. 6). The number of NPCs was counted and the density of NPCs/μm^2 was calculated (Table 1). The NPC density could not be used to discriminate between nuclei of late microspores and nuclei of vegetative or generative cells of early bicellular pollen. This was also the case when membranes of both the generative and vegetative nuclei were visible in one fractured pollen (Fig. 7). A larger standard deviation in the fraction containing late microspores and young bicellular pollen in comparison with other samples, however, indicates that there is a mixture of nuclei in this sample. The nuclear membrane of tricellular *Brassica* pollen was very lobed (Fig. 8). The NPC density significantly increased from 13 NPCs/μm^2 in the young microspore stage up to 19 NPCs/μm^2 in the vegetative nucleus during *in vivo* pollen development. The NPC density of the sperm nuclei in mature *in vivo* pollen decreased to 9 NPCs/μm^2 (Fig. 9). The cytoplasm of *in vivo* tricellular pollen contained a lot of membrane structures, often tightly packed as multilamellate bodies and stacked ER (Fig. 10).

After the first day of culture under non-embryogenic conditions the NPC density had decreased from 15.9 NPCs/μm^2 to 9.4 NPCs/μm^2. After three days of culture the NPC density had increased to 17.0 NPCs/μm^2. The tricellular pollen *in vitro* showed almost the same cytoplasmic organisation as the *in vivo* pollen. Only vacuole-like structures larger than 2 µm were found in the *in vitro* samples and the nucleus was not lobed (Fig. 11). Replicas of mature pollen both from *in vitro* and *in vivo* development often showed fracturing through the nucleoplasm instead of through the nuclear membranes. We did not succeed to fracture nuclear surfaces of dehydrated pollen collected from open flowers. Several attempts were made but the fracture always ran


v
v
n
5
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v
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gn
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Table 1; The average NPC density per μm^2 in the nuclear surface of freeze-fractured microspores, pollen, and microspore and pollen derived embryos of *Brassica napus*. The data were analysed using the Student's *t* distribution at a 0.01 level of significance (two-tailed). The same letter means no significant difference in NPC density between the stages.

	stage	x	s	N	p<0.01
In vivo:	EU	13.1	1.8	20	a
	LU/EB(stage of isolation for cultures)	15.9	2.1	26	b
	Pollen:				
	vegetative nucleus	18.7	1.5	17	c
	sperm nucleus	8.6	1.7	8	d
In vitro:	embryogenic cultures:				
	2h	13.6	1.3	18	a
	8h	8.7	1.9	15	d
	1d	13.7	4.2	20	a/b/e
	3d	12.7	3.6	17	a/f
	7d	13.6	1.7	21	a
	suspensor	9.0	1.5	4	d/e/f
	non- embryogenic cultures:				
	1d	9.4	2.9	13	d/f
	3d	17.0	2.5	18	b/c
	7d	17.2	1.9	19	b/c

Abbreviations: EB = young bicellular pollen; EU = young microspores; LU = late microspores; P = mature tricellular pollen; *d* = number of days in culture; *h* = hours in culture; x = mean NPCs/μm^2; s = standard error of the mean; N = number of nuclei analysed.

Figs. 5-9; Replicas of fractured material of different stages of *in vivo* pollen development in *B. napus*.

Fig. 5; A microspore in the middle stage. A relatively small nucleus (n), some vacuoles (v) and only a few other organelles are visible.

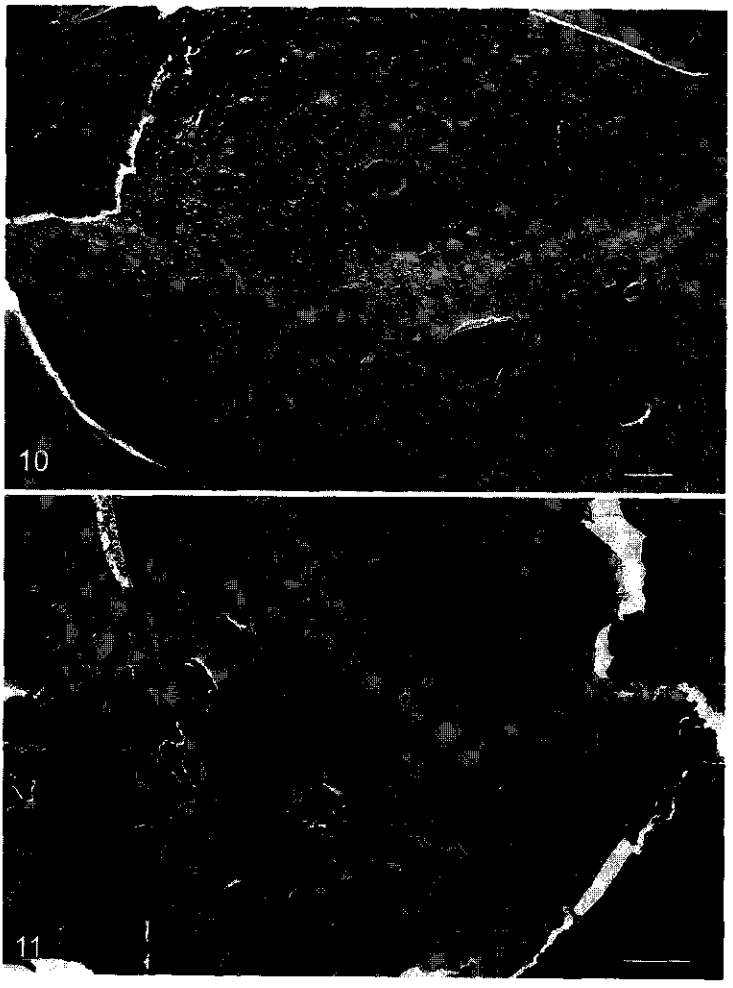
Fig. 6; A late microspore with a large vacuole (v) and a nucleus (n) pushed against the microspore wall.

Fig. 7; A replica of early bicellular pollen with both the generative nucleus (gn) and vegetative nucleus (vn) in one fracture. The cytoplasm contains more organelles than in the microspore stages.

Fig. 8; The vegetative nucleus of mature pollen was lobed. The cytoplasm showed a high amount of vesicles and membrane structures.

Fig. 9; The sperm nucleus of mature pollen with a low density of NPCs.

Bars: 2 µm (5, 6, 7), 500 nm (8, 9).



Figs. 10, 11; Replicas of *in vivo* (Fig. 10) and *in vitro* (Fig. 11) mature pollen of *Brassica napus.* Clearly visible are the larger vesicles (v) in the *in vitro* pollen. During the fracturing of the *in vivo* pollen the fracture runs through the nuclei and not in between the membranes. vn; vegetative nucleus; sn, sperm nucleus. Bars: 2 µm.

over the outside of these pollen, showing the exine layer. Therefore, it is unknown if dehydrated pollen from open flowers still have a high NPC density.

The nuclear surface was measured using DAPI and the total number of NPCs/nucleus was calculated (Table 2). An increase in NPCs/nucleus was found during pollen development from 979 in young microspores to 3901 in late microspores/young bicellular pollen but then followed by a decrease to 3247 in the vegetative nucleus of tricellular pollen *in vivo*. In *in vitro* pollen the NPC/nucleus decreased from 3901 to 2563 at the onset of culture but was increased to around the 4000 NPCs/nucleus in the vegetative nucleus within three days of culture. After one day in culture at 32 °C the NPCs/nucleus had increased from 3901 to 5136. Only after seven days in culture under embryogenic conditions, when larger cell clusters were formed, the NPCs/nucleus decreased to 3168.

Table 2; The average nuclear surface (μm^2) ± standard error measured of 100 nuclei per developmental stage using DAPI and the calculated NPCs/nucleus, nuclear volume (μm^3) and NPCs/μm^3 ± standard error of nuclei of microspores, pollen, and microspore and pollen derived embryos of *Brassica napus*.

stage	nuclear surface (μm^2)	NPCs/nucleus	nuclear volume (μm^3)	NPCs/μm^3
In vivo:				
EU	74.7 ± 20.1	979 ± 296	62 ± 25	15.7 ± 7.9
LU/EB*	245.4 ± 98.9	3901 ± 1655	384 ± 220	10.2 ± 7.2
Pollen (vegetative nucleus)	173.7 ± 45.7	3247 ± 894	220 ± 95	14.7 ± 7.5
In vitro:				
embryogenic cultures (32 °C):				
1d	374.9 ± 133.3	5136 ± 2412	715 ± 376	7.2 ± 5.1
3d	427.0 ± 219.2	5422 ± 3180	907 ± 727	6.0 ± 5.9
7d	232.9 ± 120.6	3168 ± 1687	367 ± 281	8.6 ± 8.0
non-embryogenic cultures (18 °C):				
1d	272.7 ± 115.9	2563 ± 1346	451 ± 301	5.7 ± 4.8
3d	233.7 ± 122.9	3973 ± 2169	368 ± 315	10.8 ±11.0
7d	249.9 ± 81.9	4298 ± 1486	386 ± 194	11.1 ± 6.8

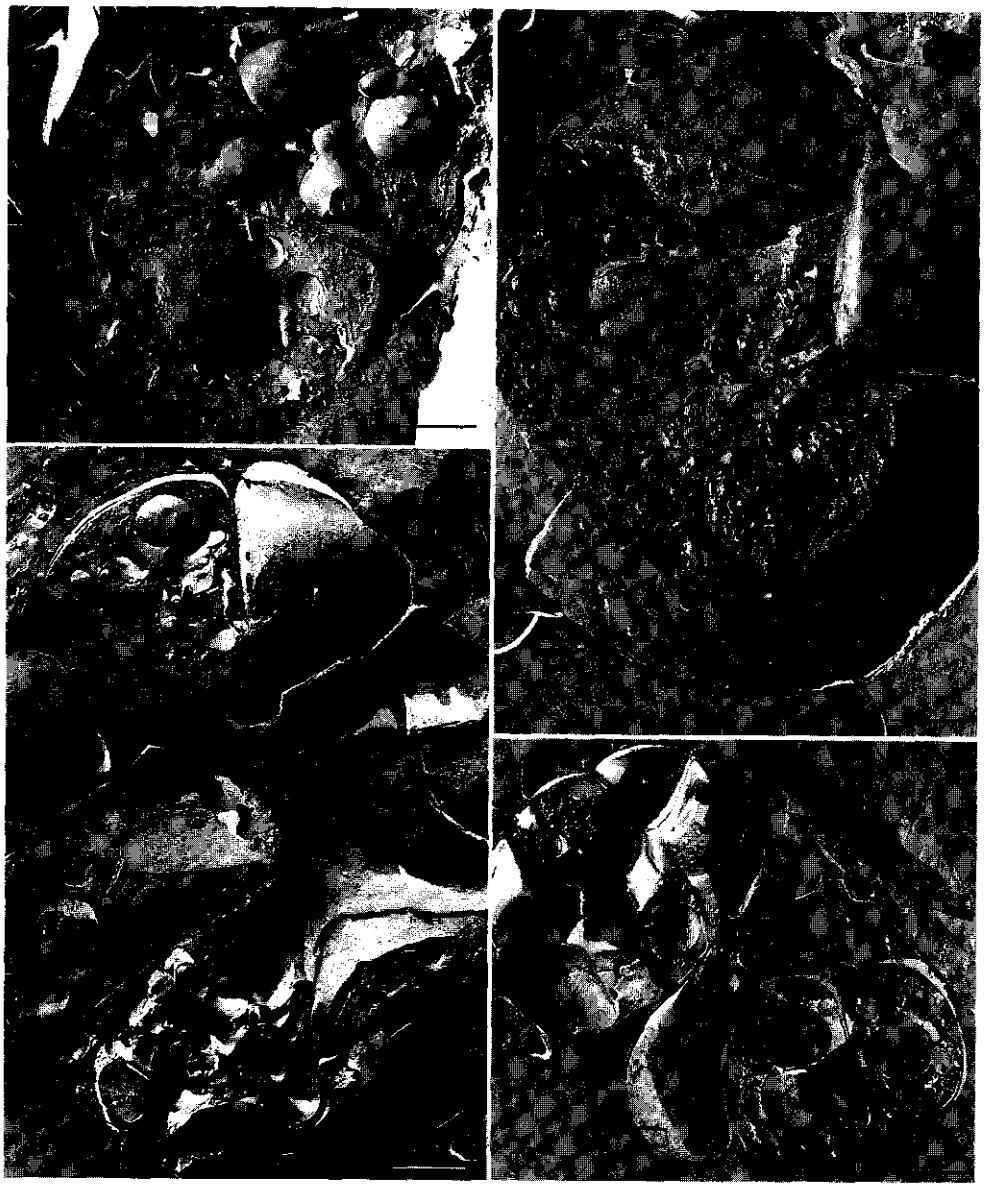
*stage of isolation for cultures

Abbreviations: EB = young bicellular pollen; EU = young microspores; LU = late microspores; d = number of days in culture.

Embryo development

The first change found in cultures at 32 °C was a drop in NPC density from 15.9 NPCs/μm^2 at the onset of culture to 9.0 NPCs/μm^2 within 8 hours. After one day of culture under embryogenic conditions (32 °C) the NPC density was restored to almost 14 NPCs/μm^2, lower than the NPC density at the stage of isolation. At this point in culture the first symmetrically divided cells were found (Fig. 12), but the majority of the analysed cells had not divided yet. After three and seven days of culture under embryogenic conditions, multicellular embryogenic structures were found in the samples (Figs. 13 and 14) and only these were analysed. Some of these structures consisted of cells which contained a few large vacuoles whereas others contained a high number of small vesicles. Most of these structures were still inside the pollen wall, since this wall had stretched. The NPC density did not change during further embryo development (Table 1).

The total number of NPCs/nucleus was measured after one, three and seven days of culture under embryogenic conditions. After one day of culture, the NPCs/nucleus was 5136, much higher than the 3901 at the moment of onset of the cultures. After three days of culture the number of NPCs/nucleus had decreased to



Figs. 12-15;: Replicas of embryogenic structures after induction of microspore and pollen embryogenesis in *Brassica napus*. n, nucleus.

Fig. 12; After one day of culture some symmetrically divided structures were found.

Fig. 13; Embryogenic structure after three days of culture, consisting of at least 4 cells.

Fig. 14; Embryogenic structure after seven days of culture; notice the stretched pollen wall (arrows).

Fig. 15; Some of the embryos had a suspensor-like structure, here seen in a seven days culture. Bars: 2 µm (12), 5 µm (13, 14), 4 µm (15).

2869 and did not change much further, whereas the NPC density had not changed between one and seven days in culture.

In some of the embryo induced cultures embryos with a suspensor-like structure were found (Fig. 15), and in a few samples the NPCs of a nucleus of a such a structure could be counted. In all cases the density of the NPCs in nuclei of suspensor-like structures was much lower than the density of the NPCs in the embryos itself (Table 1).

To see whether or not increases in NPCs/μm^2 or total number of NPCs/nucleus are related to the volume of the nucleus, the NPCs/μm^3 were calculated (Table 2). These results showed that in general during pollen development the NPCs/μm^3 is higher than in nuclei of cells in embryogenic cultures. Only after one day in culture under non-embryogenic conditions the NPCs/μm^3 had decreased from 10.2 to 5.7 but after three days of culture it was 10.8 NPCs/μm^3. Unfortunately, the high standard error makes it difficult to draw conclusions from these data.

Discussion

Using freeze-fractured material the NPCs were visualized and changes in NPC density were analysed during pollen development and after induction of microspore and pollen embryogenesis in *Brassica napus*. There was no indication from these experiments that there is a difference in NPC density between sides of the nucleus facing different parts of the cell. This was confirmed using material fixed in $KMnO_4$ for all stages of pollen development (not shown). To calculate the total number of NPCs/nucleus also the nuclear surface was measured using DAPI. Unfortunately the nuclear surface gave a large standard error what resulted in large standard errors for both the NPC/μm^2 and NPCs/μm^3. The large standard errors might be caused by the fact that there is a mixture of cells in a culture which are not synchronised meaning that all stages of the cell cycle can be found in one sample. During the cell cycle the size of nuclei changes (Maul et al., 1972).

Several groups have found an increase in RNA synthesis just prior to anthesis (reviewed by Mascarenhas, 1975, 1990). Also, the replicas showed an increase in cellular organelles during pollen development. This coincides with the increase in NPC density in the vegetative nucleus and, in most cases, an increase in total number of NPCs/nucleus during *in vivo* and *in vitro* pollen development, both indicating a high transcriptional activity. The high NPC density in nuclei of mature *in vivo* pollen, however, does not result in a relatively high total number of NPCs/nucleus. This most likely is due to an inaccuracy in measuring the nuclear surfaces using DAPI. To estimate the nuclear surface it was assumed that the nuclear diameter was spherical, but in this stage the nuclear membrane proved to be lobed resulting in a smaller

diameter and a lower nuclear surface estimation. The *in vitro* pollen did not have a lobed nuclear membrane what resulted in a relatively high total number of NPCs/nucleus.

We were not able to collect data from pollen after anthesis and therefore do not know if in the last phase of pollen dehydration the NPC density of the vegetative nucleus changed. However, in images obtained with the cryo-SEM sometimes vesicles with a hole in the membrane were found (Fig. 3), indicating that these bodies are not just bordered by membranes but that these membranes form tube-like extensions. Other vesicles were partly surrounded by membrane structures and multilamellate membranous vesicles were found. These vesicles are probably lipid bodies, bordered by parallel cisternae of rough endoplasmic reticulum (Charzynska et al., 1989). These structures are known to occur in dehydrated pollen (Tiwari et al., 1990) what suggests that the pollen collected just before flowering were already highly dehydrated and many changes in the nuclear membranes are therefore not expected. Pollen cultured at 18 °C showed less of these membrane structures in the cytoplasm and contained larger vesicles. An explanation for this might be that they were less dehydrated caused by the medium they were cultured in.

In early pollen the NPC densities in the generative and vegetative nuclei did not differ significantly, indicating that both nuclei are transcriptionally active. This was also observed for alfalfa by Shi et al. (1991). After pollen mitosis, the density of the NPCs in the sperm nuclei proved to be half of the density of NPCs in the vegetative nucleus, which indicates a low transcriptional activity. In general, the generative nucleus and sperm nuclei of mature pollen show a typical morphology of low activity in several species (Testillano et al., 1995). This is reflected by the low number of nuclear pores in the nuclear membranes of these nuclei, as was reported earlier for pollen of *Medicago sativa* (Shi et al, 1991), *Gerbera jamesonii* (Southworth, 1990) and *Plumbago zeylanica* (Southworth et al., 1997) and in germinating pollen of *Tradescantia paludosa* (LaFountain and LaFountain, 1973) and *Hordeum vulgare* (Mogensen and Wagner, 1987). However, the vegetative nucleus of mature pollen is considered to be transcriptionally inactive as well (Mascarenhas, 1975). This would imply that the high NPC density and total number of NPC/nucleus of mature pollen reflects earlier transcriptional activity.

In the microspore and pollen cultures, a decrease in NPC density was found under both embryogenic and non-embryogenic culture conditions. However, it is not sure whether or not this is caused by the same factor. Culturing the cells at 18 °C results in a lower NPC density and total number of NPCs/nucleus whereas the total nuclear surface does not change, what might be explained as a decrease in transcriptional activity. This can also be the case for the embryogenic cultures but in these cultures an increase in nuclear surface is found after one day of culture when the NPC density is already restored. This swelling of the nucleus might have resulted

in a decrease in NPC density as well in the early hours of culture, followed by NPC assembly.

In *Nicotiana tabacum* a loss of nuclear pores in the vegetative nucleus was found as a result of a lower nuclear activity when young pollen were forced to embryogenesis by starvation (Garrido et al., 1995). In our *Brassica* system it is shown that the NPC measurements can not be used to discriminate between embryogenic and non-embryogenic cells, because no loss in NPCs is found; moreover, in the embryogenic cultures about 10-20% of the cells were forming embryogenic structures while all cells showed the decrease in NPC density after eight hours.

The data also show that the number of NPCs/nucleus is a dynamic factor with active synthesis and breakdown of NPCs during development. The total number of NPCs/nucleus shows that there is a high production of new NPCs in interphase nuclei between young and late microspore stages and during the first three days of culture under non-embryogenic conditions. The increase of NPCs is higher than might be expected as a result of DNA synthesis. Also after one day of culture under embryogenic conditions the total number of NPCs/nucleus was higher. After seven days of culture this number had decreased by almost a factor two. This could mean that the increase of NPCs/nucleus is a result of DNA synthesis before cell division (Maul et al., 1972). However, the size of the nuclei was influenced by the number of cells in an embryogenic cluster with smaller nuclei in larger embryogenic clusters (Straatman and Schel, 1997) whereas the NPC density hardly changed. So part of the decrease in NPCs/nucleus must be the result of loss of NPCs.

If we take the nuclear volume as an indicator for nuclear activity (a larger nucleus is more transcriptional active) then we see that after changes in development, like after meiosis (EU) and after culturing, the nuclear volume faster changed than the NPCs/μm^3. This might be an indication that first the transcriptional activity changed before changes in NPCs occurred. The fact that the mean NPC/μm^3 is lower in the embryogenic cultures at 32 °C and 25 °C can be explained by a faster transport through the NPCs as a result of the higher culture temperature. However, there is a problem when the nuclear volume of the EU, 62 μm^3, is compared with the volume of the nuclei with the same NPC density, giving nuclear volumes between 367 and 907 μm^3 what indicates a much higher transcriptional activity. This means that the NPC density is a parameter that has to be used with care, if possible together with other parameters to get an impression of the transcriptional activity.

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Chapter 8

General discussion

K.R. Straatman

The early phase of plant embryo development has been studied for many years. The interest was mainly focused on cell development and cell division patterns (Tykarska, 1976, 1979). Recent advances in molecular biology have led to a renewed interest in the regulation of plant embryogenesis by studying the underlying pattern of gene expression, combining molecular and cytological techniques as well as experimental studies using somatic embryos (Lindsey and Topping, 1993; Raghavan, 1997). Not only the expression patterns of genes are subject of interest but also the behaviour of the nucleus in relation to transcription patterns and cellular function. The experiments described in this thesis were carried out to analyse the nuclear morphology specially in relation with transcription processes. It is difficult to study these relations in zygotic embryos because of the timing and nature of these systems *in vivo*. Therefore, pollen development and androgenesis of *Brassica napus* were chosen as a test system.

The culture system

Using bromodeoxyuridine (BrdU) nascent DNA was labelled during the S-phase of microspores and of cultured young pollen under embryogenic and non-embryogenic conditions. Labelled DNA was detected within four hours in cultured pollen under embryogenic conditions. The re-entering of the vegetative nucleus into the cell cycle coincided with a re-orientation of microtubules in the cytoplasm of the vegetative cell (Hause et al., 1993) and the occurrence of a heat shock protein of the 70-kDa class (HSP70) in the vegetative nucleus (Cordewener et al., 1995). From HSP70 it is known that it is present in the nucleus during the S-phase (Milariski and Morimoto, 1986) and that it is bound to the cytoskeleton (Tsang, 1993). This, together with the fact that also colchicine can induce microspore embryogenesis (Zaki and Dickinson, 1991; Zhao et al., 1996), suggests that the re-orientation of the microtubules and the presence of HSP70 in the vegetative nucleus result in a re-entering of the S-phase soon after the onset of culture. This also implies that the first sign of induction of embryogenesis in this system is probably not the expression of embryo-specific genes but rather a renewed mitotic activity.

Further analysis of events in embryogenic cultures gave more support to the idea that the developmental change from pollen development towards androgenesis after a heat shock is not just the induction of embryo-specific genes. This is summarized in Fig. 1. When late microspores and early pollen were cultured at 32 °C and changed their developmental pathway towards embryogenesis, multicellular structures were formed within the pollen wall, which disrupted only after five to seven days in culture. The pollen wall, however, sometimes broke too early. This resulted in three different structures: structures with one to several pollen tubes, callus-like structures and embryos with suspensor-like structures. Regarding the shape and fate of the pollen-like structures, one might suggest that cells released from the pollen wall shortly after

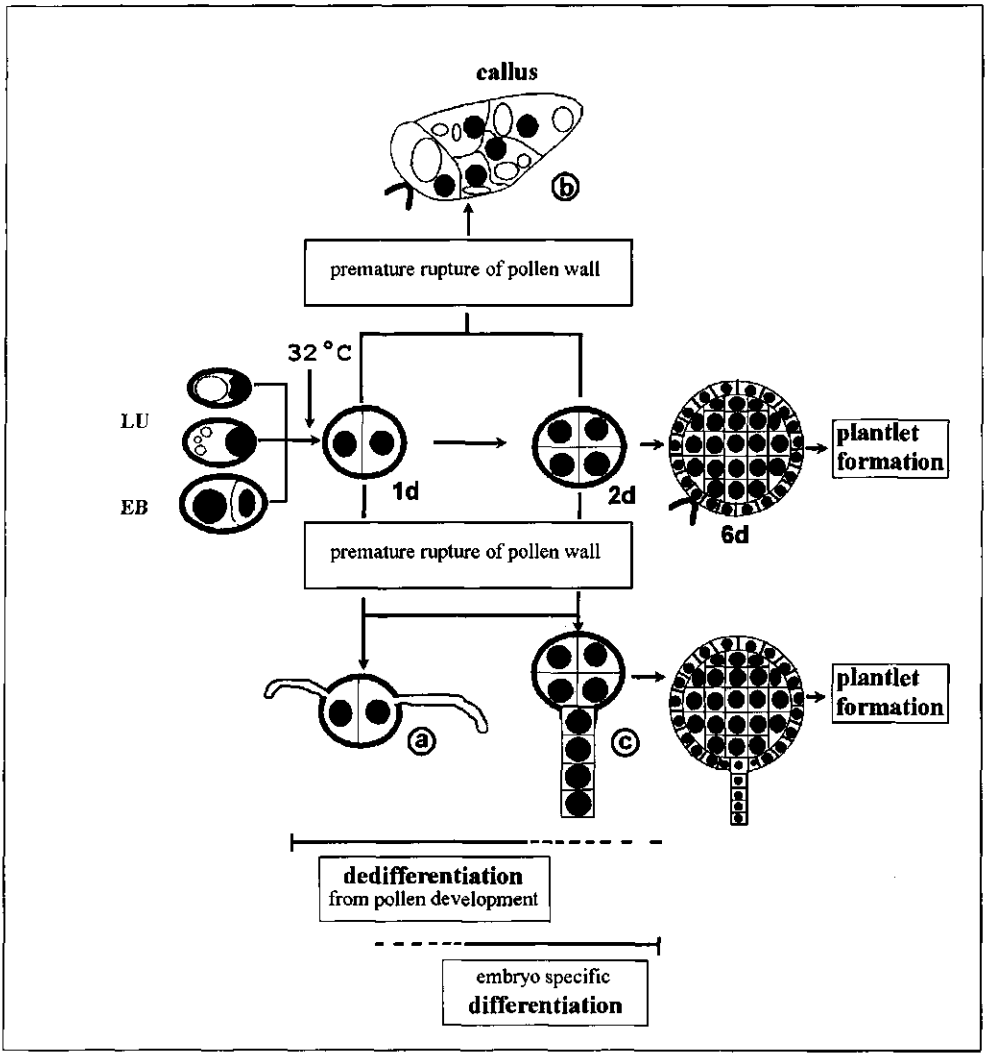

Fig. 1; The dedifferentiation from pollen development and the differentiation towards embryo development during the culture of microspore and pollen of *Brassica napus* under embryogenic conditions (32 °C). When the pollen wall was ruptured too early, three different structures could develop: a, structures with one to several pollen tubes: b, callus-like structures: c, embryos with suspensor-like structures. Normal embryogenic development occurred when the pollen wall ruptured after five to seven days. The periods of dedifferentiation of pollen development and the start of embryo-specific differentiation are shown in relation to the development of the culture (adapted from Hause and Hause, 1996).
Abbreviations: LU, late microspores; EB, young bicellular pollen; 1d, 1 day culture; 2d, 4d and 6d, 2 days, 4 days and 6 days culture; O, vacuole; ●, nucleus.

the first embryogenic division still showed a pollen fate, producing a pollen tube, sometimes even more than one, indicating that pollen-specific genes still played a major role at this stage (Fig. 1, stage a). The pollen fate was proven by Eady et al. (1995) for colchicine treated cultures of microspores of *Nicotiana* where expression of pollen-specific genes was found in both daughter cells after the first symmetrical division. Sometimes, when the pollen wall was broken prematurely, a callus-like structure was released, but neither a pollen-like nor embryo-like structure developed (Fig. 1, stage b). This points to a reduced influence of the pollen-specific genes while none or only a few embryo-specific genes are activated yet. It implies that the first period of culture can be regarded as a phase of dedifferentiation of pollen development towards a callus-like development. In other situations an early rupture of the pollen wall resulted in the formation of an embryo with a suspensor-like structure (Fig. 1, stage c; see also Chapter 7). Probably, in this stage the embryo-specific genes are already "dominant" over the pollen-specific genes. This situation would be expected using late microspores when most of the pollen-specific genes are not activated yet, but it is difficult to register whether the structures were descended from the late microspore stage or from early pollen.

The observations in all cases suggest that during the dedifferentiation of the pollen the embryo-specific differentiation already starts. This can be proved by the detection of transcription of embryo-specific genes. However, not many genes are known in plants that are expressed before the first division of the zygote. One candidate, AGL15 (for AGAMOUS-like; Heck et al, 1995), which we tested on induced microspores and pollen, showed expression in early embryogenic stages, but also in young leaves and, less abundant, in root tips (Straatman et al., 1996). So AGL 15 did not prove to be embryo-specific but rather to be expressed in proliferating cells. Another interesting candidate is the Somatic Embryogenesis Receptor-like Kinase (SERK) gene that recently has been reported to be active during early embryo development in *Daucus* cultures (Schmidt et al., 1997). It will surely be important to test the validity of this probe for the *Brassica* system.

In some cultures evidence was found that the generative nucleus can take part in the formation of the embryogenic structure. In these cases the generative nucleus was not distinguishable from the vegetative nucleus after one day in culture when analysed with 4,6-diamidino-2-phenylindole (DAPI) to stain the DNA. One example of such a culture is given in Table 1. At the onset of culture 13 percent of the cells were in the late unicellular stage and 63 percent in the early bicellular stage. After two days of culture under embryogenic conditions, 11 percent of the cells were symmetrically divided or were multicellular structures with four nuclei, while eight percent of the cells were still in the microspore stage. Therefore, the embryogenic structures showing symmetrical divisions originated at least partially from early bicellular pollen, which might indicate that the genes involved in changing the fate of the generative

Table 1; Developmental stages (in %) of freshly isolated microspores and pollen at the onset of culture (t=0), after 2 days culture at 18 °C (2d18, non-embryogenic) and after 2 days culture at 32 °C (2d32, embryogenic) conditions.

culture	lu	ml	eb	lb	p	d	sym	3	4	N
t=0	13	1	63	18	1	6	0	0	0	200
2d18	7	2	17	34	30	10	0	0	0	149
2d32	8	3	45	23	4	5	10	2	1	197

Abbreviations: d, broken and dead cells; eb, young bicellular pollen; lb, late bicellular pollen; lu, late microspores; ml, mitosis; N, total number of analysed cells; p, mature pollen; sym, symmetrically divided cells; 3, 4,: multicellular structures with 3 or 4 nuclei resp.

nucleus are not or not fully expressed yet. Some evidence for this can be found in the experiments described in this thesis. Just after microspore mitosis the nucleolus of the generative nucleus showed labelling of ribosomal RNA after *in situ* hybridization, indicating that still transcription of ribosomal genes occurred (Chapter 2). Also, the chromatin in the generative nucleus had not reached its high degree of condensation and the NPC densities of the generative and the vegetative nuclei were similar (Chapter 7) at this stage of development. The developmental time window after the microspore mitosis, during which the generative nucleus is sensitive for this change in programme is probably very small, resulting in only a few cultures in which this phenomenon was observed.

Transcription sites in plant nuclei

RNA synthesis is a key activity in the nucleus. Understanding where in the interphase nucleus transcription and further processing of pre-RNA takes place is very important if we want to understand the relation between genome activity and cellular functioning. BrUTP was therefore incorporated into nascent RNA, detected by antibodies and analyzed with confocal laser scanning microscopy to visualize the transcription sites (Chapters 4 & 5). The circumstances during these run-on experiments allowed only transcription of RNA and no further processing, resulting in a punctate labelling pattern representing only the sites of transcription. The results confirmed that, like in animal cells, the transcription sites are found throughout the whole nucleus, and often at the edges of condensed chromatin. It was also possible to distinguish between highly active nuclei (the vegetative nucleus of a pollen protoplast) and nuclei with a lower transcription activity (the vegetative nucleus in a pollen tube). So, quantification comes to view. However, due to penetration problems it was not possible to label transcription sites in plant cells with an intact cell wall.

Table 2; Summary of data on nuclear morphology, translated into marks for transcriptional activity during pollen development and androgenesis in *Brassica napus* cv. Topas combined with the expected transcriptional activity.

	pollen development								androgenesis (32 °C)				
	in vivo					*in vitro* (18 °C)							
	MS	BP		MP		1d	7d		BP		8hrs	1d	7d
		vn	gn	vn	sn	MS	vn	sn	vn	gn	vn	2n	xn
nuclear size	l	h	m	l	n	h	h/m	n	h	m	h	h	m
nucleolar size	l	h	m	-	-	h	-	-	h	m	h	h	m
chromatin condensation	l/m	m	m/l	h	n	m	h	n	m	m/l	m	m	m
CB size	l	h	m	-	-	h	-	-	h	m	m	m	l
snRNPs	+	+	+/-	-	-	+	-	-	+/-	+/-	+	+	+
NPC density	l	m	m	h	l	l	h	n/a	l	n/a	l	m	m
NPCs/nucleus	l	m	n/a	m	n/a	m	m	n/a	h	n/a	n/a	h	m
expected transcriptional activity	l	h	m/l	n	n	h?	n?	n	h	m/l	h?	h?	m

Abbreviations: BP, bicellular pollen; CB, coiled body; gn, generative nucleus; h, high transcriptional activity; l, low transcriptional activity; m, medium transcriptional activity; MP, mature pollen; MS, young microspores; n, no transcription; n/a, no data available; NPC, nuclear pore complex; snRNPs, small nuclear ribonucleoprotein particles; sn, sperm nucleus; vn, vegetative nucleus; 2n, symmetrically divided cells; xn, multicellular structures; +, present; -, not present; ?, indicates that it is not clear from literature (see text).

An indication for the transcriptional activity in the *Brassica* system can be found in the literature. For pollen development shortly after meiosis the level of transcription is low and increases towards the microspore mitosis. Shortly after the microspore mitosis the generative nucleus is silenced whereas the vegetative nucleus is transcriptionally highly active. Shortly before anthesis the RNA synthesis in the vegetative nucleus decreases (Mascarenhas, 1975; Schrauwen et al., 1990). How the transcription changes after embryo induction is less clear. According to Pechan et al. (1991) the RNA synthesis of induced and non- induced microspores and pollen within the first eight hours of culture does not differ much. Cordewener et al. (1994) showed that protein synthesis was higher in induced cultures than in non-induced pollen cultures as a result of the embryo inducing higher culture temperature. This was observed both after eight hours and 24 hours of culture. The finding that transcription sites could be visualised only in nuclei of protoplasts and not during pollen development or after induction of embryogenesis complicates the interpretation of these data.

Concerning the detection of proteins which are involved in splicing of pre-mRNA,

one to three distinct nuclear bodies could be identified (Chapter 6). These are thought to be coiled bodies, although labelling with anti-coilin p80 was negative. No speckled patterns of splicing proteins were found during pollen development which would have been useful to identify transcriptional activity in more detail. Also, interchromatin clusters, marking low transcriptional activity, were absent. Therefore, only the presence or absence of label was used as an indicator.

Blobel postulated in 1985 that active genes would be "gated" to nuclear pore complexes (NPCs) and that, therefore, the NPC patterns would mirror the underlying organization of the genome. It is clear by now that sites of transcription are found throughout the nucleus and are not linked with the NPCs. However, RNAs still have to be transported to the cytoplasm via the NPCs. Therefore the distribution, density and total number of NPCs might give information about the transcriptional activity and the direction of transport of RNA. The NPC density and total number of NPCs proved to be very dynamic (Chapter 7). In all analyzed stages no difference in distribution of the NPC on nuclear surfaces facing different parts of the cell could be found. This indicates that if there is polar transport of RNA, the distribution of the NPCs does not point to that.

Nuclear morphology and transcriptional activity

An overview of the data described in this thesis together with the expected transcriptional activity is given in Table 2. Three parameters which can be analyzed easily by using a DNA stain, the size of nucleus and nucleolus and the stage of chromatin condensation, give a rough indication of the transcriptional activity. The results are confirmed by the presence of snRNPs and the size of the coiled bodies. The size of the coiled bodies is matching with other parameters for RNA synthesis which indicates once more that this structure is somehow involved in RNA synthesis. Recently, more evidence suggesting a role of coiled bodies in the production of snRNAs in human cells became available (Schul et al., 1998); however, not all coiled bodies are closely related to snRNAs and not all snRNA genes are adjacent to coiled bodies in mammalian nuclei.

The NPC density was the only parameter that decreased directly under influence of culturing the cells, where all other data indicated a stable and high transcriptional activity. However, the decrease in NPC density occurred under both embryogenic and non-embryogenic conditions, which indicates that it is not a parameter for embryo induction. Probably, the culture of microspores and early pollen gives a shock, resulting in a decrease in nucleocytoplasmic transport. Indeed, a decrease of protein synthesis is often found after a stress treatment (Morimoto et al., 1990).

Not much is known about the time it takes for the different parameters to react on a change in transcriptional activity but from the NPC densities it is known that they

change fast if the RNA synthesis alters (Maul et al., 1972; Bussi and Wente, 1997; Belgareh and Doye, 1997). A decrease in RNA synthesis at the onset of culture is very well possible. Other parameters, as mentioned in Table 2, possibly react more delayed on changes in transcription.

Future

Many questions remain unanswered. For example:

- What is the function of the nuclear matrix in RNA processing? In some cases the transcription sites were found in rows, from one side of the nucleus to the other side, suggesting a higher structural organization.
- What causes the different behaviour of coiled bodies in plant cells as compared with the data published on mammalian cells? This difference might indicate a difference in function of the coiled bodies in plant and animal cells. Therefore, a further analysis of these bodies is needed.
- Is a specific RNA transported to a specific NPC or group of NPCs? In that case both transcription sites and NPCs may be distributed uniformly but the transport shows the polarity. It could be that RNA is transported to specific NPCs or that the direction of transport is dependent on the place of the target in the cytoplasm.
- What is the influence of the introduction of a new gene on the nuclear organization? Is its activity dependent on the presence of a transcription factory nearby or is a new transcription factory made?
- In this thesis I have tried to follow the pathway of RNA from the sites of transcription towards the NPCs, but which pathway is involved in the decision to switch on or off a gene?

These are just a few questions to be answered and I hope that I can take part in solving some of them in the future.

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Summary

To study transcription and maturation of RNA, pollen development and androgenesis of *Brassica napus* was chosen as a test system as explained in Chapter 1. Culturing microspores and young bicellular pollen at 32 °C allows to change the developmental pathway from pollen development towards embryogenesis. Culturing at 18 °C results in normal pollen development.

The ultrastructure of the nucleus was analyzed during pollen development applying various staining techniques which visualised the degree of chromatin condensation and the appearance of the interchromatin region (Chapter 2). The interchromatin region contains proteins which are necessary for the transcription and maturation of RNA. The results give a rough indication about the transcriptional activity at various pollen stages. Although difficult to interpret, they indicate an increase in transcription activity in nuclei from the young microspore stage up to the vegetative cell in almost mature pollen. The generative nucleus in early pollen initially showed all signs of a transcriptionally active nucleus, but it lost its transcriptionally active morphology prior to pollen mitosis. After pollen mitosis the chromatin of the sperm nuclei remained condensed, indicating that the sperm nuclei are transcriptionally silenced.

To get more insight into the cell cycle stages of the various nuclei of bicellular and tricellular pollen and under embryogenic and non-embryogenic culture conditions, the DNA synthesis was analysed using bromodeoxyuridine (Chapter 3). It was found that during pollen development the vegetative nucleus is arrested in the G1 and re-enters the cell cycle after embryo induction.

To visualise RNA synthesis in plant nuclei in detail, procedures were developed to label the sites of transcription using the uridine-analogue 5'-bromouridine 5'-triphosphate (BrUTP). In Chapter 4 it is assessed that this method works well on isolated leaf cell nuclei of *Brassica napus*. It enabled to visualize transcription sites throughout the plant nucleus like was shown before in nuclei of vertebrate cells. In Chapter 5 the method was further developed, using leaf and pollen protoplasts, and germinating pollen with pollen tubes. In pollen protoplasts and pollen tubes, labelling of RNA transcription was only found in the vegetative nucleus; the sperm nuclei were silenced, which is in agreement with the ultrastructural analysis in Chapter 2. It was further found that transcription in the vegetative nucleus of mature pollen protoplasts could be very intense whereas the transcription of this nucleus was relatively low in pollen tubes. However, attempts to label transcription sites during pollen development and after embryo-induction did not result in any signal.

Leaf and pollen protoplasts were also used to analyse the maturation of pre-mRNA and to understand the relation between the transcription sites and the location of small nuclear ribonucleoprotein particles (snRNPs) and small nucleolar RNPs (snoRNPs). The snRNP distribution was also studied during pollen development and

androgenesis. snRNPs were not detected in the generative nucleus of late bicellular pollen and in the sperm nuclei of tricellular pollen. This is in accordance with the ultrastructural analysis (Chapter 2) of the stages where the chromatin was more condensed in late bicellular pollen than in earlier stages indicating a low or silenced transcription. In sperm cells the chromatin was even more condensed and no incorporation of BrUTP was detected. However, also the vegetative nucleus of mature pollen did not label with antibodies against snRNPs, although the chromatin was very dispersed which points to a high transcriptional activity, as was found in the pollen protoplasts using BrUTP (Chapter 5).

A coiled body is one of the subcompartments in the nucleus containing snRNPs and fibrillarin. In general, the number of coiled bodies in nuclei of mammalian cells increases with an increase in transcriptional activity. Although there was an increase in the number of coiled bodies, the increase in size of these bodies was much more dramatic during pollen development (Chapter 6). After embryo induction a slow decrease in size of the coiled bodies was found.

All RNA leaving the nucleus and all nuclear proteins produced in the cytoplasm have to pass the nuclear pore complexes (NPCs). Therefore, the NPC density can give an indication about general nuclear activity. During pollen development the NPC density slowly increased and reached its highest level in the vegetative nucleus of mature pollen (Chapter 7). This correlates well with the fact that just before the vegetative nucleus is silenced, the transcription is high in this nucleus (Chapter 5). The NPC density of the sperm nuclei was low which confirms the state of low activity as has been described in the Chapters 2, 5 and 6. After or during embryo induction a decrease in NPC density was found. However, this occurred under both embryogenic and non-embryogenic conditions, indicating that it is not evidence for embryo induction.

Finally, the results presented in the Chapters 2-7 are discussed with emphasis on transcriptional activity in Chapter 8.

Samenvatting

Het proefschrift beschrijft onderzoek naar transcriptie, modificatie en transport van RNA waarbij het proces van pollen ontwikkeling en microspore- en pollenembryogenese in koolzaad (*Brassica napus* L.) is gekozen als onderzoekssysteem, zoals beschreven in Hoofdstuk 1. Wanneer microsporen en jonge pollen worden gekweekt voor minimaal acht uur bij 32 °C, wordt de pollen ontwikkeling omgezet in embryo ontwikkeling. Het kweken van microsporen en jonge pollen bij 18 °C resulteert in normale pollen ontwikkeling.

Een analyse van de ultrastructuur van de kern gedurende de pollen ontwikkeling, waarbij gebruik is gemaakt van verschillende technieken om chromatine condensatie en de ruimte tussen het chromatine zichtbaar te maken is beschreven in Hoofdstuk 2. De ruimte tussen het chromatine bevat eiwitten welke betrokken zijn bij modificaties van het primaire RNA transcript. De resultaten geven een globaal beeld van de transcriptie activiteit gedurende de verschillende opeenvolgende pollen stadia. De transcriptie in kernen nam toe gedurende de ontwikkeling van vroege microsporen naar de vegetatieve cel in tricellulair pollen. De generatieve kern in jong bicellulair pollen heeft alle kenmerken van een kern actief in transcriptie, maar verliest deze actieve morfologie voordat de pollen mitose begint. Na de pollen mitose blijft het chromatine van de beide spermakernen gecondenseerd, wat een indicatie is voor afwezigheid van transcriptie.

Om het stadium van de celcyclus van bicellulair en tricellulair pollen te bepalen en de invloed van embryo inductie op de celcyclus, werd de DNA synthese zichtbaar gemaakt met bromodeoxyuridine (Hoofdstuk 3). De resultaten laten zien dat gedurende de pollen ontwikkeling de vegetatieve kern in de G1-fase blijft. Na inductie van embryogenese doorloopt de kern van de vegetatieve cel opnieuw de S-fase.

Om RNA synthese in plantenkernen zichtbaar te maken, werd 5'-bromouridine 5'-trifosfaat (BrUTP) ingebouwd in nieuw gesynthetiseerd RNA. Met deze techniek wordt alleen RNA gelabeld op de plaats van synthese. In Hoofdstuk 4 is aangetoond dat deze techniek werkt op geisoleerde kernen van koolzaadbladeren. RNA transcriptie werd gevonden door de hele kern verspreid, zoals ook was aangetoond in dierlijke cellen. In Hoofdstuk 5 wordt beschreven hoe deze techniek verder is ontwikkeld. Hiervoor werden blad- en pollenprotoplasten gebruikt, alsmede kiemende pollen met pollenbuizen. In pollenprotoplasten en pollenbuizen werd alleen RNA synthese gevonden in de vegetatieve kern; de spermakernen vertoonden geen labeling, zoals was verwacht na de analyse van hun ultrastructuur (Hoofdstuk 2). Daarnaast werd gevonden dat de transcriptie in de vegetatieve kern van een pollenprotoplast veel hoger was dan in de vegetatieve kern van een pollenbuis. Het bleek echter niet mogelijk om met de gebruikte techniek RNA synthese zichtbaar te maken gedurende de pollen ontwikkeling en na embryoinductie.

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Blad- en pollenprotoplasten werden ook gebruikt om de relatie tussen RNA synthese en eiwitten betrokken bij modificatie van RNA te onderzoeken. In het nucleoplasma werd gekeken naar snRNPs, eiwitten betrokken bij modificatie van mRNA en in de nucleolus werden snoRNPs gelabeld, betrokken bij de modificatie van rRNA. De distributie van snRNPs is ook onderzocht gedurende de pollen ontwikkeling en na embryoinductie. Er werd geen labeling van snRNPs gevonden in de generatieve kern van laat bicellulaire pollen en in sperma kernen. Ook dit is in overeenstemming met de gevonden RNA synthese in Hoofdstuk 5 en de ultrastructuur in Hoofdstuk 2. In de vegetatieve kern van rijp pollen werd echter geen snRNPs gevonden, terwijl het chromatine zeer verspreid door de kern ligt en een intense labeling met BrUTP was gevonden, verwijzend naar een hoge transcriptie activiteit.

Een coiled body is een kernlichaampje dat zowel snRNPs als fibrillarine bevat. In dierlijke cellen wordt vaak gevonden dat het aantal van deze lichaampjes toe neemt naarmate de kern een hogere transcriptie activiteit vertoont. Hoewel ook in pollen en embryogene cellen een lichte toename in aantal werd gevonden, was het met name de omvang van de coiled bodies die toenam naarmate de kern meer transcriptie vertoonde. Na embryoinductie was de omvang van deze bodies weer gereduceerd (Hoofdstuk 6).

Al het RNA dat de kern verlaat en alle eiwitten nodig voor processen in de kern passeren de kernporiën. De dichtheid van de kernporiën zou daardoor een maat kunnen zijn voor de activiteit van de kern. Gedurende de pollen ontwikkeling neemt de dichtheid van kernporiën toe en bereikt de hoogste waarde in de vegetatieve kern van rijp pollen (Hoofdstuk 7). De kernporiedichtheid van de spermakernen was laag en is een verder bewijs voor de lage activiteit in deze kernen. Gedurende of vlak na inductie van embryogenese neemt de dichtheid van kernporiën af. Dit werd echter ook gevonden in de cultures onder niet-embryogene condities, en kan daarom niet gebruikt worden om geïnduceerde cellen te identificeren.

De resultaten verkregen in de Hoofdstukken 2-7 worden uiteindelijk in Hoofdstuk 8 verder besproken in relatie tot de transcriptie in de kern.

Nawoord

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Kees

Curriculum vitae

Kees Straatman werd geboren op 7 september 1966 te Appingedam. In 1984 haalde hij het HAVO diploma aan het Fivelcollege te Delfzijl, en datzelfde jaar begon hij zijn studie aan de toenmalige Rijks Middelbare en Hogere Agrarische School te Wageningen. In 1988 studeerde hij af als botanisch analist. Daarna volgde hij de studie plantenveredeling aan de Landbouwuniversiteit te Wageningen. In 1989 werd hij aangesteld bij het toenmalige Centrum voor Agrobiologisch Onderzoek (CABO) als onderzoeksassistent in de onderzoeksgroep van Dr. Ir. H. De Ruiter. Tijdens een tweejarig project deed hij onderzoek naar de opname van herbiciden door planten. Een deel van dit project werd ook gebruikt als afstudeervak bij de toenmalige vakgroep Plantenfysiologisch Onderzoek, onder begeleiding van Dr. Ir. J.J.S. van Rensen. Een tweede afstudeervak werd gevolgd bij de vakgroep Plantencytologie en -morfologie, waarbij werd gekeken naar de DNA synthese in microspore- en pollencultures van koolzaad. Dit onderzoek werd begeleidt door Dr. G. Hause en Dr. A.A.M. van Lammeren. In 1992 studeerde hij af en na een vakantie in Afrika werd dit onderzoek in maart 1993 voortgezet in de vorm van een promotie onderzoek, onder leiding van Prof. Dr. M.T.M. Willemse en Dr. J.H.N. Schel. De resultaten ervan zijn beschreven in dit proefschrift.

Op 24 maart 1998 trad hij in het huwelijk met Anna Iwanowska, die hij tijdens zijn werk op de vakgroep had leren kennen.

Sinds juni 1998 is hij werkzaam aan de University of Birmingham (Engeland) in een onderzoek naar de opslag van calcium in organellen tijdens de pollenbuisgroei bij papaver. Dit onderzoek wordt uitgevoerd in de onderzoeksgroep van Dr. V.E. Franklin-Tong.

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