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Nuclear pore dynamics during pollen development and androgenesis in *Brassica napus*

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Abstract Changes in nuclear pore complex (NPC) densities, NPCs/nucleus and NPCs/μm³, are described using freeze-fractured *Brassica napus* microspores and pollen in vivo and in vitro. Early stages of microspore- and pollen-derived embryogenic cells were also analysed. The results of in vivo and in vitro pollen development indicate an increase in activity of the vegetative nucleus during maturation of the pollen. At the onset of microspore and pollen culture, NPC density decreased from 15 NPCs/μm² at the stage of isolation to 9 NPCs/μm², under both embryogenic and non-embryogenic conditions. This implies that the drop in NPC density might be a result of culturing the microspores and pollen rather than an indication for microspore and pollen embryogenesis in *Brassica napus*. However, after 1 day in culture under embryogenic conditions, the NPC density increased again and stabilised around 13 NPCs/μm², whereas under non-embryogenic conditions the NPC density remained about 9 NPCs/μm². This low density of 9 NPCs/μm² was also found in the nuclei of sperm cells, in contrast to the 19 NPCs/μm² found in the vegetative nucleus. It means that, although both the vegetative and sperm nuclei are believed to be metabolically rather inactive in mature pollen, the NPC density of vegetative nucleus is twice as high as the NPC density of the sperm nuclei. In a few cases, embryos formed suspensor-like structures with a NPC density of 9 NPCs/μm², indicating a lower nucleocytoplasmic exchange of the nuclei of the suspensor cells than with the nuclei in the embryo proper. In addition, observations on NPCs and other organelles, obtained by high resolution cryo-scanning microscopy, are presented.

Key words *Brassica napus* · Androgenesis · Freeze fracturing · Nuclear pore complex · Pollen development

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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, pollen mitosis will take place in the pollen tube, whereas in tricellular pollen pollen mitosis takes place inside the pollen grain. However, 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. Very early events involved in the induction of embryogenesis can be studied in these androgenetic cultures. Also, for plant breeding, production of homozygous plants after spontaneous duplication of the chromosomes or after a colchicine treatment, and the possibility of producing transgenic plants make 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 be induced to androgenesis if cultured for at least 8 h at 32°C (Pechan and Keller 1988; Custers et al. 1994). Recently, Binarova et al. (1997) showed that late bicellular pollen also 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 the developmental pathway after 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 correlated with 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; Wagner et al. 1990). Further, transfer of molecules between the nucleus and the cytoplasm occurs through these NPCs (reviewed by Hicks and Raikhel 1995: Talcott and Moore 1999). NPCs are embedded in the nuclear envelope. They are composed of more than 100 different proteins and are cylindrically shaped with a diameter of approximately 100 nm (Heese-Peck and Raikhel 1998; Stoffler et al. 1999).

We studied NPC dynamics during in vivo pollen development and during the culture of microspores and young pollen under embryogenic and non-embryogenic conditions using freeze-fractured material. Besides information concerning NPC distribution, this technique also allowed us to gather information about other organelles during different developmental stages.

Materials and methods

Plant material

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

Microspore cultures were obtained from flower buds between 3.2 and 3.6 mm long containing late microspores and early bicellular pollen. After rinsing the cells in B5 medium with 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 0, 2 and 8 h and 1, 3 and 7 days of culture samples were taken.

Freeze-fracturing

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). Surfaces were shadowed with 2 nm platinum at an angle of 45°, followed by deposition of 30 nm carbon at an angle of 90° to strengthen the replicas.

To remove 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 washings in distilled water, the replicas were collected on glow-discharged 200-mesh copper grids and examined with a JEM-1200 EXII transmission electron microscope operating at 80 kV. As a control for the replica images and to obtain additional information, fresh microspores and pollen were frozen as described earlier and the sandwiches were broken in a low temperature field emission scanning electron microscope (LTFESEM; JEOL JSM-6300F) equipped with an Oxford cryochamber. After sputter coating with 3 nm platinum, the surfaces were analysed without further treatment.

Quantification

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 envelope surfaces at the different pollen stages and on the embryo induced cells; the nuclear area where the NPCs were counted was measured. After 3 and 7 days of culture under embryogenic conditions only the embryogenic structures were analysed.

To measure total nuclear surface and 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 from every sample were made with a digital camera (Panasonic WV-E550E) and the nuclear diameter was measured in two directions from the screen to get a mean diameter. The nuclear surface and volume were then calculated using this mean diameter and assuming that the nuclei were spherical.

Results

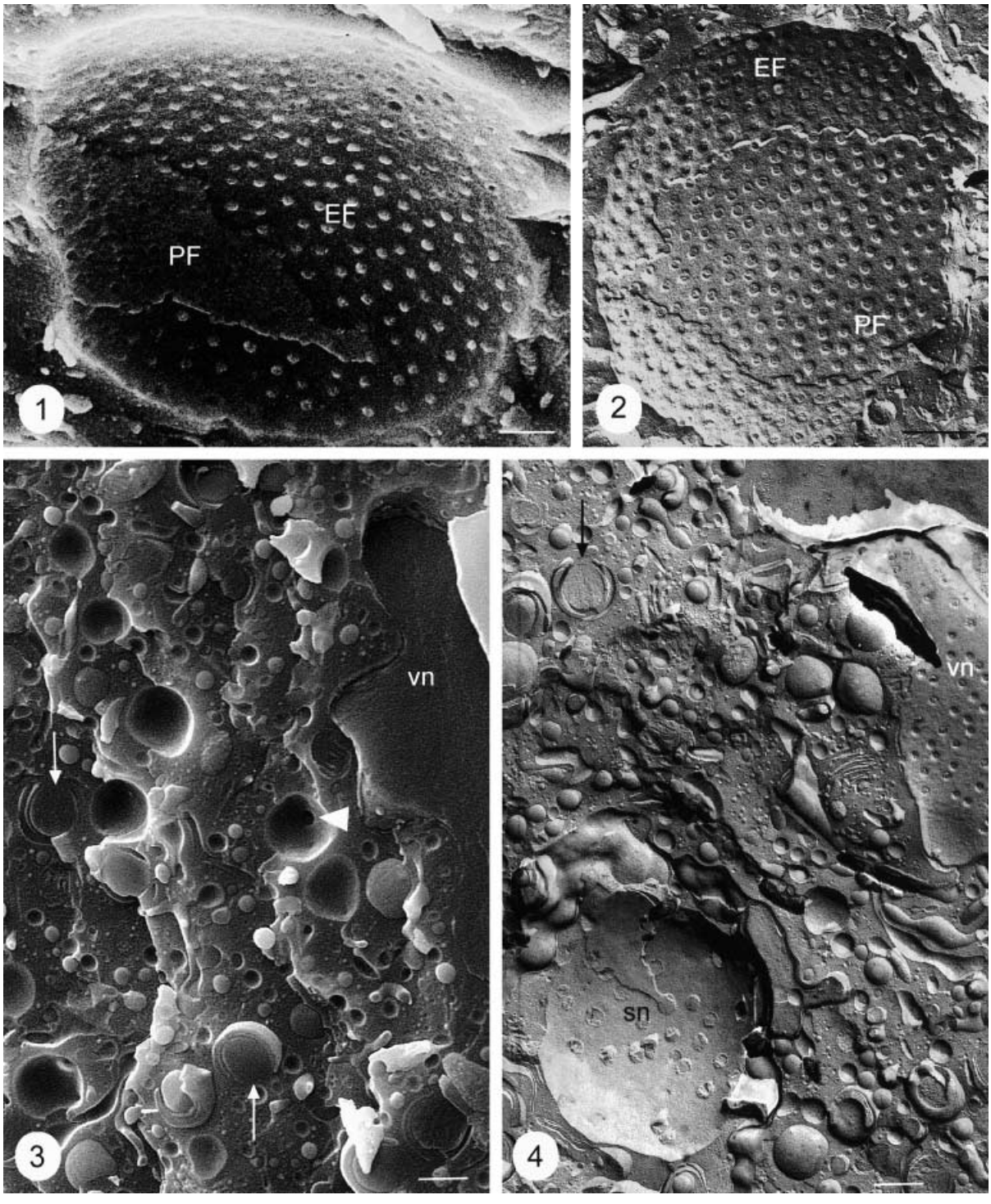
General morphology

The youngest stage of microspores found in the samples of in vivo material contained microspores with small vacuoles. This indicates that this stage was not harvested directly after release of the tetrads but somewhat further along in development. The second sample contained mostly young bicellular pollen as well as late microspores. The third sample, taken just before flowering, contained tricellular pollen.

Microspores and young bicellular pollen, cultured at 25°C for 6 days after a heat shock of 32°C for 1 day, showed embryogenic multicellular structures, large swollen structures with one or two nuclei and pollen-like structures. In some cases embryos were found with a suspensor-like structure. Microspores and bicellular pollen, cultured for 7 days at 18°C, developed into pollen.

In several replicas fractures of nuclear surfaces were found showing NPCs. Other organelles, e.g. dictyosomes, endoplasmic reticulum, vesicles, often with multilamellate membranes, and vacuoles were also clearly visible. No fractures of (pro)plastids or mitochondria were found.

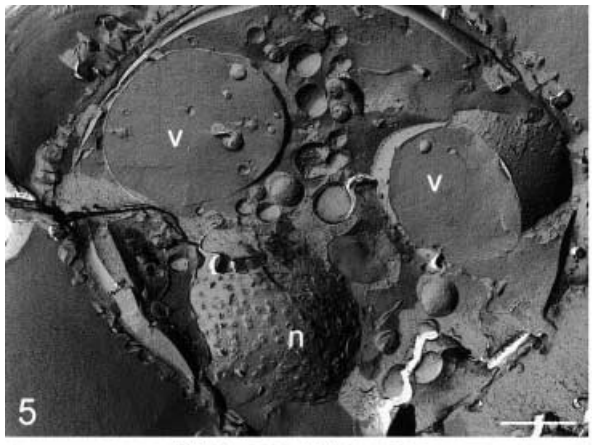
Material fractured and sputter coated in the cryoSEM showed similar nuclear surfaces (Figs. 1 and 2), indicating that the replicas are a good representation of the native material. Figure 3, obtained in the cryoSEM, gives a view inside a vesicle and shows a hole in the membranes of this vesicle, suggesting tube-like extensions. Other vesicles are not totally surrounded by membrane structures (Figs. 3 and 4, arrows).



Figs. 1–4 Comparison of fractured surfaces of pollen material of *Brassica napus* analysed directly in the LTFESEM 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**). *PF* Protoplasmic fracture face; *EF* exoplasmic fracture face. **Figs. 3–4** A comparable overview of the cytoplasm in mature *Brassica* pollen in vivo obtained using the LTFESEM (**Fig. 3**) and TEM (**Fig. 4**). Clearly visible are multilamellate bodies (*arrows*) and the hole in a membrane structure (*arrowhead*). *sn* Sperm nucleus; *vn* vegetative nucleus

Development of pollen in vivo

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). NPCs were counted and the densities, NPCs/μm^2, were calculated (Table 1). The NPC density could not be used to discriminate between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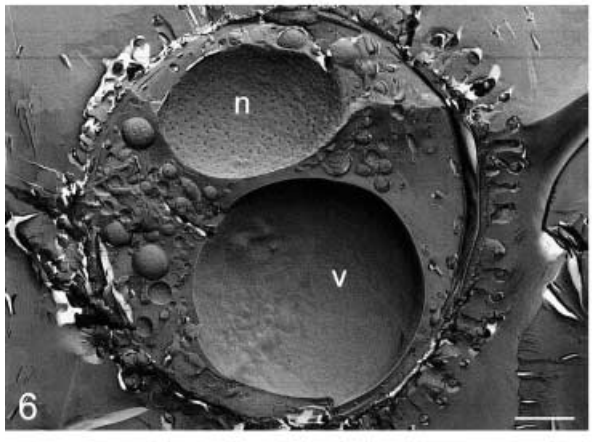
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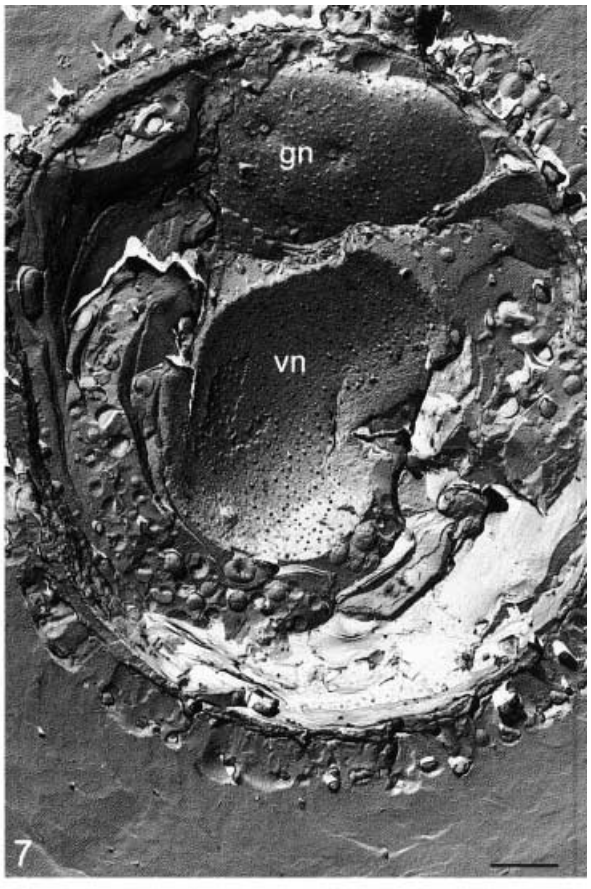
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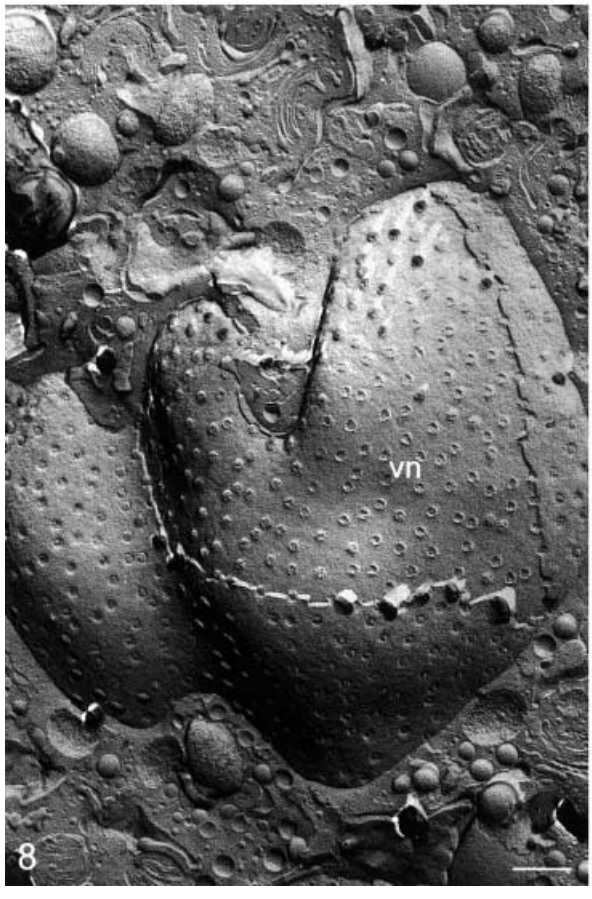
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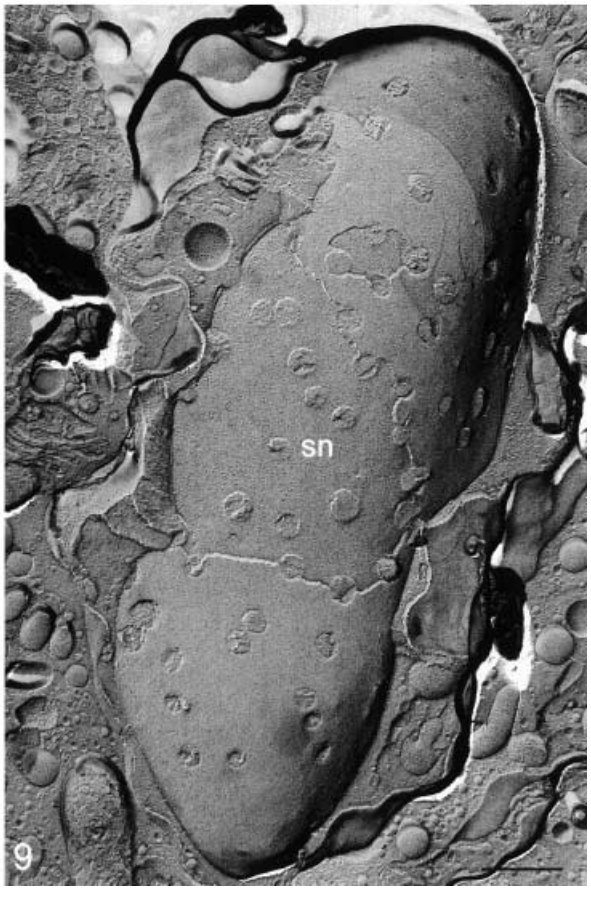
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Table 1 The average NPC density per µm² on the nuclear surface of freeze-fractured microspores, pollen, and microspore- and pollen-derived embryos of *Brassica napus*. Data were analysed using the Student's *t* distribution at a 0.01 level of significance (two-tailed). The same letter means no significant difference in NPC density between the stages. *EB*=Young bicellular pollen; *EU*=young microspores; *LU*=late microspores; *x*=mean NPCs/µm²; *n*=number of nuclei analysed

Stage		*x*	SEM	*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:	Non embryogenic cultures:				
	1 day	9.4	2.9	13	d/f
	3 day	17.0	2.5	18	b/c
	7 day	17.2	1.9	19	b/c
	Embryogenic cultures:				
	2 h	13.6	1.3	18	a
	8 h	8.7	2.5	18	d
	1 days	13.7	1.9	19	a/b/e
	3 days	12.7	3.6	17	a/f
	7 days	13.6	1.7	21	a
	Suspensor	9.0	1.5	4	d/e/f

nuclei of late microspores and nuclei of vegetative or generative cells of early bicellular pollen. This also occurred when membranes of both the generative and vegetative nuclei were visible in one fractured pollen grain (Fig. 7). The larger standard deviation in the fraction containing late microspores and young bicellular pollen than in the other samples might indicate that there is a mixture of nuclei in this sample.

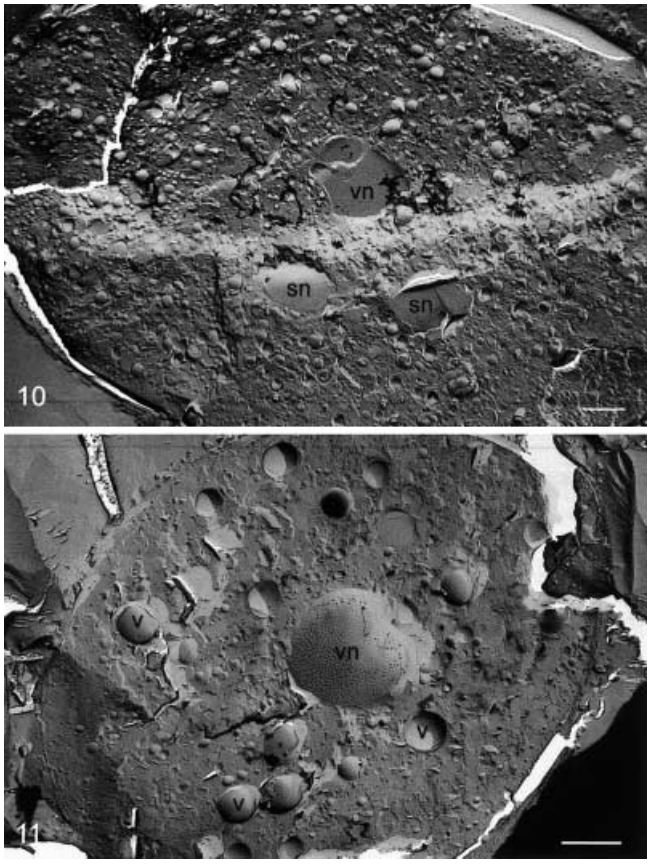
The vegetative nucleus of tricellular *Brassica* pollen was very lobed (Fig. 8). The NPC density increased significantly from 13 NPCs/µm² in the young microspore stage to 19 NPCs/µm² in the vegetative nucleus during in vivo pollen development. The NPC density of sperm nuclei in mature in vivo pollen decreased to 9 NPCs/µm² (Fig. 9). Cytoplasm of in vivo tricellular pollen contained a lot of membrane structures, often tightly packed as multilamellate bodies and stacked ER (Fig. 10).

Development of pollen in vitro under non-embryogenic conditions

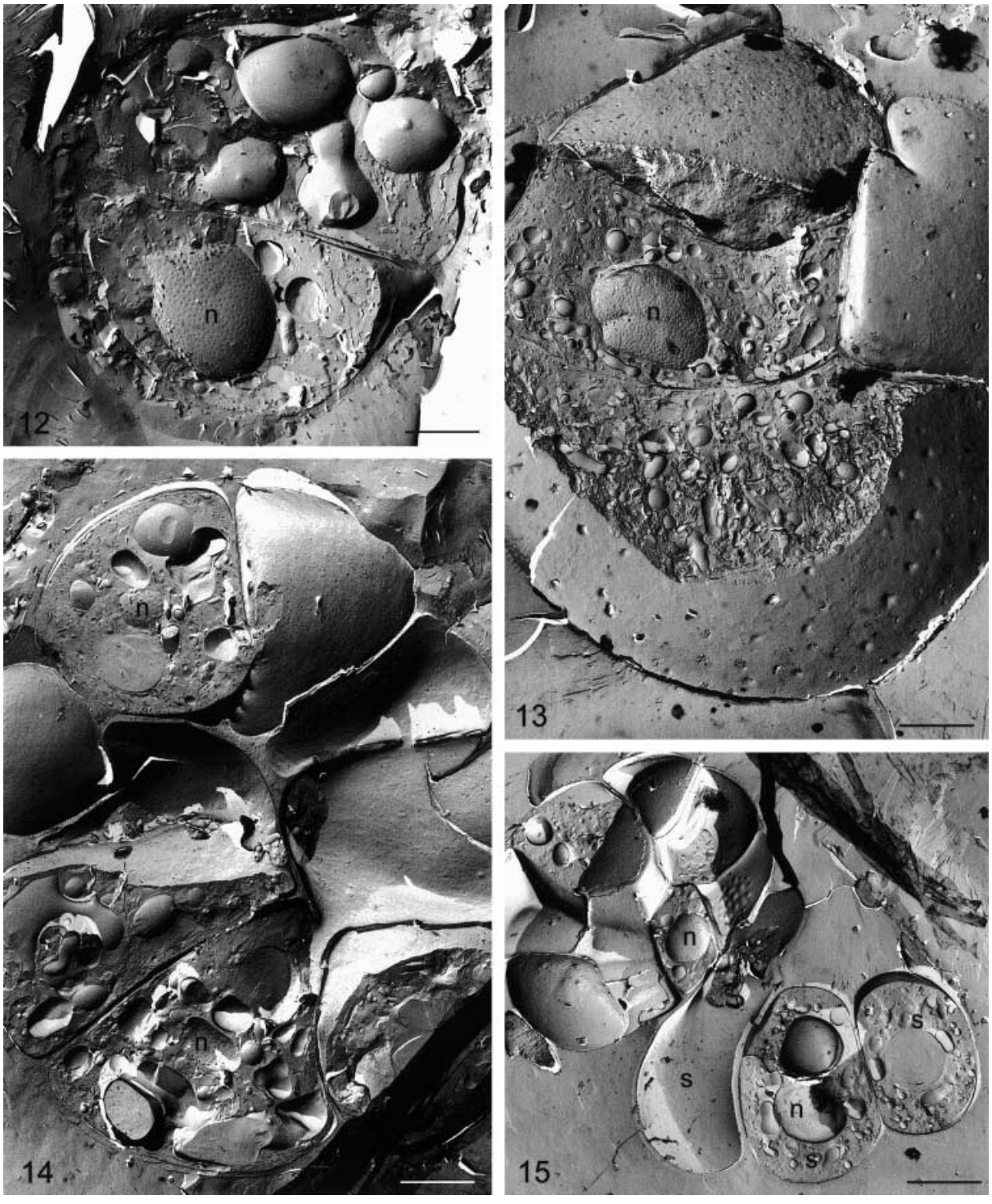
After the first day of culture under non-embryogenic conditions the NPC density decreased from 15.9 NPCs/µm² to 9.4 NPCs/µm². After 3 days the NPC density increased to 17.0 NPCs/µm². Tricellular pollen in vitro showed almost the same cytoplasmic organisation as in vivo pollen. However, vacuole-like vesicles larger than 1 µm were found only in the in vitro samples and the nucleus was not lobed (Fig. 11).

The nuclear surface areas were measured using DAPI-stained samples and the total number of NPCs/nucleus were calculated (Table 2). An increase in NPCs/

◀ **Figs. 5–9** Replicas of fractured material at different stages of in vivo pollen development in *Brassica 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 (*vn*) of mature pollen lobed. The cytoplasm contains many vesicles and membrane structures. **Fig. 9** The sperm nucleus (*sn*) 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 in the in vitro pollen. During fracturing of the in vivo pollen, the fracture often ran through the nuclei and not in between the membranes. *vn* Vegetative nucleus; *sn* sperm nucleus; *v* vesicle. *Bars*: 2 µm



Figs. 12–15 Replicas of embryogenic structures after induction of microscope and pollen embryogenesis in *Brassica napus*. *n* Nucleus. **Fig. 12** After 1 day of culture some symmetrically divided structures were found. **Fig. 13** Embryogenic structure after 3 days of culture, consisting of at least four cells. **Fig. 14** Embryogenic structure after 7 days of culture; notice the stretched pollen wall. **Fig. 15** Some of the embryos had a suspensor-like structure, (*s*), here seen in a 7-day culture. *Bars*: 2 µm (12), 5 µm (13, 14), 4 µm (15)

nucleus was found during pollen development, from 979 in young microspores to 3901 in late microspores/young bicellular pollen. This was followed by a decrease to 3247 in the vegetative nucleus of tricellular pollen in vivo. In in vitro pollen, NPCs/nucleus decreased from 3901 to 2563 at the onset of culture but increased to around the 4000 NPCs/nucleus in the vegetative nucleus within 3 days of culture. After 1 day in culture at 32°C, NPCs/nucleus increased from 3901 to 5136. However, after 7 days in culture under embryogenic conditions,

Table 2 The average nuclear surface measured on 100 DAPI-stained nuclei per developmental stage and the calculated NPCs/nucleus, nuclear volume and NPCs/μm³ of nuclei from microspores, pollen, and microspore- and pollen-derived embryos of *Brassica napus*. *EB*=young bicellular pollen; *EU*=young microspores; *LU*=late microspores; *d*=number of days in culture

Stage	Nuclear surface (μm²)±SE	NPCs/ nucleus±SE	Nuclear volume (μm³)±SE	NPCs/ μm³±SE
In vivo				
EU	74.7±20.1	979±296	62±25	15.7±7.9
LU/EB[1]	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				
Non-embryogenic cultures (18°C):				
1 day	272.7±115.9	2563±1346	715±376	5.7±4.8
3 days	233.7±122.9	3973± 2169	907±727	10.8±11.0
7 days	249.9±81.9	4298±1486	367±281	11.1±6.8
Embryogenic cultures (32°C):				
1 day	374.9±133.3	5136±2412	451±301	7.2±5.1
3 days	427.0±219.2	5422±3180	368±315	6.0±5.9
7 days	232.9±120.6	3168±1687	386±194	8.6±8.0

[1] Stage of isolation for cultures

when larger cell clusters were formed, NPCs/nucleus decreased to 3168.

Androgenesis

The first change found in cultures at 32°C was a drop in NPC density from 15.9 NPCs/μm² at the onset of culture to 9.0 NPCs/μm² within 8 h. After 1 day of culture under embryogenic conditions (32°C), NPC density was restored to almost 14 NPCs/μm², lower than the NPC density at the isolation stage. At this point in culture, the first symmetrically divided cells were found (Fig. 12), but the majority of the cells analysed had not yet divided. After 3 and 7 days of culture under embryogenic conditions, multicellular embryogenic structures were found in the samples (Figs. 13 and 14); only these structures were analysed. Some of these structures consisted of cells containing large vacuoles, whereas others had many 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 1, 3 and 7 days of culture under embryogenic conditions (Table 2). After 1 day, there were 5136 NPCs/nucleus, many more than the 3901 at the onset of culture. After 7 days the number of NPCs/nucleus decreased to 3168; the NPC density did not change between 1 and 7 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 could be counted. In all cases density of the NPCs in nuclei of suspensor-like structures was much lower than density of NPCs in the embryos themselves (Table 1).

To determine whether increases in NPCs/μm² or total number of NPCs/nucleus are related to the volume of the nucleus, NPCs/μm³ were calculated (Table 2). The results show that, in general, during pollen development the NPCs/μm³ is higher than in nuclei of cells in embryogenic cultures. After only 1 day in culture under non-embryogenic conditions NPCs/μm³ decreased from 10.2 to 5.7, but after 3 days of culture increased to 10.8 NPCs/μm³. Unfortunately, the high standard error makes it difficult to draw conclusions from these data.

Discussion

Using freeze-fractured material, NPCs were visualized and changes in NPC density were analysed during pollen development and after induction of microspore and pollen embryogenesis in *Brassica napus*. No indication was found from these experiments that there is a difference in NPC density between sides of the nucleus facing different parts of the cell, as has been reported earlier (Shi et al. 1991). This was confirmed using ultrathin sections of $KMnO_4$-fixed material for all stages of pollen development. An equal distribution of cross-sectioned nuclear pores was found (data not shown).

Several groups have found an increase in RNA synthesis just prior to anthesis (reviewed by Mascarenhas 1990). Related to this, the freeze-fracture 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 the total number of NPCs/nucleus during in vivo and in vitro pollen development, pointing at least to high metabolic 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 nucleus proved to be lobed, resulting in a smaller diameter and a lower nuclear surface estimation. The in vitro pollen did not have a lobed nucleus, which gave a relatively high total number of NPCs/nucleus.

We were not able to collect data from pollen after anthesis. No fractured nuclear surfaces were found; in all cases the fracture ran over the outside of these pollen

grains, showing the exine layer. Therefore, we do not know if, in the last phase of pollen dehydration, the NPC density of the vegetative nucleus changes. However, in specimens investigated with cryoSEM vesicles with a hole in the membrane were sometimes found (Fig. 3). This indicates 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). They are known to occur in dehydrated pollen (Tiwari et al. 1990), which suggests that the pollen collected just before flowering was already highly dehydrated. Many changes in the nuclear membranes are therefore not expected. Pollen cultured at 18°C showed fewer of these membrane structures in the cytoplasm and contained larger vesicles. An explanation for this might be that they were less dehydrated because of the liquid culture conditions.

In early pollen, NPC densities in the generative and vegetative nuclei did not differ significantly, indicating that both nuclei are equally active. This was also observed for alfalfa by Shi et al. (1991). After pollen mitosis, density of NPCs in the sperm nuclei were half of the density of NPCs in the vegetative nucleus, pointing to lower metabolic 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 NPCs/nucleus of mature pollen reflects earlier transcriptional activity or, at least, nucleocytoplasmic exchange of transcripts and/or proteins.

In the microspore and pollen cultures there was a decrease in NPC density under both embryogenic and non-embryogenic culture conditions. However, it is not certain whether this decrease is caused by the same factor in each case. Culturing the cells at 18°C results in lower NPC density and total number of NPCs/nucleus, whereas the total nuclear surface does not change. This might be explained as a decrease in transcriptional activity. It may also be true for the embryogenic cultures, but in these cultures an increase in nuclear surface is found after 1 day of culture, when NPC density is already restored. Swelling of the nucleus might have resulted in a decrease in NPC density as in the early hours of culture, followed by NPC assembly.

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 high production of new NPCs in interphase nuclei between young and late microspore stages and during the first 3 days of culture under non-embryogenic conditions. The increase of NPCs is more than might be expected as a result of DNA synthesis. Also, after 1 day of culture under embryogenic conditions the total number of NPCs/nucleus was higher. After 7 days of culture this number had decreased by one-half. This could mean that the increase of NPCs/nucleus is related to of DNA synthesis before cell division (Maul et al. 1972; Schel et al. 1978). 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 result from loss of NPCs.

In *Nicotiana tabacum* there was a loss of nuclear pores in the vegetative nucleus when young pollen were forced to embryogenesis by starvation (Garrido et al. 1995). We conclude that in *Brassica* NPC measurements cannot 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 8 h.

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