Protoplasma (2001) 216: 191–200

PROTOPLASMA

Distribution of splicing proteins and putative coiled bodies during pollen development and androgenesis in *Brassica napus* L.

K. R. Straatman* and **J. H. N. Schel**

Laboratory of Plant Cell Biology, Wageningen University, Wageningen

Received September 14, 2000
Accepted December 12, 2000

Summary. Small nuclear ribonucleoprotein particles (snRNPs) are subunits of splicing complexes, which show a transcription-dependent localization pattern. We have analyzed the labelling pattern of snRNPs during pollen development and microspore and pollen embryogenesis in *Brassica napus* with an antibody which recognizes protein D of U1, U2, U4, U5, and U6 snRNPs. It was found that nuclei were labelled almost uniformly for snRNPs in microspores and young bicellular pollen. In the generative nuclei of late-bicellular pollen and in the vegetative nuclei and sperm nuclei of mature pollen no snRNPs could be detected. The snRNP-positive nuclei contained mostly one or two brightly labelled nuclear bodies, most likely coiled bodies, often closely related to the nucleolus. These nuclear bodies increased in size from 0.5 μm in nuclei of young microspores up to 2 μm in nuclei of late microspores and the vegetative nucleus of early-bicellular pollen. Also their number increased during these developmental stages. After induction of embryogenesis the size of the coiled bodies decreased to about 0.5 μm and in several occasions the coiled body was found free in the nucleoplasm, away from the nucleolus. The results support the idea that the size and number of coiled bodies coincide with changes in general nuclear activity. They also indicate that, in nuclei of *Brassica napus*, at least assembly and disassembly of coiled bodies takes place in the nucleoplasm, whereas mature coiled bodies are located adjacent to the nucleolus.

Keywords: *Brassica napus*; Coiled body; Microspore embryogenesis; Pollen development; Spliceosome.

Abbreviations: snRNA small nuclear RNA; snRNPs small nuclear ribonucleoprotein particles.

Introduction

The nucleus is the major site for RNA synthesis. After transcription, mRNA precursors are processed and the mature mRNA is transported into the cytoplasm via nuclear-pore complexes. Processing of pre-mRNA includes 5′-end capping, splicing and 3′-end cleavage and polyadenylation. During splicing, introns are removed from the mRNA precursor and the remaining exons are bound. Splicing takes place in the presence of small nuclear ribonucleoprotein particles (snRNPs). These particles consist 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 snRNPs are mainly detected in three different subnuclear compartments: perichromatin fibrils, clusters of interchromatin granules, and coiled bodies. Perichromatin fibrils are the sites where RNA splicing takes place directly after transcription (Fakan 1994). The clusters of interchromatin granules are believed to be storage places for splicing proteins, because snRNPs accumulate in interchromatin granules when RNA synthesis is blocked (Carmo-Fonseca et al. 1992, Sleeman et al. 1998). SnRNPs also accumulate in nuclear bodies, the so-called coiled bodies, which are visible under the electron microscope in both animal and plant cells. In mammalian cells they are roughly spherical and vary in size between 0.1 and 1 μm (Lamond and Carmo-Fonseca 1993), and they can measure more than 2 μm in plant cells (Beven et al. 1995). Besides snRNPs, coiled bodies also contain a still growing list of other proteins. This includes 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; for a recent list of components of coiled bodies, see Matera 1999). The

* Correspondence and reprints (present address): Department of Genetics, University of Leicester, University Road, Leicester LE1 7RH, U.K.

coiled bodies are often found adjacent to nucleoli but are also reported free in the nucleoplasm (Lamond and Carmo-Fonseca 1993, Boudonck et al. 1999) and in the nucleoli (Ochs et al. 1994, Boudonck et al. 1999). Frey and Matera (1995) and Smith et al. (1995) found in mammalian cells an association of coiled bodies with histone genes and U1 and U2 genes. Recently, an association was also shown between coiled bodies and U4, U11, and U12 genes (Jacobs et al. 1999). 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, snRNPs no longer concentrate in coiled bodies but aggregate in large clusters of interchromatin granules (Carmo-Fonseca et al. 1992, Sleeman et al. 1998). Furthermore, inhibition of transcription after mitosis blocks the formation of new coiled bodies (Ferreira et al. 1994). All these findings indicate that the coiled bodies are somehow involved in the process of RNA transcription (for a model, see Schul et al. 1998), RNA maturation or snRNP biogenesis (for a model, see Matera 1999; for reviews, see also Lamond and Earnshaw 1998, Gonzalez-Melendi et al. 2000).

Several groups have reported snRNPs in plant systems. In *Daucus carota*, Sanchez-Pina et al. (1989) showed the presence of snRNPs as a speckled labelling pattern during somatic embryogenesis and they found brightly labelled nuclear bodies. These labelled spots were sometimes localized in the nucleolus. Beven et al. (1995) analyzed the distribution of U1, U2, and U6 snRNAs in the radicle of pea and identified the labelled nuclear bodies as coiled bodies with 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*. SnRNPs were also found in pollen of *Capsicum annuum* (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 is not clear which of the foci were coiled bodies (Glyn and Leitch 1995). Further, Kingham et al. (1995) reported the dynamics of spliceosomal proteins in the moss *Funaria hygrometrica*, while more recently, Boudonck et al. (1998) analyzed differences in the numbers of coiled bodies in the root epidermis of *Arabidopsis thaliana*. The differences were related mainly to cell cycle stage, cell type, and developmental stage (for a review, see Leitch 2000). Boudonck et al. (1999), using U2B-GFP constructs, also published evidence of movement of coiled bodies in *A. thaliana*, showing their relocalization from the periphery of the nucleus towards the nucleolus.

In this study we report on the dynamics of snRNPs and putative coiled bodies during in vivo and in vitro pollen development and after induction of microspore and pollen embryogenesis in *Brassica napus*. To change the developmental pathway from pollen development into embryo development, late microspores and early-bicellular pollen of *B. napus* cv. Topas can be cultured for at least 8 h at 32 °C (Pechan and Keller 1988). If cultured at 18 °C, normal pollen develops (Custers et al. 1994). This system allows the study of early events during embryogenesis.

Material and methods

Plant material

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

Late microspores and young 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 cultured continuously at 18 °C (nonembryogenic conditions). Samples were taken after 0, 1, 3, and 7 days of culture. Anthers, cultured microspores, cultured pollen, and embryogenic cells were collected and fixed in 3% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) (35 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4) for 3 h at room temperature. They were rinsed in PBS, and the cultured cells were immobilized in 2% low-melting-point agarose (BDH, Poole, England). After this, all samples were merged in a 13% sucrose solution, frozen in droplets of liquid propane and stored at –70 °C.

Specimen preparation and immunofluorescence labelling

Serial sections (5 µm) from frozen material, attached to a holder with M-1 embedding matrix (Lipshaw, Pittsburgh, Pa., U.S.A.), were prepared on a cryomicrotome (Micron, Biomed HM500) and mounted on aminopropyl-triethoxy-silane-coated slides (Serva, Heidelberg, Federal Republic of Germany). After the slides were air-dried and washed in PBS they were blocked in 1% bovine serum albumin (BSA) in PBS for 30 min. Then, they were washed in 0.1% acetylated BSA (BSAc; Aurion, Wageningen, the Netherlands), followed by incubation with the first antibody overnight at 4 °C. In single-labelling experiments the first antibody was the monoclonal antibody (mAb) 7.13, which was used undiluted (gift from F. Ramaekers, Department of Pathology, University of Nijmegen, the Netherlands). This is a mouse mAb which recognizes protein D of U1, U2, U4, U5, and U6 snRNPs (Billings et al. 1982, 1985; Sanchez-Pina et al. 1989). In double-labelling experiments, anti-fibrillarin P89

(kindly provided by W. Pluk, Department of Biochemistry, University of Nijmegen) was used. This antiserum recognizes in vitro translated human fibrillarin and immunoprecipitates U3RNPs (H. Pluk and W. J. van Venrooij, University of Nijmegen, unpubl. results) was used. This antibody was diluted 1 : 50 in mAb 7.13. The next day the slides were washed in 0.1% BSAc followed by incubation in the second antibody for 1 h at 37 °C. This second antibody was goat anti-mouse antibody conjugated with Bodipy (Molecular Probes, Leiden, the Netherlands) diluted 1 : 100 in 0.1% BSAc; in double-labelling experiments goat anti-human antibody conjugated with tetramethylrhodamine isothiocyanate (Sigma Chemicals Co, St. Louis, Mo., U.S.A.), diluted 1 : 20, was added. After washing in PBS, the DNA was stained with 0.1 µg of propidium iodide per ml in single-labelling experiments or with 1 µg of 4′,6-diamidino-2-phenylindole (Sigma) in double-labelling experiments. The slides were mounted in Citifluor-glycerol (Citifluor Ltd., Canterbury, U.K.) and analyzed 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 ×100 (numerical aperture, 1.3) oil immersion lens.

Results

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 when we used this antibody on root tip cells of *B. napus* or *Allium cepa*, although the controls, for which cells of *P. sativum* were used, were positive (not shown). This forced us to use another antibody raised against snRNPs. 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 nuclear bodies in our cells as coiled bodies, several antibodies against coilin p80 were tried, 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 in plants that contains both proteins. Therefore, it is most likely that these nuclear bodies are coiled bodies. All stages were analyzed with both antibodies. We are aware of the fact that there are reports of heterogeneity between coiled bodies (Alliegro and Alliegro 1998), sometimes dependent on treatment (Lamond and Carmo-Fonseca 1993, Zirbel et al. 1993), but in all the analyzed samples all nuclear bodies found were positive for both antibodies. However, it was difficult to obtain good images from fibrillarin-labelled coiled bodies of microspores and pollen because both the exine wall and the nucleolus gave a more intense labelling. As a result the coiled bodies were often difficult to see in the images (see Figs. 1 b and 2 b) and most images presented have been taken from experiments using anti-snRNPs and propidium iodide. However, double-labelling experiments for snRNPs and fibrillarin were always carried out together with these experiments.

Pollen development

During in vivo pollen development, from young-microspore stage towards late-microspore stage, the

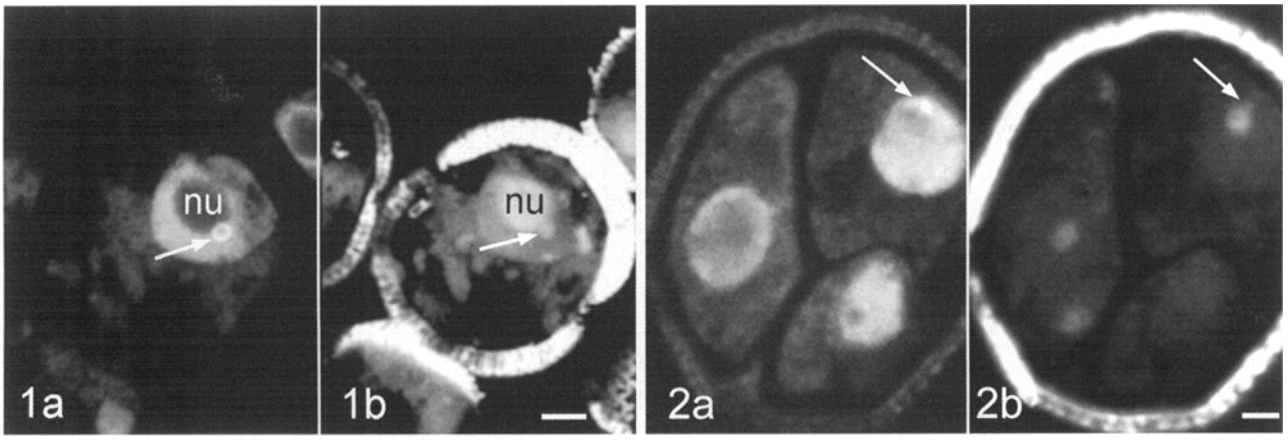


Fig. 1a, b. Confocal images of a cryosection of a late microspore from in vivo material. **a** A nuclear body labelled with an antibody against snRNPs, **b** the same nuclear body labelled with an antibody against fibrillarin. Merging the two images results in an exact correlation of both signals identifying the nuclear body as a coiled body. The labelling of snRNPs is more intense at the periphery of the coiled body, whereas the labelling of fibrillarin is homogeneous. Bar: 1 µm

Fig. 2a, b. Overview of a globular embryo after 3 days of culture. **a** SnRNP labelling, **b** fibrillarin labelling. Again, merging of the two images results in colocalization of the snRNP and fibrillarin signal. Bar: 3 µm. Note that in both Figs. 1 and 2 the autofluorescence of the exine wall and the labelling of fibrillarin in the nucleolus give a brighter signal in the left panel than the labelling in the coiled body

nucleus and nucleolus enlarged and a nucleolar vacuole appeared. The nucleoplasm of young microspores showed a relatively weak signal with a slightly speckled pattern when labelled for snRNPs, with no cytoplasmic labelling (Figs. 3 and 4). The signal became stronger in later stages and was found to be more uniform throughout the whole nucleoplasm. In addition to the nucleoplasm, the nucleolar vacuole also contained snRNPs (Fig. 5).

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. 6). In the late-bicellular pollen stage, the DNA of the generative nucleus became more condensed and the nucleolus disappeared coinciding with a loss of signal for snRNPs. The vegetative nucleus was still large and contained a large nucleolus. It remained positive for snRNPs in this stage (Fig. 7).

After the pollen mitosis, neither the vegetative nucleus nor the nuclei of the sperm cells showed any signal when labelled for snRNPs (Fig. 8). When microspores and young pollen were cultured at 18 °C, resulting in in vitro pollen development, similar results were obtained. During the development from young microspores towards young pollen the number of coiled bodies per nucleus increased slowly (Table 1). In all pollen stages the coiled bodies were only counted in the vegetative nucleus but 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. 3a and 4a) to more than 2.0 µm in late microspores and young pollen (Figs. 1a and 5a). The small coiled bodies exhibited a spherical morphology, whereas the large coiled bodies were mostly oval. In large coiled bodies the labelling of snRNPs was often observed at the periphery with less or no label in the center, whereas fibrillarin was detected uniformly throughout the coiled bodies (Fig. 1).

After the microspore mitosis, coiled bodies were found in both the generative and vegetative nuclei (Figs. 5a and 6a). In a few cases, three to four small nuclear bodies were present in the generative nucleus shortly after this division, whilst both the vegetative and generative nuclei were still situated closely to the pollen wall (Fig. 6a). Between the early- and late-bicellular pollen stage, the generative nucleus lost its signal for snRNPs. The number of coiled bodies in the generative nucleus, if still labelled with anti-snRNP, decreased to approximately one coiled body per nucleus. The number of coiled bodies in the vegetative nucleus had increased after the microspore mitosis, while the average size of the coiled bodies did not change after this division. From the early- to the late-bicellular stage, however, the size of the coiled bodies decreased to 1.5 µm (Fig. 7a) and the number of coiled bodies diminished (Table 1). After the pollen mitosis no snRNPs could be detected by the antibodies, so no labelled coiled bodies were found (Fig. 8).

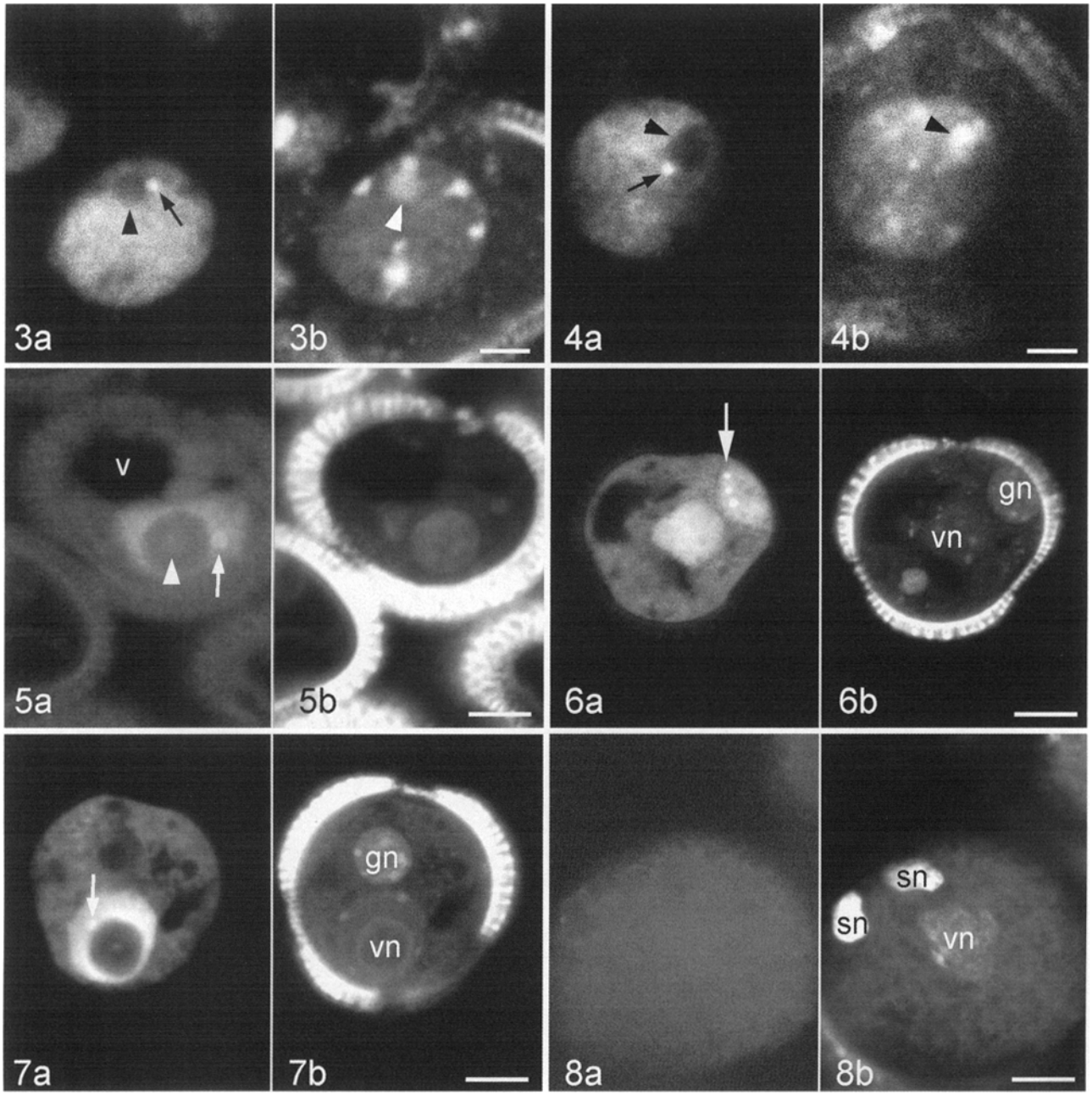
Microspore and pollen embryogenesis

When late microspores and young pollen are cultured for two days at 32 °C, they can change their developmental pathway from pollen development towards

Table 1. Distribution of coiled bodies in cryosections of nuclei of *B. napus* at different stages of pollen development (in vivo and in vitro) and after induction of microspore and pollen embryogenesis

Developmental stage	% Nuclei with the following nr. of coiled bodies:				N[a]	Maximum size (µm) of coiled body
	0	1	2	3		
Young microspores	65	33	2	0	101	<0.5
Late microspores	51	48	1	0	132	2.0
Young bicellular pollen	44	41	14	1	71	2.0
Late bicellular pollen	38	58	4	0	146	1.5
1 day 18 °C	41	56	2	0	94	2.0
3 days 18 °C	63	37	0	0	41	1.8
1 day 32 °C	59	41	0	0	46	1.5
7 day embryos	44	50	4	1	93	0.8

[a] Number of sectioned nuclei examined to calculate the number of coiled bodies. In pollen the coiled bodies were only counted in the vegetative nucleus



Figs. 3–8. Labelling of snRNPs during in vivo pollen development. Optical sections through nuclei showing the labelling with anti-snRNP (**a**) and the DNA stained with propidium iodide (**b**). The brighter spots in the nucleoplasm of the propidium iodide-stained sections are heterochromatin. Bars: Figs. 3 and 4, 2 µm; Figs. 5, 7, and 8, 5 µm; Fig. 6, 7 µm

Figs. 3 and **4.** In young microspores the nuclei are relatively weakly labelled for snRNPs in the nucleoplasm, with a small coiled body (arrow) closely related to a small nucleolus (arrowheads)

Fig. 5. In late microspores the nucleus has moved to the exine wall near to the central vacuole (*v*). Both the nucleolus and the coiled body (arrow) have increased in size compared with young microspores. It is also notable that the so-called nucleolar vacuole (arrowhead) is positive for snRNPs

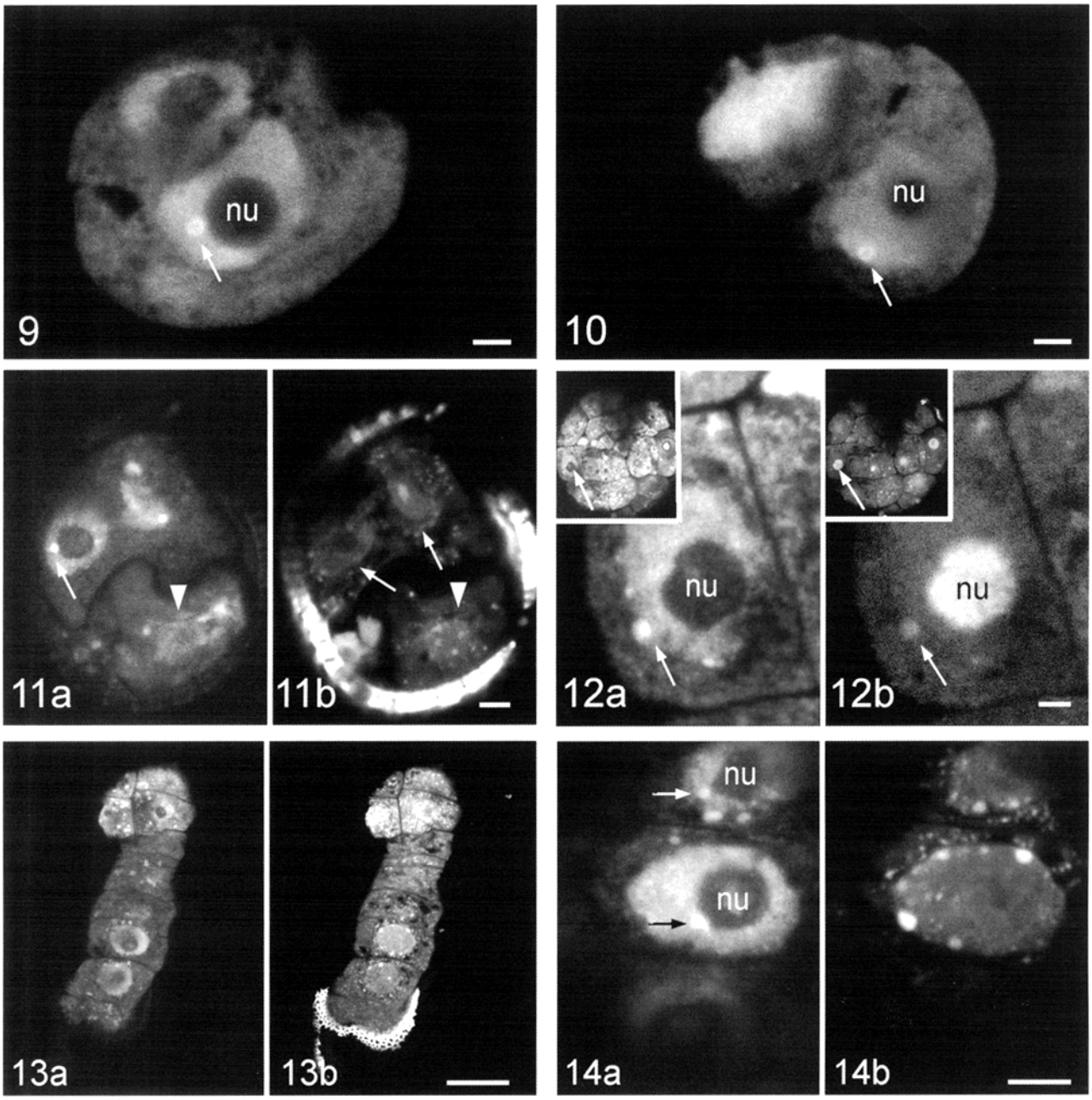
Fig. 6. After the microspore mitosis both the generative (*gn*) and vegetative (*vn*) nuclei are positively labelled for snRNPs. Note the nuclear bodies in the nucleoplasm of the generative nucleus (arrow)

Fig. 7. In late-bicellular pollen the generative nucleus (*gn*) lost its signal for snRNPs, whereas the vegetative nucleus (*vn*) remained positively labelled and still contained a coiled body (arrow)

Fig. 8. After the pollen mitosis both vegetative nucleus (*vn*) and the sperm nuclei (*sn*) did not show any label for snRNPs

embryo development. This change means that, instead of the normal asymmetrical pollen division that 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. 9 and 10). After 3 and 7 days of culturing under embryogenic conditions, several nuclei showed only a faint or no labelling with this antibody. However, in an embryogenic structure, labelled and unlabelled nuclei could be found (Fig. 11).

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 associated with the nucleolus but was located towards the periphery of the nucleus (Fig. 10). After the second embryogenic division only a few coiled bodies, with a size of 0.5 µm, could be visualized (Fig. 11 a). After 7 days of culture, when the embryo is globular, the coiled bodies ranged in size from 0.5 to 0.8 µm. The number of coiled bodies increased slightly during the embryo development.



In some cultures, suspensorlike structures with a globular embryo were found (Fig. 13). The nuclei in these structures, often with a nucleolar vacuole, were positively labelled for snRNPs, both in the suspensorlike structure as well as in the globular part of the embryo. They contained coiled bodies ranging in size between 0.5 and 1 μm (Fig. 14), situated closely to the nucleolus.

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 similar to that found during in vivo pollen development and in pollen cultured at 18 °C.

Discussion

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 increased nuclear activity (Testillano et al. 1993, Boudonck et al. 1998). Also during this period, the size of the coiled bodies increased from less than 0.5 μm up to 2 μm. Glyn and Leitch (1995), using an antibody against the D-polypeptide, found in wheat also an increase in total foci area related to an increase of cellular activity. From earlier experiments (Binarova et al. 1993) we know that late microspores are in the G_2 and that the vegetative nucleus of pollen is arrested in the G_1 of the cell cycle. So, the size of the coiled bodies during *B. napus* pollen development most likely is not cell cycle dependent, as was found in several other systems (see, e.g., Carmo-Fonseca et al. 1993, Glyn and Leitch 1995, Boudonck et al. 1998).

From the sections that were viewed it is difficult to say if the number of coiled bodies increased during this period. Because sections of 5 μm thickness 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 there is also an increased chance that a single coiled body will be cut and appear in two sections. This makes it difficult to estimate the number of coiled bodies per nucleus. However, the maximum size of coiled bodies found in late microspores, early-bicellular pollen, and cells cultured for one day at 18 °C is 2 μm and from the slides analyzed we know that in these stages many of the coiled bodies were relatively large. Therefore, it is possible to use a G-test of independence (Sokal and Rohlf 1969) to test the hypothesis that the number of coiled bodies is independent or dependent on the developmental stage. The result shows $G = 18.2$; using the χ^2-distribution with $(a-1)(b-1)$ degrees of freedom, where a is the number of columns and b the number of rows, we find that $\chi^2_{3,0.05} = 12.83$. This indicates that with a $P < 0.005$ the number of coiled bodies in late-unicellular microspores is smaller than in early-bicellular pollen. Using the same test between early-bicellular pollen and cells cultured for one day at 18 °C (a mixture of late microspores and early-bicellular pollen when isolated) it was found that, with $P < 0.01$, the number of coiled bodies has decreased in culture.

Figs. 9–14. Labelling of snRNPs after induction of microspore and pollen embryogenesis. Bars: Figs. 9 and 10, 2 μm; Figs. 11 and 12, 3 μm; Fig. 13, 5 μm; Fig. 14, 25 μm

Figs. 9 and **10.** After embryo induction, microspores divide symmetrically instead of asymmetrically. In both nuclei snRNP labelling is found in the nucleoplasm. Coiled bodies (arrows) were found in close relation to the nucleolus (*nu*) (Fig. 9) as well as free in the nucleoplasm (Fig. 10)

Fig. 11 a, b. Embryogenic structure, probably after the second embryogenic division, three days in culture. **b** Three nuclei appear labelled with propidium iodide (arrows and arrowhead); **a** snRNP labelling shows clearly only two nuclei; one of the nuclei shown in **b** (arrowhead) is only faintly labelled for snRNPs. Coiled bodies were still found in close relation to the nucleolus and free in the nucleoplasm

Fig. 12. In early embryogenic structures the coiled bodies were labelled with both the antibodies against snRNPs (arrow) (**a**) and fibrillarin (arrow) (**b**). **Inset** Overview of the embryogenic structure, the arrow points to the enlarged nucleus

Figs. 13 and **14.** Embryos with a suspensorlike structure as found in some cultures. **a** Labelling with antibodies against snRNPs, **b** propidium iodide staining. The brighter propidium iodide-positive spots in the nucleoplasm are heterochromatin

Fig. 13. The nuclei of the suspensorlike structures label with antibodies against snRNPs

Fig. 14. The nuclei often contain one coiled body in close relation to the nucleolus (*nu*) (arrows)

Taken into account the number of sections from one nucleus we can say that 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. After 1 day in culture the number decreases to one coiled body per nucleus. Raska et al. (1991) found up to eight and Zirbel et al. (1993) found up to nine coiled bodies per nucleus, depending on the species or cell type examined. Boudonck et al. (1998) found in *A. thaliana* root cells also an increase in the numbers of coiled body reflecting the "gross metabolic status" of the cells but not necessarily the nucleolar transcriptional activity. Our results suggest that, during pollen development of *B. napus*, increases in size and number of coiled bodies coincide with an increase in overall nuclear activity. This is also supported by earlier data, showing an increase in nuclear activity during pollen development and a decrease when the cells were cultured, using the nuclear-pore complex density as an indicator (Straatman et al. 2000).

It was found that in early-bicellular pollen both the generative and vegetative nuclei 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 small coiled bodies were found in a generative nucleus. In later stages of the generative nucleus, only one small coiled body was found before all signal was lost. The high number of coiled bodies therefore can be an indication of a decrease in activity of the generative nucleus before and after mitosis. Furthermore, in the highly active vegetative nucleus this high number of coiled bodies was observed only once.

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 of the pollen.

Microspore and pollen embryogenesis

In our experiments, inducing embryogenesis at 32 °C caused a slight decrease in the number and size of coiled bodies at the onset of culture. This decrease was not seen in nonembryogenic cells cultured 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). However, in root tip cells from pea subjected to a heat shock of 42 °C for 1 h, there were no coiled bodies detected with either a U2 antisense probe or an antibody against U2 (Beven et al. 1995). Similar results were found in HeLa cells (Carmo-Fonseca 1993, Zirbel 1993). This indicates that the decrease found in our experiments might be due to the culture temperature.

Seven days after induction of microspore and pollen embryogenesis, several nuclei in multicellular structures did not show labelling of snRNPs. Furthermore, in one embryogenic structure, nuclei labelled for snRNPs were found together with unlabelled nuclei. We think that this might be caused by poor penetration of the antibody or that binding sites for the antibody were not accessible. Several cells in these embryogenic structures showed very condensed cytoplasm (see Fig. 12a, inset). When nonembryogenic structures in these embryogenic cultures were examined, labelling of snRNPs was no problem, indicating that this is a specific problem for the embryogenic structures and not a result of the higher culture temperature of 25 °C in comparison with 18 °C of nonembryogenic cultures.

After the first symmetrical division of the induced microspores and pollen, large snRNP-positive nuclei were observed with relatively small nucleoli. Also the mean size of the coiled bodies was reduced and several coiled bodies were found in the nucleoplasm, no longer in close proximity to the nucleolus. This phenomenon was also reported by other authors and is often seen in some cells types (Lamond and Carmo-Fonseca 1993, Boudonck et al. 1999, Matera 1999). The fact that more coiled bodies were found at some distance from the nucleolus might be an indication that they were transported away from the nucleolus and disintegrated, resulting in one small remaining coiled body. However, Boudonck et al. (1999) reported only unidirectional movements of coiled bodies from the nuclear periphery towards the nucleolus. The detection of so many "free" coiled bodies at this stage could indicate that the coiled bodies in these early embryogenic structures have a function different from that of the coiled bodies observed during pollen development.

In the moss *Funaria hygrometrica* the number of coiled bodies is cell type dependent (Leitch 2000). This was also found in different cell types of *A. thaliana*

roots (Boudonck et al. 1998). The developmental change from pollen towards androgenesis in *B. napus* does not result in such a relation between cell types and the numbers of coiled bodies but shows a change in the size of coiled bodies.

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 at these stages. Although there were changes in the number of coiled bodies during pollen development and androgenesis, the changes in size of the coiled bodies were more dramatically. These changes in size and number of the coiled bodies coincide with changes in markers for overall nuclear activity and are not related to the cell cycle. However, the function of these coiled bodies needs to be further elucidated.

Acknowledgments

We thank Mr. S. Massalt and Mr. P. Snippenburg for preparing the photographs and Mr. L. Peterse for culturing the plants. The experiments comply with the Dutch laws.

References

Alliegro MC, Alliegro MA (1998) Protein heterogeneity in the coiled body compartment. Exp Cell Res 239: 60–68

Andrade LEC, Tan EM, Chan EKL (1993) Immunocytochemical analysis of the coiled body in the cell cycle and during proliferation. Proc Natl Acad Sci USA 90: 1947–1951

Beven AF, Simpson GG, Brown JWS, Shaw PJ (1995) The organization of spliceosomal components in the nuclei of higher plants. J Cell Sci 108: 509–518

Billings PB, Allen RW, Jensen FC, Hoch SO (1982) Anti-RNP monoclonal antibodies derived from mouse strain with lupus-like autoimmunity. J Immunol 128: 1176–1180

– Barton JR, Hoch SO (1985) A murine monoclonal antibody recognizes the 13,000 molecular weight polypeptide of the Sm small nuclear ribonucleoprotein complex. J Immunol 135: 428–432

Binarova P, Straatman K, Hause B, Hause G, van Lammeren AAM (1993) Nuclear DNA synthesis during the induction of embryogenesis in cultured microspores and pollen of *Brassica napus* L. Theor Appl Genet 87: 9–16

Boudonck K, Dolan L, Shaw PJ (1998) Coiled body numbers in the *Arabidopsis* root epidermis are regulated by cell type, developmental stage and cell cycle parameters. J Cell Sci 111: 3687–3694

– – – (1999) The movement of coiled bodies visualized in living plant cells by the green fluorescent protein. Mol Biol Cell 10: 2297–2307

Brasch K, Ochs RL (1992) Nuclear bodies (NB): a newly "rediscovered" organelle. Exp Cell Res 202: 211–223

Carmo-Fonseca M, Pepperkok R, Carvalho MT, Lamond AI (1992) Transcription-dependent co-localization of the U1, U2, U4/U6 and U5 snRNPs in coiled bodies. J Cell Biol 117: 1–14

– Ferreira J, Lamond AI (1993) Assembly of snRNP-containing coiled bodies is regulated in interphase and mitosis: evidence that the coiled body is a kinetic nuclear structure. J Cell Biol 120: 841–852

Christensen AH, Sharrock RA, Quail PA (1992) Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol Biol 18: 675–689

Concha II, Mansilla J, Riveros M, Burzio LO (1995) U1 snRNP components are present in the vegetative and generative nuclei of the pollen grain. Sex Plant Reprod 8: 339–344

Custers JBM, Cordewener JHG, Nöllen Y, Dons HJM, van Lookeren Campagne MM (1994) Temperature controls both gametophytic and sporophytic development in microspore cultures of *Brassica napus*. Plant Cell Rep 13: 267–271

Fakan S (1994) Perichromatin fibrils are in situ forms of nascent transcripts. Trends Cell Biol 4: 86–96

Ferreira JA, Carmo-Fonseca M, Lamond AI (1994) Differential interaction of splicing snRNPs with coiled bodies and interchromatin granules during mitosis and assembly of daughter cell nuclei. J Cell Biol 126: 11–23

Frey MR, Matera G (1995) Coiled bodies contain U7 small nuclear RNA and associate with specific DNA sequences in interphase human cells. Proc Natl Acad Sci USA 92: 5915–5919

Glyn MCP, Leitch AR (1995) The distribution of a spliceosome protein in cereal (Triticeae) interphase nuclei from cells with different metabolic activities and through the cell cycle. Plant J 8: 531–540

González-Melendi P, Testillano PS, Ahmadian P, Fadon B, Vicente O, Risueño MC (1995) In situ characterization of the late vacuolate microspore as a convenient stage to induce embryogenesis in *Capsicum*. Protoplasma 187: 60–71

– – – – Risueño MC (1996) New in situ approaches to study the induction of pollen embryogenesis in *Capsicum annuum* L. Eur J Cell Biol 69: 373–386

– Beven A, Boudonck K, Abranches R, Wells B, Dolan L, Shaw P (2000) The nucleus: a highly organized but dynamic structure. J Microsc 198: 199–207

Jacobs EY, Frey MR, Wu W, Ingledue TC, Gebuhr TC, Gao L, Marzluff WF, Matera AG (1999) Coiled bodies preferentially associate with U4, U11, and U12 small nuclear RNA genes in interphase HeLa cells but not with U6 and U7 genes. Mol Biol Cell 10: 1653–1663

Kingham KI, Duckett JG, Glyn MCP, Leitch AR (1995) Nuclear differentiation in the filamentous caulonema of the moss *Funaria hygrometrica*. New Phytol 131: 543–556

Lamond AI, Carmo-Fonseca M (1993) The coiled body. Trends Cell Biol 3: 198–204

– Earnshaw WC (1998) Structure and function in the nucleus. Science 280: 547–553

Leitch AR (2000) Higher levels of organization in the interphase nucleus of cycling and differentiated cells. Microbiol Mol Biol Rev 64: 138–152

Lichter R (1982) Induction of haploid plants from isolated pollen of *Brassica napus*. Z Pflanzenphysiol 105: 427–434

Lischwe MA, Ochs RL, Reddy R, Cook RG, Yeoman LC, Tan EM, Reichlin M, Busch H (1985) Purification and partial characterization of a nucleolar scleroderma antigen (Mr = 34,000; pI 8.5) rich in N^G,N^G-dimethylarginine. J Biol Chem 260: 14304–14310

Mascarenhas JP (1975) The biochemistry of angiosperm pollen development. Bot Rev 41: 259–314

Matera AG (1999) Nuclear bodies: multifaceted subdomains of the interchromatin space. Trends Cell Biol 9: 302–309

Ochs RL, Lischwe MA, Spohn WH, Busch H (1985) Fibrillarin: a new protein of the nucleolus identified by autoimmune sera. Biol Cell 54: 123–134

Ochs RL, Stein TJ, Tan EM (1994) Coiled bodies in the nucleolus of breast cancer cells. J Cell Sci 107: 385–399

Osteryoung KW, Sundberg H, Vierling E (1993) Poly(A) tail length of a heat shock protein RNA is increased by severe heat stress, but intron splicing is unaffected. Mol Gen Genet 239: 323–333

Pechan P, Keller WA (1988) Identification of potentially embryogenic microspores in *Brassica napus*. Physiol Plant 74: 377–384

Raska I, Andrade LE, Ochs RL, Chan EK, Chang CM, Roos G, Tan EM (1991) Immunological and ultrastructural studies of the nuclear coiled body with autoimmune antibodies. Exp Cell Res 195: 27–37

Sánchez-Pina MA, Kieft H, Schel JHN (1989) Immunocytochemical detection of non-histone nuclear antigens in cryosections of developing somatic embryos from *Daucus carota* L. J Cell Sci 93: 615–622

Schul W, van Driel R, de Jong L (1998) Coiled bodies and U2 snRNA genes adjacent to coiled bodies are enriched in factors required for snRNA transcription. Mol Biol Cell 9: 1025–1036

Sleeman J, Lyon CE, Platani M, Kreivi JP, Lamond AI (1998) Dynamic interactions between splicing snRNPs, coiled bodies and nucleoli revealed using snRNP protein fusions to the green fluorescent protein. Exp Cell Res 243: 290–304

Smith KP, KC Carter, CV Johnson, JB Lawrence (1995) U2 and U1 snRNA gene loci associated with coiled bodies. J Cell Biochem 59: 473–485

Sokal RR, Rohlf FJ (1969) Biometry: the principles and practice of statistics in biological research. W H Freeman, San Francisco

Solymosy F, Pollak T (1993) Uridylate-rich small nuclear RNAs (UsnRNAs), their genes and pseudogenes, and UsnRNPs in plants – structure and function: a comparative approach. Crit Rev Plant Sci 12: 275–369

Straatman KR, Nijsse J, Kieft H, van Aelst AC, Schel JHN (2000) Nuclear pore dynamics during pollen development and androgenesis in *Brassica napus*. Sex Plant Reprod 13: 43–51

Testillano PS, Sánchez-Pina MA, Olmedilla A, Fuchs JP, Risueño MC (1993) Characterization of the interchromatin region as the nuclear domain containing snRNPs in plant cells: a cytochemical and immunoelectron microscopy study. Eur J Cell Biol 61: 349–361

– González-Melendi P, Ahmadian P, Fadon B, Risueño MC (1995) The immunolocalization of nuclear antigens during the pollen developmental program and the induction of pollen embryogenesis. Exp Cell Res 221: 41–54

Zirbel RM, Mathieu UR, Kurz A, Cremer T, Lichter P (1993) Evidence for a nuclear compartment of transcription and splicing located at the chromosome boundaries. Chromosome Res 1: 93–106