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RESEARCH ARTICLE

mRNA accumulation in the Cajal bodies of the diplotene larch microsporocyte

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Abstract In microsporocytes of the European larch, we demonstrated the presence of several mRNAs in spherical nuclear bodies. In the nuclei of microsporocytes, we observed up to 12 bodies, ranging from 0.5 to 6 μm in diameter, during the prophase of the first meiotic division. Our previous studies revealed the presence of polyadenylated RNA (poly(A) RNA) in these bodies, but did not confirm the presence of nascent transcripts or splicing factors of the SR family. The lack of these molecules precludes the bodies from being the sites of synthesis and early maturation of primary transcripts (Kołowerzo et al., Protoplasma 236:13–19, 2009). However, the bodies serve as sites for the accumulation of splicing machinery, including the Sm proteins and small nuclear RNAs. Characteristic ultrastructures and the molecular composition of the nuclear bodies, which contain poly(A) RNA, are indicative of Cajal bodies (CBs). Here, we demonstrated the presence of several housekeeping gene transcripts—α-tubulin, pectin methylesterase, peroxidase and catalase, ATPase, and inositol-3-phosphate synthase—in CBs. Additionally, we observed transcripts of the RNA polymerase II subunits RPB2 and RPB10 RNA pol II and the core spliceosome proteins mRNA SmD1, SmD2, and SmE. The co-localization of nascent transcripts and mRNAs indicates that mRNA accumulation/storage, particularly in CBs, occurs in the nucleus of microsporocytes.

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Introduction

Coiled bodies were discovered in mammalian nervous tissue by a Spanish researcher, Ramon y Cajal (Cajal 1903). Coiled bodies, revealed by studies at the ultrastructural level, are composed of coiled fibrils that resemble a coil of twisted thread. Due to their convoluted structure, they were named coiled bodies by Monneron and Bernhard (1969) in mammalian cells. Later, the same architecture of these bodies in plant cells was confirmed by Moreno Diaz de la Espina et al. (1982). A hundred years after their discovery, they were finally named Cajal bodies (CBs) to honor their discoverer (Gall et al. 1999). They are evolutionarily conserved structures present both in animal cells (Cioce and Lamond 2005) and plant cells (Shaw and Brown 2004), which indicates their fundamental role in the nuclei metabolism of eukaryotic cells. CBs participate in the storage and maturation of both snRNPs and small nucleolar RNAs, as well as other splicing factors necessary for mRNA and pre-rRNA processing, but they do not directly participate in transcription. Despite the presence of splicing machinery elements, numerous transcription factors (Schul et al. 1998), and even RNA polymerase II (Xie and Pombo 2006) in CBs, they are not considered to be a direct site of transcription and splicing. DNA (Moreno Díaz de la Espina et al. 1982; Thiry 1994; Wróbel and Smoliński 2003), newly formed transcripts (Biggiogera and Fakan 1998; Kołowerzo et al. 2009), and several crucial splicing factors, such as SC35 and U2AF (Gama-Carvalho et al. 1997), were not observed in CBs. CBs are involved in tRNA

maturation (Jarrous et al. 2001), histone mRNA maturation (Wagner and Marzluff 2006), and telomere synthesis (Venteicher et al. 2009). Therefore, CBs are currently thought to provide a location where various components and subcomplexes can be assembled before they are released to the site of function (Shaw and Brown 2004).

In plant cells, nuclear structures that morphologically resemble CBs have been observed for a long time (for a review, see Risueño and Medina 1986). The term "plant coiled body" was first used by Moreno Díaz de la Espina et al. (1980), which was based primarily on the ultrastructural similarity of the nuclear bodies of onion cells to animal cell CBs. Plant CBs are characterized as being similar to animal CBs. In plant cells, both free CBs and perinucleolar CBs were observed to accumulate splicing system elements, such as small nuclear RNAs (snRNAs) and Sm proteins (Chamberland and Lafontaine 1993; Beven et al. 1995; Gulemetova et al. 1998; Jennane et al. 1999; Wróbel and Smoliński 2003; Kołowerzo et al. 2009). The investigations of Boudonck et al. (1999), using a U2B″–GFP fusion protein that illustrated the CB movement within the cell nucleus, demonstrated that nucleoplasmic CBs and nucleolar Cajal bodies presented different locations of the same structure. Plant CBs were shown to contain the rRNA processing machinery components, U3 snoRNA and fibrillarin (Olmedilla et al. 1997; Boudonck et al. 1999; Acevedo et al. 2002; Wróbel and Smoliński 2003), but are devoid of rDNA (Lafontaine and Chamberland 1995) and rRNA (Wróbel and Smoliński 2003). Visualization of CBs in living plant cells revealed also that plant CBs exhibit dynamic movement, fusing together and splitting apart within the nucleus (Boudonck et al. 1999). Moreover, the number of CBs in plant cells was observed to change during the cell cycle and differentiation (Boudonck et al. 1998; Straatman and Schel 2001; Seguí-Simarro et al. 2006; Zienkiewicz and Bednarska 2009). Recently, plant CBs were shown to contain also Atcoilin (Collier et al. 2006; Koroleva et al. 2009), which is a homolog of the mammalian protein coilin, a marker of CB in animal cells.

In summary, CBs are highly conserved nuclear structures that perform similar functions in all eukaryotic cells, although plant CBs have been implicated as sites of siRNA biogenesis whereas siRNA dicing occurs in the cytoplasm in animals (for a review, see Pontes and Pikaard 2008).

In our earlier studies on the diplotene microsporocytes of larch, we observed that polyadenylated RNA (poly(A) RNA) localizes to CBs (Kołowerzo et al. 2009), which had not yet been observed in animal cells (Visa et al. 1993; Huang et al. 1994). In this study, we aimed to clarify the type of poly(A) RNA that is present in CBs to determine whether it is protein-coding. The large microsporocyte nuclei size facilitated our study of the mRNA in the larch by in situ hybridization.

Materials and methods

Plant material and isolation of meiotic protoplasts

To maintain constant experimental conditions, *Larix decidua* Mill. anthers were collected from the same tree in successive meiotic prophase stages of middle diplotene for 4 weeks. The whole diplotene stage in larch microsporocytes lasts 15 weeks. Anthers were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, for 6 h and squashed to obtain free meiocytes. Meiotic protoplasts were isolated from these cells (Kołowerzo et al. 2009) and were then used for immunodetection of Sm and m3G snRNA and detection of poly(A) RNA, U2 snRNA, 5S rRNA, 26S rRNA, and mRNAs by fluorescence in situ hybridization (FISH).

Immunodetection of bromouridine incorporation before FISH detection of poly(A) RNA

Bromouridine (BrU) incorporation was performed according to Smoliński et al. (2007) with a 90-min incubation time. The long incubation allowed BrU to penetrate through all the layers of the anther and enter the microsporocytes. It facilitated the comparison of transcripts at all analyzed stages, which differed in RNA synthesis intensity. Under these conditions, part of the product moved from the synthesis site to the cytoplasm at some stages. After incubating the anthers with BrU, the material was prepared as described above and incubated with a mouse anti-BrU primary antibody (F. Hoffmann-LaRoche Ltd., Rotkreuz, Switzerland) in 1% bovine serum albumin (BSA) in PBS (diluted 1:100), pH 7.2, overnight at 4°C. The protoplasts were incubated with a goat anti-mouse secondary antibody conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) in 0.2% BSA in PBS (diluted 1:1,000) for 1 h at 37°C, washed in PBS, and assessed for poly(A) RNA presence using FISH. Finally, the slides were stained using DAPI (1 μg/ml for 3 min), washed in double-distilled water, and mounted in Citifluor glycerol solution (Agar Scientific, Essex, UK). Some additional experiments were performed using longer (2.5–6 h) incubation times, while other conditions were kept the same.

Preparation of probes

Sequences of mRNA for 40 protein-coding larch genes were chosen for this study (Table 1), which were composed of 25 housekeeping genes and 15 genes coding for proteins that are cyclically very highly expressed during the meiotic prophase, including six mRNAs coding for RNA polymerase II subunits and transcription factors and nine mRNAs of snRNP proteins involved with snRNA. The study was

Table 1 Localization of the following larch microsporocyte mRNAs to Cajal bodies was analyzed using FISH

mRNA type	Presence in CB
Housekeeping genes	
mRNA pectin methylesterase (PME1)	Present in CB
mRNA for peroxidase (px1 gene)	
mRNA for alpha-tubulin	
mRNA for catalase	
mRNA for ATPase	
mRNA for inositol-3-phosphate synthase	
mRNA for NADH-glutamate synthase	Doubtful or unconfirmed localization
mRNA for 60S ribosomal protein L3	
mRNA for cinnamyl alcohol dehydrogenase (CAD)	
mRNA for phytochrome	
mRNA for coumarate CoA ligase	
mRNA for cytochrome P450 CYPA1	
mRNA for (1-3)-beta-glucanase	
mRNA for transcription factor MYB5	
mRNA for transcription factor MYB10	
mRNA for homeodomain transcription factor	
mRNA for AP2-related transcription factor AP2L3	
mRNA for histone deacetylase HDA101 (HDac)	
mRNA for ATP binding/nucleotide kinase/phosphotransferase protein (ADK)	
mRNA for cellulose synthase-like H2 (ClS_H2)	
mRNA for phosphoribulokinase	
mRNA for translation initiation factor (eIF5A)	
mRNA for aquaporin (Aqp)	
mRNA for DNA ligase	
mRNA for argonaute/Zwille-like protein	
snRNP mRNA	
mRNA for SmD1	Present in CB
mRNA for SmD2	
mRNA for SmE	
mRNA for U1a protein	Doubtful or unconfirmed localization
mRNA for U1c protein	
mRNA for Dim 1 protein	
mRNA for Dim 2 protein	
mRNA for like Sm 8 protein	
RNA pol II genes and TF	
mRNA for: RPB2 of Pol II RNA	Present in CB
mRNA for: RPB10 of Pol II RNA	
mRNA for transcription factor MYB5	Doubtful or unconfirmed localization
mRNA for transcription factor MYB10	
mRNA for homeodomain transcription factor	
mRNA for AP2-related transcription factor AP2L3	

performed using 63 antisense DNA oligonucleotide probes, 15 of which positively localized to CBs and were used in further investigations (Table 2).

For mono-labeling and double labeling of single mRNAs, probes were labeled at the 5′ end with digoxigenin (Genomed) and at the 3′ end with digoxigenin-11-dUTP (Roche) and ChromaTide Alexa Fluor 488-5-dUTP nucleotides (Invitrogen) using a tailing reaction by terminal deoxynucleotidyl transferase TdT (Roche). For double labeling with single mRNAs, a U2 snRNA DNA oligo probe was used—5′ ATATTAAACTGATAAGAACAGA TACTACACTTG 3′ (Genomed)—which was labeled at

Table 2 Probe sequences for mRNAs that positively localized to Cajal bodies

mRNA type	Probe sequence
Housekeeping genes	
mRNA for pectin methylesterase	5′ ATAAACTCCTGCTTTTATATRAATTACGTATCT 3′
mRNA for peroxidase (px1 gene)	5′ GTAGTTTAAACTTGGTAGTAAGAGTCTGGAG 3′
mRNA for α-tubulin (exon 1)	5′ GATCTAGACAGAGGTCCACAATCTC 3′
mRNA for α-tubulin (exon 3)	5′ GACTTTTTACCGTAGTCCACTGAAA 3′
mRNA for α-tubulin (3′ UTR fragment)	5′ GAAGTCCAAAACAACACAATCTAAGA 3′
mRNA for catalase	5′ GTAACRTCTAAAGGATCAAAGTCATATTTATCCT 3′
mRNA for ATPase	5′ GATTCCAGAGTACAGACACATAAATAAAAAC 3′
mRNA for inositol-3-phosphate synthase	5′ CTTTTTAAGTTGAATCCTGGTACAGAG 3′
snRNP mRNA	
mRNA for SmD1	5′ AGGAGTTGACATAAGTATGAATACACATCT
mRNA for SmD2	5′ CTATTCACTTTGGATTTCTAAGAACAATAAT 3′
mRNA for SmD2	5′ GTATATAATTTCCTCTTCAAAAACTACTTTTCTCT 3′
mRNA for SmE	5′ AATACAAGGTTCATGTACTCATCAAATC 3′
RNA pol II genes	
mRNA for RPB2 of Pol II RNA (exon 2)	5′ GACCTAGAAATATCATAGCAGATAATTTTCTAC 3′
mRNA for RPB2 of Pol II RNA (exon 3)	5′ CTGTGGATAATAGAGTACATATGCTAAGGTAT 3′
mRNA for RPB10 of Pol II RNA	5′ GTAATTCAAAAGCTTCTCRATGAGATC 3′

the 3′ end with ChromaTide Alexa Fluor 594-5-dUTP (Invitrogen) by the TdT reaction (Roche).

mRNA probes for multiplex FISH were prepared with one residue of Cy3 at the 5′ end (Genomed, Warsaw, Poland). For double labeling with mix mRNA, a U2 snRNA DNA oligo probe was labeled at the 5′ end with Alexa 488 (Sigma-Proligo, Poznań, Poland). The mRNA probe for RPB2 of Pol II RNA was prepared with one residue of Cy3 at the 5′ end (Genomed) for double labeling with U2 snRNA (probe was labeled at the 5′ end with Alexa 488).

For poly(A) RNA (Sigma-Proligo), 26S rRNA, and 5S rRNA (IBB PAN, Warsaw, Poland), the following DNA oligonucleotides were used:

Poly(A) RNA 5′ Alexa 546 TTTTTTTTTTTTTTTTTT TTTTTTTTTTTT 3′
26S rRNA 5′ Cy3 CTACTTTTAACGTTATTTTACTT ATTCCGTG 3′
5S rRNA 5′ Cy3 GGTGCATTAACGCTGGTATGATC 3′

In high-resolution in situ hybridization (HISH), the mRNA probes were labeled chemically at the 5′ end with digoxigenin (Genomed) and at the 3′ end with digoxigenin nucleotides using TdT (Roche).

FISH in mono-labeling, double-labeling, and multiplex reactions

For hybridization, the probes were resuspended in hybridization buffer (30%, *v*/*v*, formamide, 4× SSC, 5× Denhardt's buffer, 1 mM EDTA, and 50 mM phosphate buffer) at a concentration of 50 pmol/ml. In situ hybridization of meiotic protoplasts was conducted as described previously (Smoliński et al. 2007). Hybridization was performed overnight at 28°C with gentle agitation.

For multiplex hybridizations, all 15 probes (Table 1) that localized to CBs were mixed with U2 snRNA and placed into a hybridization buffer at a concentration of 50 pmol/ml for each probe. Digoxygenine (DIG) probes were detected after hybridization using mouse anti-DIG (Roche) and anti-mouse Alexa 488 (Invitrogen) antibodies in 0.01% acetylated BSA in PBS (1:100 and 1:1,000, respectively) in a humidified chamber for 1 h at 37°C. In double/multi-labeling FISH (mRNA–U2 snRNA), the probes were added simultaneously to the hybridization medium.

HISH immunogold labeling of mRNA, U2 snRNA, and immunogold labeling of Sm proteins and m3G snRNA

After fixation in 4% PA in PBS overnight, the anthers were rinsed in PBS, dehydrated, and mounted in LR Gold resin (Sigma) according to Majewska-Sawka and Rodriguez-Garcia's (1996) protocol. Ultrathin sections were collected on formvar-coated nickel or gold grids, pretreated with pre-hybridization buffer (hybridization buffer without probe) for 1 h at room temperature, and incubated with mRNA probes in the hybridization buffer. Hybridization was performed in a sealed, humidified chamber for 20 h at room temperature at a concentration of 50 pmol/ml for each probe. Post-hybridization washing was performed according to

Smoliński et al. (2007). Nonspecific antigens were blocked with PBS buffer containing 0.05% acetylated BSA for 0.5 h. DNA–RNA hybrids were localized by incubation with a sheep anti-digoxigenin antibody coupled to 10-nm diameter colloidal gold particles (Orion Diagnostica, Espoo, Finland; 1:30 in PBS containing 0.02% acetylated BSA for 1 h at room temperature). Grids were rinsed and contrasted according to Smoliński et al. (2007). Immunogold labeling of Sm proteins and m3G snRNA was performed according to Wróbel and Smoliński (2003).

Immunodetection of m3G snRNA and Sm proteins

For detecting the m3G cap, we used an anti-m3G antibody (Calbiochem, Bad Soden, Germany) according to the protocols provided by Zienkiewicz et al. (2006). Sm proteins were also detected by incubating with a primary anti-Sm Y12 mouse antibody, which recognizes Sm B′/B and D1 proteins in plants (Hirakata et al. 1993; Echeverría et al. 2007), according to the method in Zienkiewicz et al. (2008b), in 0.1% acetylated BSA in PBS (1:10) in a humidified chamber at 8°C overnight and an anti-mouse Alexa 488 (Invitrogen) secondary antibody. For all experiments (Sm–mRNA, m3G snRNA–mRNA), immunocytochemistry always preceded in situ hybridization because immunofluorescent signals were extremely weak if ISH was applied first.

Optical and electron microscopy

The results were analyzed with a Nikon C1 confocal microscope with an argon ion laser emitting a wavelength of 488 nm (blue excitation) and a He–Ne laser emitting wavelengths of 543 nm (green excitation) and 594 nm. A mid pinhole, long exposure time (75 μs), and a ×60 (numerical aperture, 1.4) Plan Apochromat DIC H oil and CFI Plan Apochromat ×60 oil immersion lens were used. Images were collected simultaneously in the green (Alexa 488 fluorescence) and yellow–red (Cy3, Alexa 546 or Alexa 594) channels. To minimize bleed-through between the fluorescence channels, low argon laser power (3–10% of maximum power) and control single-channel collections were applied. For bleed-through analysis and control experiments, Lucia G software was used (Laboratory Imaging, Prague, Czech Republic). For image processing and analysis, the EZ Viewer software package (Nikon Europe BV, Badhoevedorp, the Netherlands) was used. For DAPI staining, a fluorescence inverted Nikon Eclipse TE 2000-E microscope equipped with a mercury lamp, UV-2EC UV narrow band filter, Nikon DXM 1200 FX and Nikon DS-5Mc digital cameras were used. Nikon NIS-elements deconvolution software was used. The material was examined and photomicrographs were taken under a TEM Jeol 1010 (Japan) electron microscope at 80 kV.

Control reactions

For the immunofluorescence and immunogold methods, control incubations lacking the first antibody were performed. For the in situ hybridizations, high-resolution and fluorescent, sense-labeled probes and ribonuclease-treated samples were used. For the detection of new transcripts, control reactions were performed on sections incubated without bromouridine. All control reactions produced negative results, or the result of the control reaction was undetectably low compared with the standard reactions.

Results

Out of the 40 tested mRNAs, 11 positively hybridized to spherical nuclear. For the other mRNAs, their localization was not observed in nuclear bodies or was disputable (Table 1). Probes for the mRNAs that localized to nuclear bodies were used in further analysis (Table 2). A high accumulation of mRNA was observed in the spherical nuclear bodies, which ranged in diameter from 0.5 to 6 μm (Fig. 1). Several nuclear bodies were frequently observed in one microsporocyte (Fig. 1). These probes demonstrated the nuclear body localization for mRNA of six housekeeping genes, including pectin methylesterase, peroxidase, α-tubulin, catalase, ATPase, and inositol-3-phosphate synthase (Fig. 1a–f); three Sm protein-coding genes (Fig. 1g–i); and two genes coding for the second and tenth subunits of RNA polymerase II (Fig. 1j, k). The oligo probes were designed to prevent their reciprocal hybridization, which would weaken the level of hybridization to the target sequences in multiplex experiments. Multiplex reactions significantly increased the signal detection (Fig. 1l) and were used in double-labeling reactions with splicing factors (Electronic supplementary material (ESM) Fig. S1), which are considered to be markers of CBs (ESM Fig. S1a, b, c).

Simultaneous localization of core spliceosomal Sm proteins (ESM Fig. S1d) and mRNA (ESM Fig. S1e) revealed that the CBs, which contain large amounts of Sm proteins (ESM Fig. S1a), are the same structures that house mRNA. Additionally, double labeling of U2 snRNA (ESM Fig. S1g) and a mix of mRNA (ESM Fig. S1h) revealed that CBs co-localized with the mRNA-containing bodies. Similarly, the mature forms of small nuclear RNA, detected by anti-snRNA m3G cap antibodies (ESM Fig. S1j) with a mix of mRNA (ESM Fig. S1k), co-localized at CBs. In

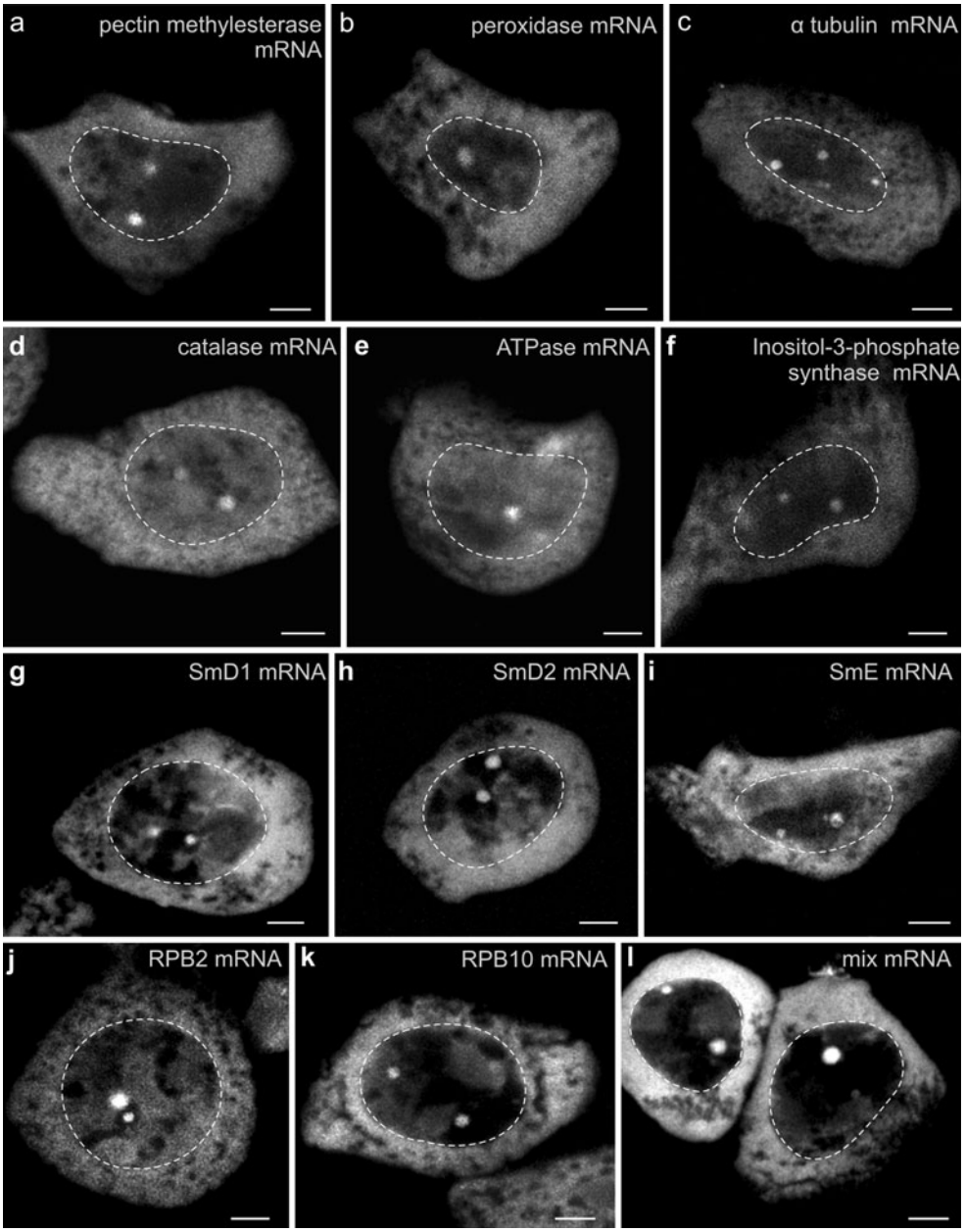

Fig. 1 **a–k** FISH reaction using probes complementary to 11 larch mRNAs reveal high concentrations of the studied mRNAs in the oval, regular nuclear bodies. In the nucleus outside the nuclear bodies and in the cytoplasm, the mRNA is dispersed. **l** Multiplex FISH reaction using 15 probes complementary to 11 larch mRNAs (see MM, Table 2). *Bars*, 10 μm

addition to localizing to the CBs, m3G snRNA was dispersed in the nucleoplasm (ESM Fig. S1j).

The double-labeling analysis demonstrated that the nuclear bodies, which contain mRNA and splicing molecules, were occasionally heterogeneous. Sometimes different levels of individual mRNAs in CBs were observed (ESM Fig. S2a–c). Sometimes CBs lacked individual mRNAs (an example of labeling the mRNA of the second subunit of RNA polymerase II at ultrastructural level; ESM Fig. S2e).

The strongest hybridization signals were obtained in double FISH with a mix of mRNA and U2 snRNA, so an attempt was made to use double FISH for U2 snRNA as a marker of CB and mRNA of distinct genes. U2 snRNA co-localized with CBs for all 11 studied mRNAs. Pectin methylesterase mRNA co-localization with U2 snRNA (Fig. 2a–d) served as a positive control for the set of housekeeping genes. However, CBs in the cell occasionally lacked these mRNAs (data not shown). Figure 2e–h illustrates an example of the heterogeneous localization of an mRNA of the second subunit of RNA polymerase II in different CBs after double labeling with U2 snRNA. In addition to the concentrated localization to CBs, a high level of signal proximal to these structures was observed. In such cases, the localization of the studied mRNA to CBs was not clearly visible until the signals from the U2 snRNA and mRNA were merged. The mRNA of the Sm protein group was present in the majority of

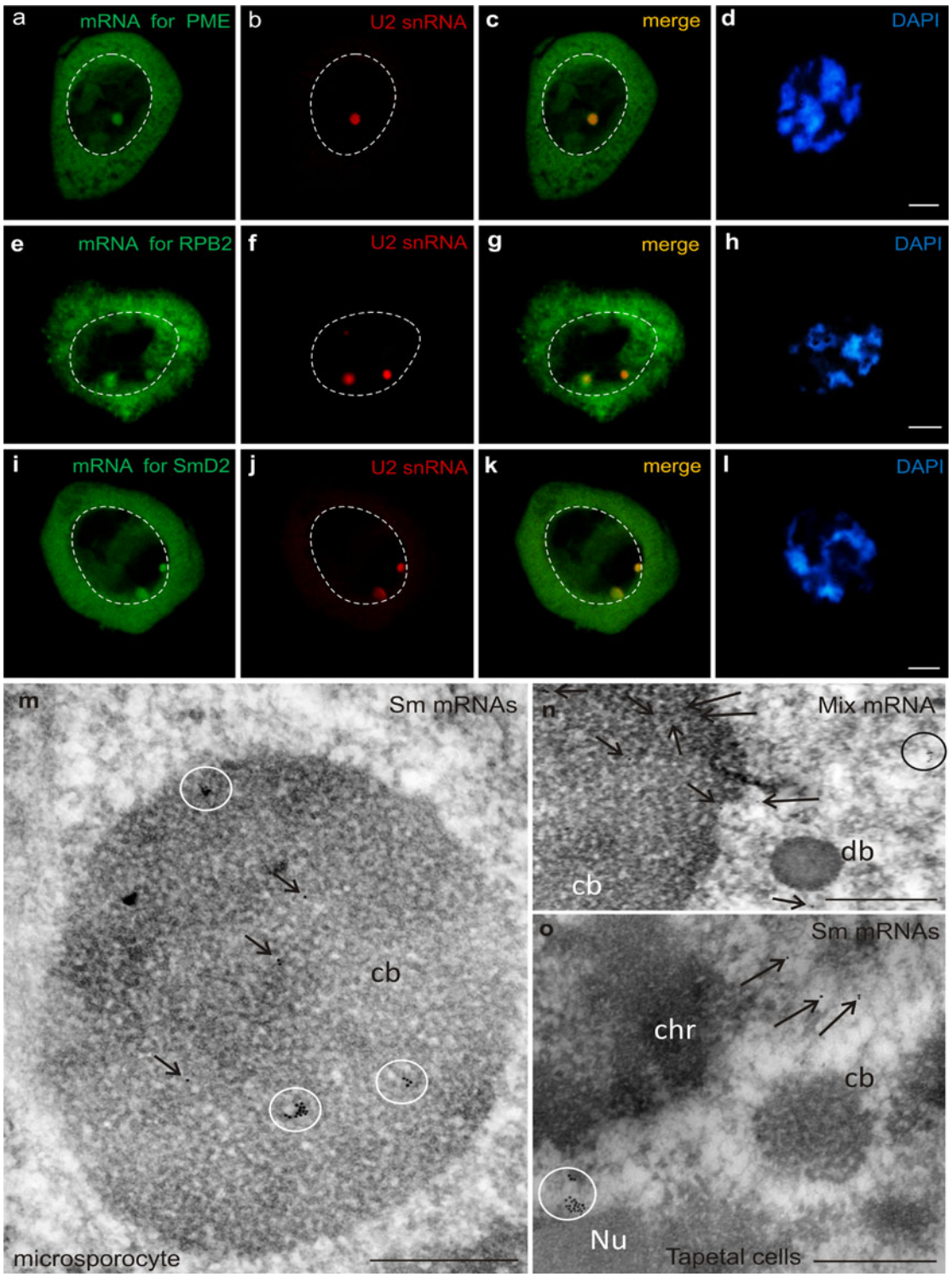

Fig. 2 a–l Double labeling of mRNA of distinct genes: pectin methylesterase (*PME*) mRNA (**a**), mRNA of the second subunit of RNA polymerase II (*RPB2*) (**e**), mRNA of the core spliceosomal Sm protein D2 (**i**), and U2 snRNA (**b**, **f**, **j**, respectively). Merge—mRNAs present in CBs (**c**, **g**, **k**). Nuclei were stained with DAPI. All corresponding DAPI images were collected using widefield fluorescence and deconvolution software. *Bars*, 10 μm. **m–o** Ultrastructural localization of mRNA. For better visualization of mRNA localization at the ultrastructural level, results of in situ hybridization, in which several probes for mRNA to three genes coding Sm protein (**m**, **o**) or a multiplex reaction (**n**) were shown. **m** A high level of labeling in the microporocytes was observed primarily in a Cajal body (*cb*) (*arrows*, *outline*). **n** A lack of mRNA in dense nuclear bodies (*db*). The gold particles are present in the nucleoplasm and in CBs in single form (*arrows*). **o** Studied mRNA was not present in CBs in the somatic cells of tapetum, which surround the microsporocytes; the signal was only observed in the nucleoplasm (*arrows*, *outline*), outside dense chromatin (*chr*), and the nucleolus (*Nu*). *Bars*, 0.5 μm

CBs containing U2 snRNA (Fig. 2i–l); however, their levels were also heterogeneous (data not shown).

In situ hybridization and electron microscopy analysis confirmed that larch microsporocyte nuclei exhibited an accumulation of the studied mRNAs in CBs. In situ hybridization results, with two to three probes for mRNA of the same protein group (Sm mRNA as an example), are shown in Fig. 2m.

mRNA localized exclusively to the CBs; moreover, their localization was not confirmed in other types of nuclear bodies, such as dense bodies, which were also observed in the nuclei of larch microsporocytes (Fig. 2n). In somatic tapetal cells, which surround microsporocytes, numerous fine CBs (with a diameter of 0.1–0.8 μm) were present. Detectable amounts of mRNA were not observed in these structures (Fig. 2o). Labeling was observed only in the nucleoplasm outside the CBs.

Double labeling of newly formed transcripts and poly(A) RNA, which can be considered as an indicator of mature mRNA transcript levels, enabled us to analyze the dynamics of occurrence of CBs that contained mRNA in comparison to the microsporocyte transcriptional activity. Characteristic changes in newly formed transcript levels and in the distribution of poly(A) RNA in microsporocytes were observed during the 4 weeks of the middle diplotene stage. Seven stages were distinguished in the studied period (Fig. 3a–n).

At the beginning of the middle diplotene (stage I), the newly formed transcript levels were low, and a low poly(A) RNA signal was observed in the nucleus. The cytoplasm exhibited a much higher and more dispersed poly(A) signal (Fig. 3a, b). At the second stage, a high level of newly synthesized RNA was observed, and elevated levels of poly (A) RNA were visible in the nucleus as small clusters (Fig. 3c, d). The third stage exhibited still a high level of newly formed transcripts in the nucleus, and poly(A) RNA was visible in single CBs and was dispersed in the nucleoplasm (Fig. 3e, f). During this stage, we rarely observed transcripts at the periphery of CBs during prolonged incubations (2.5–6 h; ESM Fig. S3a–d). In stages II and III, FISH with the oligo T probe gave a strong signal (ESM Fig. S3e, h). ESM Fig. S3f, i exhibits Fig. 3c, e "uncovered" newly formed transcripts at stages II and III. At stage IV, a decrease in the level of newly formed transcripts was observed, and poly(A) RNA accumulated primarily in numerous CBs (Fig. 3g, h). In the next stage, the levels of newly formed transcripts remained low, whereas poly(A) RNA levels increased in both the nucleoplasm and the cytoplasm while decreasing in the CBs in the nucleus (Fig. 3i, j). At stage VI, the low level of newly formed transcripts persisted in the nucleus. No CBs that contain poly (A) RNA were observed. Poly(A) RNA was primarily dispersed in the nucleus and was observed at small concentrations in the cytoplasm (Fig. 3k, l). At the last stage, newly formed transcripts became extremely elevated, particularly in the nucleolus, and the poly(A) RNA localized predominantly to the cytoplasm as large spots (Fig. 3m, n).

During this diplotene stage (stage VII), we have previously observed a rapid increase in the nucleolar volume and a restart of the transcriptional activity of the nucleolus (Smoliński et al. 2007). The microsporocytes in this period possessed a high content of 26S rRNA (Fig. 3o) and 5S rRNA (Fig. 3p) in the nucleolus and cytoplasm.

Discussion

Previous reports regarding the spatial organization of successive pre-mRNA maturation stages indicate that the main sites of poly(A) RNA and mRNA concentration in the nucleus are dynamic, irregular structures termed speckles (Carter et al. 1991, 1993; Wei et al. 1999; Molenaar et al. 2004; Xie and Pombo 2006; Ishihama et al. 2008; Bogolyubova et al. 2009; Batalova et al. 2010). We have previously demonstrated that in larch, poly(A) RNA localizes in speckles, but this occurred independently from the RNA accumulation in nuclear bodies (Kołowerzo et al. 2009).

Detailed analysis showed that the oval, regular concentrations of poly(A) RNA localized to CBs. In this study, we aimed to categorize the types of poly(A) RNA that localize to the CBs in larch. We confirmed that the poly(A) RNA detected in CBs is mRNA. Part of the poly(A) RNA that is present in the CBs in larch microsporocytes may be mRNA-like non-coding RNA that are, like mRNAs, spliced, capped, and polyadenylated (Erdmann et al. 1999; Hiller et al. 2009). Their function is not fully elucidated, but they likely regulate genes and play important roles in organogenesis and cell differentiation (Inagaki et al. 2005).

Based on mRNA sequence libraries, we aimed to localize, in situ, several dozen mRNAs and verify their localization to larch CBs. Detection of mRNA using in situ methods has been technologically difficult due to detection sensitivity. Specifically, lowly expressed gene transcript levels have been difficult to properly assess. Therefore, we selected genes coding for primary metabolism proteins, which are ubiquitously and constitutively expressed; genes coding for subunits of RNA polymerase II and transcription factors, which are highly and cyclically expressed during diplotene in larch (data not published; Kołowerzo 2010); and genes coding for various splicing factors, which are also highly and cyclically expressed during the meiotic prophase in larch (Smoliński et al. 2011). Analysis using the double-labeling method, with snRNA and snRNP spliceosomal factors being the markers for CBs in plants

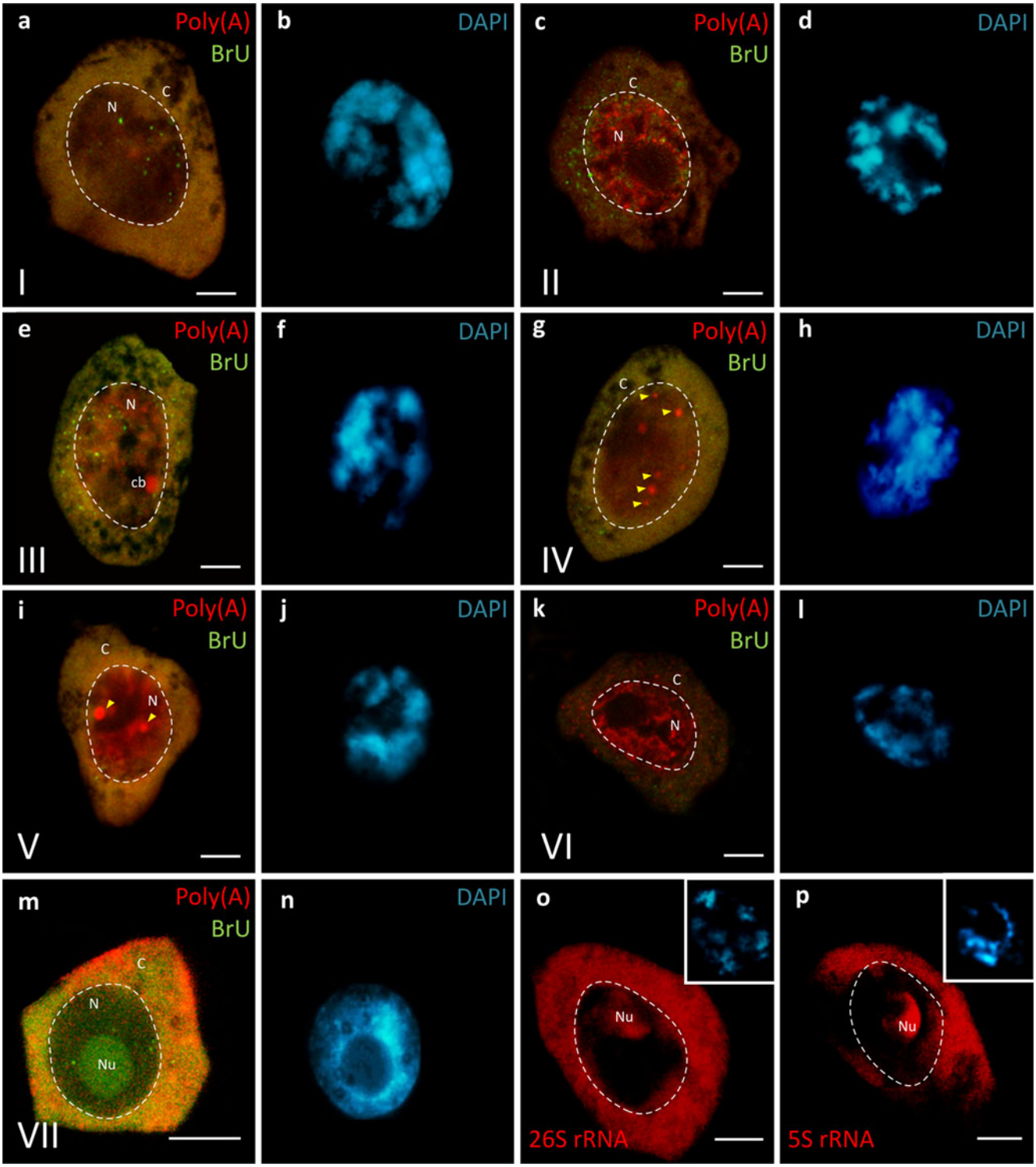

Fig. 3 **a–n** Double labeling of a newly formed transcript (*green*, 90-min BrU incubation time) and poly(A) RNA (*red*) in microsporocytes during the middle diplotene (stages I–VII). Nuclei were stained with DAPI (*blue*). The DAPI images were collected using widefield fluorescence and deconvolution software. Cajal bodies (*cb*, *arrowheads*), nucleus (*N*), cytoplasm (*C*). Detection of ribosomal RNA 26S (**o**) and 5S (**p**) conducted in the last period highly accumulated these RNAs both in the nucleolus (*Nu*) and the cytoplasm. *Bars*, 10 μm

(Beven et al. 1995; Straatman and Schel 2001; Lorković et al. 2004), confirmed that mRNA accumulated in CBs. Eleven out of the 40 mRNAs studied were present in CBs.

In addition to CBs, other types of plant nuclear bodies have been extensively analyzed, including karyosomes and argyrophilic intranuclear bodies or dense

bodies (Barlow 1981, 1983). They are easily distinguished from CBs by their dense structure and smaller diameter. The ribonucleoprotein nature of those bodies has been strongly suggested. An EDTA-regressive staining technique revealed the presence of a ribonucleoprotein material (Docquier et al. 2004), but a lack of splicing factors (Niedojadło and Górska-Brylass 2003) and fibrillarin (Jennane et al. 1999), in dense bodies (DBs). Their dense, compact structure place them in a class of nuclear bodies distinct from CBs. Due to the lack of good DB markers, verification relies on the presence of accumulated mRNA in the DB, on an ultrastructural level. The localization of mRNA observed by electron microscopy confirmed the presence of the studied mRNA in CBs and its absence in other types of nuclear bodies.

The confirmation of the presence of the mRNA transcripts is the first report of its kind, both in plants and in animals. Studies conducted so far have not demonstrated the presence of mRNA or poly(A) RNA in CBs (Visa et al. 1993; Huang et al. 1994). Those studies were performed only in animals, primarily in somatic cells; moreover, such studies in plants have been absent. Our analysis of mRNA localization to CBs in the somatic cells of larch anthers demonstrates a lack of the studied mRNA in these bodies. Whether the presence of mRNA in CBs in the microsporocytes directly correlates to its elevated synthesis or whether the bodies are storage sites remains unknown.

To answer those questions, we analyzed the distribution of the pool of mRNAs with a simultaneous detection of newly formed transcripts using a double-labeling technique. The studies encompassed microsporocytes in middle diplotene, which lasts approximately 4 weeks in larch. This period included three phases of microsporocytes development: (1) a lack of CBs that contained mRNA, (2) an abundance of CBs in the nucleus, and (3) the disappearance of CBs that contained mRNA at the end of the middle diplotene. The entire larch diplotene lasts for approximately 15 weeks. The developmental synchronization of larch meiocytes allowed for the sequential tracking of the level and distribution of poly(A) RNA in comparison to changes in the level of total transcription.

The increase in transcriptional activity, which was observed in the first stage of microsporocyte development, was accompanied by an elevation in the mRNA levels in the nucleus. These molecules were primarily dispersed in the nucleus. Significant increases in mRNA were not observed in the cytoplasm. The first CBs that contained poly(A) RNA were observed during the period of decreased microsporocyte transcriptional activity. Similar to other studies (Biggiogera and Fakan 1998), newly formed transcripts were absent in CBs even during the long 90-min incubations. We observed transcripts at the periphery of several CBs when we used extremely long incubations ranging from 2.5 to 6 h. Therefore, long incubations may reveal newly formed snRNAs or U3 snoRNA at CBs. In the last stages of the studied period, in the presence of low levels of total transcription, a gradual loss of mRNA accumulation in CBs and an increase in mRNA in the cytoplasm were observed. The cyclical appearance and disappearance of CBs that contain poly(A) RNA indicates that, in larch microsporocytes, CBs serve as a storage site for mRNA transcripts, which are subsequently transported to the cytoplasm.

After the disappearance of CBs that contain mRNAs, and the increase of RNA levels in the cytoplasm, the transcriptional activity of the nucleolus, which was suppressed from pachytene, reinitiates, resulting in the expansion of the nucleolus size (Smoliński et al. 2007) and in the increase of ribosomal RNA in the cytoplasm. The larch microsporocytes exhibit high protein biosynthesis activity during this period (Chwirot and Górska-Brylass 1981). The RNA, previously synthesized in the nucleus and stored in CBs, is likely gradually released and translated after its transport to the cytoplasm.

Whether mRNA accumulation in nuclear CBs results from the atypical developmental strategy of the larch or whether it is a common process is unknown. The localization of poly(A) RNA in the nucleus of the lily (Chandra Sekhar and Williams 1992) may indicate an answer. They observed the accumulation of poly(A) RNA in the nucleus during meiotic prophase in the microsporocytes of lily (Chandra Sekhar and Williams 1992). In this work, in situ hybridization techniques with immunogold–silver detection were used, which, because of low resolution, do not allow the determination of whether this RNA type is present in the structures of nuclear bodies. High levels of poly(A) RNA were found in the pollen grain of the hyacinth (Zienkiewicz et al. 2006); however, it accumulated in the cytoplasm, not in the nucleus (Zienkiewicz et al. 2008a). In generative plant cells, mRNA storage may occur both in the nucleus and in the cytoplasm. Recognition of this new role of CBs in mRNA metabolism during larch microsporocyte development requires further, detailed study.

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References

Acevedo R, Samaniego R, Moreno Díaz de la Espina S (2002) Coiled bodies in nuclei from plant cells evolving from dormancy to proliferation. Chromosoma 110:559–569

Barlow PW (1981) Argyrophilic intranuclear bodies of plant cells. Experientia 37:1017–1018

Barlow PW (1983) Nucleolus-associated bodies (karyosomes) in dividing and differentiating plant cells. Protoplasma 115:1–10

Batalova FM, Bogolyubov DS, Parfenov VN (2010) Interchromatin granule clusters of the scorpionfly oocytescontain poly(A)$^+$ RNA, heterogeneous ribonucleoproteins A/B and mRNA export factor NXF1. Cell Biol Int 34:1163–1170

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

Biggiogera M, Fakan S (1998) Fine structural specific visualization of RNA on ultrathin sections. J Histochem Cytochem 46:389–395

Bogolyubova I, Bogolyubov D, Parfenov V (2009) Localization of poly(A)$^+$ RNA and mRNA export factors in interchromatin granule clusters of two-cell mouse embryos. Cell Tissue Res 338:271–281

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 18:3687–3694

Boudonck K, Dolan L, Shaw PJ (1999) The movement of coiled bodies visualized in living plant cells by the green fluorescent protein. Mol Biol Cell 10:2297–2307

Cajal SRY (1903) Un sencillo metodo de coloracion seletiva del reticulo protoplasmatico y sus efectos en los diversos organos nerviosos de vertebrados e invertebrados. Trab Lab Invest Biol (Madrid) 2:129–221

Carter K, Taneja K, Lawrence JB (1991) Discrete nuclear domains of poly(A) RNA and their relationship to the functional organization of the nucleus. J Cell Biol 115:1191–1202

Carter K, Bowman D, Carrington W, Fogarty K, McNeil JA, Fay FS, Lawrence JB (1993) A three-dimensional view of precursor messenger RNA metabolism within the mammalian nucleus. Science 259:1330–1335

Chamberland H, Lafontaine JG (1993) Localization of snRNP antigens in nucleous-associated bodies: study of plant interphase nuclei by confocal and electron microscopy. Chromosoma 102:220–226

Chandra Sekhar KN, Williams EG (1992) Nonradioactive in situ localization of poly(A)$^+$ RNA during pollen development in anthers of tobacco (*Nicotiana tabacum* L.). Protoplasma 169:9–17

Chwirot WB, Górska-Brylass A (1981) Variations of total protein content and protein synthesisduring microsporogenesis in *Larix europeaea* DC. Acta Coc Bot Pol 50:33–38

Cioce M, Lamond AI (2005) Cajal bodies: a long history of discovery. Annu Rev Cell Dev Biol 21:105–131

Collier S, Pendle A, Boudonck K, van Rij T, Dolan L, Shaw P (2006) A distant coilin homologue is required for the formation of Cajal bodies in *Arabidopsis*. Mol Biol Cell 17:2942–2951

Docquier S, Tillemans V, Deltour R, Motte P (2004) Nuclear bodies and compartmentalization of pre-mRNA splicing factors in higher plants. Chromosoma 112:255–266

Echeverría O, Vázquez-Nin G, Juárez-Chavero S, Moreno Díaz de la Espina S (2007) Firing of transcription and compartmentalization of splicing factors in tomato radicle nuclei during germination. Biol Cell 99:519–530

Erdmann VA, Szymanski M, Hochberg A, de Groot N, Barciszewski J (1999) Collection of mRNA-like non-coding RNAs. Nucleic Acids Res 27:192–195

Gall JG, Bellini M, Wu Z, Murphy CH (1999) Assembly of the nuclear transcription and processing machinery: Cajal bodies (coiled bodies) and transcriptosomes. Mol Biol Cell 10:4385–4402

Gama-Carvalho M, Krauss RD, Chiang L, Valcárcel J, Green MR, Carmo-Fonseca M (1997) Targeting of U2AF65 to sites of active splicing in the nucleus. J Cell Biol 137:975–987

Gulemetova R, Chamberland H, Gugg S, Plante M, Lafontaine JG (1998) Presence of small-nuclear-ribonucleoprotein-containing nuclear bodies in quiescent and early germinating *Zea mays* embryos. Protoplasma 202:3–4

Hiller M, Findeiss S, Lein S, Marz M, Nickel C, Rose D, Schulz C, Backofen R, Prohaska SJ, Reuter G, Stadler PF (2009) Conserved introns reveal novel transcripts in *Drosophila melanogaster*. Genome Res 19:1289–1300

Hirakata M, Craft J, Hardin JA (1993) Autoantigenic epitopes of the B and D polypeptides of the U1 snRNP. Analysis of domains recognized by the Y12 monoclonal anti-Sm antibody and by patient sera. J Immunol 150:3592–3601

Huang S, Deerinck TJ, Ellisman MH, Spector DL (1994) In vivo analysis of the stability and transport of nuclear poly(A)$^+$ RNA. J Cell Biol 4:877–899

Inagaki S, Numata K, Kondo T, Tomista M, Yasuda K, Kanai A, Kageyama Y (2005) Identification and expression analysis of putative mRNA-like non-coding RNA in *Drosophila*. Genes Cells 10:1163–1173

Ishihama Y, Tadakuma H, Tani T, Funatsu T (2008) The dynamics of pre-mRNAs and poly(A)$^+$ RNA at speckles in living cells revealed by iFRAP studies. Exp Cell Res 314:748–762

Jarrous N, Reiner R, Wesolowski D, Mann H, Guerrier-Takada C, Altman S (2001) Function and subnuclear distribution of Rpp 21, a protein subunit of the human ribonucleoprotein ribonuclease P. RNA 7:1153–1164

Jennane A, Thiry M, Goessens G (1999) Identification of coiled bodylike structures in meristematic cells of *Pisum sativum* cotyledonary buds. Chromosoma 108:132–142

Kołowerzo A (2010) Distributions of splicing factors and nuclear transcription activity of diplotene microsporocytes of *Larix deciduas* Mill. Dissertation. Nicolas Copernicus University, Toruń

Kołowerzo A, Smoliński DJ, Bednarska E (2009) Poly(A) RNA a new component of Cajal bodies. Protoplasma 236:13–19

Koroleva OA, Calder G, Pendle AF, Kim SH, Lewandowska D, Simpson CG, Jones IM, Brown JW, Shaw PJ (2009) Dynamic behavior of *Arabidopsis* eIF4A-III, putative core protein of exon junction complex: fast relocation to nucleolus and splicing speckles under hypoxia. Plant Cell 21:1592–1606

Lafontaine JG, Chamberland H (1995) Relationship of nucleoleus-associated bobies with nucleolar organizer tracks in plant interphase nuclei (*Pisum sativum*). Chromosoma 103:545–553

Lorković ZJ, Hilscher J, Barta A (2004) Use of fluorescent protein tags to study nuclear organization of the spliceosomal machinery in transiently transformed living plant cells. Mol Biol Cell 15:3233–3243

Majewska-Sawka A, Rodriguez-Garcia MI (1996) rRNA distribution during microspore development in anthers of *Beta vulgaris* L.: quantitative in situ hybridization analysis. J Cell Sci 109:859–866

Molenaar C, Abdulle A, Gena A, Tanke HJ, Dirks RW (2004) Poly(A) RNAs roam the cell nucleus and pass through speckle domains in transcriptionally active and inactive cells. J Cell Biol 165:191–202

Monneron A, Bernhard W (1969) Fine structural organization of the interphase cell nucleus of some mammalian cells. J Ultrastruct Res 27:266–288

Moreno Díaz de la Espina S, Sanchez Pina A, Risueño MC, Medina FJ, Fernandez-Gomez ME (1980) The role of plant coiled bodies in the nuclear RNA metabolism. Electron Microsc 2:240–241

Moreno Díaz de la Espina S, Risueño MC, Medina FJ (1982) Ultrastructural cytochemical and autoradiographic characterization of coiled bodies and in the plant cell nucleus. Biol Cell 44:229–238

Niedojadło J, Górska-Brylass A (2003) New type of snRNP containing nuclear bodies in plant cells. Biol Cell 95:303–310

Olmedilla A, de Dios AJ, Rodríguez-García MI (1997) Nucleolar evolution and coiled bodies during meiotic prophase in *Olea europaea*: differential localization of nucleic acids. Eur J Cell Biol 74:181–189

Pontes O, Pikaard CS (2008) siRNA and miNA processing: new functions for Cajal bodies. Curr Opin Genet 18:197–203

Risueño MC, Medina FJ (1986) The nucleolar structure in plant cells. Revis Biol Celular 7:1–154

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

Seguí-Simarro JM, Bárány I, Suárez R, Fadón B, Testillano PS, Risueño MC (2006) Nuclear bodies domain changes with microspore reprogramming to embryogenesis. Eur J Histochem 50:35–44

Shaw PJ, Brown JW (2004) Plant nuclear bodies. Curr Opin Plant Biol 7:614–620

Smoliński DJ, Niedojadło J, Noble A, Górska-Brylass A (2007) Additional nucleoli and NOR activity during meiotic prophase I in larch (*Larix decidua* Mill.). Protoplasma 232:109–120

Smoliński DJ, Wróbel B, Noble A, Zienkiewicz A, Górska-Brylass A (2011) Periodic expression of Sm proteins parallels formation of nuclear Cajal bodies and cytoplasmic snRNP-rich bodies. Histochem Cell Biol. doi:10.1007/s00418-011-0861-8

Straatman KR, Schel JH (2001) Distribution of splicing proteins and putative coiled bodies during pollen development and androgenesis in *Brassica napus* L. Protoplasma 216:191–200

Thiry M (1994) Cytochemical and immunocytochemical study of coiled bodies in different cultured cell lines. Chromosoma 103:268–276

Venteicher AS, Abreu EB, Meng Z, McCann KE, Terns RM, Veenstra TD, Terns MP, Artandi SE (2009) A human telomerase holoenzyme protein required for Cajal body localization and telomere synthesis. Science 323:644–648

Visa N, Puvion-Dutilleul F, Harper F, Bachellerie JP, Puvion E (1993) Intranuclear distribution of poly(A) RNA determined by electron microscope in situ hybridization. Exp Cell Res 1:19–34

Wagner EJ, Marzluff WF (2006) ZFP100, a component of the active U7 snRNP limiting for histone pre-mRNA processing, is required for entry into S phase. Mol Cell Biol 26:6702–6712

Wei X, Somanathan S, Samarabandu J, Berezney R (1999) Three-dimensional visualization of transcription sites and their association with splicing factor-rich nuclear speckles. J Cell Biol 146:543–558

Wróbel B, Smoliński DJ (2003) Coiled bodies in the meristematic cells of the root of *Lupinus luteus* L. Biol Plant 46:223–232

Xie SQ, Pombo A (2006) Distribution of different phosphorylated forms of RNA polymerase II in relation to Cajal and PML bodies in human cells: an ultrastructural study. Histochem Cell Biol 125:21–31

Zienkiewicz K, Bednarska E (2009) snRNP: rich nuclear bodies in *Hyacinthus orientalis* L. microspores and developing pollen cells. Int J Cell Biol. doi:10.1155/2009/209303

Zienkiewicz K, Smoliński DJ, Bednarska E (2006) Distribution of poly(A) RNA and splicing machinery elements in mature *Hyacinthus orientalis* L. pollen grains and pollen tubes growing in vitro. Protoplasma 227:95–103

Zienkiewicz K, Zienkiewicz A, Smoliński DJ, Rafińska K, Świdziński M, Bednarska E (2008a) Transcriptional state and distribution of poly(A) RNA and RNA polymerase II in differentiating *Hyacinthus orientalis* L. pollen grains. Sex Plant Reprod 21:233–245

Zienkiewicz K, Zienkiewicz A, Smoliński DJ, Świdziński M, Bednarska E (2008b) Intracellular organization of the pre-mRNA splicing machinery during *Hyacinthus orientalis* L. pollen development. Sex Plant Reprod 21:217–231