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U1 snRNP components are present in the vegetative and generative nuclei of the pollen grain

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Abstract In eucaryotes, the nuclei contain small ribonucleoprotein particles (snRNPs) which mediate splicing of RNA precursors. The male gametophytes of *Pinus radiata* and *Viburnum tinus* have more than one U1 snRNA isospecies and some snRNP polypeptides detected by northern and western blotting with probes complementary to tomato U1 snRNA and by protein immunodetection. The snRNP components were localized in both vegetative and generative nuclei of the pollen grain by in situ hybridization and immunocytochemistry. This study suggests that vegetative and generative nuclei contain the splicing apparatus for the post-transcriptional RNA processing in the pollen grain.

Key words snRNA · snRNP polypeptides · Nuclear splicing · Pollen

Introduction

The mature pollen grain contains a store of presynthesized mRNAs meeting the requirements for protein synthesis during pollen germination (for review see Mascarenhas 1993); however, new mRNA synthesis occurs during pollen germination and tube growth (Mascarenhas 1975; Mascarenhas and Mermelstein 1981). Biochemical analyses have shown that both vegetative and generative nuclei of pollen are transcriptionally active during pollen germination (Mascarenhas 1966; LaFountain and Mascarenhas 1972; Frankis 1990), but most of the new mRNA synthesis is thought to be directed by the vegetative nucleus (Mascarenhas 1990). In situ hybridization showed that Zmc 13 mRNA is localized in the cytoplasm of the vegetative cell of the maize pollen grain, and, after germination, is distributed throughout the pollen tube cytoplasm (Hanson et al. 1989; Mascarenhas 1990; Mascarenhas 1993). Similar in situ hybridizations with tomato cDNA clones as probes also showed localization of the mRNAs in the vegetative cell cytoplasm (Ursin et al. 1989). Conclusive experiments have not been done to determine whether the mRNAs are present in the generative cell or sperm cells, in addition to being present in the vegetative cell, and conflicting information is available regarding the synthesis of RNA in sperm cells. In the tricellular pollen of *Secale cereale*, [5-^{3}H]-uridine was incorporated into both the sperm and the vegetative nuclei during in vitro pollen tube growth (Haskell and Rogers 1985; Russel 1991). However, in the bicellular pollen of *Hyoscyamus niger*, exposure to short pulses of [5-^{3}H]-uridine did not reveal incorporation (Reynolds and Raghavan 1982). Recently, Zhang et al. (1993) demonstrated that the sperm cells isolated from *Zea mays* are engaged in the synthesis of RNA and protein.

Taken together, these findings suggest that the vegetative nucleus and probably the generative nucleus of mature pollen contain components of the spliceosomal small ribonucleoprotein particles, which are involved in the processing of pre-mRNA (Steitz et al. 1988). These particles are ubiquitously localized in the nuclei of all eukaryotes and contain a set of five major, uridylate-rich small nuclear RNAs (snRNAs), U1 to U6 (Reddy and Busch 1988), each associated with some six to ten polypeptides. In an effort to elucidate the presence of small ribonucleoprotein components in both nuclei in the pollen grain, we examined the localization of snRNA and snRNP polypeptides in the male gametophyte of *Pinus radiata* and *Viburnum tinus*.

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Materials and methods

Plant material

Pollen of *Pinus radiata* D. Don was kindly provided by Erika Pérez (Faculty of Forestry, Universidad Austral de Chile). The

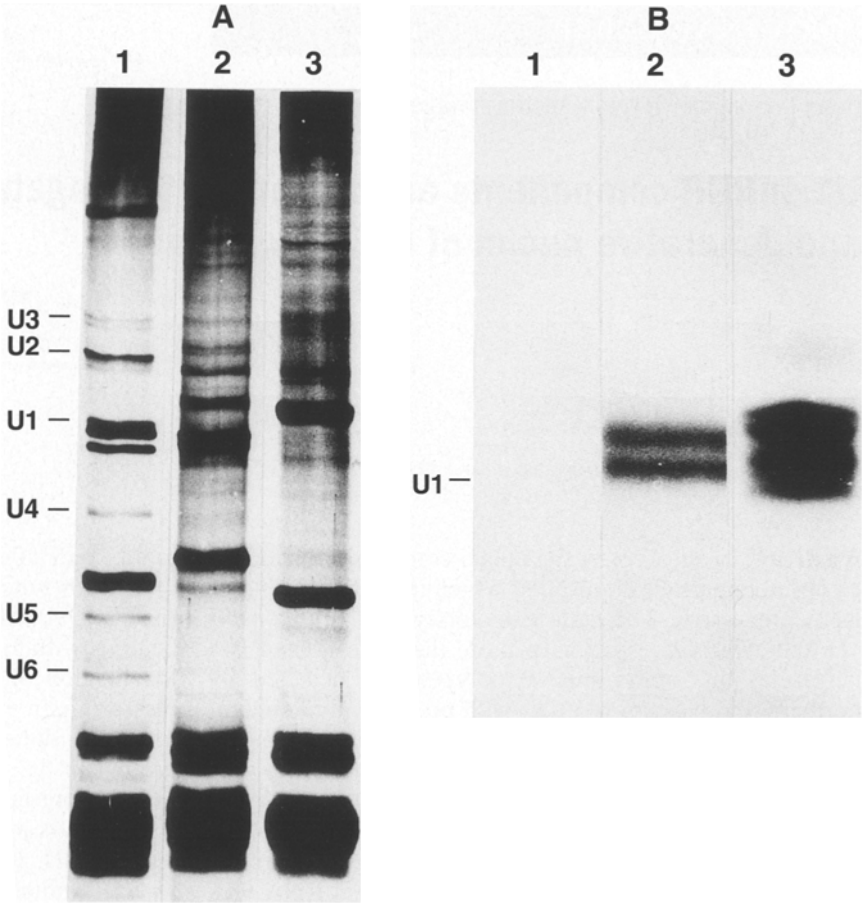

Fig. 1A, B Identification of U1 snRNA in *Pinus radiata* and *Viburnum tinus* pollen grain. **A** Silver-stained profile of 2 µg of HeLa cell RNA (*lane 1*) and 2 µg of *P. radiata* and *V. tinus* pollen grain RNA (*lanes 2 and 3*, respectively). The position of snRNAs from HeLa cells is indicated. **B** About 5 µg of HeLa cells RNA (*lane 1*) and 15 µg of *P. radiata* and *V. tinus* pollen grain RNA (*lanes 2 and 3*, respectively) were analyzed on a denaturing polyacrylamide gel, transferred to Hybond-N membranes and hybridized with U1 snRNA antisense probe labeled with ^{32}P-dCTP

pollen from *Viburnum tinus* L. was obtained from flowers soon after anther dehiscence and stored in sealed vials at –20°C.

Reagents

Terminal transferase and α^{32}P-dCTP (3000 Ci/mmol) were from Amersham (Buckinghamshire, England). Digoxigenin-11-dUTP and anti-digoxigenin-AP, Fab fragments were from Boehringer (Mannheim, Germany). Anti-human IgG (Fc)-alkaline phosphatase conjugate was from GIBCO BRL (Gaithersburg, Md., USA), and 4',6'-diamidino-2-phenylindole (DAPI) and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma (St. Louis, Mo., USA).

Isolation of RNA

Pollen grain was ground in liquid nitrogen and homogenized in a 4 M thiocyanate solution as described by Chomczynski and Sacchi (1987) and modified by Puissant and Houdebine (1990). Isolation of nuclear RNA from HeLa cells was performed according to Concha et al. (1993).

Electrophoresis of RNA

Fifteen µg of total pollen RNA was fractionated on denaturing 10% polyacrylamide-8 M urea (Pessot et al.1989). After electrophoresis, the RNA was electrotransferred for 24 h at 40 mA to a nylon membrane (Hybond-N, Amersham), essentially as described by Sambrook et al. (1989). Northern blots were probed with a synthetic probe 3' labeled with α^{32}P-dCTP and terminal transferase (Concha et al.1993). The probe sequence was 5'TTGACCCCG-TCCAGGTAAGTA3', complementary to nucleotides 2–22 of tomato U1 snRNA (Reddy and Gupta 1990). Blots were hybridized at 52°C for 22 h in 1×SSPE [150 mM NaCl, 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA] containing 0.5% SDS (Henderson et al. 1991). After hybridization, the filters were washed once at room temperature for 30 min with 1×SSPE plus 0.5% SDS, twice with 0.4×SSPE plus 1% SDS, and once with 0.1×SSPE plus 0.5% SDS at 42°C. Autoradiograms of the dried filters were obtained by exposure of Hyperfilm β-max (Amersham) for 1–5 days at –70°C using intensifying screens.

In situ hybridization

Pollen grains were fixed in buffered 4% paraformaldehyde, rinsed in phosphate-buffered saline, dehydrated with increasing concentrations of ethanol and embedded in paraffin. Sections of 7 µm on gelatin-coated slides were deparaffinized and hybridized with antisense or sense oligoprobes labeled at the 3' end with digoxigenin-11-dUTP (Baldino and Lewis 1989; Concha et al. 1993). The sections were poststained with 0.1 g/ml DAPI in saline phosphate buffer at 37°C in the dark. Slides were examined using a Nikon type 104 microscope equipped for epifluorescence illumination.

Isolation of pollen proteins

Pollen was ground in liquid nitrogen and suspended in 10 mM TRIS-HCl (pH 8.0), 1 mM PMSF, 2% SDS, 10% 2-mercaptoetha-

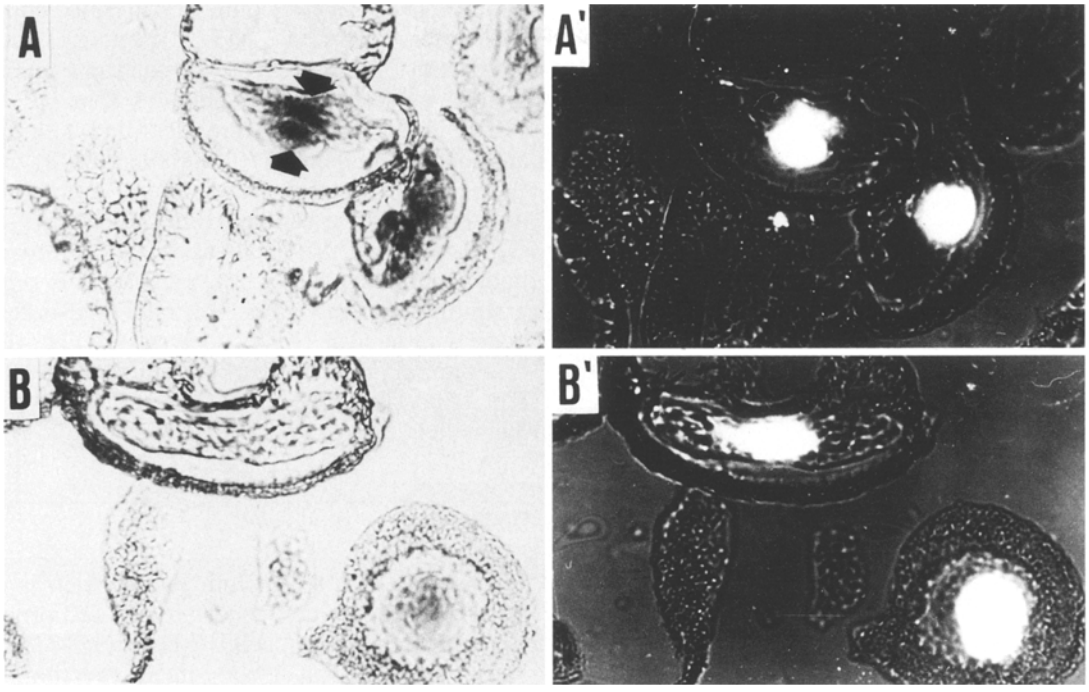

Fig. 2A, B In situ localization of U1 snRNA in *Pinus radiata* pollen grain. **A** Sections of *P. radiata* pollen grain were hybridized with the probe complementary to U1 snRNA and labeled with digoxigenin. Both vegetative and generative nuclei were stained (*arrows*). **B** Control sections hybridized with the labeled sense probe revealed no staining. Post-staining with DAPI is shown in **A'** and **B'**, respectively. The bright-field and fluorescence images are superimposed (×400)

nol and 1 mM EGTA and incubated at room temperature for 30 min and thereafter at 100°C for 2 min. After centrifugation at 100 000 *g* for 3 h to eliminate most DNA and pollen debris, the protein extract was stored at –70°C as total pollen proteins. HeLa cell nuclear proteins were prepared from snRNP extract (Delgado et al. 1994).

Western blotting

Anti-Sm sera were obtained from patients positively identified as carrying systemic lupus erythematosus (SLE). These patients produce autoantibodies that recognize snRNP polypeptides B, B' and D, known as Sm antigens (Tan 1982; Lührmann 1988). Samples of pollen proteins and nuclear proteins of HeLa cells were separated by SDS-polyacrylamide gel electrophoresis according to the procedure described by Laemmli (1970). After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Hybond C, 0.45 µm pore, Amersham) as described by Tsang et al. (1983). The membrane was air dried and washed for 15 min at 50°C in a saline-milk-Tween buffer (10 mM sodium phosphate (pH 7.4), 150 mM NaCl, 5% unfatted milk and 0.3% Tween 80), followed by incubation at 37°C for 3 h with the anti-Sm serum diluted 1:300 in the saline-milk-Tween solution. The membrane was washed three times with the above solution and then incubated at 37°C for 2 h with goat anti-human IgG conjugated with alkaline phosphatase (diluted 1:300). Finally, the membrane was washed three times with saline-milk-Tween solution and incubated for about 10 min in darkness at room temperature for color development (Blake et al. 1984).

Immunocytochemistry

Pollen grains were fixed in buffered 4% paraformaldehyde, rinsed in phosphate-buffered saline, dehydrated with increasing concentrations of ethanol and embedded in paraffin. Sections of 7 µm on gelatin-coated slides were deparaffinized and incubated first with TCT (40 mM TRIS-HCl, 3.5 mM KH_2PO_4, 10.5 mM Na_2HPO_4, 120 mM NaCl, 3 mM NaN_3, 0.5% carragenine (Sigma), 0.7% v/v Triton X-100) for 1 h, and then with the anti-Sm serum, the anti-Sm serum preadsorbed with nuclear HeLa cell proteins (Delgado et al. 1994) or normal serum diluted 1:100 with TCT. The pollen sections were washed with buffered saline and incubated with goat anti-human IgG coupled to alkaline phosphatase (diluted 1:300 with TCT) for 1 h, followed by subsequent saline washes. Color development was performed as described by Blake et al. (1984). Post-staining with DAPI was performed essentially as described for the in situ hybridization experiments.

Results

To determine whether U1 snRNA is contained in the male gametophyte, total RNA was extracted. Figure 1A shows the electrophoretic profiles of *P. radiata* and *V. tinus* pollen RNA (lanes 2 and 3, respectively), ranging from high-molecular-weight RNA to 4S RNA. Some of the bands share the same electrophoretic mobility as the snRNAs U3, U2 and U1 extracted from HeLa cell nuclei (lane 1).

To show that small nuclear RNAs are expressed in the pollen grain, a synthetic DNA probe complementary to nucleotides 2–22 of tomato U1 snRNA was hybridized to RNA extracted from *P. radiata* pollen, and two bands were identified (Fig. 1B, lane 2). With the same oligoprobe, multiple U1 snRNA forms were observed in *V. tinus* pollen grain RNA (lane 3). No positive signal was detected with RNA extracted from HeLa cell nuclei (lane 1). In situ hybridization experiments were performed,

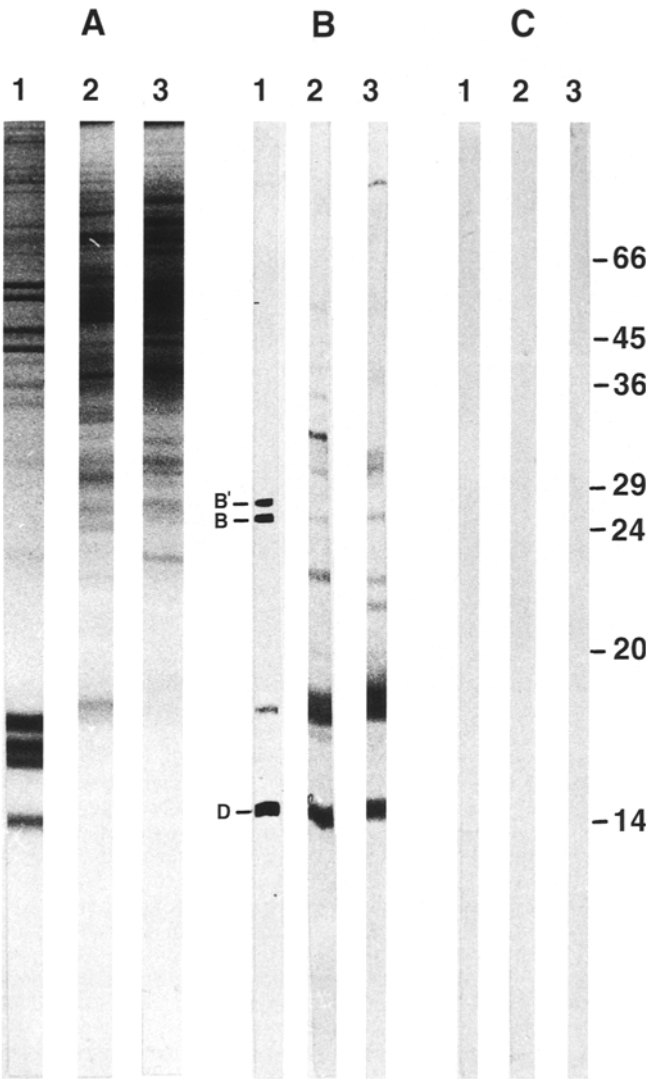

Fig. 3A–C Identification of Sm polypeptides in *Pinus radiata* and *Viburnum tinus* pollen grains. **A** Electrophoretic profile of total pollen proteins: 25 μg of HeLa cells proteins (*1*), 25 μg of *P. radiata* pollen proteins (*2*) and 25 μg of *V. tinus* pollen proteins (*3*) were loaded to the gel. **B, C** About 5 μg of HeLa cell proteins (*1*), 40 μg of *P. radiata* pollen proteins (*2*) and 40 μg of *V. tinus* pollen proteins (*3*) were electrophoresed and transferred to nitrocellulose. The membranes were challenged with either anti-Sm antibody diluted 1:300 (**B**) or serum from a normal donor at the same dilution (**C**). The molecular weights ($\times 10^{-3}$) of the standards used and the identification of the polypeptides *B'*, *B* and *D* of HeLa cell proteins are shown

and U1 snRNA was confined to the generative and vegetative nuclei of *P. radiata* pollen grains (Fig. 2A). As expected, the control with the sense probe did not reveal positive staining (Fig. 2B). In order to confirm the nuclear localization of U1 snRNA, post-staining of the sections with the fluorescent DNA-binding dye DAPI was performed. In A' and B' the bright field and the nuclear fluorescence images are superimposed.

To define the polypeptides associated with snRNAs in the *P. radiata* and *V. tinus* pollen, total extracted proteins were subjected to denaturing polyacrylamide gel electrophoresis (Fig. 3A). A complex pattern of pollen proteins was observed (lanes 2 and 3). Proteins with molecular weights of 27 and 16 kDa with the same mobility as HeLa cell nuclear B and D antigens (Fig. 3B, lane 1), as well as other proteins, were identified with the anti Sm-antibody in pollen of *P. radiata* (lane 2) and *V. tinus* (lane 3). The control with serum of a normal donor did not show positive banding (Fig. 3C, lanes 1–3). Finally, Fig. 4A shows the localization of the Sm antigens in both generative and vegetative nuclei in *P. radiata* pollen grains by immunocytochemistry. Preadsorbed Sm antibody with nuclear HeLa cell proteins (Fig. 4B) or serum of a normal donor (Fig. 4C) eliminated the signal. As in Fig. 2, post-staining with DAPI revealed the position of the nuclei.

Discussion

Considering that the mature pollen grain is engaged in RNA synthesis in the vegetative cell and probably in the generative or sperm cells (Mascarenhas 1990; Russel 1991; Zhang et al. 1993), the expression of snRNPs which mediate post-transcriptional processing of the primary RNA polymerase II transcripts is unprecedented in pollen grain.

The results presented in this paper yield, first of all, compelling evidence for the presence of spliceosomal U1 snRNA and Sm antigens in gymnosperm and angiosperm pollen. They show the localization of both components in the vegetative as well as in the generative nuclei in *P. radiata* pollen. Similar localization studies were obtained with *V. tinus* pollen (data not shown). The presence of these ribonucleoprotein particle components in the generative cell is surprising, considering that the chromatin of the generative nucleus is highly condensed and has been postulated as transcriptionally inactive or less active than the chromatin in the vegetative nucleus (La Fountain and La Fountain 1973; Wagner et al. 1990). Northern blot analysis revealed two U1 snRNA bands in *P. radiata* pollen, whereas approximately six isoforms were revealed in *V. tinus*. The existence of more than one isoform for some snRNAs is consistent with published observations in flowering plants such as pea (Hanley and Schuler 1991), tomato and others (Reddy and Gupta 1990). In contrast, U6 snRNA appears as a single form in pea (Hanley and Schuler 1991) and also in RNA extracted from pollen of *P. radiata, V. tinus* and tomato (unpublished results). As expected, the stringent hybridization conditions employed did not favor positive hybridization with HeLa cell RNA. The significance of different snRNA species is not clearly understood at the present time. It has been postulated that selected subsets of plant snRNAs are expressed at particular stages of development, due either to differential transcription or to post-transcriptional snRNA processing (Hanley and Schuler 1991).

Using the anti-Sm antibody, proteins with the same mobility as Sm antigens B and D were clearly identified

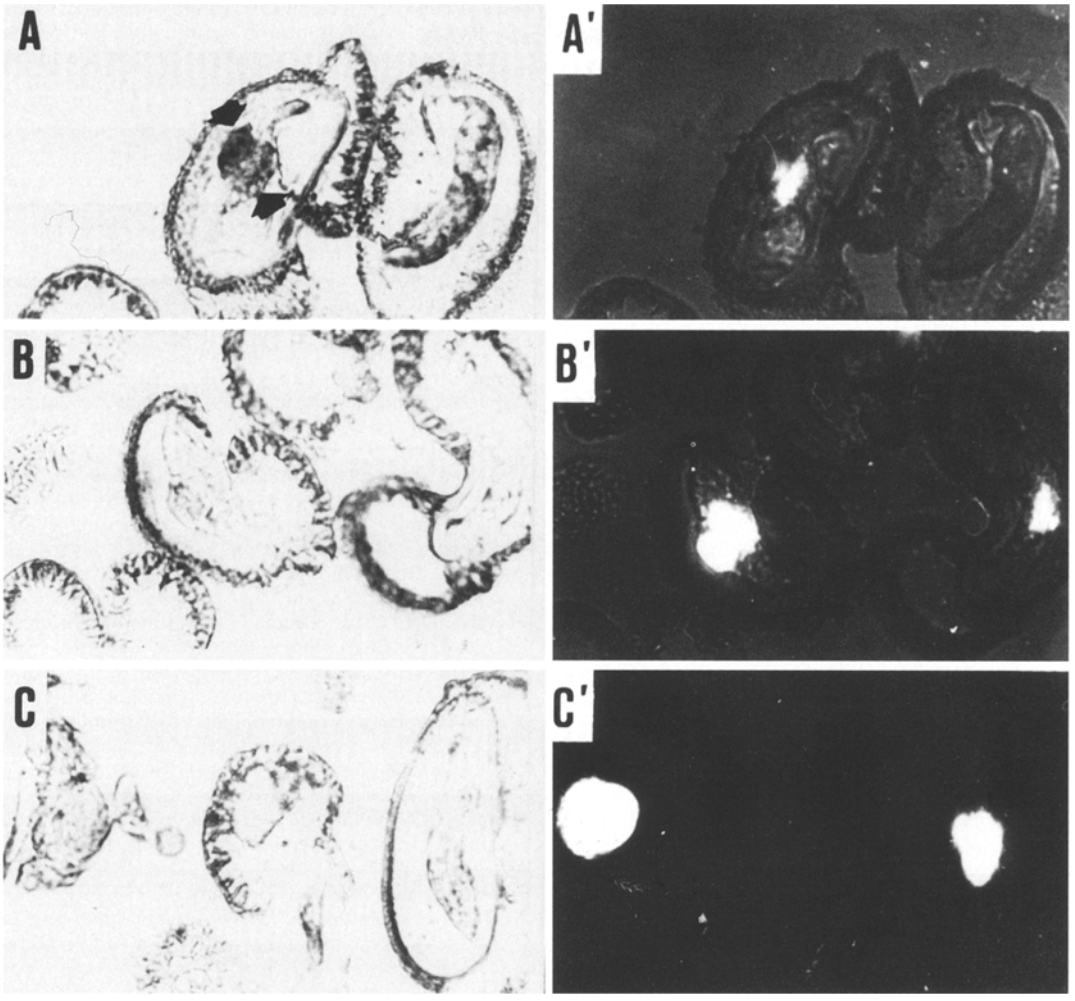

Fig. 4A–C Nuclear localization of Sm antigens in *Pinus radiata* pollen grain. Sections of *P. radiata* pollen grain (**A**) were probed with an anti-Sm serum diluted 1:100 and developed as described in Materials and methods. Parallel sections were incubated with the serum preadsorbed with HeLa cell nuclear proteins (**B**) or serum from a normal donor (**C**). *Arrows* indicate the stained nuclei (**A**). Post-staining with DAPI is shown in **A'**, **B'** and **C'**, respectively. The bright-field and fluorescence images are superimposed (×400)

(Tan 1982; Lührmann 1988; Tan 1989). The signal was weak, considering that the snRNP polypeptides are highly conserved in eukaryotes as demonstrated by immunoprecipitation with anti-Sm serum (Tollervey and Mattaj 1987; Bullard-Dillard et al. 1992). Nevertheless, it is reasonable to think that these plant proteins may not share exactly the same epitopes as their human counterparts.

The significance of these snRNP particles in the generative nuclei is interesting and may suggest a functional role in primary transcript processing. Although pollen contains ribosomes, tRNAs, and presynthesized mRNAs, new RNA synthesis occurs during pollen germination and tube growth (Mascarenhas 1993). The evidence indicates that the RNA synthesized during pollen germination and pollen tube growth are mRNAs or mRNA precursors synthesized by the vegetative nucleus (Mascarenhas et al. 1974; Mascarenhas and Mermelstein 1981). Future strategies directed towards the isolation of genes from the generative or sperm cells expressed in the pollen or pollen tube would provide new insights into the participation of the splicing apparatus at this stage of male gametophyte development.

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