Vicia faba germination: Synchronized cell growth and localization of nucleolin and α-tubulin.

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Abstract

The first cell cycle of *Vicia faba* L. seeds, which begins upon imbibition of dry seeds and is completed at the first mitosis after radicle protrusion, was characterised by the flow cytometry and immunodetection of nucleolin and tubulins in root tip meristems. Flow cytometry revealed highly synchronised profiles from the quiescent G_1 phase to the late G_2 phase, indicating uniform cell cycle progression within a root tip until the first mitosis. Using immunoblotting, nucleolin was detected in two distinct bands with the apparent molecular masses of 89 and 99 kD; the former was detected only in seeds imbibed at 4°C for 1 day whereas the latter was found at all stages examined, suggesting that the 89 kD nucleolin may be seed-specific. Unusual localization of nucleolin in cold-imbibed seeds, undetectable in half of the cells and present in nucleoplasm, was revealed by immunofluorescence microscopy. While α- and β-tubulin were detected at all stages and no significant changes in accumulation of the proteins were observed, few microtubules were detected at the beginning of germination when cells were still in the G_1 phase, suggesting that microtubules may be depolymerized in the dry seeds.

Keywords: Cell cycle, flow cytometry, germination, immunofluorescence microscopy, microtubules, nucleolin, Vicia faba L.

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Introduction

In many species, embryo cells of dry seeds are arrested mostly in the G_1 phase (Bewley and Black, 1994), and the first mitosis in the root tip meristems normally occurs shortly after radicle protrusion. Therefore, the germination period coincides roughly with the first cell cycle. However, the first cell cycle is distinctly different from subsequent cell cycles as it also presents the transition from the dry quiescent state to the actively growing state. In *Vicia faba* L. the root starts growing by elongation of the basal cells after radicle protrusion; when the radicle is 5–7 mm long and the hypocotyl is fully elongated, mitotic activity in the root tip meristem begins (Obroucheva *et al.*, 1995). The report showed that timing of the entry into mitosis may be detected by measuring radicle length.

Valuable information on cell cycle synchrony during seed germination has been obtained by flow cytometry. In many species, most embryo cells of dry seeds have been found to be arrested in G_1 (Bino *et al.*, 1993). Appearance of cells in G_2 in the root tip was observed as germination progressed in tomato (Bino *et al.*, 1992) and pepper (Lanteri *et al.*, 1993), and the increase of cells in G_2 was observed before radicle protrusion, indicating that DNA replication preceded visible germination in both species. Cell cycle synchronisation in *V. faba* root tip meristems has been obtained by hydroxyurea treatment and analysed by flow cytometry (Doležel *et al.*, 1992). However, the root tips were collected from radicles more than 20 mm long, i.e., after meristem cells had undergone the first mitosis.

Nucleolin is a major nucleolar protein of growing eucaryotic cells. It has been found to have

Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; DMF, dimethylformamide; MTSB, microtubule stabilizing buffer; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; TTBS, Tris-buffered saline with Tween-20.

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multifunctional roles in rDNA transcription and in pre-ribosomal RNA formation (Shaw and Jordan, 1995) as well as in cytoplasmic-nucleolar transport of preribosomal particles (Borer *et al.*, 1989). Nucleolin may have unique features in early germination; immediately upon imbibition it may have roles in assembling the machinery for protein synthesis in previously dry quiescent cells. Nucleolin has an apparent molecular mass of 90–110 kD in SDS-PAGE (Lapeyre *et al.*, 1987; Meßmer and Dreyer, 1993; Shaw and Jordan, 1995). It has a highly conserved tripartite structure, consisting of N-terminal acidic repeats, central RNA recognition motifs, and a Gly- and Arg-rich domain at the C-terminus. Nucleolin in plants has been identified by characterization of an alfalfa cDNA encoding nucleolin (*nuc*Ms1) (Bögre *et al.*, 1996). The alfalfa nucleolin in suspension cell cultures was detected by immunoblotting as a double band with the apparent molecular mass between 90 and 95 kD, and its mRNA expression was found to be tightly linked to cell proliferation but not to a particular cell cycle phase. In addition, mitosis-specific phosphorylation of nucleolin (Belenguer *et al.*, 1990) and translocation of nucleolin during growth (Meßmer and Dreyer, 1993) have been reported.

Microtubules are assembled from heterodimers containing one α-tubulin polypeptide and one β-tubulin polypeptide, each with a molecular mass of approximately 50 kD. Microtubules are continually disassembled, assembled, and rearranged into new configurations or arrays as a cell progresses through division and differentiation (Goddard *et al.*, 1994). In general, cortical microtubules and radial cytoplasmic arrays in the interphase, preprophase band in late G_2, and spindle and phragmoplast in mitosis are stepwise rearranged during cell cycle progression (Lambert and Lloyd, 1994).

It is in our interest to characterise germination from the aspect of cell cycle progression as well as of growth. In the present study, based on the Feulgen test which visualizes condensed chromosomes and thus detects mitotic cells (Doležel and Novak, 1984), appropriate time points or radicle lengths were chosen for *V. faba* root tip meristems to investigate the transition from the quiescent state to the first mitosis. Flow cytometry was first conducted to assess the synchrony of cells in each stage during the progression of the first cell cycle. Immunolocalization of *V. faba* nucleolin in differently prepared cells has been studied by the authors and here, stages within the first cell cycle of *V. faba* seeds were examined. Also cell cycle progression during *V. faba* germination was monitored by a survey of microtubule arrays.

Materials and methods

Plant material and sample stages

Seeds of *Vicia faba* L. cv. Inovec (provided by Dr. Vavák, Horna Streda, Slovakia) were normally submerged in water at 23°C for 1d, then transferred onto two layers of fully wet filter papers in a 20 cm Petri dish. The incubation at 23°C was continued up to 96 h with occasional supplies of water until the seeds reached the desired growth stages, which were decided by the Feulgen staining. No mitosis was observed in root tip meristems at 48 h of imbibition (stage 2, 0–2 mm radicles), nor in 10 mm radicles (stage 3); in 15 mm radicles (stage 4) just a few mitotic cells were found; in 20 mm radicles (stage 5) many cells were undergoing mitosis. Root tip meristems were also collected from seeds kept on artificial soil at 23°C when their radicles were more than 30 mm long, as a post-first mitosis sample (stage 6). As a control to approximate the dry state, prechilled *V. faba* seeds were submerged in water at 4°C for 1d (stage 1), minimizing biochemical processes which would commence upon imbibition at 23°C. Alfalfa (*Medicago sativa* L.) seeds were prepared in the same way except that the initial immersion was for 2 h.

Flow cytometry

Relative DNA content of nuclei from *V. faba* root tip meristems was analyzed as described previously (Doležel *et al.*, 1992) with minor modifications. Ten root tips (1–1.5 mm) were isolated for each stage and immediately fixed in 4% (v/v) formaldehyde in Tris buffer (10 mM Tris pH 7.5, 10 mM Na_2EDTA, and 100 mM NaCl) with 0.1% (v/v) Triton X-100 at 5°C for 25 min. After three washes with Tris buffer, the root tips were homogenized with a Polytron PT1200C (Kinematica AG, Switzerland) homogenizer with 1 ml LB01 lysis buffer (15 mM Tris pH 7.5, 2 mM Na_2EDTA, 80 mM KCl, 20 mM NaCl, 0.5 mM spermine, 15 mM mercaptoethanol, and 0.1% Triton X-100). The suspension was filtered through 50 µm nylon mesh and stained with DAPI at a final concentration of 4 µg/ml. Relative fluorescence intensity was analyzed with a PAS II flow cytometer (Partec GmbH, kindly provided by Prof. W. Göhde, Münster, Germany). The histograms were recorded over 512 channels and analyzed for cell cycle distribution using Partec software.

Primary antibodies

Nucleolin was detected by a rabbit antibody raised against a synthetic peptide derived from an alfalfa nucleolin cDNA (Bögre *et al.*, 1996). Tubulins were detected by mouse monoclonal antibodies: clone DM1A for α-tubulin and clone TUB 2.1 for β-tubulin (both from Sigma).

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Protein extraction and immunoblotting

Approximately 60 root tip meristems, 1–1.5 mm pieces of yellowish tissue, from each *V. faba* stage were isolated, frozen in liquid nitrogen, and ground with a prechilled mortar and pestle. The ground powder was mixed with a small amount of polyvinylpolypyrrolidone and transferred to a tube with 300 µl of extraction buffer (50 mM Pipes, pH 6.9, 2 mM DTT, and 1 mM EGTA) containing protease inhibitors (0.5 mM PMSF and 1 µg/ml each of prefabloc, leupeptine, and antipain) and phosphatase inhibitors (1 mM NaF, 15 mM beta-glycerophosphate, 15 mM p-nitrophenol, 0.5 mM Na_3VO_4) (Linhartová *et al.*, 1992). After three cycles of freezing-thawing, a clear supernatant was obtained by centrifugation (14,000 g for 5 min at 4°C). Protein content was determined by the Coomassie Blue method (Bradford, 1976).

For each sample, 20 µg of protein was separated by SDS-PAGE (Laemmli, 1970) and blotted onto a polyvinylidene difluoride membrane at 1mA/cm^2 for three hours or at 0.8 mA/cm overnight with a Novablot electrophoretic transfer unit (Pharmacia) using transfer buffer [25 mM Tris, 192 mM glycine and 20% (v/v) methanol]. After a brief wash with TTBS [10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% (v/v) Tween-20], the membranes were incubated with blocking buffer [5% (w/v) non-fat dry milk and 0.05% (w/v) NaN_3 in TTBS] at 23°C for 1–2 h and subsequently with the primary antibody at about 0.2 µg/ml in TTBS for at least 16 h at 4°C. Alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse IgG (Sigma) was used as the secondary antibody, and the mixture of 10% (w/v) nitro blue tetrazolium chloride in 70% (v/v) DMF, 1.5% (w/v) of 5-bromo-4-chloro-3-indol-phosphate in DMF, and 100 mM $NaHCO_3$, pH 9.8, containing 1 mM $MgCl_2$ (1:1:160 v/v) was used for visualization of the antigen.

Immunofluorescence microscopy

Root tips were fixed in 3.7% formaldehyde in MTSB (100 mM Pipes, pH 6.9, 1 mM $MgSO_4$, and 2 mM EGTA) for 1 h. After washing with MTSB, the root tips were digested in 1% (w/v) cellulysin (Calbiochem) in MTSB containing protease inhibitors (0.3 mM leupeptin and 1.0 mM PMSF). After the wash the root tips were squashed on poly-L-Lys coated slides. Then, cells were fixed in methanol at −20°C for 10 min, followed by incubation with 1% Triton in MTSB at 23°C for 30 min. After washing 3 times for 5 min with PBS, the slides were incubated with primary antibodies at 32°C for 1 h or at 4°C overnight (Binarová *et al.*, 1993). The primary antibodies were diluted with 2% (w/v) BSA in PBS: 1:500 for DM1A and 1:200 for the anti-nucleolin antibody. After washing 3 times for 5 min with PBS, the slides were incubated for 45 min with appropriate secondary antibodies (FITC- and TRITC-conjugated anti-mouse and Cy3-conjugated anti-rabbit antibodies, all from Sigma). After washing 3 times for 5 min with PBS, the slides were incubated in 1 µg/ml DAPI in PBS for 10 min to visualize DNA, mounted in Mowiol 4-88 (Calbiochem), and then examined with an Olympus BX 60 microscope by epifluorescence.

Results

Flow cytometric analysis

Cells from stage 6, at which cells are asynchronously dividing, were initially analysed to locate peaks of the 2C DNA content (the sharp higher peak) which represents nuclei in G_1 and of the 4C DNA content (the broad lower peak) which represents nuclei in G_2 (Fig. 1, control). In stage 1 cells, about 80% of the nuclei were in G_1. No changes in peak distribution were seen in stage 2 cells. A transition through S phase towards G_2 was obtained, and 52 and 61% of G_2 nuclei were detected in stage 3 and 4 cells, respectively. The peak distribution in stage 5 resembled that of asynchronously dividing cells (control). The shoulder at the left side of the histogram of stage 3 and 4 cells was caused by the presence of small cellular fragments in the sample which are non-specifically stained.

Immunoblotting and immunofluorescence microscopy of nucleolin

The nucleolin antibody detected a double band larger than 99 kD from an extract of alfalfa root tips (Fig. 2A, 7). The apparent molecular masses appeared to be slightly larger than those of nucleolins detected in alfalfa suspension culture (90–95 kD) (Bögre *et al.*, 1996). The antibody detected a 99 kD protein in extracts from all *V. faba* stages and a 89 kD protein only in the stage 1 extract (Fig. 2A, 1–6). The apparent molecular mass of the 99 kD *V. faba* nucleolin appeared to fluctuate during the growth period after visible germination, especially from stages 4–6.

In Fig. 3 (A and a), nucleolin was detected in about half of the cells in stage 1; there were cells with DNA signals but without nucleolin signals. In half of the cells in which nucleolin was detected, the signals were found both in the interchromatin region of nuclei (nucleoplasm) and in nucleoli, and in the other half only in small compact nucleoli. This observation is intriguing as nucleolin was detected in every cell in G_1 but only in small compact nucleoli when cell synchrony of *V. faba* root tip meristems, obtained from radicles more than 20 mm long, was obtained by hydroxyurea treatment (Binarova *et al.*, unpublished

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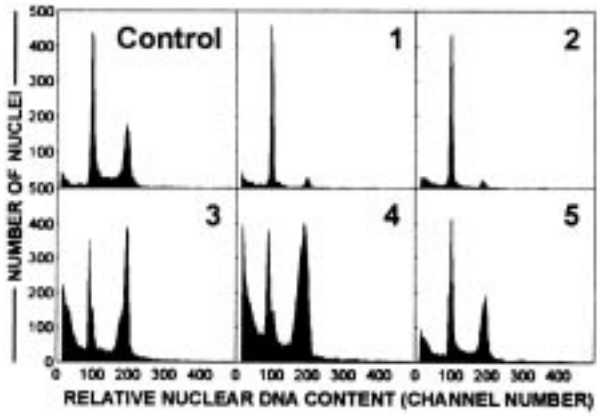

Figure 1. Histograms of relative nuclear DNA contents obtained by flow cytometric analysis of *Vicia faba* root tip meristems. (Control) stage 6, meristems from radicles more than 30 mm long; (1) stage 1, cold-imbibed seeds; (2) stage 2, 0–2 mm radicles; (3) stage 3, 10 mm radicles; (4) stage 4, 15 mm radicles; (5) stage 5, 20 mm radicles.

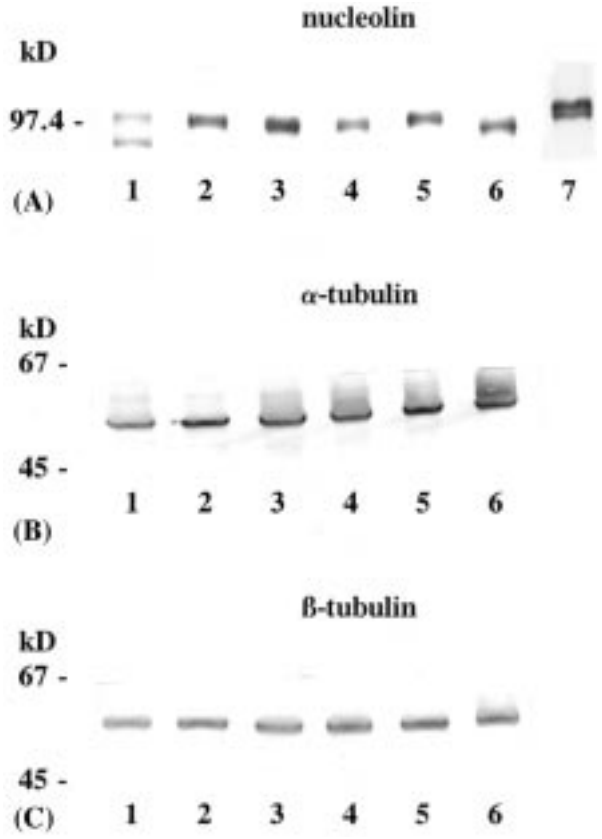

Figure 2. Immunoblotting of nucleolin and α- and β-tubulin in *Vicia faba* root tip meristems. Numbers 1 to 6 correspond to stage numbers. (7), alfalfa root tips from radicles more than 10 mm long. Only parts of the gels where proteins of interest were located are shown. Nucleolin (A) and β-tubulin (C) are from a single gel of 7.5% SDS-PAGE. α-Tubulin (B) was separated in 10% SDS-PAGE.

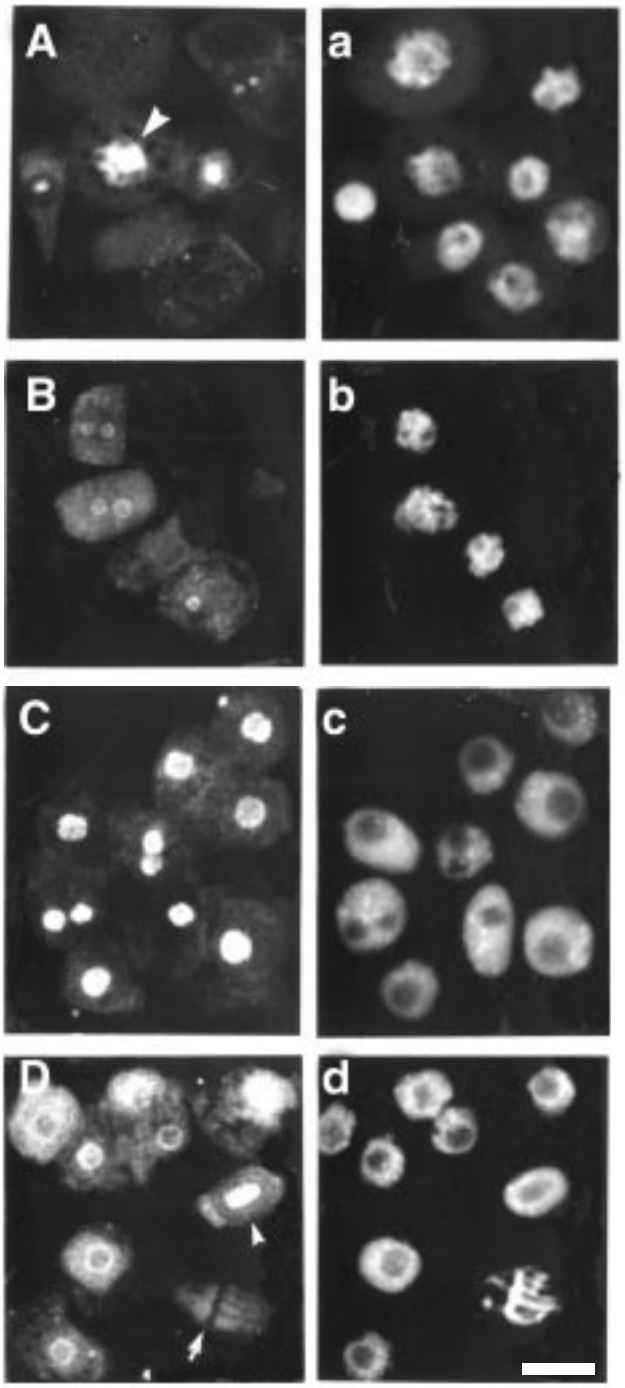

Figure 3. Immunofluorescence localization of nucleolin in *Vicia faba* root tip meristems. Double staining: immunostaining of nucleolin (A,B,C,D) and DAPI staining of DNA (a,b,c,d). (A) and (a), stage 1, cold-imbibed seeds; (B) and (b), stage 2, 0–2 mm radicles; (C) and (c), stage 3, 10 mm radicles; (D) and (d), stage 5, 20 mm radicles; Arrowhead in (A) indicates nucleolin in nucleoplasm and nucleolus. Arrowhead in (D) indicates nucleolin in an irregular-shaped nucleolus at preprophase. Arrow in (D) indicates nucleolin in cytoplasm of a mitotic cell. (Bar = 10 μm)

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results). In stage 2, while nucleolin was detected in small nucleoli in the majority of the cells (Fig. 3, B and b), it no longer showed nucleoplasmic localization. In stage 3, when DNA signals showed increased size of nucleoli and the granular structure of chromatin, indicating cells in G_2, nucleolin was detected in the enlarged nucleoli of all cells (Fig. 3, C and c). In stage 5, various cell cycle stages were found. Nucleolin was detected in G_2-type enlarged nucleoli, in nucleoli with irregular shapes at preprophase, and in cytoplasm of mitotic cells (Fig. 3, D and d).

Immunoblotting of tubulins and Immunofluorescence microscopy of α-tubulin

The commercial monoclonal tubulin antibodies, which have been used to detect tubulins from animals as well as plants, detected α- and β-tubulin as a 54 and 56 kD protein, respectively, in extracts from stage 1 (cold-imbibed seeds) to stage 6 (Fig. 2, B and C). No significant changes in their steady state protein level were observed throughout. The smear bands above the tubulins and the apparently increasing molecular mass of α-tubulin were regarded as artifacts.

In Fig. 4 (A and a), α-tubulin was mostly detected in stage 1 as diffused fluorescence spread evenly through the cytoplasm, suggesting that microtubules were depolymerized, while DNA signals indicated that the cells were in G_1. In stage 2, α-tubulin was still detected mainly as diffused fluorescence and DNA signals still indicated G_1 (Fig. 4, B and b), but short cortical microtubules were found in about 40% of the cells. In stage 3, randomly arranged dense cortical microtubules were detected and DNA signals still indicated the interphase (Fig. 4, C and c). In stage 5, various mitotic microtubular arrays such as the preprophase band (Fig. 4, D and d) and mitotic spindle were detected, clearly indicating mitotic activity in the cells. From stage 3 onward, both microtubules and DNA were detected in a pattern similar to that of *V. faba* hydroxyurea-synchronized cells (Binarova *et al.*, 1993).

Discussion

The high percentage of G_1 cells, more than 80%, in stage 1 (cold-imbibed seeds) is in accord with the observation by Brunori (1967) that *V. faba* root tip cells are mostly in G_1 during the last phase of seed ripening when the water content is about 20%. The cells in stage 1 may correspond to *Pisum sativum* root tip meristem cells at 15 h of imbibition, which are non-cycling cells arrested in G_1 and G_2 phases in which nuclei have not reached the nuclear protein threshold value necessary to enter the next phase (Sgorbati *et al.*, 1989). The large proportion of G_1 nuclei in stage 2 as

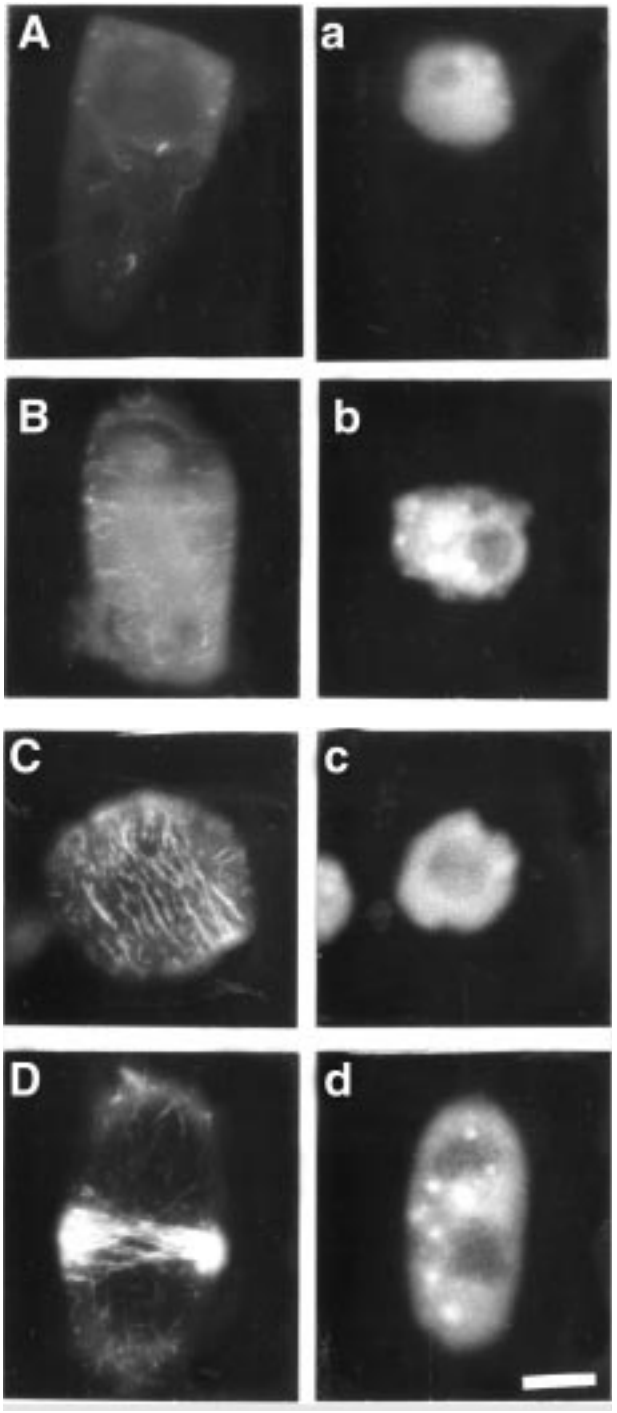

Figure 4. Immunofluorescence localization of α-tubulin in *Vicia faba* root tip meristems. Double staining: immunostaining of α-tubulin (A,B,C,D) and DAPI staining of DNA (a,b,c,d). Sample descriptions are the same as in Fig. 3. (Bar = 5 μm)

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well as of G_2 nuclei in stages 3 and 4 suggests that the cells had highly synchronous cell cycle progression at these stages. The natural cell synchrony appeared to be lost in stage 5. The results suggest that it may be possible to obtain cells at a specific phase of the cell cycle by collecting *V. faba* root tip meristems from radicles of the same length: stage 1 (cold-imbibed seeds) is in G_1; stage 2 at 2 mm radicle length is in late G_1; stage 3, at 10 mm in S/G_2; stage 4, at 15 mm in late G_2.

A high degree of synchrony in the cell cycle progression from G_1 to late G_2 was detected in *V. faba* root tip meristems over a 96 h period (Fig. 1). More than 48 h of germination is necessary for *V. faba* cells to exit the G_1 phase. Moreover, the transition to M phase seemed to require a similar length of time, during which the signaling of the cell cycle check point controls took place. Thus, signaling might be easier to study during this long time span in germinating seeds than in artificially synchronized systems. For example, in hydroxyurea-synchronized *V. faba* root tips, the cells travel from G_1/S to M phase in only about 8 h (Doležel *et al.*, 1992). In addition, the synchrony in the germinating seed system is obtained without the aid of drugs that might disturb natural physiological conditions. However, the cell cycle synchrony of imbibed seeds may not be common. Alfalfa seeds were also investigated in the same way but no synchrony was detected with flow cytometry (Fujikura *et al.*, unpublished results).

The presence of the 89 kD nucleolin only in stage 1 is intriguing (Fig. 2A). The 89 kD nucleolin is either seed specific, thus formed during seed development, newly synthesized, or modified from the 99 kD protein upon imbibition. In the Northern analysis with an alfalfa nucleolin cDNA probe, the *V. faba* nucleolin transcript appeared to be absent in stage 1 and 2, and present only from stage 3 onward (data not shown), suggesting that newly synthesized nucleolin might appear only from stage 3. However, the Northern blot result was indecisive because of the weakness of the signals, which was probably due to heterogeneity in nucleolin DNA sequence between the two species. Yet, possibilities such as cold-induced synthesis of the 89 kD nucleolin as well as enzymatic modification of the 99 kD nucleolin to the 89 kD protein by the cold immersion are less likely upon imbibition of dry seeds. The disappearance of the 89 kD nucleolin coincided with the increase in intensity of the 99 kD nucleolin in stage 2, indicating that the former might be converted to the latter since *de novo* synthesis of nucleolin might not have started in stage 2. The mobility shift of nucleolin in SDS-PAGE from 89 kD to 99 kD might be caused by modifications such as phosphorylation. Phosphorylation of nucleolin accompanying an increase in the apparent molecular mass has been reported (Meßmer and Dreyer, 1993). In a human nucleolar protein p130, the interconversion of 130 and 95 kD forms by phosphatase and cellular protein kinases has been demonstrated (Pai *et al.*, 1995). Similarly, in alfalfa a single nucleolin band, with a smaller molecular mass than the previously known alfalfa nucleolins, was detected only in 2 h cold-imbibed seeds, and only larger bands were detected at later stages (Fujikura *et al.*, unpublished results).

The absence of the nucleolin signal in half of the cells (Fig. 3, A) suggests that the epitope was physically masked for the undetected nucleolin, since uneven distribution of nucleolin among the meristem cells is unlikely. Also, the signal in the nucleoplasm is intriguing. It has been reported that the nuclear location signal (NLS) in nucleolin is sufficient for nuclear location but not for nucleolar accumulation, and that nucleolar accumulation requires the presence of domains which interact with RNA in addition to the NLS (Meßmer and Dreyer, 1993). Therefore, the domains necessary to interact with RNA might not have been available to some of *V. faba* nucleolin at this stage due to structural strain or interaction with other molecules. The appearance of the nucleolin signal in small nucleoli in the majority of the cells and the disappearance of the signal in the nucleoplasm in stage 2 suggest that both unmasking of the epitope and translocation to nucleoli had occurred by this time, since newly synthesized nucleolin might not appear at this stage. Therefore, the presence of the 89 kD nucleolin and the unusual localization of the nucleolin signal in stage 1 cells may be connected. It is possible that some of the nucleolin is modified and stored in a seed-specific manner at some point during seed development, processed into a mature form upon imbibition, and localized accordingly. The benefit of the alteration could be safe storage.

Both α- and β-tubulin were detected at all *V. faba* stages without significant changes in their steady state protein level (Fig. 2, B and C). In contrast, β-tubulin was not detected in dry tomato seeds or at 24 h of imbibition; the accumulation of β-tubulin appeared to coincide with the replication of DNA (de Castro *et al.*, 1995). The differences between tomato and *V. faba* might be due to species-specific regulation of tubulin expression. Immunolocalization of α-tubulin indicated that most microtubules might be depolymerized in seeds (Fig. 4, A and B). It is known that microtubules can disassemble at low temperature (Wallin and Stromberg, 1995). Therefore, the depolymerization of *V. faba* microtubules could have been caused by the cold immersion. However, the detection of α-tubulin in mainly depolymerized microtubules from *V. faba* still in stage 2 (0–2 mm radicles) (Fig. 4B), after 48 h of germination at 23°C, and thus without cold immersion, suggests that the microtubules may be depolymerized in the seeds until visible germination. The observation was also confirmed by similar results

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from the seeds imbibed in water at 23°C for 12 h (data not shown). Depolymerization of microtubules was also caused by treatments with antimicrotubular drugs (colchicine and oryzalin) in cells of the quiescent center of maize root apices whose nuclei are mostly in G_1 (Baluska and Barlow, 1993). Interestingly, the depolymerization of the maize tubulins resulted in an increased proportion of S and G_2 nuclei which was detected by cytophotometry, suggesting that there may be a link between depolymerization of microtubules and DNA replication. The former might be a prerequisite to the latter in quiescent cells. In animal cells, depolymerization of microtubules is sufficient to initiate DNA synthesis (Crossin and Carney, 1981). The finding of depolymerized microtubules in cold-imbibed *V. faba* seeds suggests that observation of microtubules in the embryo from late embryogenesis to early germination may provide valuable information on the reorganization of microtubules during deactivation/activation of cell cycle.

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