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SHORT COMMUNICATION

Association of γ-tubulin with kinetochore/centromeric region of plant chromosomes

Pavla Binarová[1,*,†], Bettina Hause[2], Jaroslav Doležel[1] and Pavel Dráber[3]

[1]*De Montfort University, Norman Borlaug Centre of Plant Science, Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Olomouc, Czech Republic,*

[2]*Institute of Plant Biochemistry, Halle, Germany, and*

[3]*Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic*

Summary

Monoclonal antibodies raised against a phylogenetically conserved peptide from the C-terminal domain of γ-tubulin molecule were used for immunofluorescence detection of γ-tubulin in acentriolar mitotic spindles of plant cells. The antibodies stained kinetochore fibres along their whole length, including the close vicinity of kinetochores. After microtubule disassembly by the antimicrotubular drugs amiprophos-methyl, oryzalin and colchicine, γ-tubulin was found on remnants of kinetochore fibres attached to chromosomes. In cells recovering from the amiprophos-methyl treatment, γ-tubulin was localized with the re-growing kinetochore microtubule fibres nucleated or captured by kinetochore/centromeric regions. On isolated chromosomes, γ-tubulin co-localized with α-tubulin in the kinetochore/centromeric region. The data presented suggest that in acentriolar higher plant cells γ-tubulin might be directly or indirectly involved in modulation and/or stabilization of kinetochore–microtubule interactions.

Introduction

The assembly of the mitotic spindle is an example of polymeric self-organization of microtubules from tubulin subunits. In the majority of eukaryotic cells, the duplication and movement of microtubule-organizing centres such as centrosomes or spindle pole bodies ensure the nucleation of bipolar mitotic spindles and proper segregation of chromosomes. However, many meiotic animal cells lack centrosomes and the chromosomes themselves appear to promote the assembly of microtubules in their vicinity, which then organize into bipolar spindles (Vernos and Karsenti, 1995). Similarly, higher plants also lack centrosomes, but the mechanisms of spindle assembly in these cells are poorly understood. The plant nuclear envelope was shown to promote microtubule nucleation in an *in vitro* assay and putative homologues of animal centrosomal proteins were consistently immunolocalized on nuclear envelope (Chevrier *et al.*, 1992). Transformation of a prophase spindle, which is initiated in the perinuclear region before the nuclear envelope breakdown, to a prometaphase spindle, which consists mainly of kinetochore microtubule fibres, is unclear (Lambert, 1993; Palevitz, 1993). Plant cells express specific features of microtubule self-organization, such as the presence of microtubule-converging centres (Smirnova and Bajer, 1994). It was reported recently that microtubules self-organized around chromatin-coated beads could form bipolar spindles in the presence of microtubule motor proteins (Heald *et al.*, 1996).

γ-Tubulin has been shown to be an ubiquitous component of microtubule-organizing centres such as centrosomes or spindle pole bodies, where it performs an essential nucleation function (Joshi *et al.*, 1992). Data on the presence of cytosolic γ-tubulin complexes, and γ-tubulin localization with spindle microtubules or midbody (Moudjou *et al.*, 1996; Nováková *et al.*, 1996) imply that the relationship between γ-tubulin and the microtubular cytoskeleton could be more complex than was initially suggested. Immunolocalization of γ-tubulin with polyclonal antibodies in acentriolar plant cells implies that it is associated with all microtubular arrays except kinetochore microtubule ends in the vicinity of kinetochores and phragmoplast microtubule ends in the vicinity of the cell plate where plus ends of microtubules are supposed to be localized (Joshi and Palevitz, 1996; Liu *et al.*, 1993). Here we present the evidence for the association of γ-tubulin with the kinetochore/centromeric region of chromosomes in acentriolar plant cells.

Results

Localization of γ-tubulin with kinetochore microtubule fibres

In whole-cell lysates of *V. faba* meristem cells, the monoclonal antibodies against γ-tubulin TU-30, TU-31, TU-32

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*For correspondence (fax +42 2 4752384; e-mail binarova@biomed.cas.cz).

†Present address: Institute of Microbiology, Academy of Sciences of the Czech Republic, Videòská 1083, 142 20 Prague 4, Czech Republic.

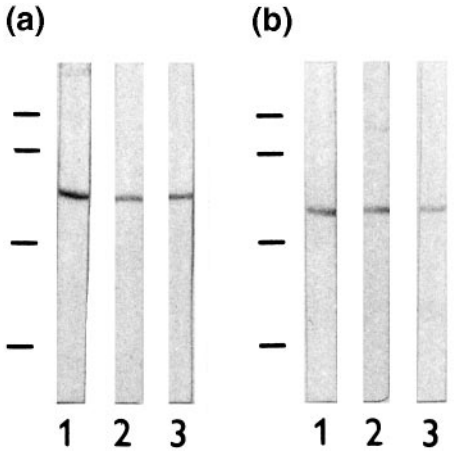


Figure 1. Immunoblot analysis of cell extracts from root tip meristems with monoclonal antibody TU-32 against γ-tubulin and monoclonal antibody DM1A against α-tubulin.
(a) Immunoblot with TU-32 antibody; (b) immunoblot with DM1A antibody. Lane 1, *Medicago sativa*; lane 2, *Vicia faba*; lane 3, *Zea mays*. Bars on the left indicate the positions of specific molecular mass markers (97.4, 67, 45 and 29 kDa).

as well as the affinity-purified polyclonal anti γ-tubulin antibody reacted with proteins of relative electrophoretic mobility corresponding to an M_r of approximatley 58 000 (Figure 1).

In immunofluorescence experiments performed on cells during the cell cycle, the anti γ- tubulin antibodies specifically decorated microtubule structures. The immunostaining with TU-32 antibody was prevented by preabsorbing the antibody with the 16-amino-acid EYHAATRPDYISWGTQ peptide used for immunization; this peptide corresponds to human γ-tubulin sequence 434–449 (Zheng *et al.*, 1991). Moreover, the immunostaining was not prevented when the 16-amino-acid peptide EEFATEGTDRKDVFFY corresponding to the human γ-tubulin sequence 38–53 (Zheng *et al.*, 1991) or purified porcine brain tubulin were used for preabsorption (not shown).

The staining pattern of mitotic cells was similar to that obtained with anti γ-tubulin polyclonal antibodies (for example Liu *et al.*, 1993). The only difference observed was a different staining of kinetochore microtubule fibres. Both standard fluorescence microscopy and confocal laser microscopy (CLMS) analyses showed that the signal for γ-tubulin was localized along the whole length of kinetochore fibres, including the close vicinity of kinetochores (Figure 2a). Additionally, analysis of thin optical sections (about 0.2 μm) using a co-localization program confirmed the spatial nearness of γ-tubulin and DNA (blue pixels in Figure 2b).

Localization of γ-tubulin in cells treated with antimicrotubule drugs

In mitotic meristem cells treated with amiprophos-methyl (APM), oryzalin or colchicine, remnants of kinetochore fibres (kinetochore stubs) remained attached to chromosomes as demonstrated for APM in Figure 3(a–c). Triple-label staining with monoclonal anti-γ-tubulin antibody, polyclonal antibody against αβ-tubulin heterodimer and DNA-binding dye revealed that γ-tubulin was located on these remnants (Figure 3b). When mitotic cells were released from the treatment with antimicrotubular drugs, microtubules regrew from kinetochore stubs and γ-tubulin was distributed along their whole length (Figure 3d–f). Analysis of the co-localization of γ-tubulin and DNA by CLSM using a co-localization program confirmed the presence of γ-tubulin within the kinetochore region of chromosomes (Figure 2c,d). γ-Tubulin signal associated with kinetochores and kinetochore stubs was not significantly reduced after treatment with high-salt extraction buffer (not shown).

It has been shown previously that MPM-2 antibody, recognizing a phosphoprotein epitope, is a good marker of kinetochore/centromeric regions of chromosomes in higher plant cells (Binarová *et al.*, 1994). Triple-label staining with polyclonal anti-γ-tubulin antibody, MPM-2 antibody and DNA-binding dye, showed that whereas the γ-tubulin was associated with kinetochores and with microtubule kinetochore stubs, the MPM-2 antigen was found only in the kinetochore/centromeric region of chromosomes (Figure 3g–i).

Localization of γ-tubulin on isolated chromosomes

After labelling with monoclonal antibody against α-tubulin or with polyclonal antibodies against αβ-tubulin heterodimer, the immunofluorescence signal was observed in the kinetochore/centromeric region of chromosomes isolated from *V. faba* mitotic cells (Figure 4a). Polyclonal as well as monoclonal antibodies against γ-tubulin stained the same regions of isolated chromosomes. The antibodies decorated sister kinetochores on all chromosomes as documented by CLSM analysis of double-label staining with antibody TU-32 and DNA-binding dye (Figure 4b). Polyclonal antibodies against αβ-tubulin heterodimer as well as monoclonal antibodies to α-tubulin stained relatively large areas of various shapes in kinetochore regions, whereas the staining with γ-tubulin antibodies occupied smaller regions. Nevertheless, both α- and γ-tubulin were distributed more on the outer face of kinetochores. Confocal analysis using a co-localization program showed localization throughout the kinetochore/centromeric region of chromosome for both α-and γ-tubulin (inserts in Figure 4a,b). α-Tubulin was often bound both on the corona of the two sister kinetochores and between the kinetochores in the centromere region (not shown). Staining of the kinetochore/centromeric region with MPM-2 antibody is shown in Figure 4(c). A more centromeric distribution of the phosphoepitope recognized by MPM-2

compared to the predominantly kinetochore distribution of α- and γ-tubulin was also confirmed with the co-localization program (insert in Figure 4c).

Similar staining patterns for α- and γ-tubulin were observed when isolated chromosomes were extracted with high-salt buffer. Localization of γ-tubulin to the kinetochore/centromeric region of isolated chromosomes was also found in other plant species (*Hordeum vulgare* and *Pisum sativum*, data not shown). The antibodies against abundant plant proteins such as actin and nucleolin gave no staining of kinetochores.

Discussion

Uniform labelling of kinetochore fibres with anti-γ-tubulin antibodies and the localization of γ-tubulin-immunopositive material in the immediate vicinity of kinetochores has not been reported previously (reviewed by Joshi and Palevitz, 1996). It is unlikely that differences in the staining of kinetochore fibres and kinetochores are caused by variations in fixation conditions and immunolabelling procedures, since we have applied very similar procedures. On the other hand, the antibodies used in the present study were raised against a peptide with an amino acid sequence different from that employed for preparing the antipeptide polyclonal antibodies in previous studies (for example Liu *et al.*, 1993). For immunization, we used the conserved C-terminal peptide sequence corresponding to the human γ-tubulin sequence 434–449 (Zheng *et al.*, 1991) and it is possible that these epitopes are more easily accessible to antibodies applied to fixed kinetochore fibres and kinetochores. Differential accessibility of α- and β-tubulin epitopes on polymerized microtubules has been reported (Dráber *et al.*, 1989).

Labelling of kinetochore fibres was even more accentuated after antimicrotubular drug treatment. γ-Tubulin was found on kinetochore microtubule stubs, which are known to be resistant to drug-induced depolymerization. Contrary to our observations, γ-tubulin labelling was noticeably absent in the kinetochore fibre stubs after antimicrotubular drug treatment in experiments by Liu *et al.* (1995). As depolymerization of microtubules could cause a relocalization of γ-tubulin and since the protein appears to have some affinity to polymerized tubulin, this might lead to an artifactual localization on the tubulin stubs. Data from experiments with antimicrotubule drug treatment must therefore be interpreted with some caution. On the other hand, immunolocalization of the major kinetochore/centromere-associated proteins, such as centromeric protein (CENP-E) (Brown *et al.*, 1996) or MCAK (Wordeman and Mitchinson, 1995) and many others, was performed on chromosomes isolated from cells which had been arrested in mitosis by antimicrotubular drugs, as in our experiments. It seems unlikely that the accumulation of γ-tubulin in the kinetochore region reflects only a non-specific association of the soluble pool of protein, because the γ-tubulin staining of kinetochores was not significantly reduced after extraction with high-salt buffer, which was used to confirm the association of γ-tubulin with centrosomes in experiments by Moudjou *et al.* (1991).

Data on self-organization of plant microtubules and microtubule converging centres (Smirnova and Bajer, 1992, 1994) as well as data on differences between animal and acentriolar plant cells with regard to stage-specific forces that act along the chromosomes (Khodjakov *et al.*, 1996) appear to indicate a certain uniqueness of plant microtubules and plant acentriolar spindle organization. It is highly probable that kinetochores in plants have autonomous and specific mechanisms of microtubule assembly and anchoring of microtubules in the absence of focused polar activity. In spite of their important role in organizing the kinetochore fibres in higher plants, it is still not clear whether kinetochores nucleate microtubule assembly and/or capture the already formed microtubules (Baskin and Cande, 1990; Brinkley *et al.*, 1992). Positive staining of the kinetochore/centromeric region in plants was obtained with CREST autoantibodies (for example Houben *et al.*, 1995), monoclonal antibody 6C6 (Schmit *et al.*, 1994) and monoclonal antibody MPM-2 (Binarová *et al.*, 1994). All these antibodies react in animals with centrosomes. The presence of these proteins both in plant kinetochores and animal centrosomes indicates that some components of microtubule nucleation machinery are evolutionarily conserved and may have a similar function in various structures. The present data show that γ-tubulin, another ubiquitous component of mitotic centrosomes, is also present in plants in the kinetochore region.

Recent data on an important role of γ-tubulin in γ-tubulin mutants of acentriolar *Drosophila* cells (Tavosanis *et al.*, 1997) support the hypothesis that the difference in microtubule-organizing centres of centriolar and acentriolar cells is not primarily in their composition but rather in their structure, shape and subcellular localization. This is consistent with the arguments of Mazia (1984) whose hypothesis of dispersed microtubule-organizing centres is often discussed in the context of plant cells lacking centrosomes (Lambert, 1993). There are no γ-tubulin mutants known in higher plant cells to provide direct evidence of the role of γ-tubulin in spindle organization, but the data presented here indicate that γ-tubulin could be directly or indirectly involved in chromosome-mediated organization of microtubules in plant cells.

Experimental procedures

Plant material and treatment of roots

Seeds of *Vicia faba*, *Zea mays* and *Medicago sativa* were germinated at 25°C in Hoagland solution. After 3–5 days, 1–2 mm long

Figure 2

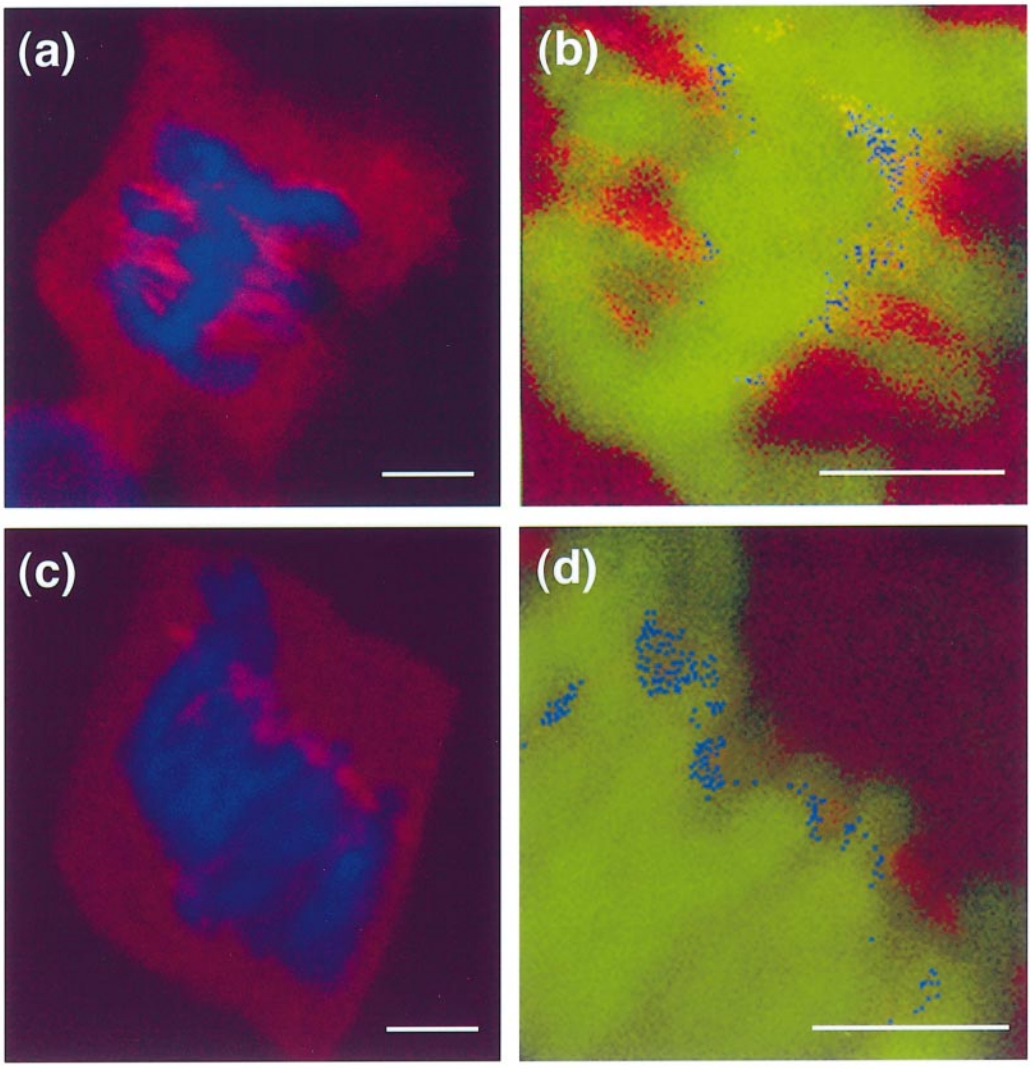
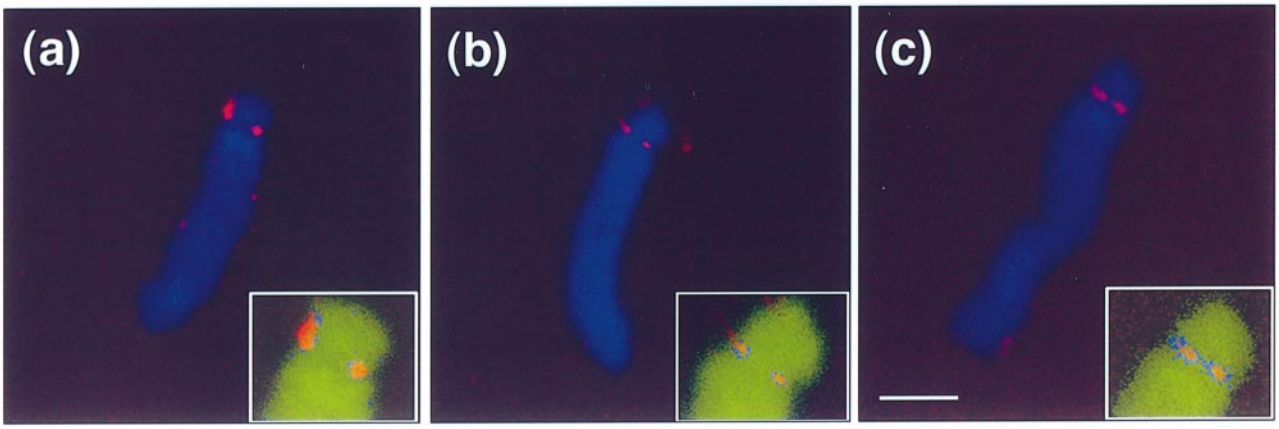


Figure 4



root tips were harvested for immunofluorescence staining and for immunoblotting. Details of culture conditions and synchronization have been described by Dolezel *et al.* (1992). The anti-microtubule drugs amiprophos-methyl (APM) and oryzalin (Mobay Co., Kansas City, Kansas, USA) were dissolved in DMSO and colchicine (Sigma Chemical Co., St Louis, Missouri, USA) was dissolved in water to obtain 20 mM stock solutions. Roots were treated with 5 μM APM or 10 μM of oryzaline or colchicine for 2 h.

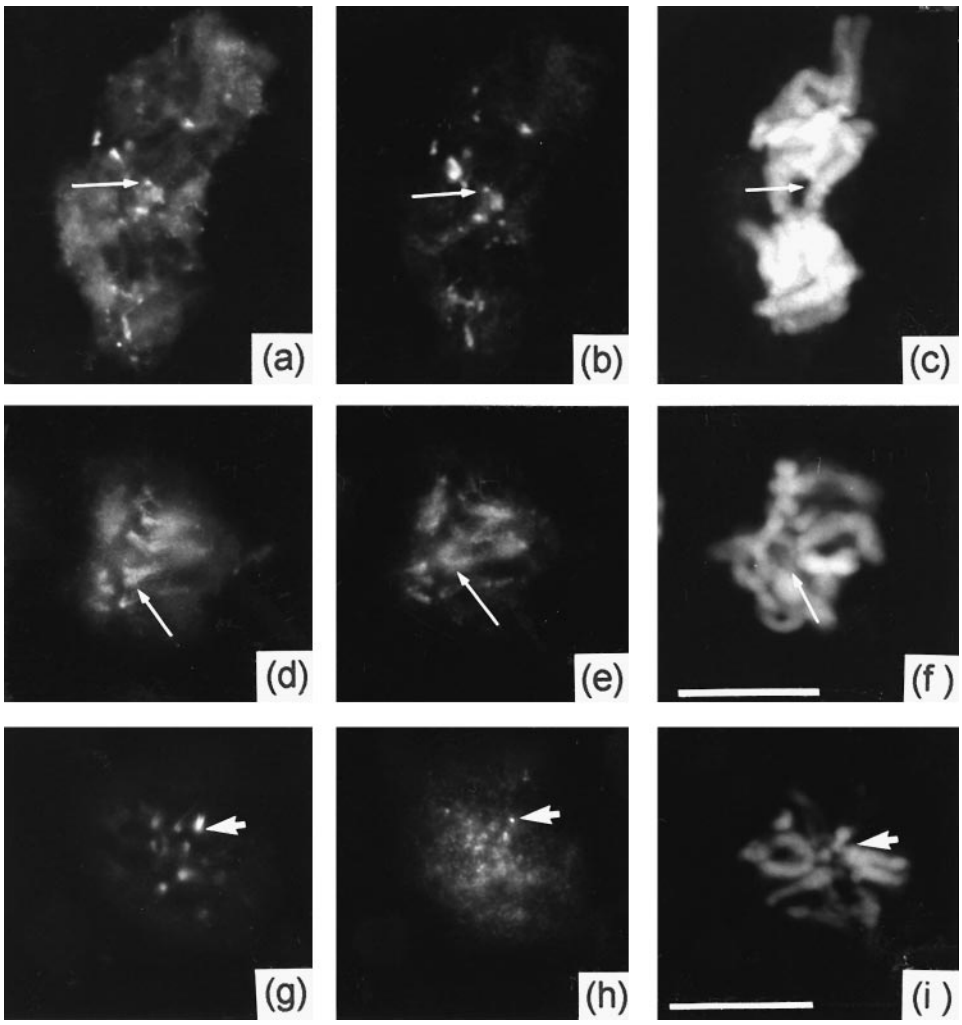


Figure 3. Distribution of γ-tubulin in APM-treated cells of *Vicia faba*.
Cells were stained with polyclonal antibody against αβ-tubulin heterodimer (a,d), TU-32 antibody against γ-tubulin (b,e,g), MPM-2 antibody (h) and DNA-binding dye (c,f,i).
(a–c) Cells treated with APM and fixed in the presence of the drug. Note γ-tubulin labelling associated with kinetochores and kinetochore microtubule stubs (b).
(d–f) Cells recovered for 60 min from APM treatment. Note co-localization of γ-tubulin with regrowing kinetochore microtubules.
(g–i) Distribution of γ-tubulin and MPM-2 antigen. Note that MPM-2 signal is associated with the kinetochore/centromeric region and absent in microtubule stubs, while γ-tubulin staining is associated with kinetochore microtubule stubs. Arrows indicate the same positions in each group of three. Bar represents 12 μm for all micrographs.

Figure 2. CSLM images of γ-tubulin and DNA staining obtained by optical sectioning and progression through the co-localization program in control cells (a, b) and APM-treated cells (c, d).
(a,c) Optical sections of about 0.2 μm thickness exhibit γ-tubulin labelling (red) and DNA staining (blue).
(b,d) Images obtained by the co-localization program show staining of γ-tubulin in red and staining of DNA in green. All pixels with the same intensity of red and green colour are expressed in blue. All bars represent 5 μm.

Figure 4. CLSM images of chromosomes isolated from *V. faba* meristem cells and labelled with antibodies against α-tubulin (a), γ-tubulin (b) or with MPM-2 (c). Inserts contain twofold magnifications of the same pictures which were processed by the co-localization program (for colours see Figure 2). The bar represents 10 μm for all micrographs.

Antibodies

The γ-tubulin was detected with mouse monoclonal antibodies TU-30 (IgG2b), TU-31 (IgG2b), TU-32 (IgG1) and with rabbit affinity-purified polyclonal antibodies (Nováková *et al.*, 1996). The antibodies were prepared against a conserved 16-amino acid peptide EYHAATRPDYISWGTQ corresponding to the human γ-tubulin sequence 434–449 (Zheng *et al.*, 1991). Microtubule structures were detected with mouse monoclonal antibody DM1A (IgG1) against α-tubulin (Sigma Chemical Co.) or with rabbit affinity-purified antibody against αβ-tubulin heterodimer (Dráber *et al.*, 1991). The MPM-2 monoclonal antibody (IgG1) was from DAKO (Carpinteria, California, USA). Fluorescein isothiocyanate (FITC) and indocarbocyanate (Cy3)-conjugated anti-mouse and anti-rabbit IgG antibodies were from Sigma. Anti-mouse IgG antibody conjugated with alkaline phosphatase was purchased from Promega Biotec (Madison, Wisconsin, USA).

Isolation of chromosomes

Metaphase chromosomes were isolated from hydroxyurea synchronized *V. faba* meristem cells accumulated in metaphase after treatment with APM as described by Dolezel *et al.* (1992). Ten microlitres of the chromosome suspension were dropped onto poly-L-lysine-coated glass slides and left to dry at room temperature. Chromosomes were processed for immunofluorescence labelling experiments directly or after extraction with high-salt buffer (50 mM Tris–HCl, pH 7.4, 5 mM EDTA, 0.25 M NaCl, 0.5% Nonidet P40) for 1 h at room temperature (Moudjou *et al.*, 1991).

Electrophoresis and immunoblotting

SDS–polyacrylamide gel electrophoresis was carried out according to Laemmli (1970). Details of the immunostaining procedure using secondary antibody labelled with alkaline phosphatase are given elsewhere (Dráber *et al.*, 1988).

Immunofluorescence

Root tips were fixed for 1 h in 3.7% paraformaldehyde, digested, and squashed as described by Binarová *et al.* (1993). Thereafter, cells were post-fixed for 10 min in methanol at –20°C, and processed for immunostaining after rehydration in PBS. Isolated chromosomes attached to poly-L-lysine-coated slides were also fixed for 10 min in methanol at –20°C, and processed for immunostaining. Antibodies TU-30, TU-31 and TU-32 were used as undiluted supernatants; antibodies DM1A, TUB 2.1, MPM-2 were diluted 1:500. Rabbit affinity-purified polyclonal antibodies against γ-tubulin and rabbit affinity-purified polyclonal antibodies against αβ-tubulin heterodimer were used at dilutions of 1:5 and 1:10, respectively. For double-label immunofluorescence with rabbit polyclonal antibodies and mouse monoclonal antibodies, slides with adhering cells were first incubated with polyclonal antibodies, washed and then incubated with monoclonal antibody. Samples were then incubated simultaneously with a mixture of FITC- and Cy3-conjugated secondary antibodies. DAPI staining of DNA, mounting of slides and observation were performed as described by Binarová *et al.* (1993).

Confocal microscopy

Slides were examined with the confocal laser scanning microscope (CLSM) LSM410 invert (Zeiss, Germany) equipped with a He/Ne laser (543 nm) and a UV laser (351/364 nm, Enterprise II, Coherent, Auburn, California, USA). Pinholes were nearly closed to obtain thin optical sections (around 0.2 μm thickness) Co-localization of antibodies and DNA was analysed using the co-localization program of the LSM 410 by selecting pixels of the same intensity higher than the relative brightness of 20%. Z-series of isolated chromosomes were taken by 15 optical sections and 3D-reconstructed using the 3D software of the LSM.

Acknowledgements

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