The plant Spc98p homologue colocalizes with γ-tubulin at microtubule nucleation sites and is required for microtubule nucleation

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Summary

The molecular basis of microtubule nucleation is still not known in higher plant cells. This process is better understood in yeast and animals cells. In the yeast spindle pole body and the centrosome in animal cells, γ-tubulin small complexes and γ-tubulin ring complexes, respectively, nucleate all microtubules. In addition to γ-tubulin, Spc98p or its homologues plays an essential role. We report here the characterization of rice and *Arabidopsis* homologues of *SPC98*. Spc98p colocalizes with γ-tubulin at the nuclear surface where microtubules are nucleated on isolated tobacco nuclei and in living cells. AtSpc98p-GFP also localizes at the cell cortex. Spc98p is not associated with γ-tubulin along microtubules. These data suggest that multiple microtubule-nucleating sites are active in plant cells. Microtubule nucleation involving Spc98p-containing γ-tubulin complexes could then be conserved among all eukaryotes, despite differences in structure and spatial distribution of microtubule organizing centers.

Key words: γ-TuSCs, γ-TuRCs, *Arabidopsis*, Tobacco BY-2 cells

Introduction

The molecular mechanisms responsible for microtubule (MT) nucleation have not yet been identified in higher plants. Unlike other eukaryotic cells, no centrosome-like organelles are present to nucleate MT assembly. In animal cells, the centrosome functions as a microtubule-organizing center (MTOC), and in fungi, the spindle pole body (SPB) plays this role. MT nucleation is initiated by γ-tubulin ring complexes, or γ-TuRCs (Zheng et al., 1995), which are recruited from the cytoplasm to the MTOC and activated (Schiebel, 2000). The smallest complex unit capable of MT nucleation, the γ-tubulin small complex (γ-TuSC), was identified in yeast (Knop and Schiebel, 1997) and in *Drosophila* (Oegema et al., 1999; Gunawardane et al., 2000). The γ-TuSC, which is thought to be a γ-TuRC precursor, contains γ-tubulin, known as a universal nucleator (Oakley, 1992) and two additional proteins, spindle pole body components Spc98p and Spc97p or their homologues. These proteins are essential for the nucleation activity of the complex. *SPC98* was characterized as a dosage-dependent suppressor of a mutant of *TUB4* (Geissler et al., 1996), the *S. cerevisiae* γ-tubulin homologue (Sobel and Snyder, 1995). Spc98p interacts with γ-tubulin, and Spc97p interacts with Spc98p. Spc98p or its homologue can therefore be considered as a marker for MT nucleation complexes (Martin et al., 1998; Tassin et al., 1998).

Compared with other eukaryotes, the situation in higher plants is unique and surprising. Two major features of plant cells should be underlined. First, diverse MT arrays assemble successively, with different orientations during the cell cycle and/or developmental controls. The cortical MTs, the preprophase band and the phragmoplast are not found in other eukaryotic cells (Staiger and Lloyd, 1991; Lambert and Lloyd, 1994; Shibaoka and Nagai, 1994). It is not known whether a single MTOC or whether multiple MTOCs are involved in generating the plant MT cytoskeleton (Canaday et al., 2000; Vantard et al., 2000), and it is not clear how assembly of an acentrosomal spindle could be regulated (Marc, 1997; Vaughn and Harper, 1998).

Secondly, in higher plant cells, γ-tubulin is distributed along all MT arrays (Liu et al., 1993; Joshi and Palevitz, 1996; Endlé et al., 1997; Canaday et al., 2000). This localization is enigmatic as γ-tubulin, which is considered to be a universal nucleator in other eukaryotes (Oakley, 1992), would not be expected to nucleate along MTs. The nuclear surface is the only functionally characterized MT nucleation site in plants (Mizuno, 1993; Stoppin et al., 1994), and γ-tubulin is detected there. Our aim was to identify proteins involved in MT nucleation in higher plant cells and to use them as markers of plant MT nucleation sites.

In the present report, we have identified and characterized *SPC98* orthologues in rice (*Oryza sativa*) and *Arabidopsis thaliana*, indicating that higher plants possess γ-tubulin complex components, although no centrosome-like organelle is present. Unlike γ-tubulin, plant Spc98p is not detected along

MTs, suggesting that plant γ-tubulin may have a role that is independent of MT nucleation at these sites. We show that Spc98p and γ-tubulin colocalize at MT nucleation sites on the nuclear surface. In addition, both proteins are also found close to the cell membrane, suggesting that cortical MTs are either nucleated at these sites or stabilized by a minus-end anchorage complex as suggested for pericentriolar MTs in animal cells (Mogensen et al., 2000).

Materials and Methods

Cloning and sequencing of the plant SPC98 and fusion to GFP

To identify plant homologues of *SPC98*, database searches were done using BLASTN programs. A rice (*O. sativa*)-expressed sequence tag (EST) (GenBank accession number c26482) was detected, but sequencing showed that this EST clone was incomplete at the 5′ end. A complete *A. thaliana* gene, *PGC95* (GenBank accession number BAB09802), was identified and amplified by PCR from *A. thaliana* (ecotype Columbia) genomic DNA. To obtain clone pCK.AtSPC98::GFP, the gene was amplified by PCR using primers 1 (CCATGGAAGACGACGATCAGCAG) and 2 (CCATGGCTCCT-TTGGAATGCAATCG), which introduced *Nco*I sites at each end of the *AtSPC98* gene. The 2600 nucleotide fragment amplified was digested by *Nco*I and inserted into a pUC-based plasmid, pCK.EGFP, which is a modified version of pCK.GFPS65C. In our construct, pCK.AtSPC98::GFP, the *PGC95* initiation codon was conserved, and the GFP termination codon was replaced by GCC.

Antibody production

To generate rabbit polyclonal antibodies against plant γ-tubulin, two peptides were selected. The first was a maize peptide EDFATQGGDRKDVFFYQ, (p1), which is conserved in eukaryotes (Joshi et al., 1992), and the second, CESPDYIKWGMEDP, (p2), is a plant-specific sequence from the C-terminus. Antibody specificity was shown using extracts from tobacco BY-2 cells, mammalian 3T3 cells and from *Escherichia Coli* expressing plant γ-tubulin.

Rabbit polyclonal antibodies were raised against three rice Spc98p peptides. The first peptide, (pA), LETAIRASNAQYDDRDIL, is from the central domain, which is conserved in eukaryotes (Fig. 1, residue 625 to 647). The second peptide, (pB), DLDSIAKDYTSSLDA, is a plant-specific peptide from the C-terminus. The third peptide, (pC), FRLDFTEYYSRVSSNK, is from the C-terminal domain and is less conserved than pA or pB. All antibodies were affinity purified against the corresponding peptide.

For MT labeling, commercial mouse monoclonal antibodies against α-tubulin (Amersham RPN 356) were used. Alexa-488- and -568-labeled anti-rabbit or anti-mouse IgGs were purchased from Molecular Probes (A-11029 and A-11036).

Cell extracts and immunoblotting

A suspension of the tobacco BY-2 cell line derived from *Nicotiana tabaccum* L. cv. Bright Yellow 2 was subcultured as described by Nagata et al. (Nagata et al., 1981). Transient expression of *SPC98::GFP* was obtained after bombardment of cDNA-coated tungsten particles using a particle inflow gun. 1 μg of cDNA, mixed with 1 mg of particles in the presence of 0.75 M $CaCl_2$ and 1.5 M spermidin, was used per assay. Cells expressing Spc98p-GFP fusion proteins were collected under a fluorescence-equipped binocular microscope. Cells were centrifuged at 60 ***g*** for 3 minutes and mixed (v/v) with sample buffer (250 mM Tris, 20% glycerol, 100 mM DTT and 1.5 M SDS). After 3 minutes of sonication, the sample was boiled for 5 minutes and stored in aliquots at –20°C. Cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (Laemmli, 1970) using 0.75 or 1 mm thick mini-slab gels and an acrylamide concentration of 6.5% or 12.5%. After electrotransfer to Immobilon (Millipore) membranes, samples were probed using various antibodies. Non-specific binding sites were saturated with 5% dry milk, 1% acetylated BSA (Aurion) and 0.2% Triton X-100 in 20 mM Tris-HCl pH 7.4 for 1 hour at 37°C. Primary antibodies were diluted in the saturating buffer. Secondary horseradish-peroxidase-conjugated antibodies (Pierce) were detected using a chemiluminescence kit (Roche).

Nuclei isolation and in vitro MT nucleation assays

Nuclei were isolated from 3.5-day-old cultured tobacco BY-2 cells (Stoppin et al., 1996). BY-2 cells were grown with 1.5% sucrose for 24 hours. To avoid plastid contamination, cells were grown without sucrose for the last 12 hours before harvesting. Protoplasts were then prepared using 3% cellulase RS (Onozuka), 0.2% macerozyme R10 (Serva 28302) and 0.15% pectolyase Y23 (Seishin), 0.45 M mannitol, 8 mM $CaCl_2$ in 25 mM MES buffer pH 5.5 for 2 hours at 30°C with slow agitation. After three washes with 250 mM sucrose, 0.5 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$ in 25 mM MES pH 5.5, protoplasts were suspended in washing medium supplemented with 0.025% NP40, 10 μM leupeptin, 10 μM pepstatin, 1 mM PMSF, 1 mM DTT, 10 μg/ml aprotinin for 20 minutes at 4°C and agitated slowly. Protoplasts were then broken by passage through a 10 μm nylon mesh. Purified nuclei were centrifuged for 5 minutes at 100 ***g*** at 4°C and conserved in 50% (v/v) glycerol in liquid nitrogen.

MT nucleation assays were done (Stoppin et al., 1994; Stoppin et al., 1996) in the presence of 10^{-5} M oryzalin to avoid elongation of plant MT seeds on the nuclei. Oryzalin is a potent inhibitor of the assembly of plant MTs but does not interfere with neurotubulin assembly. The concentration of purified neurotubulin used (7 to 10 μM) was below the critical concentration for autoassembly. Isolated nuclei were observed by DIC and fluorescence microscopy.

Antibody inhibition assays

Isolated nuclei were incubated in the presence of anti-Spc98pA, pB or anti-γ-tubulin antibodies (diluted 1/50-1/200) for 20 minutes at 4°C before the nucleation assays. Incubation with anti-GFP or anti-Spc98pC antibodies, which do not crossreact with tobacco Spc98p, were used as negative controls. Competition was performed by incubation of anti-Spc98p antibodies together with their corresponding peptides for 1 hour at room temperature before the nucleation assays. Each experiment was done three times. 200 nuclei were counted in each assay and compared with controls done simultaneously using the same set of tubulin and nuclei. The proportion of nuclei unable to nucleate MTs in controls was subtracted from the inhibition measurements. This allows direct comparison between the 100% nucleation in controls and the various nucleation rates shown in inhibition assays.

Indirect immunofluorescence microscopy

Arabidopsis or BY-2 cells were fixed for 15 minutes in 3.7% formaldehyde in MT-stabilizing buffer (5 mM EGTA, 2 mM $MgCl_2$, 50 mM PIPES, pH 6.9), post-fixed for 5 minutes in cold methanol and washed three times with stabilizing buffer. Cell walls were permeabilized for 5 minutes in a 1/10 dilution of the enzyme mixture used for protoplast isolation and incubated overnight with anti-γ-tubulin (1/1000 in PBS: 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8,1 mM Na_2HPO_4, pH 7.4) or anti-Spc98p antibodies (1/500), followed by secondary antibodies (1/300 in PBS supplemented with 0.1% acetylated bovine serum albumin from Aurion). Controls were performed both using preimmune sera and by preincubation of the antibodies with their respective corresponding peptides.

Immunofluorescence images were obtained using a Sony camera connected to Visolab 200 software adapted to a Leica DMBR microscope equipped with various filters (SP505-550 and LP560),

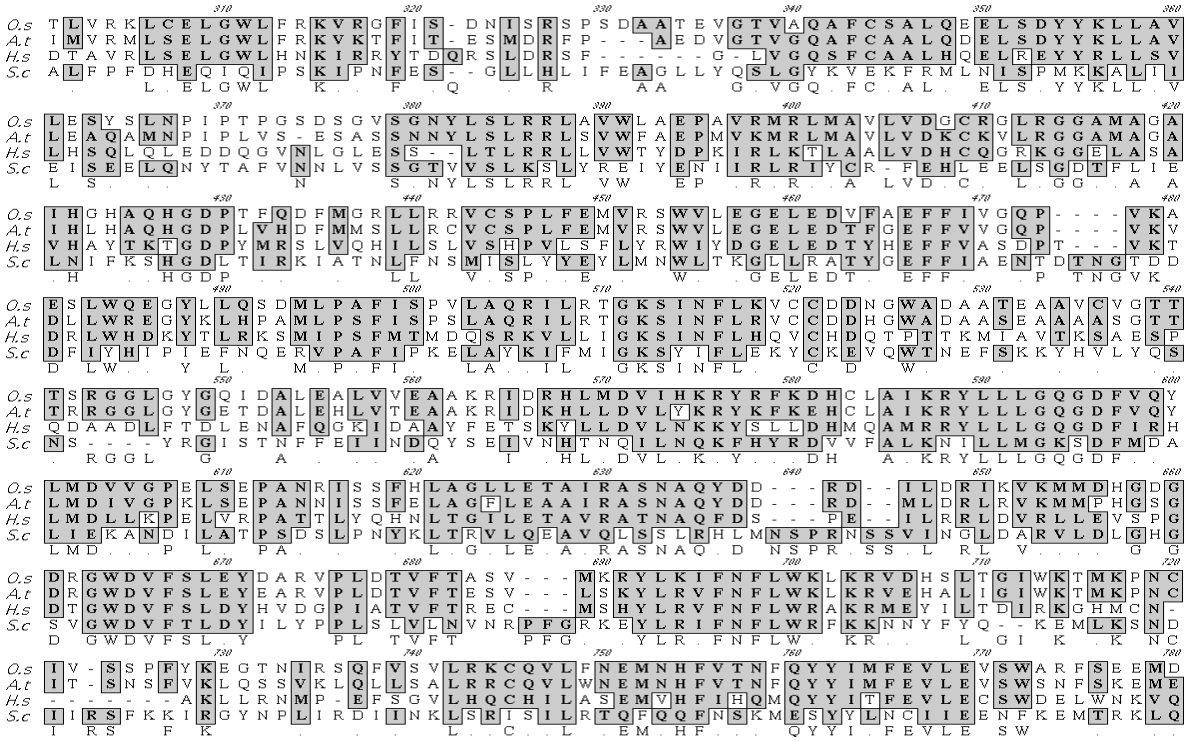

Fig. 1. Multiple sequence alignment of Spc98p homologues using CLUSTAL W (Thompson et al., 1994). Comparison of plant (Os, *Oryza sativa* and At, *Arabidopsis thaliana*) Spc98p, Hs, *Homo sapiens* GCP3/HsSpc98p and Sc, *Saccharomyces cerevisiae* Spc98p amino-acid sequences in the most conserved part of the protein. Identical and similar amino acids are shadowed, and the consensus sequence is shown on the bottom line.

which avoid crosstalk. TIFF images were treated using Adobe Photoshop software.

Microscopy imaging

Living BY-2 cells were attached to polylysine-coated glass coverslips, mounted in a perfusion chamber and perfused for 15 to 30 minutes with culture medium in the presence or absence of 0.2 M mannitol. Cells were monitored during plasmolysis and recovery. Distribution of AtSpc98p-GFP fusion protein in living or fixed cells was analysed using a Zeiss LSM 510 Confocal Laser Scanning Microscope equipped with argon and helium/neon lasers. Pinholes were adjusted to obtain 0.4 μm optical sections. Projections of serial optical sections using Z-series of 0.4 μm intervals were obtained using the LSM software. Optical planes were scanned with the 488 nm ray of the Argon laser and using a 505-550 nm barrier filter to detect GFP fluorescence. For dual imaging of AtSpc98p-GFP and Alexa-568-labeled MTs, the 543 nm ray of the helium/neon laser was used in a multitrack configuration to avoid crosstalk. A dichroic mirror at 545 nm separated GFP (short pass filter, 505-545 nm) and Alexa (long pass filter >560 nm) channels. Time-lapse series of the same confocal plane were taken during 30 minutes with 10 second intervals. LSM images were converted into TIFFs for treatment with Adobe Photoshop software. A Zeiss microscope equipped for conventional fluorescence microscopy was used to capture the corresponding Dapi images using a 3CCD digital camera (Axiocam) associated with Axiovision software.

Results

Cloning plant *SPC98* homologues

O. sativa EST c26482 was partially sequenced, and the 630 amino-acid C-terminal sequence was compared using the BLAST program to the NCBI database, which revealed homology with *A. thaliana* and Spc98p-like proteins. For *A. thaliana*, a unique mRNA of 2514 nucleotides obtained by splicing of an intron of 116 nucleotides codes for a presumptive 95 kDa protein we called AtSpc98p. Analysis by the CLUSTAL W program showed that plant Spc98p-like proteins (*A. thaliana* and *O. sativa*) show homology to γ-tubulin-interacting proteins from other organisms (*Homo sapiens*, *Xenopus laevis*, *Drosophila melanogaster* and *Saccharomyces cerevisiae*). The 95 kDa AtSpc98p shows 64.5% similarity to human GCP3 and 57% similarity to yeast Spc98. The multiple sequence alignment (Fig. 1) shows that the region from position 319 to 809 is conserved in different species (45% similarity), whereas the N-terminal region is divergent (24% similarity).

Spc98p and γ-tubulin antibodies recognize specific bands by immunoblotting

Nicotiana tabaccum L. cv. Bright Yellow 2 (BY-2) culture cells were used for transient expression of Spc98p-GFP. Protein extracts were prepared from wild-type and bombarded populations of cells. Immunoblots were probed with antibodies directed against α, β and γ-tubulin, and polypeptides of the expected sizes (50 to 55 kDa) were detected (Fig. 2A, lane 1-3). Monoclonal anti-GFP antibodies labeled the 125 kDa Spc98p-GFP fusion protein (Fig. 2A, lane 4). Free GFP was not detected, indicating the absence of proteolysis in Spc98p-GFP cells. Antibodies were raised against three rice peptides (pA, pB and pC) and used to probe

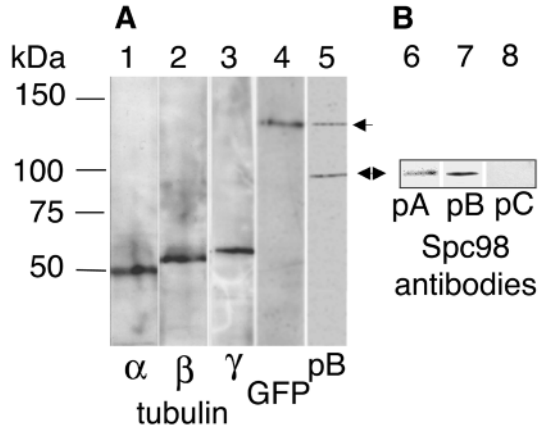

Fig. 2. Immunoblot analysis of tobacco BY-2 cell extracts. (A) Extracts from cells expressing the Spc98p-GFP fusion protein. Lane 1, anti-α-tubulin (1/5000); lane 2, anti-β-tubulin (1/5000); lane 3, anti-γ-tubulin (1/1000), which does not crossreact with α- or with β-tubulin; lane 4, anti-GFP (1/2000) reveals one band at the expected size of the fusion protein; lane 5, anti-Spc98pB (1/1000) reveals the endogenous Spc98p (95 kDa, double arrow) as well as the fusion protein (125 kDa, arrow). (B) Lanes 6 to 8, affinity-purified antibodies directed against three Spc98 peptides (pA, pB and pC) were tested on control BY-2 extracts; best results were obtained using the pB peptide (DLDSIAKDYTSSLDA), a plant/animal consensus sequence. Antibodies directed against the C-terminal peptide pC did not crossreact with BY-2 extracts.

BY-2 cell extracts (Fig. 2B) and extracts from cells expressing Spc98p-GFP (Fig. 2A, lane 5). Anti-Spc98pB labeled the 125 kDa Spc98p-GFP fusion protein and revealed endogenous tobacco Spc98p (Fig. 2A, lane 5; Fig. 2B, lanes 7), as did the anti-Spc98pA antibodies (Fig. 2B, lanes 6). The third one, anti-Spc98pC, did not crossreact with the tobacco Spc98p orthologue and was used as negative control (Fig. 2B, lanes 8). Anti-Spc98pA labels centrosomes in human HEP cells (data not shown).

An Spc98p homologue is co-distributed with γ-tubulin at the nuclear surface

To investigate the role of plant Spc98p, we used a functional assay for MT nucleation. Isolated tobacco BY-2 nuclei were incubated with purified neurotubulin, which was below the critical concentration for MT autoassembly in the absence of stabilizing agents such as taxol. Oryzalin, a specific inhibitor of plant MT assembly, was used to prevent elongation from MT seeds, which could be present on the nuclear surface after nuclei isolation. Neurotubulin assembly is not affected by oryzalin at the concentration used. In our in vitro assay, MTs

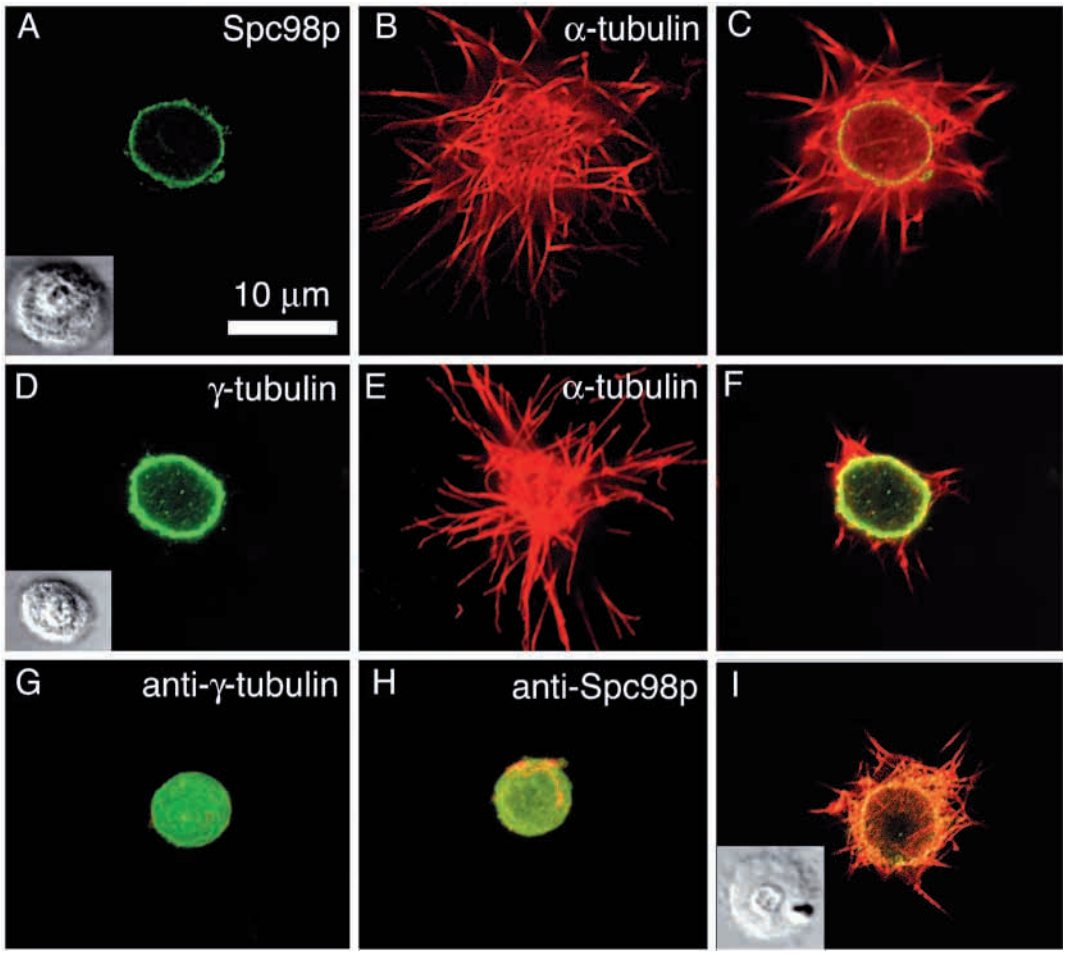

Fig. 3. Plant Spc98p and γ-tubulin localization at the surface of isolated tobacco BY-2 nuclei after in vitro MT nucleation. (A) Mid-section of a confocal z-stack labeled with anti-Spc98pB/Alexa 488. (B) Top section at the surface of the same nucleus labeled with anti-α-tubulin/Alexa 568. (C) Merged mid-section showing the perinuclear sites of MT nucleation. (D) Mid-section of a nucleus revealed with anti-γ-tubulin/Alexa 488. (E) Top-section at the surface of the same nucleus labeled with anti-α-tubulin/Alexa568. (F) Merged mid-section using a Zeiss LSM 510 microscope. Insets, Nomarski images of the isolated nuclei. (G) Inhibition of MT nucleation by preincubation with anti-γ-tubulin antibodies. Merged projection of an entire nucleus. No MTs are polymerized. (H) Inhibition by anti-Spc98pB antibodies: a few short MT remnants are observed at the surface of the nucleus in this merged projection, but no MT nucleation was observed. (I) Following a competition assay for 1 hour with preincubation using anti-Spc98pB antibodies and Spc98pB peptide, nucleation was not inhibited. Bar, 10 μm.

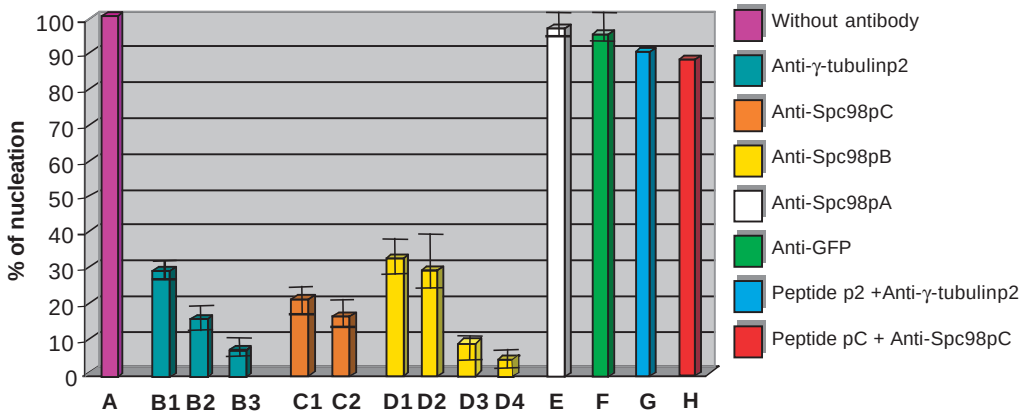

Fig. 4. In vitro MT nucleation activity on BY-2 nuclei in control and inhibition assays. (A) Without the antibody, the relative nucleation activity was 100%, and this was compared with the observed after incubation in the presence of anti-γ-tubulin 1/200 (B_1), 1/100 (B_2), 1/50 (B_3); anti-Spc98pA 1/200 (C_1), 1/50 (C_2); anti-Spc98pB 1/200 (D_1), 1/50 for 20 minutes (D_2), 40 minutes (D_3), 60 minutes (D_4); non-specific anti-Spc98pC 1/200 (E) and anti-GFP 1/200 (F). A competition assay incubating anti-Spc98pA 1/200 with the corresponding peptide 1μM for 1 hour before nucleation is shown (G). 70 to 95% inhibition of MT nucleation was observed using specific antibodies, indicating that both Spc98p and γ-tubulin are directly involved in MT nucleation.

are specifically nucleated on the surface of plant nuclei and form a sun-like MT pattern (Fig. 3B,C,E,F). The average nucleation efficiency reached 68%. MT-nucleated nuclei were labeled with the same antibodies used for immunoblots, either polyclonal antibodies raised against plant γ-tubulin or a mixture of anti-Spc98pA and pB. MTs were labeled simultaneously using commercial monoclonal anti-α-tubulin. Optical sections obtained by laser scanning microscopy showed that the plant Spc98p homologue (Fig. 3A) as well as γ-tubulin (Fig. 3D) are distributed over the entire surface of the plant nucleus. Both proteins are found at sites where MTs are nucleated, showing that they remain at the nuclear surface during nuclei isolation and that their MT nucleation activity is conserved (Fig. 3C,F). However, Spc98p and γ-tubulin immunolabeling is decreased by treating nuclei with detergent or salt (data not shown). When isolated nuclei were preincubated with antibodies against γ-tubulin (Fig. 3G) or against Spc98pB (Fig. 3H,I), sun-like nucleated nuclei decreased dramatically, showing 63 to 96% inhibition (Fig. 4). Each inhibition assay was simultaneously performed, without antibodies, using the same batch of purified brain tubulin and isolated tobacco nuclei. 200 nuclei were analysed in each experiment, and the proportion of nucleated nuclei was compared with the corresponding controls in which inactive nuclei were subtracted from active ones. Thus, the control nucleation rate was adjusted to 100%, and nucleation inhibition appeared as a decrease in the nucleation rate.

Increasing antibody concentration proportionally decreased the nucleation capacity. 70 to 93% inhibition was observed using γ-tubulin antibodies, and 80 to 85% inhibition was observed using Spc98pA antibodies. Increasing the time of incubation of antibodies before nucleation assays also enhanced the inhibition of MT nucleation, which varied from 72% after 20 minutes of incubation to 96% after 60 minutes. Controls using either non-crossreactive anti-Spc98pC antibody or an anti-GFP antibody at comparable levels of concentration and time of incubation did not significantly affect the nucleation process.

The plant homologue of Spc98p is localized at MT nucleation sites in situ but is not co-distributed with γ-tubulin along plant MTs

To determine whether the plant Spc98p is located at MT nucleation sites, we labeled tobacco BY-2 and cultured *A. thaliana* cells at different stages of the cell cycle with anti-Spc98p, anti-γ-tubulin and anti-α-tubulin antibodies, as shown for BY-2 cells in Fig. 5. Both anti-Spc98p (Fig. 5A) and anti-γ-tubulin (Fig. 5C) densely label the nuclear surface that functions as a MT nucleation site in plants. The anti-Spc98p antibody gives some punctate cytoplasmic staining. Neither preimmune sera nor antibody depletion by peptide competition produced such labeling.

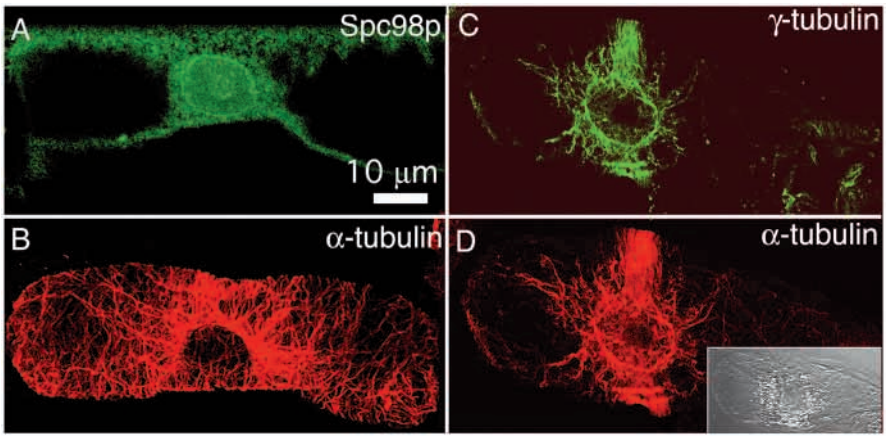

Fig. 5. Distribution of plant Spc98p, γ-tubulin and MTs in G2 tobacco BY-2 cells. Immunolocalization with (A) anti-Spc98p/Alexa 488 and (B) anti-α-tubulin/Alexa 568 in the same cell. In (A), a slightly oblique cut through the z-stack clearly shows the nucleus (n), vacuole (v) and surrounding cytoplasm. In (B), the projection of three confocal views shows the preprophase band in formation. Immunolocalization with (C) anti-γ-tubulin/Alexa 488 and (D) anti-α-tubulin/Alexa 568 in the same cell. DIC image of the cell (D, inset). Both Spc98p and γ-tubulin accumulate densely at the nuclear surface. Spc98p is not present along MTs, whereas γ-tubulin is detected. Spc98p spots are also present inside the nucleus, suggesting import of the protein. Bar, 10 μm.

γ-tubulin was found at the nuclear surface and along all MT arrays: nuclear-associated MTs, cortical MTs and the preprophase band (Fig. 5C,D). Spc98p is not co-distributed with the γ-tubulin associated with MTs. The same results were obtained in both tobacco and *Arabidopsis* cells.

AtSpc98p-GFP in vivo localization

The *AtSPC98::GFP* fusion construct was introduced by bombarding tobacco BY-2 cells or by electroporating protoplasts. In both BY-2 cells and protoplasts, the fusion protein was found mainly on the nuclear surface (Fig. 6C), whereas, when unfused GFP is transiently expressed in BY-2 cells, a diffuse distribution is detected in both the cytoplasm and the nucleus (Fig. 6A). In addition to perinuclear labeling, AtSpc98p-GFP was regularly distributed in the cortical cytoplasm, close to the plasma membrane (Fig. 6C,G-L). These cortical labelings were particularly visible in elongated cells where the cortical MTs are well organized. To determine whether the cortical labelling is linked to the plasma membrane, we followed the movement of fluorescent fusion proteins. Time-lapse images were taken every 10 seconds for 30 minutes on turgescent cells. Then, cells were plasmolyzed for 15 minutes, and recovery was analysed during the following 15 minutes. The GFP fluorescent signals moved slightly during plasmolysis within the focal area and followed the speed of membrane displacement during the 6 minutes necessary for turgescence recovery, as shown by the increasing distance between fluorescent dots marked by an arrow and an arrowhead (Fig. 6G-M). Some of the GFP signals observed using confocal microscopy coincide with ends of MT bundles as observed after immunolabeling (Fig. 6M,N). These data suggest that Spc98p is linked to the plasma membrane and localized at sites that could be involved in cortical MT nucleation and/or anchoring.

The subcellular distribution of Spc98p-GFP confirms the results obtained by immunolabeling, although some fusion protein aggregates were observed when the protein was overexpressed. Using both techniques, a punctate labeling is observed at the nuclear surface and in the cytoplasm. The cortical labeling at the plasma membrane is clearer when the Spc98p-GFP fusion protein is expressed than when immunolabeled, which is mainly because of membrane permeabilization in the latter case.

Discussion

Plant MT nucleation and γ-tubulin containing complexes: identification of Spc98p homologues

We show that there are plant homologues of yeast Spc98, a

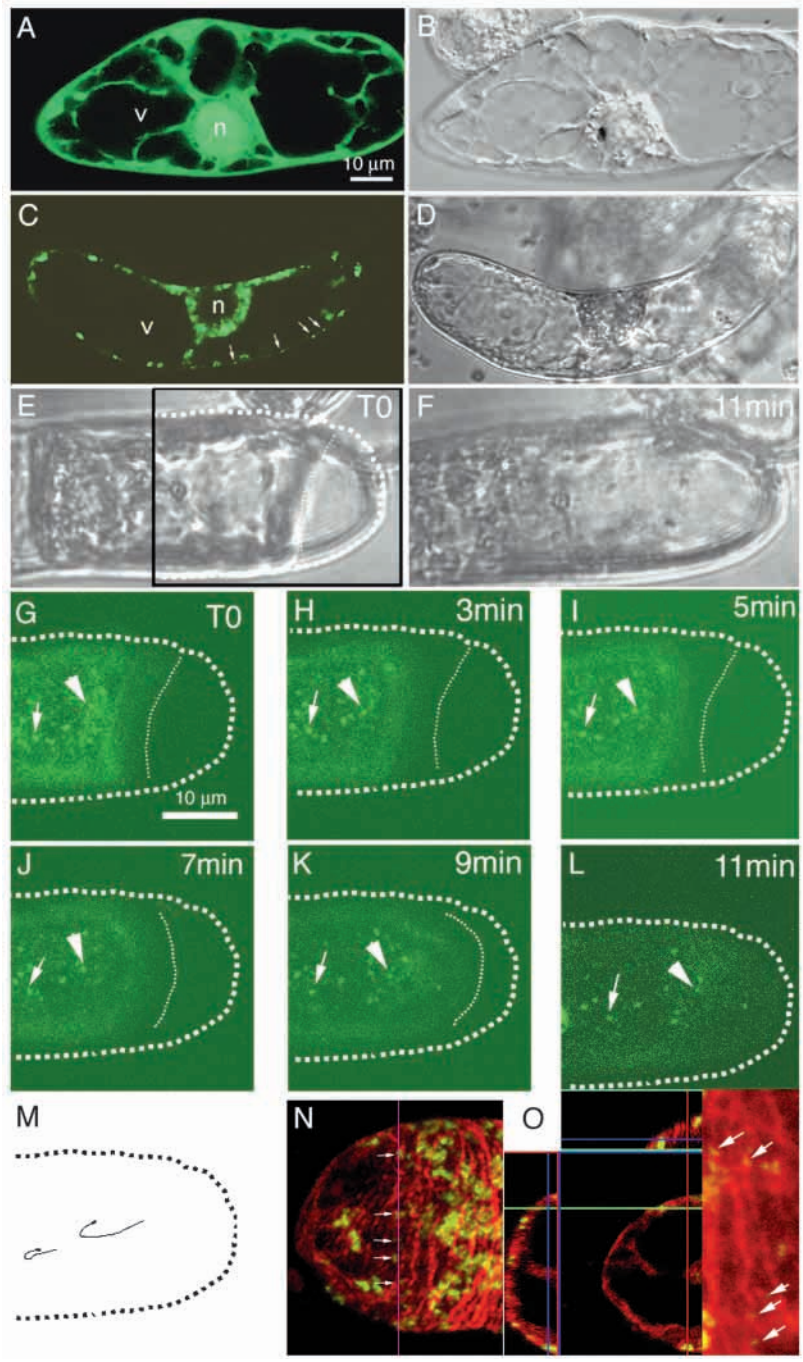

Fig. 6. GFP and Spc98p-GFP fusion protein in live tobacco BY-2 cells observed in confocal microscopy. (A) GFP, detected by fluorescence, is diffuse in the cytoplasm and the nucleus. (B) DIC microscopy of the same cell. The Spc98p-GFP fusion protein (C) is detected on the nuclear surface and as regularly spaced cortical signals close to the plasma membrane (arrows). (D) DIC microscopy of the same cell. (E,F) Plasmolysis recovery in DIC microscopy. (G-L) The same cell expressing Spc98p-GFP fusion protein. The fluorescent signals observed using time-lapse microscopy in the cortical area detailed in E, close to the plasma membrane shows slight displacements. During recovery (J-L), the fluorescent signals moved according to the plasma membrane movements, indicating a close relationship. (Large dotted line, cell wall; small dotted line, edge of the plasma membrane). (M) Movements of both GFP dots labelled by an arrow and an arrowhead from G to L are drawn. (N,O) Microtubule immunolabeling in a cell expressing Spc98p-GFP. The reconstructed cross-section shows that GFP fluorescence localizes at microtubule bundle ends. n, nucleus. v, vacuole. Bar, 10 μm.

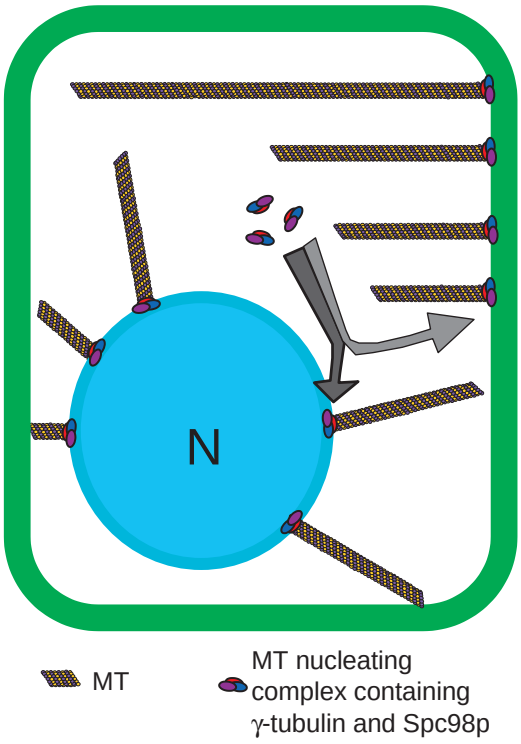

Fig. 7. A working model of plant MT nucleation and organization. The soluble cytoplasmic γ-tubulin/Spc98p-containing complexes are recruited to two different MT nucleation sites (arrows): the plasma membrane and the nuclear surface. Recruitment and activation of these soluble complexes would be responsible for nucleation of the cortical MTs involved in cell growth and for the nucleation of the perinuclear and preprophase band MTs, which determine polarity before mitosis. The activation of MT nucleation sites and coordinated regulation of the MT assembly would be controlled by cell cycle and/or developmental signals. Such signals would enter into cell cycle check points in higher plants.

component of the γ-TuSC which is the minimal unit for MT nucleation in yeast and *Drosophila* (Knop and Schiebel, 1997; Oegema et al., 1999; Gunawardane et al., 2000).

The presence of both γ-tubulin and Spc98p suggests that plant cells contain γ-TuSC-like nucleation complexes. In addition, Spc97, another γ-TuSC complex component, has a putative homologue in the *Arabidopsis thaliana* database. Do plant cells possess larger MT nucleating complexes, such as the γ-TuRCs (Murphy et al., 1998; Jeng and Stearns, 1999; Wiese and Zheng, 1999; Moritz and Agard, 2001), found in other eukaryotes? Large soluble γ-tubulin-containing complexes were identified in plants (Stoppin-Mellet et al., 2000), but their activity has not yet been characterized. Further experiments will be necessary to isolate and characterize γ-tubulin complexes containing plant Spc97p and Spc98p and to determine whether additional plant-specific proteins are present in these nucleation complexes.

Colocalization of Spc98p and γ-tubulin at MT nucleation sites on isolated nuclei

The colocalization of Spc98p and γ-tubulin on isolated BY-2 nuclei where MT nucleation is initiated favors the hypothesis that MT nucleation complexes are present at the nuclear surface. Inhibition of MT nucleation by Spc98p and γ-tubulin antibodies argues that Spc98p/γ-tubulin-containing complexes are involved in MT nucleation. Isolated centrosomes, like plant nuclei, are capable of MT nucleation in vitro. Addition of plant cellular extracts to urea-inactivated mammalian centrosomes rescues nucleation activity (Stoppin-Mellet et al., 1999), suggesting that cytosolic γ-tubulin complexes containing Spc98p may be recruited and activated at MT nucleation sites, as in mammalian cells (Moudjou et al., 1996).

AtSpc98p-GFP in living cells: identification of cortical MT nucleation sites

AtSpc98p-GFP was detected in living tobacco BY-2 cells on the nuclear surface as expected. In addition, in G1 phase and in elongated cells, a fluorescent signal is detected close to the plasma membrane, at ends of MT bundles. γ-tubulin is also present at sites where cortical MTs contact the cell membrane (McDonald et al., 1993; Canaday et al., 2000), and perinuclear MTs do not directly form cortical arrays (Nagata et al., 1994). The co-distribution of Spc98p and γ-tubulin suggests that cortical MTs are nucleated in the cortex at the plasma membrane. A 49 kDa component of the centrosphere, identified as elongating factor E2F-1α, is also found both at the nuclear envelope and at the plasmalemma (Hasezawa and Nagata, 1993). This result is indicative of multiple MT nucleation sites, but E2F-1α could be involved in signal transduction rather than nucleation.

Cortical MTs turn over more rapidly than centrosome-nucleated MTs in animal cells, suggesting that cortical MTs are assembled by de novo nucleation (Wasteneys et al., 1993; Yuan et al., 1994; Hush et al., 1994). This cortical MT instability and the localization of AtSpc98p-GFP at sites where MT bundles start, strongly argue for nucleation at the cortex. Spc98p was not clearly immunodetected at cortical sites, but the fixation and permeabilization procedures used could affect the plasma membrane, leading to the loss of small structures such as γ-tubulin-nucleating complexes. In vivo detection using GFP fusion protein enhances the resolution of membrane nucleation sites and provides evidence to support the hypothesis that there are multiple MT nucleation sites in plant cells.

Plant Spc98p is not co-distributed with γ-tubulin along MTs

Neither AtSpc98p-GFP nor antibody-labeled endogenous Spc98p are co-distributed with γ-tubulin along MT arrays. This indicates that the γ-tubulin associated along the length of MTs has an alternative activity that may affect MT properties. Cytosol plant γ-tubulin is present in high molecular weight complexes containing Hsp70 and TCP1 chaperones (Stoppin-Mellet et al., 2000), suggesting that the different γ-tubulin complexes present in higher plant cells may have different functions. γ-tubulin may be involved in MT stabilization instead of nucleation or be maintained in a storage form before its recruitment at nucleation sites (Dibbayawan et al., 2001). Perinuclear and perhaps cortical sites where γ-tubulin and Spc98p colocalize would correspond to activatable nucleation sites.

A functional model for recruitment and activation of Spc98p/γ-tubulin complexes in higher plants

On the basis of our present data, we suggest that plant γ-tubulin/Spc98p-containing complexes are involved in nucleation of plant MTs and are functionally homologous to the γ-TuRCs found in metazoans and fungi. The localization of Spc98p-GFP in vivo in combination with the results of our MT nucleation assays leads us to propose a model for the dynamics of plant MT nucleating complexes during the cell cycle and development (Fig. 7).

Cytoplasmic MT-nucleating complexes containing γ-tubulin and plant Spc98p could be recruited to various MT nucleation sites during the cell cycle or development. Complexes situated at cortical sites could be activated during G0 and G1, that is, at stages where cortical MTs are assembled. The complexes located at perinuclear sites would be activated in G2, when MTs radiating from the nuclear surface are predominant. The pre-prophase band assembled at this stage could originate from perinuclear and/or cortical nucleation sites. In most eukaryotes, the γ-TuRC is targeted to a structured organelle such as the centrosome or the SPB, which nucleates and organizes all MTs. In higher plants, γ-TuRC-like complexes could be recruited to different sites and coordinately activated to organize the successive MT arrays.

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