

## Changes in $\beta$ -Tubulin Isoforms and their RNA Level in Synchronized Tobacco Cells

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**ABSTRACT.** Tobacco BY-2 cells were synchronized by an aphidicolin treatment, and their  $\beta$ -tubulin isoforms and their mRNA were analyzed by Western, Northern and dot blottings. The relative ratio of the  $\beta$ -tubulin isoforms changed with the progress of cell cycle stage. By Northern blot hybridization of poly(A)<sup>+</sup> RNAs with a cloned carrot  $\beta$ -tubulin cDNA probe, a single band of about 1.6 kb was detected throughout the cell cycle. Dot blot hybridization showed that  $\beta$ -tubulin mRNA existed in all stages in the cell cycle at a relatively constant level, though it accumulated slightly more than average at M phase and decreased during G1 phase.

Microtubules play a key role in cellular fundamental functions such as cell division and differentiation. The distribution and function of microtubules change with the progress of the cell cycle stage (6). In plant cells, four major types of microtubules, i.e. spindle, phragmoplast, cortical microtubules and preprophase band appear sequentially. In interphase plant cells, the orientation of cortical microtubules is related to the cell's morphological differentiation (14).

$\alpha$ - and  $\beta$ -tubulins are major subunit proteins of microtubules. Most eukaryotic cells have multiple isoforms of both tubulin subunits. Six or seven functional  $\beta$ -tubulin isotypes exist in vertebrate genomes (8). Amino acid sequences of these isotypes, even in the carboxy-terminal variable region, have been evolutionarily conserved. At least six isotypes of  $\beta$ -tubulin genes were also identified in higher plant, *Arabidopsis* (15).

If repeated cycles of the microtubule formation and its degradation are performed according to the program at the transcriptional level, the level of tubulin RNA may oscillate during cell cycle as reported for histone genes (4). The oscillation of the level of tubulin RNA during cell cycle has been reported on a slime mold and an alga. In the plasmodium of *Physarum*, microtubules

are formed only at mitosis and the cytoplasmic microtubules which usually exist in the interphase cells of other organisms are absent. The level of tubulin RNA increased at G2 to M phase and decreased immediately after the mitosis (13). In a green alga, *Chlamydomonas*, tubulin RNA also increased at M phase, though the level of increase was less than the case of *Physarum* (1).

There are no reports concerning tubulin RNA levels in the cell cycle of higher organisms. Two contradictory observations were reported concerning tubulin protein level at M phase in HeLa cells. Brave and Celis reported that tubulin level increased at M phase in synchronous cultured cells (2). On the contrary, no differences were detected between exponentially growing and mitotic enriched populations in tubulin content, in its activity of polymerization or colchicine binding, in tyrosination and in microtubule associated protein composition (3). In non-dividing carrot cells in which only cortical microtubules were observed, multiple tubulin isoforms were detected (7). All the isoforms existing in either dividing or non-dividing cell populations were assembled into microtubules both *in vitro* and *in vivo* (5, 7).

Cultured tobacco BY2 cells have been shown to be efficiently synchronized by aphidicolin treatment (10). Using this culture system, we examined the isoform composition and variation of mRNA for  $\beta$ -tubulin.

### MATERIALS AND METHODS

**Cells and cultivation.** Tobacco BY-2 cells derived from the seedling of *Nicotiana tabacum* L. cv. Bright Yellow 2 was a generous gift from Dr. T. Matsumoto of Japan Tobacco Inc. Cells were grown in a liquid medium of Murashige and

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Abbreviations used: PMg, 10 mM potassium phosphate, 1 mM MgSO<sub>4</sub>, pH 6.5; TBS-milk, 10 mM Tris-HCl, 0.5 M NaCl, 3% skimmed milk, 0.1% Nonidet P40, 0.02% sodium azide, pH 8.0; DEPC, diethylpyrocarbonate; MOPS, N-morpholinoethanesulfonic acid; MEA, 0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0; SSC, 150 mM NaCl, 15 mM sodium citrate; SSPE, 150 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4.

Skoog (9) supplemented with 3% sucrose and 1 mg/l of 2,4-D (pH 5.8). Two-milliliter aliquots were transferred to 100 ml of fresh medium in a 500 ml flask and were agitated on a reciprocal shaker at a speed of 127 strokes per minute at 26°C.

**Determination of mitotic index and labeled index.** For the measurement of mitotic index, cells from 1 ml of culture were collected by centrifugation, fixed with Carnoy's solution (EtOH:AcOH=3:1) for 5 minutes at room temperature and stained with 1% propionic orcein by heating over a small flame. Cells at mitotic phase in 2000 randomly selected cells were scored and the percentage of these cells were calculated.

For the determination of labeled index, 0.5 ml aliquots from the culture were incubated with 7.5  $\mu$ l (281 kBq.) of [<sup>3</sup>H-methyl] thymidine (NEN, specific activity; 3.06 TBq./mmol) at 27°C for 20 minutes. The cells were collected by centrifugation and fixed with Carnoy's solution. After fixation, cells were rinsed twice with 1 ml of 70% ethanol, and incubated in 1 N HCl for 30 minutes at 37°C. After rinsing twice with 1 ml of water, they were placed on a slide glass which had been coated with 0.2% gelatin. Cells on the slide glass were then coated with photographic emulsion (Konica NR-M2) and exposed for 3 days at -30°C. They were developed by Rendol (Fuji Photo Film Co.) at 20°C for 6 minutes, fixed, washed and dried. The nuclei were stained by Giemsa solution and 500 randomly selected cells were observed under a microscope. Nuclei with silver grains were scored and expressed as the percentage to the total number of the cells.

**Synchronization of the BY-2 cells.** Cells were synchronized by the method of Nagata *et al.* (10). Ten milliliters of tobacco BY-2 cells which were at the second day of the stationary phase were added to 90 ml of the fresh medium containing aphidicolin (5  $\mu$ g/ml in final) and incubated at 26°C for 24 hours. After the treatment with the drug, cells were washed quickly with 1 liter of the fresh medium on a sintered glass filter. The cells were then suspended in 100 ml of fresh medium and cultured at 26°C.

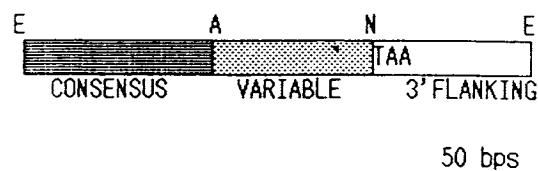
**Immunoblotting of  $\beta$ -tubulins.** An aliquot of the culture was taken periodically and cooled in an ice bath for 1 hour to promote depolymerization of microtubules. Cells were collected by suction, washed with cold saline and weighed. Two grams of the cells were frozen in liquid nitrogen and ground with 2 ml of frozen grinding buffer (1 M sucrose, 0.2 M sodium tartrate, 10 mM potassium phosphate, 1 mM MgSO<sub>4</sub>, 1.67  $\mu$ g/ml leupeptin, 5%  $\beta$ -mercaptoethanol, 15% polyvinylpyrrolidone, pH 6.5). The ground cell powder was thawed and centrifuged at 20,000  $\times$  G for 5 min. A suspension of DEAE-Sepharcel was added to the supernatant, and the suspension was washed with 0.4 M ammonium sulfate in PMg (PMg=10 mM potassium phosphate (pH 6.5) containing 1 mM MgSO<sub>4</sub>) twice and eluted with 0.8 M ammonium sulfate in PMg. The eluted proteins were precipitated with cold TCA (10% final concentration). The TCA precipitate was washed twice with acetone, suspended in lysis buffer (8.5 M urea, 2% Nonidet P-40, 2% Servalyt (pH 4-6), 2.5  $\mu$ g/ml each of leupeptin and pepstatin), and applied onto a gel for isoelectric

focusing. After focusing, proteins were blotted on a nitrocellulose filter electrically in 0.7% acetic acid and 20% methanol at 40 V for 20 min. The blotted membrane was treated with TBS-milk (10 mM Tris-HCl, 0.5 M NaCl, 3% skimmed milk, 0.1% Nonidet P40, 0.02% sodium azide, pH 8.0) at room temperature for 1 hour. After that, it was incubated with anti-chick  $\beta$ -tubulin monoclonal antibody DM1B (Amersham Japan, Tokyo) at 4°C overnight, washed with TBS-milk 5 times and incubated with peroxidase-conjugated anti-mouse Ig (G+M) for 3 hours at room temperature. The filter was processed for peroxidase-mediated color development with Immunostain (Konica, Tokyo).

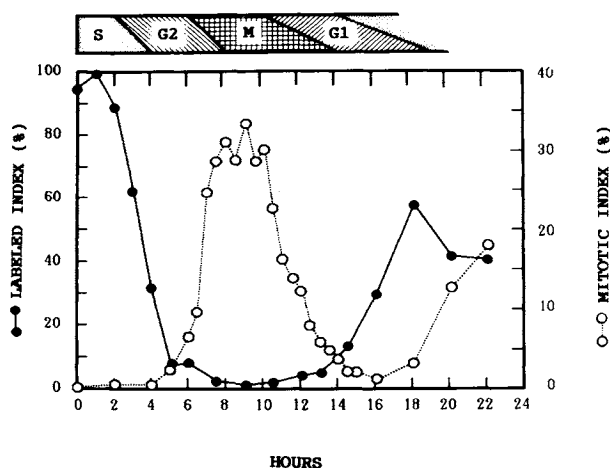
**Extraction of RNA.** Three grams of cells collected by filtration were frozen quickly in liquid nitrogen, and ground with mortar and pestle with 3 ml each of frozen phenol and extraction buffer (50 mM Tris, 1% SDS, pH 9.0). After thawing with an additional 3 ml each of phenol and extraction buffer, RNA was extracted by phenol and chloroform treatment and precipitated by adding two volumes of ethanol in the presence of 0.1 M LiCl (pH 6.5). The precipitate was resuspended in DEPC (diethylpyrocarbonate)-treated water and reprecipitated under 2 M LiCl overnight. The precipitated RNA was rinsed with 80% EtOH and used either directly for dot blot analysis or for Northern blotting, after preparing poly(A)<sup>+</sup> RNA with oligo-dT cellulose column.

**Northern blot.** Ten micrograms of poly(A)<sup>+</sup> RNAs from each time point were loaded on a 2.2 M formaldehyde-1% agarose gel and electrophoresed at 50 V for 4.5 hours in MEA (0.2 M N-morpholinoethanesulfonic acid (MOPS), 50 mM sodium acetate, 10 mM EDTA, pH 7.0). After electrophoresis, RNAs were transferred to a nitrocellulose filter and the filter was dried at 80°C for 2 hours.

**RNA dot blot.** Twenty micrograms of total RNAs were treated with denaturation solution (34(v/v)% glyoxal, 20 mM sodium phosphate buffer pH 6.5) at 50°C for 1 hour. A 1/2 dilution series of the denatured RNA suspension was made with 1% SDS. The diluted RNA suspensions were placed on a nitrocellulose filter which had been equilibrated with 20  $\times$  SSC (1  $\times$  SSC is 150 mM NaCl, 15 mM sodium citrate) and dried. The RNA was fixed on a nitrocellulose paper by heating at



**Fig. 1.** Schematic representation of the carrot cDNA, CB1 that corresponds to 168 nucleotides around the translation terminal region of a  $\beta$ -tubulin mRNA. The DNA can be separated into three fragments by the restriction enzymes, AluI (A) and NspI (N). The EcoRI (E) linkers have been ligated to both ends. The E-A fragment codes for highly conservative amino acid sequence, the A-N fragment for variable amino acids at the carboxy-terminal and the N-E fragment is the 3' flanking sequence.



**Fig. 2.** Change in the labeled index and the mitotic index of tobacco BY2 cells after aphidicolin treatment. Stage of the cell cycle is shown at the top of the figure.

80°C for 2 hours.

**Hybridization.** Filters were prehybridized for 1 hour at 30°C in 5 × SSPE (1 × SSPE is 150 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA (pH=7.4)), 1 mg/ml polyvinylpyrrolidone, 1 mg/ml Ficoll, 1 mg/ml bovine serum albumin, 50% formamide and 100 µg/ml sonicated salmon sperm DNA

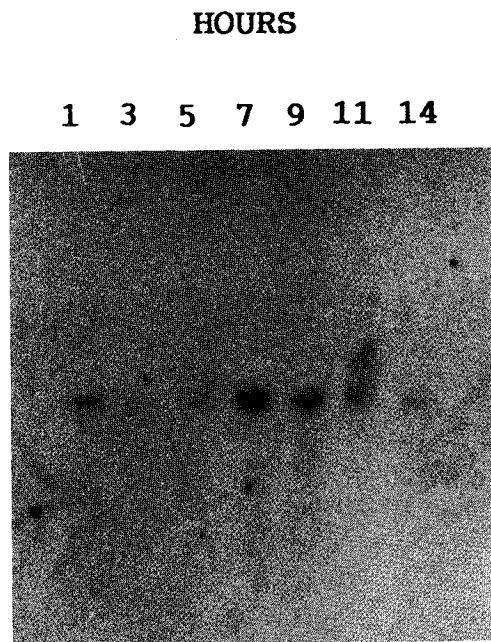
in a sealed bag. The prehybridizing solution was removed and identical solution containing denatured,  $^{32}$ P-labeled probe was added and hybridized at 30°C overnight. Filters were washed four times with agitation with 2 × SSPE, 0.1% SDS at 42°C and exposed to XAR-5 film (Eastman Kodak Co. Rochester, N.Y.) using a Lightning-Plus intensifying screen (DuPont Instruments, Wilmington, DE.).

**Preparation of  $\beta$ -tubulin and TAB20 probes.** Carrot cDNA clone, CB1 consists of three different regions, i.e. consensus, following variable amino acid codes for C-terminal region of a  $\beta$ -tubulin and 3'-flanking sequence (12) (Fig. 1).  $^{32}$ P-labeled probe was prepared by primer extension with whole CB1 sequence as a template and the larger fragment of the AluI-digested CB1 as a primer so as to predominantly synthesize radioactive antisense strand.

A randomly selected cDNA clone, TAB20, from the tobacco BY2 cDNA library was labeled with  $^{32}$ P by random primer method and used as a control probe.

## RESULTS

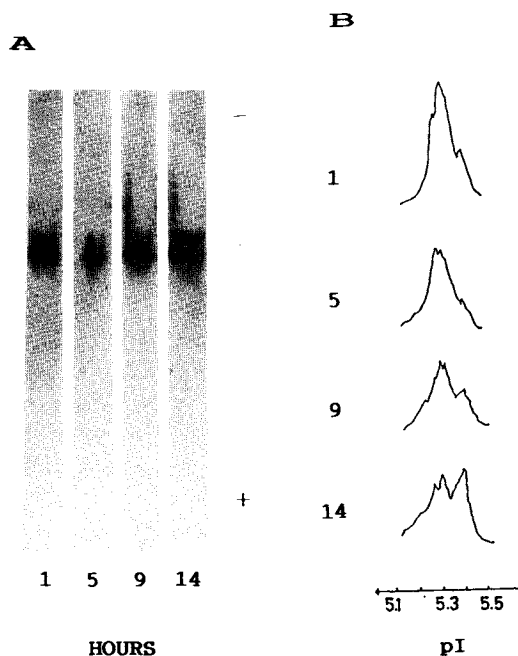
**Synchronization of tobacco BY2 cells.** Changes in the mitotic and labeled indices after the treatment of aphidicolin is shown in Fig. 2. One hour after the treatment, the first peak in the labeled index was observed. At this time 99% of the nuclei were labeled with  $^3$ H-thymidine. The labeled index declined thereafter and decreased to a very low level about 5 hours after the treatment. After that, the mitotic index began to increase



**Fig. 3.** Composition of  $\beta$ -tubulin isoforms.

**A:** Acidic proteins from the tobacco BY2 cells at 1, 5, 9 and 14 hours after the release of aphidicolin were separated by isoelectric focusing and blotted on nitrocellulose paper.  $\beta$ -tubulin isoforms were detected by DMIB.

**B:** Densitometric tracing of the bands of each lane in A.



**Fig. 4.** Northern blotting of the poly(A)<sup>+</sup>RNA from the cells at the indicated time after the release from the aphidicolin treatment. RNAs were hybridized with CB1 probe.

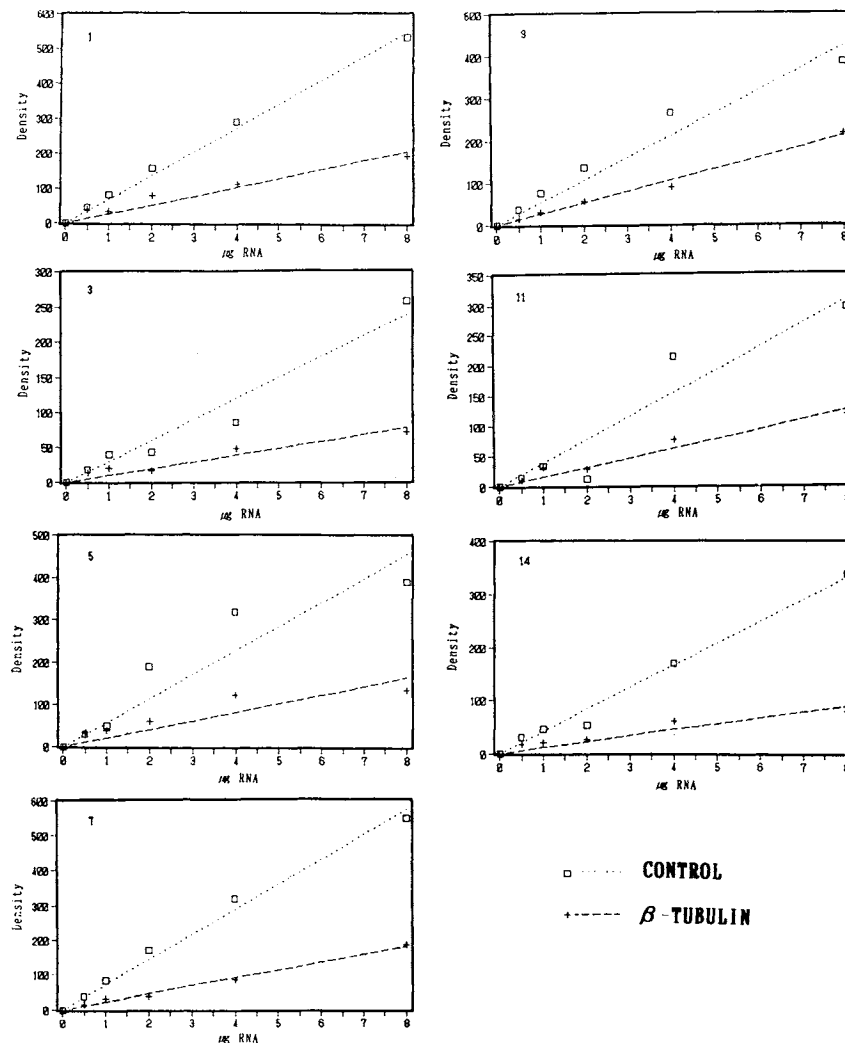
and peaked at 9 hours. It decreased to a minimum level at 14 hours and from this time, the labeled index increased again to form the second peak.

**Isoform pattern of  $\beta$ -tubulin during cell cycle.** The acidic proteins in the extract of tobacco cells harvested at 1, 5, 9 and 14 hours after the removal of aphidicolin were separated by isoelectric focusing. After blotting to nitrocellulose filter, isoforms of  $\beta$ -tubulin were detected by monoclonal antibody against  $\beta$ -tubulin, DM1B. At least three isoforms of  $\beta$ -tubulin were observed (Fig. 3). The relative ratio of these isoforms appear to change with the progress of stage of cell cycle. Change was especially evident in relative amount of the most basic isoform. This isoform was relatively poor at S (1 hr) and G2 (5 hr) phase, but increased when the cell cycle stage

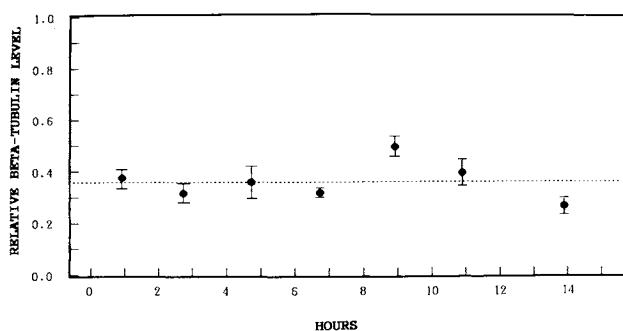
proceeded from M (9 hr) to G1 (14 hr) phase.

**Northern blot analysis of  $\beta$ -tubulin mRNAs.** In order to examine the size and heterogeneity of  $\beta$ -tubulin transcripts, poly(A)<sup>+</sup> RNAs were prepared and separated on a formalin-agarose gel. The RNAs were blotted on a nitrocellulose filter and hybridized with carrot  $\beta$ -tubulin cDNA probe, CB1 (Fig. 4). A single band of RNA, with the size of approximately 1.6 kb, was detected throughout the cell cycle. No other hybridizable RNA band was detected in any stage of cell cycle.

**Change in  $\beta$ -tubulin RNA level during the cell cycle.** RNA was extracted from the cells at 1, 3, 5, 7, 9, 11 and 14 hours after the removal of aphidicolin. A series of dilutions of the denatured RNA was fixed on a nitrocellulose filter and hybridized with the CB1 probe. After the



**Fig. 5.** Dot blot hybridization analysis of RNAs isolated 1, 3, 5, 9, 11 and 14 hours after the release of aphidicolin treatment. CB1 probe was hybridized to various amounts of total RNA dotted on a nitrocellulose filter and autoradiographed ( $\beta$ -tubulin). After the CB1 probe was thoroughly washed out, the filter was rehybridized with TAB20 and autoradiographed (control). The density of each dot was measured by a densitometer and plotted. The regression lines were obtained by the least-squares method.



**Fig. 6.** Relative  $\beta$ -tubulin RNA levels during the cell cycle of tobacco BY2 cells. The ratio of the slopes of the lines for CB1 and TAB20 in Fig. 6 was calculated and plotted. The standard error of each point was indicated by the vertical bar. The mean value of the seven measured levels was 0.35 (broken, line).

probe was thoroughly washed out, it was rehybridized with randomly selected tobacco cDNA probe, TAB20, as a control. The autoradiograms of the filter after the hybridizations were subjected to densitometry and the density of each spot was plotted against the amount of the applied RNA (Fig. 5). The regression lines were obtained from each series by the least-squares method and the ratio of the slope of CB1 and TAB20 was calculated (Fig. 6). Relative ratio of the content of  $\beta$ -tubulin RNA to TAB20 RNA did not vary significantly, but it was slightly higher than the average level at 9 hours (M phase) and it decreased towards G1 phase.

## DISCUSSION

Aphidicolin treatment aligned tobacco BY2 cells at the start of S phase and induced synchronization of culture. Since this drug acts on DNA polymerase  $\alpha$  specifically and reversibly (11), the influence to the microtubule system would be less compared with other methods such as by using spindle poison or temperature shift. The degree of synchrony at the S phase was high, but it declined gradually as the cell cycle proceeded. At 14 hours after the drug release, which was the final sampling time, cells in other phases than G1 (sum of S and M; G2 population seemed to be negligible) comprised about 15% of the population.

$\beta$ -tubulin RNA existed throughout the cell cycle, and its level did not vary significantly. However, the level of  $\beta$ -tubulin RNA increased slightly at M phase (about 30% higher than the average level), but decreased at G1 phase. In *Physarum* and *Chlamydomonas*, a relatively high level of tubulin RNA accumulated during G2, and reached a maximum level at metaphase and then decreased rapidly (13, 1). The level of  $\alpha$ -tubulin RNA at the peak was 40-fold and 8-fold compared with the minimum level in *Physarum* and *Chlamydomonas*, respectively. The time of the peak of the  $\beta$ -tubulin RNA level

in this study coincided with these observations, although the increase in  $\beta$ -tubulin RNA level was not so large as in *Physarum* and *Chlamydomonas*. As suggested by Shedl *et al.* (13), tubulin RNA may be stabilized at mitosis and destabilized after M phase in tobacco. The observed small change in the mRNA level does not exclude the possibility that only a certain fraction of  $\beta$ -tubulin mRNA contributed to the oscillation. At least three different isoforms of  $\beta$ -tubulin were detected and their relative amounts varied during the cell cycle. At present, however, we have no information to correlate the isoform pattern and tubulin mRNA isotypes in tobacco cells. Post-translational modification of tubulin molecule may be responsible for the change in the isoforms. Characterization of each isoform protein and mRNA are, therefore, necessary to pursue the possibility of participation of the different type of the tubulin molecules in controlled construction of each type of microtubules during a cell cycle.

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