

# Tyrosine phosphorylation of plant tubulin

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Received: 5 June 2008 / Accepted: 15 August 2008 / Published online: 18 September 2008  
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**Abstract** Phosphorylation of  $\alpha\beta$ -tubulins dimers by protein tyrosine kinases plays an important role in the regulation of cellular growth and differentiation in animal cells. In plants, however, the role of tubulin tyrosine phosphorylation is unknown and data on this tubulin modification are limited. In this study, we used an immunochemical approach to demonstrate that tubulin isolated by both immunoprecipitation and DEAE-chromatography is phosphorylated on tyrosine residues in cultured cells of *Nicotiana tabacum*. This opens up the possibility that tyrosine phosphorylation of tubulin could be involved in modulating the properties of plant microtubules.

**Keywords** Plant microtubules ·  
Post-translational modifications · Phosphorylation ·  
Tubulin · Tyrosine residues

## Introduction

Microtubules are necessary for a wide spectrum of cellular functions, including cell division, cell wall deposition, intracellular transport, organelle positioning, cell motility, and establishment of cell polarity. Distinct types of microtubular arrays form transiently in higher plant cells as they proceed through the cell cycle: the interphase network of cortical microtubules, the preprophase band, mitotic spindle, and phragmoplast. The main components of microtubules,  $\alpha$ - and  $\beta$ -tubulins, are known to be polymerized from a multiplicity of isoforms. This originates from expression of a multigene family (Breviario 2000), but isoform heterogeneity is increased by a variety of post-translational modifications. In animals, such modifications give rise to a large number of  $\alpha$ - and  $\beta$ -tubulin isoforms (Westermann and Weber 2003), generating populations of microtubules with different functions.

Although there is evidence that plant tubulin is post-translationally modified (Duckett and Lloyd 1994; Blume et al. 1997; Smertenko et al. 1997; Gilmer et al. 1999a, b; Huang and Lloyd 1999; Wang et al. 2004), the functional role of these modifications is not known. Some modifications have not been described for plant tubulin: e.g., nitrosylation and palmitoylation. In animals, tyrosine phosphorylation has been found on  $\alpha$ -tubulin (Akiyama et al. 1986; Wandosell et al. 1987; Maness and Matten 1990; Matten et al. 1990; Ley et al. 1994; Ishibashi et al. 1999) and  $\beta$ -tubulin (Akiyama et al. 1986; Wandosell et al. 1987; Maness and Matten 1990; Matten et al. 1990), and is known to play a key role in cellular growth and differentiation. Recent large-scale phosphorylation mapping projects revealed several peptides phosphorylated on serine or threonine in *Arabidopsis*  $\alpha$ - and  $\beta$ -tubulins (Heazlewood et al. 2007; Sugiyama et al. 2008; De la Fuente van Bentem et al.

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2008). Data on plant tubulin phosphorylation on tyrosine are very limited. A phosphorylated peptide of *Arabidopsis*  $\alpha$ -tubulin which contained two tyrosine residues but no serine or threonine residues has recently been identified (Nühse et al. 2007).

In higher plants, it has been found that tyrosine phosphorylation plays a vital role in the signal transduction of plant phytohormones, in plant cell cycle control, and in MAP kinase cascade pathways, which are important for plant defense against pathogens and other environmental stresses (Hirt 1997, 2000; Walker-Simmons 1998; Hardie 1999; Heberle-Bors 2001; Luan 2002). Tyrosine phosphorylated proteins have been detected in various plant tissues (Torruella et al. 1986; Trojanek et al. 1996; Barizza et al. 1999; Kameyama et al. 2000). On the other hand, typical protein tyrosine kinases are rare in plants (Ingram and Waites 2006; Shimotohno et al. 2006) and tyrosine phosphorylation is usually carried out by dual specificity kinases (Trojanek et al. 2004; Rudrabhatla et al. 2006). Such plant dual-specificity serine/threonine and tyrosine kinases have been isolated and sequenced (Ali et al. 1994; Reddy and Rajasekharan 2007) in parallel to a putative protein tyrosine phosphatases (Xu et al. 1998). Despite this, there is no evidence so far that plant tubulin is modified in this way. Here, we demonstrate that both  $\alpha$ - and  $\beta$ -subunits of plant tubulin undergo phosphorylation on tyrosine residues.

## Materials and methods

### Antibodies

The following monoclonal anti-tubulin antibodies were used: TU-01 (IgG1) (Viklický et al. 1982) and TU-16 (IgM) (Dráberová and Dráber 1998) against  $\alpha$ -subunit and TUB 2.1 (IgG1) against  $\beta$ -subunit (Sigma-Aldrich, Prague, Czech Republic). Phosphotyrosine was detected with monoclonal antibodies PY-20 (IgG2b) conjugated with horseradish peroxidase, biotinylated PY-20 (Transduction Laboratories, Lexington, KY, USA), biotinylated PT-66 (IgG1) (Sigma-Aldrich) and 4G10 (IgG2b) conjugated with horseradish peroxidase (Upstate Laboratories, Lake Placid, NY, USA). Affinity purified rabbit antibody against phosphotyrosine was from Upstate Laboratories. Anti-vimentin antibody VI-10 (IgM) (Dráberová et al. 1999) was used as control antibody for precipitation experiments. Antibody NF-01 against phosphorylated epitope on heavy chain of neurofilament (Lukáš et al. 1993) was used as control antibody for dephosphorylation experiments. Anti-mouse and anti-rabbit Ig antibodies conjugated with horseradish peroxidase were purchased from Promega Biotech (Madison, WI, USA).

### Preparation of *Nicotiana tabacum* cell extract and tubulin

For preparation of cell extracts for immunoblotting and immunoprecipitation, 5 to 8-day-old rapidly dividing suspension cells of *Nicotiana tabacum* strain VBI-0 (kindly provided by Professor Z. Opatrný, Department of Plant Physiology, Charles University, Prague, Czech Republic) growing in V-4 medium (Opatrný and Opatrná 1976) were used. Cells were washed in ice cold PEM buffer (50 mM Pipes-KOH, pH 6.9, 1 mM EGTA, 0.5 mM MgCl<sub>2</sub>, 1 mM DTT) and the washed cell pellet was resuspended in 0.5 volume of PEM buffer supplemented with phosphatase inhibitors (1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF) and protease inhibitor cocktail tablet (Complete EDTA-free; Roche Molecular Biochemicals, Mannheim, Germany; supplemented PEM) containing 1 mM GTP. The suspension was blended using ETA 1-012 mixer (ETA Corp., Hlinsko, Czech Republic) at maximal speed for 1 min at 4°C. The homogenate was spun down at 42,000g for 10 min at 4°C and the supernatant was further centrifuged at 100,000g for 45 min at 4°C.

Tubulin was purified by DEAE-chromatography according to (Morejohn et al. 1984) with modifications. The high-speed supernatant was mixed with 0.3 vol of DEAE-Sepharcel (Sigma-Aldrich) equilibrated with PEM supplemented with 0.1 mM GTP and incubated for 30 min at 4°C under slow swirling. The slurry was poured into a column and washed with 10 vol of 0.3 M KCl in PEM supplemented with 0.1 mM GTP. Bound proteins were eluted with 0.8 M KCl in PEM supplemented with 0.1 mM GTP. The presence of tubulin in fractions was detected by dot-immunobinding assay with anti- $\alpha$ -tubulin antibody TU-01. Fractions containing the highest amount of tubulin were pooled, dialyzed against cold PEM buffer and centrifuged at 100,000g for 20 min at 4°C.

### Protein phosphatase treatment

For phosphatase treatment, protein extracts were prepared in Tris buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM ZnCl<sub>2</sub>) supplemented with protease inhibitor cocktail. Alkaline phosphatase from *Escherichia coli* (type III, Sigma, St Louis, MO, USA) was dialyzed against the same buffer. The extracts were incubated with alkaline phosphatase at concentration 1 U/10  $\mu$ g protein for 30 min at 37°C. Control samples were treated under identical conditions except that alkaline phosphatase was omitted from the reaction mixture. The enzymatic reaction was terminated by adding phosphatase inhibitors Na<sub>3</sub>VO<sub>4</sub> and NaF to final concentration 1 mM and  $\beta$ -glycerophosphate to final concentrations 15 mM. Samples were boiled in SDS-sample buffer. Dephosphorylation of high-speed extract from porcine brain, followed by detection of dephosphorylated heavy chain of neurofilament with NF-01 antibody

directed to phosphorylated epitope (Lukáš et al. 1993) confirmed activity of used alkaline phosphatase. Alternatively, tubulin-enriched fraction was used for dephosphorylation. After enzymatic reaction tubulin samples were boiled in SDS-sample buffer or diluted in sample buffer for two-dimensional electrophoresis (2D-PAGE).

### Immunoprecipitation

Immunoprecipitation was performed according to (Drábová and Dráber 1993). Cell extracts were incubated with beads of (1) protein L (Pierce, Rockford, IL, USA) saturated with anti-tubulin antibody TU-16, (2) protein L saturated with negative control anti-vimentin antibody VI-10, and (3) protein L alone. Antibodies were used in the form of ten times concentrated spent culture supernatants from hybridoma cells to avoid binding of the other mouse antibodies. About 50 µl of sedimented beads with immobilized protein L were incubated under rocking at 4°C for 2 h with 1 ml of corresponding antibody [0.4 ml of concentrated supernatant mixed with 0.8 ml of TBST (10 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween-20)]. The beads were pelleted, washed four times in TBST, and further incubated under rocking for 3 h at 4°C with 0.5 ml of cell extract diluted 1:1 with TBST. The beads were pelleted and washed four times before boiling for 5 min in 100 µl of SDS-sample buffer with 2-mercaptoethanol to release the bound proteins.

### Gel electrophoresis and immunoblotting

One-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). The 2D-PAGE was performed essentially as described by Görg et al. (1999). Sample buffer contained 7 M urea, 2 M thiourea, 20 mM Tris, 4% (w/v) CHAPS, 1% (w/v) DTT, and 2% (v/v) IPG buffer, pH 4–7 (Amersham Pharmacia Biotech, Vienna, Austria). Immobiline DryStrip gels with pH 4–7 gradient, 11-cm long (Amersham Pharmacia Biotech) were rehydrated using 250 µl of prepared samples. Each strip was overlaid with mineral oil and left overnight at room temperature. Strips were focused for a total of 27 kWh on a Ettan IPGphor II apparatus (Amersham).

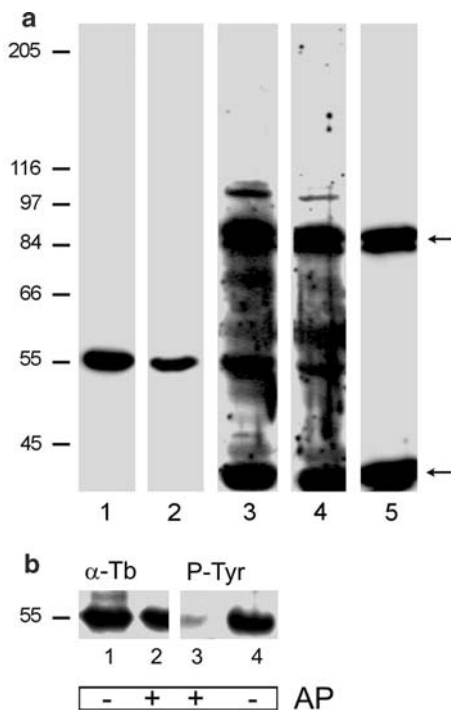
Proteins were electrophoretically transferred from gels onto nitrocellulose sheets according to Towbin et al. (1979). Proteins on nitrocellulose were stained by Ponceaus S. Nitrocellulose was blocked by 3% BSA in TBST. Details of the immunostaining procedure are described in Dráber et al. (1988). The antibodies TU-01 and TU-16 in the form of spent culture media were used undiluted; biotinylated antibodies PY-20 and PT-66 were used at dilutions 1:1,000 and 1:30,000, respectively. The antibodies TUB 2.1, PY-20 conjugated with horseradish peroxidase and P-Tyr were diluted 1:1,000. Bound antibodies were detected by incuba-

tion of blots with horseradish peroxidase conjugated secondary antibody, diluted 1:10,000 in 1% instant non-fat milk in TBST. Biotinylated antibodies were detected with ExtrAvidine conjugated with horseradish peroxidase (Sigma-Aldrich) diluted 1:5,000 in TBST. Alternatively, proteins phosphorylated on tyrosine were detected with 4G10 antibody conjugated with horseradish peroxidase diluted 1:10,000. Blots were washed and thereafter incubated with SuperSignal WestPico Chemiluminescent reagents (Pierce) according to the manufacturer's directions. Autoradiography films X-Omat AR (Eastman Kodak, Rochester, NY, USA) were evaluated using gel documentation system GDS 7500 (UVP, Upland, CA, USA).

### Results

First, we used the well-characterized TU-01 and TU-16 antibodies to detect tubulin in immunoblots of total cell extracts from cultured cells of *N. tabacum* (Fig. 1a, lanes 1, 2). No cross-reaction with other proteins was observed and secondary anti-mouse antibody applied alone gave no staining. Two biotinylated anti-phosphotyrosine antibodies PY-20 (Fig. 1a, lane 3) and PT-66 (Fig. 1a, lane 4) were then shown to react with the 55 kDa and other bands. The two most prominent other reactions (at 90 and 40 kDa) were found to stain with extravidin used for detecting biotinylated antibodies and therefore do not represent phosphotyrosinated proteins (Fig. 1a, lane 5). To confirm that 55 kDa protein in position of tubulin is indeed phosphoprotein, immunoblotting on *N. tabacum* extracts treated with alkaline phosphatase was performed. The anti- $\alpha$ -tubulin antibody TU-01 and anti-phosphotyrosine antibody 4G10 conjugated with horseradish peroxidase were used as probes. Treatment with alkaline phosphatase did not affect staining with anti-tubulin antibody (Fig. 1b, lanes 1, 2); however, dephosphorylation substantially diminished staining of 55 kDa protein with anti-phosphotyrosine antibody (Fig. 1b, lane 3). The anti-phosphotyrosine antibody gave strong signal in untreated cells (Fig. 1b, lane 4). Blot was cut along the middle of the lane containing dephosphorylated material in order to precisely locate band for double immunolabeling. Diminished reaction after phosphatase treatment was also observed with the other tested anti-phosphotyrosine antibodies (PY-20, PT-66).

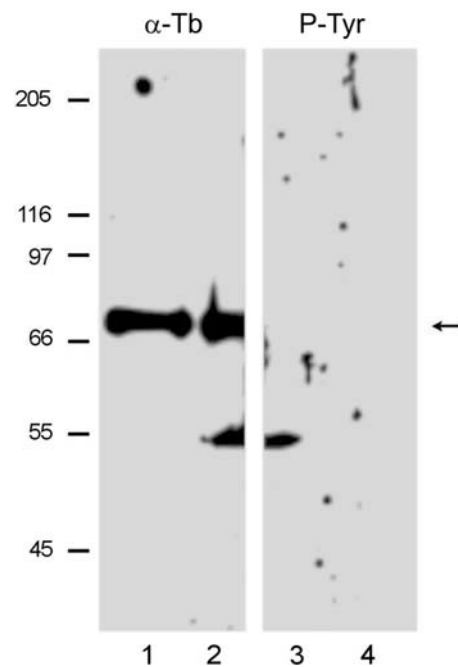
Next, we used immunoprecipitation for enrichment of the tubulin from total cell extracts of *N. tabacum* cultured cells. The anti- $\alpha$ -tubulin antibody TU-16 (IgM) immobilized on protein L was capable of precipitating tubulin. Several lines of evidence indicate that precipitation was specific. When beads containing protein L or beads containing protein L saturated with negative control antibody VI-01 (of the same class as TU-16) were incubated with *N.*



**Fig. 1** Immunoblot analysis of *N. tabacum* cell extract. Extracted proteins (**a**) or extracted proteins treated with alkaline phosphatase (AP) (**b**) were probed with anti-tubulin or anti-phosphotyrosine antibodies. **a** Staining with antibody TU-01 to  $\alpha$ -tubulin (lane 1), TU-16 to  $\alpha$ -tubulin (lane 2), biotinylated PY-20 to phosphotyrosine (lane 3) and biotinylated PT-66 to phosphotyrosine (lane 4). Staining with extravidin only (lane 5). Arrows indicate positions of extravidin-binding proteins in the cell extract. **b** Untreated (minus) or AP-treated (plus) extracts were stained with antibody TU-01 to  $\alpha$ -tubulin ( $\alpha$ -Tb) or antibody 4G10 to phosphotyrosine (P-Tyr). Molecular mass markers (in kDa) are indicated on the left. 7.5% SDS-PAGE

*tabacum* extract, tubulin was not precipitated (not shown). When secondary anti-mouse-antibody stained carrier with precipitating antibody (without extract), only heavy chains of precipitating antibodies were detected (Fig. 2, lane 1). This demonstrates that the band in the position of tubulin does not represent a proteolytic fragment of the antibody. Blot was cut along the middle of the lane containing precipitated material in order to precisely locate bands for double immunolabelling. Figure 2 (lanes 2, 3) shows that the anti-phosphotyrosine antibody (biotinylated PY-20) recognized the same band as the anti-tubulin antibody.

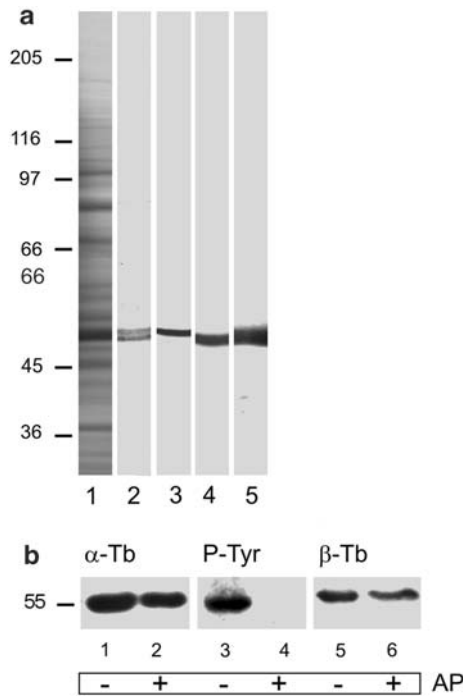
To verify that the 55-kDa phosphotyrosine protein could represent tubulin, highly enriched tubulin fraction was prepared by DEAE chromatography from high-speed supernatant of *N. tabacum* cultured cells. SDS-PAGE revealed substantial enrichment of 55 kDa protein, as documented by Coomassie Blue staining of preparation before and after purification (Fig. 3a, lanes 1, 2). Under the used separation conditions two very tightly spaced bands were detected. Immunoblot analysis confirmed that protein with lower electrophoretic mobility represented  $\beta$ -tubulin (Fig. 3a,



**Fig. 2** Immunoprecipitation of tubulin from *N. tabacum* cell extract. Antibody TU-16 (IgM) to  $\alpha$ -tubulin immobilized on protein L carrier (lanes 1 and 4), immunoprecipitated proteins (lanes 2 and 3). Staining with antibody TU-01 to  $\alpha$ -tubulin (lanes 1, 2), staining with biotinylated antibody PY-20 to phosphotyrosine (lanes 3, 4). 7.5% SDS-PAGE. Molecular mass markers (in kDa) are indicated to the left. Arrow indicates position of heavy chain of TU-16 antibody

lane 3) while  $\alpha$ -tubulin formed the higher mobility fraction (Fig. 3a, lane 4). Polyclonal anti-phosphotyrosine antibody stained both subunits (Fig. 3a, lane 5). Close inspection of autoradiographs revealed that intense phosphotyrosine staining appeared in the position of  $\alpha$ -tubulin and weak in the position of  $\beta$ -tubulin, as also documented in Fig. 3b. Treatment with alkaline phosphatase did not affect staining with anti- $\alpha$ -tubulin antibody (Fig. 3b, lanes 1, 2) and anti- $\beta$ -tubulin antibody (Fig. 3b, lanes 5, 6), yet dephosphorylation substantially diminished the staining intensity of tubulin with anti-phosphotyrosine antibody (Fig. 3b, lanes 3, 4).

To further confirm that tubulin-subunits are phosphorylated on tyrosine, untreated or phosphatase-treated tubulin samples were separated by 2D-PAGE and immunoblotted with anti-phosphotyrosine antibody. After stripping the blots were re-probed with antibodies against tubulin subunits. To make the results comparable, the same protein amounts were used and the corresponding blots were exposed on the same autoradiography film. It is known that after high-resolution isoelectric focusing  $\alpha$ -tubulin isoforms shift to the more basic region of the gel compared with  $\beta$ -tubulin isoforms (Linhartová et al. 1992; Smertenko et al. 1997). Staining a separate set of blots with antibodies against individual tubulin subunits confirmed this relative position after separation of *N. tabacum* tubulin-enriched fraction on 11 cm long Immobiline DryStrip gels with pH 4–7 gradient (data

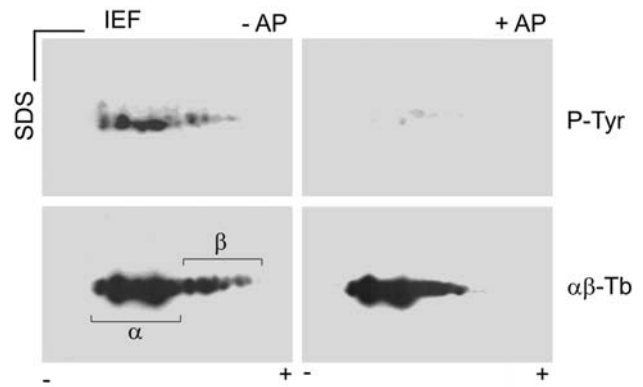


**Fig. 3** Immunoblot of *N. tabacum* tubulin-enriched fraction. Tubulin-enriched fraction (**a**) or tubulin-enriched fraction treated with alkaline phosphatase (*AP*; **b**) were probed with anti-tubulin or anti-phosphotyrosine antibodies. **a** Coomassie Blue staining of cell extract (*lane 1*) or tubulin-enriched fraction (*lane 2*). Immunostaining of tubulin-enriched fraction with antibody TUB 2.1 to  $\beta$ -tubulin (*lane 3*), TU-01 to  $\alpha$ -tubulin (*lane 4*) and polyclonal antibody to phosphotyrosine (*lane 5*). **b** Untreated (*minus*) or *AP*-treated (*plus*) tubulin samples were stained with antibody TU-01 to  $\alpha$ -tubulin ( $\alpha$ -Tb), polyclonal antibody to phosphotyrosine (*P-Tyr*) or antibody TUB 2.1 to  $\beta$ -tubulin. Molecular mass markers (in kDa) are indicated on the left. 7.0% SDS-PAGE

not shown). Polyclonal affinity-purified antibody to phosphotyrosine revealed labeling of both tubulin subunits in untreated samples. Stronger reaction was seen in position of  $\alpha$ -tubulin (Fig. 4,  $-AP$ ). On the other hand, dephosphorylation substantially diminished staining of tubulin subunits. The presence of tubulin was confirmed by staining the same blot with anti-tubulin antibodies (Fig. 4,  $+AP$ ). Sequential staining a separate blot with antibody TUB 2.1 to  $\beta$ -tubulin followed after autoradiography by staining with antibody TU-01 enabled to mark the positions of tubulin isoforms corresponding to  $\alpha$ - and  $\beta$ -tubulin subunits (Fig. 4, left lower panel). Close inspection failed to detect the most acidic  $\beta$ -tubulin isoforms in phosphatase-treated samples. Collectively, the presented data can be interpreted as an evidence of tyrosine phosphorylation of *N. tabacum* tubulin.

**Discussion**

Tyrosine phosphorylation of tubulin is well established for animal models but not for plants. In this work we used three



**Fig. 4** Immunoblot of *N. tabacum* tubulin-enriched fraction after separation by two-dimensional electrophoresis. Untreated ( $-AP$ ) or phosphatase-treated ( $+AP$ ) samples were stained with polyclonal anti-phosphotyrosine antibody (*P-Tyr*) and after stripping with a mixture of antibodies TU-01 and TUB2.1 to  $\alpha$ - and  $\beta$ -tubulin, respectively ( $\alpha\beta$ -Tb). Anti-phosphotyrosine antibody stained tubulin subunits and its signal diminished after treatment with *AP*. Position of  $\alpha$ - and  $\beta$ -tubulin isoforms is denoted in the left lower panel

monoclonal anti-phosphotyrosine antibodies (PY-20, PT-66, 4G10) and one polyclonal affinity-purified anti-phosphotyrosine antibody to demonstrate the phosphotyrosination of plant tubulins. In extracts from *N. tabacum* cultured cells the antibodies reacted with polypeptides migrating with mobilities corresponding to tubulin dimers.

To immunoprecipitate tubulin we took advantage of the monoclonal antibody TU-16 (IgM), whose heavy chains under reducing conditions migrate at 70 kDa, separate from the tubulin bands; the antibody binds to protein L and has been successfully used for precipitating tubulin from *A. thaliana* (Dryková et al. 2003). In the present experiments, the immunoprecipitated tobacco tubulin was recognized by the anti-phosphotyrosine antibody. In order to demonstrate that tyrosine phosphorylation indeed occurs on tubulin, highly enriched tubulin fraction from ionex chromatography was analyzed by immunoblotting after 1D and 2D electrophoresis. In both cases anti-phosphotyrosine antibody reacted with  $\alpha$ - and  $\beta$ -tubulin subunits as confirmed by blotting with anti-tubulin antibodies. When *N. tabacum* microtubules were prepared from tubulin-enriched fraction by taxol polymerization, and microtubules were thereafter immunoprobed after 2D-PAGE, phosphotyrosine was also present on tubulin. This suggests that phosphorylated tubulin can form microtubules in vitro by taxol-driven polymerization (unpublished results). Finally, substantially diminished reactivity with anti-phosphotyrosine antibody was observed when samples from cell extracts or purified tubulin were treated with alkaline phosphatase. These data strongly suggest that *N. tabacum* tubulin subunits are phosphorylated on tyrosine. We have also examined the tyrosine phosphorylation in different cellular fractions prepared from *Daucus carrota* suspension cells as described by Barroso et al. (2000). The anti-phos-

phosphotyrosine antibody PY-20 stained in cold-stable microtubule fraction the dominant proteins corresponding to the position of  $\alpha\beta$ -tubulin dimers (unpublished results). This indicates that tubulin phosphorylation on tyrosine need not be limited to *N. tabacum*. Further experiments on highly purified *D. carota* tubulin are needed to confirm this result.

Interestingly, more tubulin isoforms were detected by immunoblotting with anti-phosphotyrosine antibody after 2D-PAGE. It is well established that high heterogeneity of tubulin charge variants reflects the presence of several expressed genes for each subunit and extensive posttranslational modification of gene products. We have previously shown that up to 22 tubulin isoforms can be discriminated in *N. tabacum* tubulin after 1D high-resolution isoelectric focusing (HRIF) on 22-cm long gels. On the other hand, when proteins separated by HRIF were subsequently separated by SDS-PAGE, the number of tubulin isoforms was substantially reduced due to diffusion of separated isoforms in gel strips during equilibration step (Smertenko et al. 1997). Tubulin spots on 2D-PAGE thus contain more than one tubulin charge variants under the separation conditions used in presented study. One tubulin spot could comprise several individual charge variants due to expression of multiple tubulin genes and multiple posttranslational modifications. Combination of phosphorylation on tyrosine with other posttranslational modifications and/or tyrosine phosphorylation of different tubulin gene products could generate the heterogeneity in phosphotyrosine immunostaining. In such case dephosphorylation might not substantially influence the positions of detected tubulin spots, and non-phosphorylated and phosphorylated forms could not be easily separated by isoelectric focusing. Clear discrimination of phosphorylated variants was possible only in the case of the most acidic  $\beta$ -tubulin forms (Fig. 4). It was reported previously that several tubulin isoforms react with antibody directed against glutamylated tubulin (Smertenko et al. 1997).

The proteome-wide mapping of in vivo phosphorylation sites in *Arabidopsis* by using complementary phosphopeptide enrichment techniques coupled with high-accuracy mass spectrometry revealed that the proportion of phosphotyrosines among the phospho-residues in *Arabidopsis* is similar to that in humans (Sugiyama et al. 2008). In humans tubulin phosphorylation on tyrosine was detected on multiple  $\alpha$ - and  $\beta$ -tubulin isotypes encoded by different genes (Guo et al. 2008). Up to now phosphoproteome approaches in *Arabidopsis* revealed one phosphotyrosine peptide that originated from  $\alpha 2/\alpha 4$ -tubulin (Nühse et al. 2007). Our immunological studies on *N. tabacum* tubulin detected high-level phosphorylation of  $\alpha$ -tubulin, but low-level phosphorylation was also found on  $\beta$ -tubulin. This suggests that tyrosine phosphorylation of both tubulin subunits could be involved in modulating the properties of plant microtubules, similarly as in animal cells.

In future work, it will be important to focus on the specificities of various tyrosine kinases of the kind known to tyrosine-phosphorylate animal tubulin (Kadowaki et al. 1985; Akiyama et al. 1986; Peters et al. 1996). In plants it is thought that tyrosine is phosphorylated by dual-specificity serine/threonine and tyrosine kinases (Rudrabhatla et al. 2006) and bona fide tyrosine-specific protein phosphatases are already known (Xu et al. 1998; Fordham-Skelton et al. 1999). At present the location of phosphorylated tyrosine residue in plant  $\alpha$ -tubulin molecule is unknown. It is, therefore, unclear whether the tyrosination/detyrosination cycle on C-terminus of  $\alpha$ -tubulin could regulate the phosphorylation of tubulin and affect the properties of plant microtubules as was shown for animal cells (Wandosell et al. 1987).

**Acknowledgments** The work was funded partially by NATO LST.CLG 979212 for Alla Yemets and Clive Lloyd, and by INTAS Grant 03-51-6459 for Alla Yemets, Yaroslav Blume, Pavel Dráber and Vadym Sulimenko. The work of Tetyana Sulimenko, Vadym Sulimenko and Pavel Dráber was also supported from project LC545 (Ministry of Education, Youth and Sport of Czech Republic) Grant No. 204/05/2375 from GACR and by Institutional Research support AVOZ 50520514. Alla Yemets was supported from INTAS Experienced Postdoctoral Fellowship for Young Scientists YSF 00-184. Clive Lloyd and Jordi Chan were funded by the BBSRC.

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