

## RESEARCH PAPER

# Plant cell division is specifically affected by nitrotyrosine

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## Abstract

Virtually all eukaryotic  $\alpha$ -tubulins harbour a C-terminal tyrosine that can be reversibly removed and religated, catalysed by a specific tubulin-tyrosine carboxypeptidase (TTC) and a specific tubulin-tyrosine ligase (TTL), respectively. The biological function of this post-translational modification has remained enigmatic. 3-nitro-L-tyrosine (nitrotyrosine, NO<sub>2</sub>Tyr), can be incorporated into detyrosinated  $\alpha$ -tubulin instead of tyrosine, producing irreversibly nitrotyrosinated  $\alpha$ -tubulin. To gain insight into the possible function of detyrosination, the effect of NO<sub>2</sub>Tyr has been assessed in two plant model organisms (rice and tobacco). NO<sub>2</sub>Tyr causes a specific, sensitive, and dose-dependent inhibition of cell division that becomes detectable from 1 h after treatment and which is not observed with non-nitrosylated tyrosine. These effects are most pronounced in cycling tobacco BY-2 cells, where the inhibition of cell division is accompanied by a stimulation of cell length, and a misorientation of cross walls. NO<sub>2</sub>Tyr reduces the abundance of the detyrosinated form of  $\alpha$ -tubulin whereas the tyrosinated  $\alpha$ -tubulin is not affected. These findings are discussed with respect to a model where NO<sub>2</sub>Tyr is accepted as substrate by TTL and subsequently blocks TTC activity. The irreversibly tyrosinated  $\alpha$ -tubulin impairs microtubular functions that are relevant to cell division in general, and cell wall deposition in particular.

**Key words:** Microtubules, nitrotyrosine, post-translational modification, rice, tobacco BY-2, tubulin.

## Introduction

Plant tubulins, as well as all eukaryotic tubulins, undergo various post-translational modifications, such as acetylation, glutamylation, phosphorylation, and cyclic detyrosination/tyrosination (Hammond *et al.*, 2008). These post-translational modifications are considered as mechanisms to define functionally different subpopulations of one protein without the need to synthesize the respective proteins *de novo*. Although several post-translational modifications have been known for years, their precise function has remained relatively elusive.

One of the best investigated post-translational modifications is the reversible tubulin tyrosination cycle (Barra *et al.*, 1973), which is most likely to be unique for  $\alpha$ -tubulin (MacRae, 1997). Most of the eukaryotic  $\alpha$ -tubulin genes encode a C-terminal tyrosine, which can be removed by a specific and as yet not identified tubulin-tyrosine carboxypeptidase (TTC, also referred to as TCP or TTCP),

preferring polymers rather than  $\alpha/\beta$ -tubulin heterodimers (Ersfeld *et al.*, 1993). Thus, TTC creates detyrosinated  $\alpha$ -tubulin (detyr-tub), which hence exposes a C-terminal glutamic acid (therefore often designated as Glu-tubulin). After depolymerization of the microtubule, the  $\alpha$ -subunit of the soluble  $\alpha/\beta$ -tubulin heterodimers can be (re-)tyrosinated by a tubulin-tyrosine ligase [TTL, first isolated and cloned from porcine brain by Ersfeld *et al.* (1993)], creating the initial tyrosinated  $\alpha$ -tubulin (tyr-tub). TTL prefers the  $\alpha$ -tubulin of soluble  $\alpha/\beta$ -tubulin heterodimers as substrate and has a lower affinity for assembled microtubules (for a review, see Westermann and Weber, 2003).

The degree of tubulin detyrosination is correlated with the stability of microtubules: the higher the degree of detyrosination, the more stable the microtubules. However, *in vitro* studies showed that detyrosination is neither the cause of increased microtubule lifetime nor of increased

Abbreviations: BY-2, tobacco (*Nicotiana tabacum* L.) cell culture Bright Yellow 2; detyr-tub, detyrosinated  $\alpha$ -tubulin; MI, mitotic index; NO<sub>2</sub>Tyr, 3-nitro-L-tyrosine; TTC, tubulin-tyrosine carboxypeptidase; TTL, tubulin-tyrosine ligase; tyr-tub, tyrosinated  $\alpha$ -tubulin.

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microtubule length (Skoufias and Wilson, 1998), but is rather the consequence of stability, the real cause of which is not known. Detyrosination therefore conveys a signal meaning increased microtubule lifetime. Possible targets for this signal are the kinesin motors, which preferentially bind to detyrosinated microtubules, with ~2.8-fold increased affinity for detyrosinated over tyrosinated microtubules (Liao and Gundersen, 1998). In animal cells, a proper balance between tyrosinated and detyrosinated  $\alpha$ -tubulin is thought to be used as a cell cycle checkpoint to monitor abnormalities (Idriss, 2004).

Cellular malfunctions in animal cells, for instance in cancer cells or during infection by pathogens, are known to increase the abundance of nitric oxide (NO) that will produce, in addition to a couple of other nitrosylated products, 3-nitro-L-tyrosine (nitrotyrosine, NO<sub>2</sub>Tyr), created by nitration of tyrosine (for a review see Beckman and Koppenol, 1996). NO<sub>2</sub>Tyr is a substrate for TTL and can therefore be incorporated into detyr-tub. However, nitrotyrosination is an irreversible process; once nitrotyrosinated,  $\alpha$ -tubulin cannot be detyrosinated again by TTC, although opinions differ on this point (Bisig *et al.*, 2002; Chang *et al.*, 2002). Thus, the elevated levels of NO<sub>2</sub>Tyr in malignant or stressed cells will result in elevated levels of irreversibly nitrotyrosinated  $\alpha$ -tubulin. This leads to microtubule malfunctions and inaccurate microtubule-motor interactions, which will normally culminate in apoptosis. However, cancer cells are able to escape this cell death by phosphorylating TTL, which suppresses the function of the ligase and therefore reduces the quantity of nitrotyrosinated  $\alpha$ -tubulin (Idriss, 2004).

In plant cells, nitrosative stress is known to exist, although the metabolism and sources have not been investigated as successfully as in the animal field and are still not completely understood (Corpas *et al.*, 2007). However, NO has been identified as an important plant signalling molecule. Nitric oxide synthase (NOS) is discussed to be a major source of NO in plants, with L-arginine acting as nitrogen donor. NOS enzymatic activity has been demonstrated in plant extracts, and NOS inhibitors reduced the release of NO; however, so far no genes encoding NOS in the *Arabidopsis* genome have been identified. As a very likely enzymatic alternative for NOS, nitrate reductase (NR) is considered to be a candidate; the production of NO by NR could be demonstrated *in vitro* and *in vivo*. Once generated, NO is able to move within a cell and also from cell to cell, due to its volatility. As it is not expected that NO has a specific receptor, but as the cell undoubtedly senses the presence of NO, there must be other target molecules or proteins. It is well known that NO can interact with amino acids (such as cysteine and tyrosine), and with thiol groups (for a review, see Neill *et al.*, 2003; Planchet and Kaiser, 2006). NO<sub>2</sub>Tyr is created from the reaction of NO with tyrosine.

Since plant homologues of TTL exist, as well as the tubulin tyrosination cycle (Wiesler *et al.*, 2002), it can be expected that NO<sub>2</sub>Tyr should produce specific effects in plant cells. However, to our knowledge, this has not been tested yet.

Therefore, in this work the effects of NO<sub>2</sub>Tyr in two plant model organisms (rice and tobacco) have been investigated. It can be reported that NO<sub>2</sub>Tyr produces a specific, sensitive, and dose-dependent inhibition of cell division that is not observed after treatment with non-nitrosylated tyrosine. This inhibition is accompanied by a stimulation of cell length, and a misorientation of cross walls. It could further be shown that NO<sub>2</sub>Tyr increases the sensitivity of BY-2 cells to the microtubule assembly blocker oryzalin and reduces the abundance of the detyrosinated form of  $\alpha$ -tubulin, whereas the abundance of tyrosinated  $\alpha$ -tubulin is not affected, defining microtubule-dependent cell plate formation as very sensitive target of NO<sub>2</sub>Tyr.

## Materials and methods

### *Tobacco cell culture*

The tobacco cell culture BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2; Nagata *et al.*, 1992) was cultivated in liquid medium containing 4.3 g l<sup>-1</sup> Murashige and Skoog salts (Duchefa, Haarlem, The Netherlands), 30 g l<sup>-1</sup> sucrose, 0.2 g l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 0.1 g l<sup>-1</sup> myo-inositol, 1 mg l<sup>-1</sup> thiamine, and 0.2 mg l<sup>-1</sup> 2,4-dichlorophenoxyacetic acid (2,4-D), pH 5.8. For subcultivation, 1–2 ml of 7-day-old stationary phase cells were transferred to a 100 ml Erlenmeyer flask containing 30 ml of fresh medium and cultured on a rotary shaker (KS 260 basic, IKA Labortechnik, Germany) at 150 rpm and 25 °C, in the dark. Stock BY-2 calli were kept on medium solidified with 0.8% (w/v) agar and subcultured every 4–6 weeks.

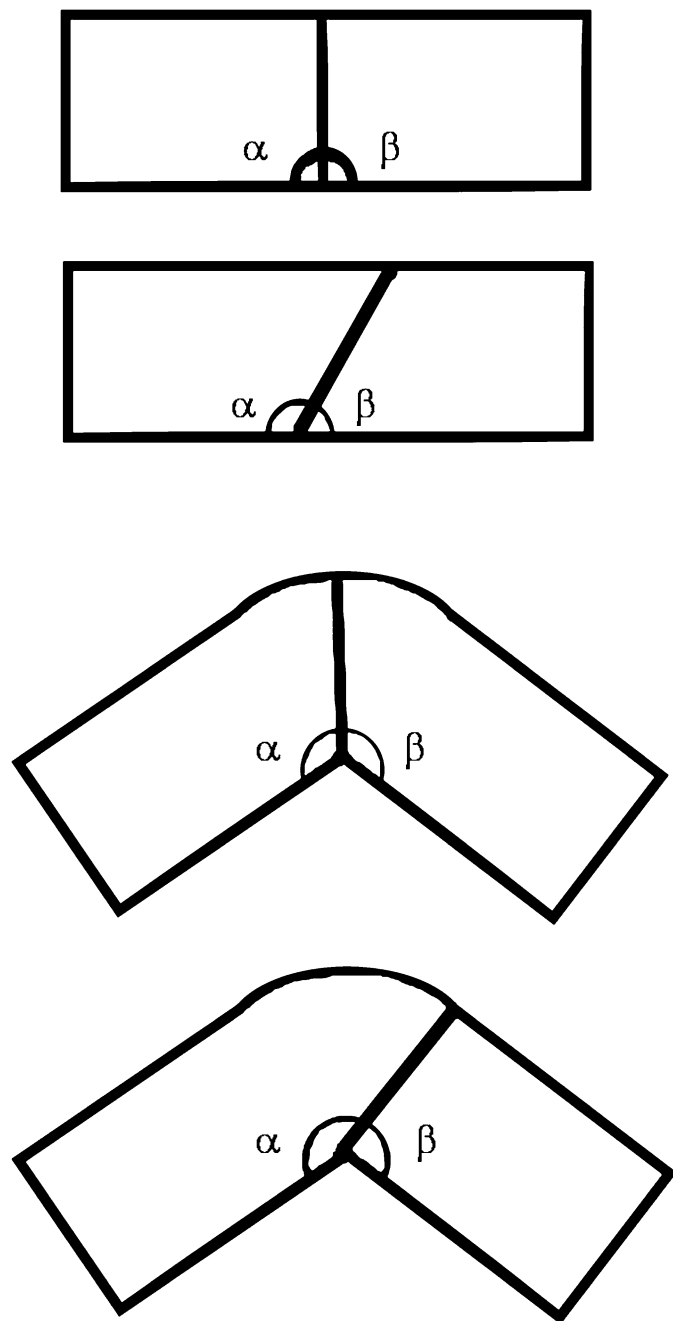
To test the response to NO<sub>2</sub>Tyr (Sigma-Aldrich, München, Germany), a sterile stock solution of NO<sub>2</sub>Tyr was diluted directly in the medium to yield the desired concentration. NO<sub>2</sub>Tyr was added to fresh medium either at the time of subcultivation and incubated for 3 d [to assess the mitotic index (MI)] or 4 d (for the measurement of cell density), or at 3 d after subcultivation and incubated for a further 24 h (for measurements of cell length and cell wall angles, and protein extraction followed by western analysis). Controls were either not treated with NO<sub>2</sub>Tyr or treated with equivalent concentrations of tyrosine or 4-nitro-L-phenylalanine (NO<sub>2</sub>Phe, Sigma-Aldrich). In a third set of experiments, NO<sub>2</sub>Tyr was administered in combination with oryzalin.

### *Quantification of the cellular response to NO<sub>2</sub>Tyr in BY-2*

The growth of the BY-2 cell culture was quantified by measuring the sedimented cell volume at day 4 after subcultivation in a sample of 15 ml. To determine the MI, cells were fixed at day 3 after subcultivation in Carnoy fixative [3:1 (v/v) 96% (v/v) ethanol:acetic acid] complemented by 0.5% (w/v) Triton X-100 (Campanoni *et al.*, 2003), stained with 2'-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi(1H-benzimidazole) trihydrochloride (Hoechst 33258, Sigma-Aldrich, final concentration 1 µg ml<sup>-1</sup>), and observed under the fluorescence microscope (see below).

The response of cell length and cross wall orientation was scored after 24 h of treatment with NO<sub>2</sub>Tyr that has been added at day 3 after subcultivation. Cells were examined by differential interference contrast under an Axioskop (Zeiss Axioskop 2 FS, Jena, Germany) and images were analysed by AxioVision 4.7 software (Zeiss, Jena, Germany). Cell length was defined as the distance between the midpoints of the two cross walls of a cell.

To quantify the orientation of cross walls, the two angles between cross wall and side wall were measured and the ratio between the larger and the smaller angle was calculated (see Fig. 1). For symmetric cross walls, this ratio is between 1 and 1.09; for asymmetric orientations, this ratio is  $\geq 1.1$  (representing a difference of angles of at least 10%).



**Fig. 1.** Schematic diagram of the determination of the cross wall angle ratio in tobacco BY-2 cell files. The uppermost cell file shows a symmetric cross wall with two equal angles  $\alpha$  and  $\beta$ ; the ratio is 1. The second cell file shows an asymmetric cross wall with  $\alpha$  bigger than  $\beta$ ; the ratio derives from 1. The third and fourth cell files correspond to the first and second; the difference is the form of the cell file, which is curved instead of straight.

#### Protein extraction and western blot analysis

Protein extracts were prepared from 4-day-old BY-2 cells after 24 h of treatment with  $\text{NO}_2\text{Tyr}$ . After sedimentation in 15 ml test tubes (10 min, 1500 g, Hettich Centrifuge Typ 1300, Tuttlingen, Germany), cells were homogenized in the same volume of extraction buffer [prepared according to Nick *et al.* (1995)] with some modifications, containing 25 mM MES, 5 mM EGTA, 5 mM  $\text{MgCl}_2$ , 1 M glycerol, pH 6.9, supplemented with 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF) by using a glass Potter.

Insoluble tissue debris was removed by centrifugation (5 min at 13 000 g; Heraeus Instruments, Biofuge pico, Osterode, Germany, rotor PP 1/96 #3324), followed by ultracentrifugation (15 min at 100 000 g, 20 °C; Beckman, USA, TL-100, rotor TLA 100.2). All steps were carried out at room temperature to avoid depolymerization of microtubules and to collect only the soluble proteins including soluble tubulin heterodimers.

Proteins were concentrated and sedimented by precipitation with trichloroacetic acid (Bensadoun and Weinstein, 1976), with minor modifications. Samples were dissolved in 200  $\mu\text{l}$  of sample buffer, incubated for 10 min at 95 °C, loaded onto a standard 10% SDS-mini gel, and subjected to western blotting as described in Nick *et al.* (1995). A pre-stained broad range protein marker (P7708S, New England Biolabs) was used as a molecular weight standard. For detection of tyrosinated  $\alpha$ -tubulin, monoclonal antibody TUB-1A2 (Sigma-Aldrich; Kreis, 1987), and for detection of detyrosinated  $\alpha$ -tubulin, monoclonal antibody DM1A (Sigma-Aldrich; Breitling and Little, 1986) were used at a dilution of 1:400 in TRIS-buffered saline containing Triton X-100 (TBST; 20 mM TRIS-HCl, 150 mM NaCl, 1% Triton, pH 7.4), respectively. Signal development was obtained by a goat secondary anti-mouse IgA, conjugated with alkaline phosphatase (Sigma-Aldrich) at a dilution of 1:2500 in TBST with 3% low-fat milk powder. A parallel set of lanes loaded in exactly the same manner was visualized by staining with Coomassie Brilliant Blue (CBB) to control the loading.

#### Quantification of the response to $\text{NO}_2\text{Tyr}$ in rice seedlings

Caryopses of rice (*Oryza sativa* L. ssp. *japonica* cv. Nihonmasari) were deposited equidistantly on plastic meshes, floating on 100 ml of deionized water in plastic boxes. Seedlings were raised in photobiological darkness (plastic boxes wrapped in black cloth, stored in light-proof boxes) at 25 °C for 6 d.

For the  $\text{NO}_2\text{Tyr}$  treatment,  $\text{NO}_2\text{Tyr}$  was added to the deionized water to the respective concentration at the time of sowing (for growth measurements and MI) or after 6 d and incubated for 1 h (for the determination of MI). Controls were either not treated with  $\text{NO}_2\text{Tyr}$  or treated with tyrosine at the equivalent concentration. To measure growth, the length of coleoptiles and roots was determined. To define the MI, root tips were fixed for 1 h in 3.7% (w/v) paraformaldehyde in phosphate-buffered saline (PBS; 0.15 M NaCl, 2.7 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 6.5 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.2), washed three times with PBS, minced into small pieces, stained with Hoechst 33258 (Sigma-Aldrich, final concentration 1  $\mu\text{g ml}^{-1}$ ), and observed under the fluorescence microscope (see below).

#### Microscopy

All samples were investigated with an AxioImager Z1 microscope (Zeiss, Jena, Germany); images were analysed by the AxioVision 4.7 software (Zeiss).

For detection of mitotic cells, samples were observed using the filter set 49 (excitation at 365 nm, beamsplitter at 395 nm, and emission at 445 nm) using a  $\times 40$  objective (EC Plan-Neofluar 40 $\times$ /0.75 M27) for BY-2 and a  $\times 63$  oil-immersion objective (Plan-Apochromat 63 $\times$ /1.40 Oil DIC M27) for rice cells.

For measurement of cell length or cell wall angles in BY-2, samples were observed in the differential interference contrast using a  $\times 20$  objective (Plan-Apochromat 20 $\times$ /0.75) and the MosaiX imaging software (Zeiss). Images were processed and analysed using the AxioVision software 4.7 (Zeiss).

## Results

### *NO<sub>2</sub>Tyr* inhibits plant mitosis in a dose-dependent manner

In order to identify targets for  $\text{NO}_2\text{Tyr}$  in plant cells, the growth response of rice coleoptiles (consisting of

non-cycling cells that grow exclusively by cell expansion), rice seedling roots (consisting of cycling cells that subsequently expand), and tobacco BY-2 cells (with strong cycling activity) to the modified amino acid was investigated by dose–response studies.

In root growth of rice seedlings, measured at day 6 after germination, NO<sub>2</sub>Tyr was found to inhibit growth strongly, with a threshold of ~10 μM (Fig. 2A). Already at 20 μM NO<sub>2</sub>Tyr growth was reduced by ~70% as compared with control seedlings. At these concentrations, coleoptile growth was still unaffected; inhibition became detectable from 60 μM NO<sub>2</sub>Tyr, reaching a plateau (of 60% inhibition as compared with control seedlings) only at high concentrations >500 μM NO<sub>2</sub>Tyr. Thus, the dose–response curve for coleoptile growth over NO<sub>2</sub>Tyr was shifted by almost an order of magnitude as compared with root growth, suggesting that the main target of NO<sub>2</sub>Tyr is not the mechanism of cell elongation but rather other mechanisms involved in cell division.

BY-2 cells (in contrast to rice coleoptiles, a model for cycling cells) treated for 3 d with NO<sub>2</sub>Tyr also displayed growth inhibition, in this experiment measured as a decrease in packed volume cell density (Fig. 2B). The growth inhibition was observed already from 50 nM NO<sub>2</sub>Tyr and increased strongly to ~40% at 0.1 μM NO<sub>2</sub>Tyr, reaching saturation at 0.5 μM NO<sub>2</sub>Tyr with ~90% inhibition as compared with untreated controls. BY-2 cells treated with NO<sub>2</sub>Phe instead of NO<sub>2</sub>Tyr showed no significant effect on packed volume. Thus, the inhibition of cell division is not a general effect of nitrosylated amino acids, but rather is specific for NO<sub>2</sub>Tyr.

For a deeper insight, the MIs of rice root tips and tobacco BY-2 were determined. Figure 2C shows the increase of mitotic inhibition for rice root tips either after 6 d of continuous cultivation (filled diamonds) on NO<sub>2</sub>Tyr or after 6 d of cultivation on water followed by incubation with NO<sub>2</sub>Tyr for 1 h (open diamonds). Interestingly, 1 h incubation produced almost the same inhibition as continuous treatment for 6 d, indicating rapid uptake and metabolism or incorporation of NO<sub>2</sub>Tyr. Similarly, BY-2 cells treated with NO<sub>2</sub>Tyr showed a decline of the MI (Fig. 2D), whereby the inhibition occurred at much lower concentrations of the applied agent as compared with rice root tips, confirming the higher sensitivity to NO<sub>2</sub>Tyr in BY-2 cells, as already observed for the dose–response of packed volume.

#### *The inhibition of mitosis is specific for nitrosylated tyrosine*

To test whether mitotic inhibition was specific for the nitrosylated form of tyrosine, dose–response curves were recorded for non-nitrosylated tyrosine (Fig. 2E–H, open symbols). In Fig. 2E, growth inhibition of NO<sub>2</sub>Tyr and tyrosine in rice root tips is compared; in Fig. 2F the reduction of packed volume in BY-2 cells as a measure of culture growth is shown. A significant inhibition by tyrosine was not observed either for root growth in rice or for

culture growth in BY-2. Furthermore, there was no significant inhibition of MIs in rice and tobacco even at the highest concentrations where strong inhibition was observed for NO<sub>2</sub>Tyr (Fig. 2G, H). Thus, the inhibition of mitosis is specific for the nitrosylated form of tyrosine.

#### *NO<sub>2</sub>Tyr stimulates cell length and affects the formation of cross walls*

Cell length in tobacco cells is a sensitive indicator for reduced mitotic activity (Holweg *et al.*, 2003). The dose–response relationship in tobacco BY-2 cells that had been treated for 24 h with NO<sub>2</sub>Tyr at day 3 after subcultivation was therefore recorded, and an increase in cell length of almost 20% from 5 μM NO<sub>2</sub>Tyr was found (Fig. 3A).

To gain insight into the cellular events that accompany the observed inhibition of cell division in BY-2 cells, the deposition of cross walls that appeared to be affected upon treatment with NO<sub>2</sub>Tyr was investigated. The ratio of the angles between the cross wall and the side walls was determined as a measure of orientation symmetry (see Fig. 1). This ratio is 1 in the case of symmetrical cross walls, but deviates from 1 when the cross wall is laid down asymmetrically. In untreated cells, the cross walls were mostly symmetrical (manifest as a high frequency of ratios <1.1). With increasing NO<sub>2</sub>Tyr concentration, the number of incorrectly oriented cross walls with oblique orientation (ratio ≥1.1) increased (Fig. 3B). As for the stimulation of cell length, this effect was saturated from 5 μM NO<sub>2</sub>Tyr.

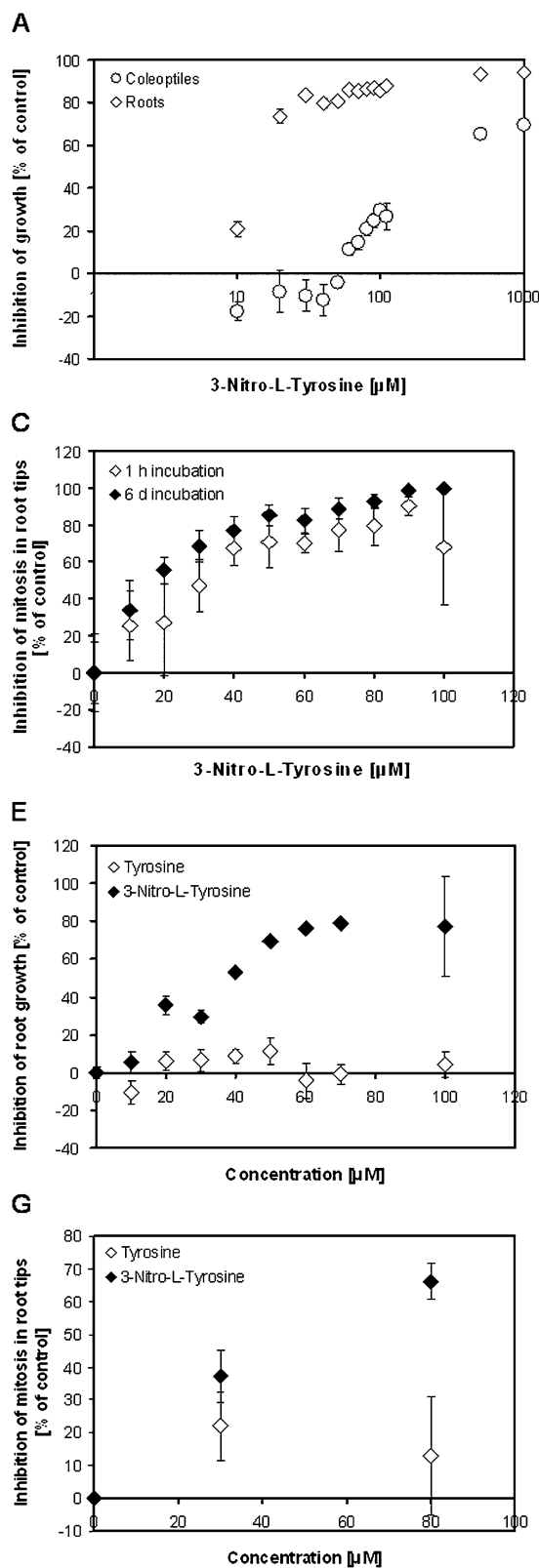
#### *NO<sub>2</sub>Tyr increases the sensitivity to oryzalin*

Oryzalin inhibits the polymerization of plant microtubules by sequestering tubulin heterodimers from their integration into the polymer such that microtubules are eliminated dependent on the extent of their innate turnover. To analyse the interaction between oryzalin and NO<sub>2</sub>Tyr, dose–response curves over packed volume of BY-2 cells were recorded in the absence or presence of 0.1 μM NO<sub>2</sub>Tyr (Fig. 4). The excess inhibition caused by the combination of NO<sub>2</sub>Tyr and oryzalin over the inhibition caused by oryzalin alone was not constant, but was dependent on the concentration of oryzalin. At lower concentrations of oryzalin, the effect of NO<sub>2</sub>Tyr was more pronounced than at higher concentrations. In contrast, at higher concentration of oryzalin, the additional effect of NO<sub>2</sub>Tyr was small. If oryzalin and NO<sub>2</sub>Tyr acted on culture growth independently, their interaction should be additive; that is, the inhibition by NO<sub>2</sub>Tyr should be a constant. In contrast, a multiplicative interaction is observed, which is expected, when the two compounds share the same target.

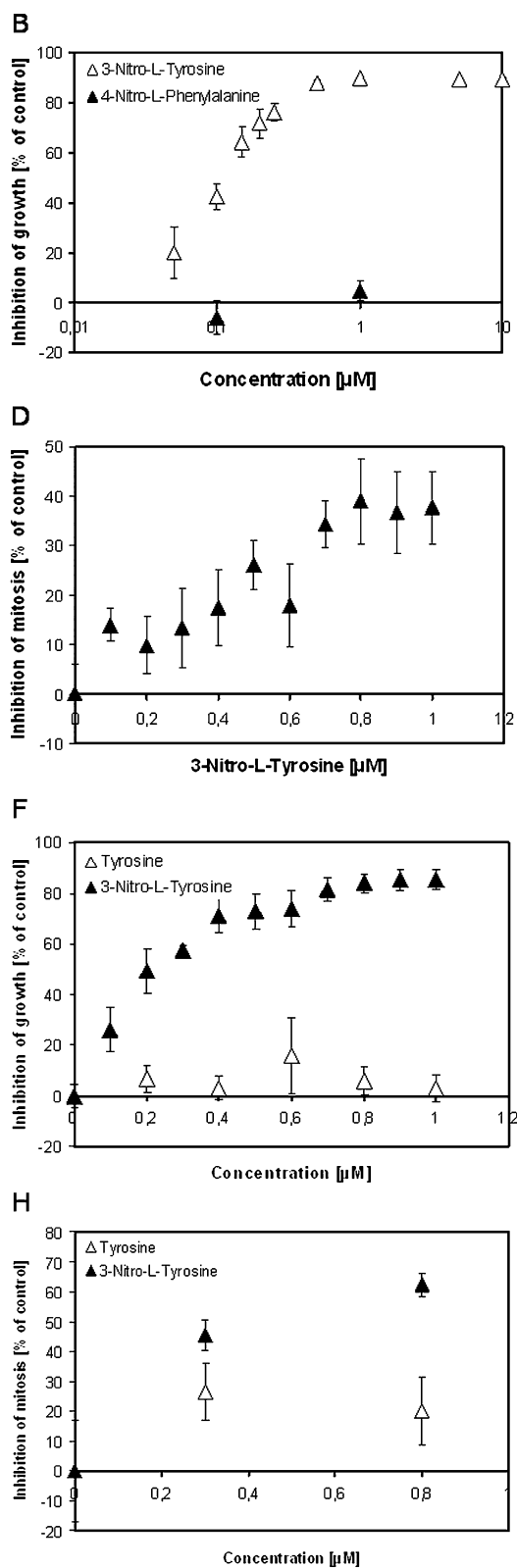
#### *NO<sub>2</sub>Tyr induces a decrease in the proportion of detyrosinated α-tubulin*

To detect potential changes in the proportion of detyrosinated versus tyrosinated α-tubulin, the total soluble protein

## Rice seedlings



## Tobacco BY-2



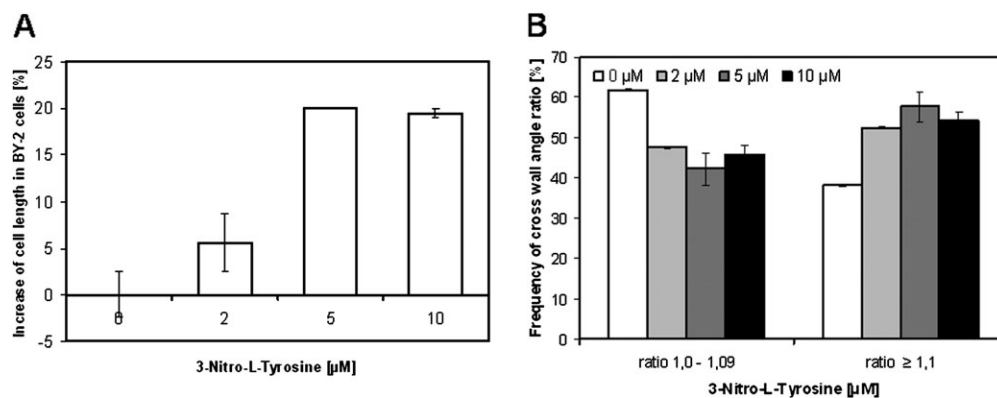
**Fig. 2.** Quantification of the effects of  $\text{NO}_2\text{Tyr}$  on 6-day-old rice seedlings (left column, A, C, E, and G) and BY-2 cell culture (right column, B, D, F, and H). (A) Inhibition (percentage of control) of coleoptile (open circles) and root (open diamonds) growth in rice seedlings ( $n \geq 18$ , error bar=SE). (B) Inhibition of growth (given as a percentage of packed volume) in BY-2 cells grown with  $\text{NO}_2\text{Tyr}$  (open triangles) or  $\text{NO}_2\text{Phe}$  (filled triangles;  $n \geq 4$ , error bar=SE). (C) Inhibition of mitosis (given as a percentage of control) in rice root tips, incubated for 1 h (open diamonds) or for 6 d with  $\text{NO}_2\text{Tyr}$  (filled diamonds;  $n=3$  independent experiments with 300 cells each,

fraction of BY-2 cells was extracted and probed for signals of tyrosinated and detyrosinated  $\alpha$ -tubulin after 24 h of treatment with 10  $\mu$ M NO<sub>2</sub>Tyr. Preliminary experiments testing the dose–response of detyrosination/tyrosination ratios showed that this concentration of NO<sub>2</sub>Tyr yielded the maximal effect (data not shown). The equal loading of protein samples was adjusted and verified by staining a replicate gel with CBB (Fig. 5, CBB). For detection of tyr-tub, one replicate was probed with the antibody TUB-1A2; for detection of detyr-tub, a second replicate was probed with the antibody DM1A (for detection and specificity, see Wiesler *et al.*, 2002). The signal for tyr-tub was the same in untreated and treated samples (Fig. 5, TUB-1A2). In contrast, the signal for detyr-tub (Fig. 5, DM1A) differed between untreated and treated samples. The untreated cells displayed a double band, with the upper band corresponding to the band detected by the TUB-1A2 antibody, consistent with previous results (Wiesler *et al.*, 2002). The lower band corresponds to detyr-tub that is characteristically shifted in apparent molecular weight (Wiesler *et al.*, 2002). In samples treated with 10  $\mu$ M NO<sub>2</sub>Tyr, this lower band was hardly detectable, whereas the upper band remained unaltered. Thus, the abundance of tyr-tub is not significantly altered by NO<sub>2</sub>Tyr (also indicating that the affinity of the antibody is not affected by the incorporated NO<sub>2</sub>Tyr), whereas the *bona fide* signal for detyr-tub is eliminated, consistent with an inhibition of tubulin detyrosination. A similar decrease of detyr-tub was observed after treatment with NO<sub>2</sub>Tyr in rice seedlings, however at 10-fold higher concentration.

## Discussion

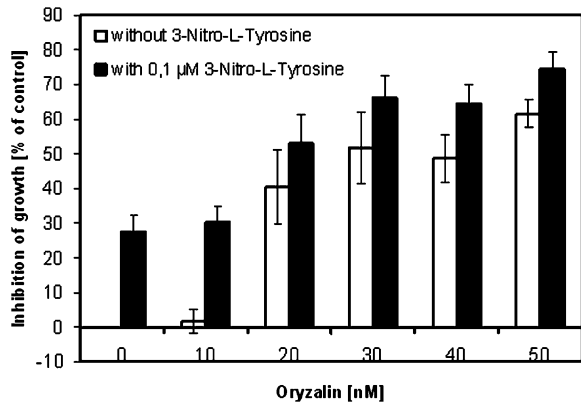
To gain insight into the possible function of detyrosination in plants, NO<sub>2</sub>Tyr was administered in two plant model organisms (rice and tobacco). A specific, sensitive, and dose-dependent inhibition of cell division became manifest that was not observed after treatment with non-nitrosylated tyrosine and became detectable from 1 h after treatment. This effect was most pronounced in cycling tobacco BY-2 cells. Here, the inhibition of cell division was accompanied by a stimulation of cell length, and a misorientation of cross walls. NO<sub>2</sub>Tyr acted multiplicatively with the microtubule assembly blocker oryzalin (which is an indirect assay, merely indicating that both drugs act on microtubules). It is evident that this indication has to be confirmed by direct biochemical assays using putative TTLs, which is already in progress. Moreover, NO<sub>2</sub>Tyr reduced the abundance of the detyrosinated form of  $\alpha$ -tubulin, whereas the abundance of tyrosinated  $\alpha$ -tubulin was not affected.

The sensitivity to NO<sub>2</sub>Tyr was more pronounced in tobacco BY-2 cells as compared with rice roots. Differences in sensitivity between different cell types have also been reported for other antimicrotubular drugs and depend not only on permeability (tissue versus single cells) but also on microtubular dynamics. Dynamic microtubules are expected to be more seriously affected by irreversible tyrosination as compared with microtubules with a lower cycling activity. It has been demonstrated for a couple of microtubule-eliminating compounds (that act by sequestering tubulin heterodimers from integration into growing microtubules

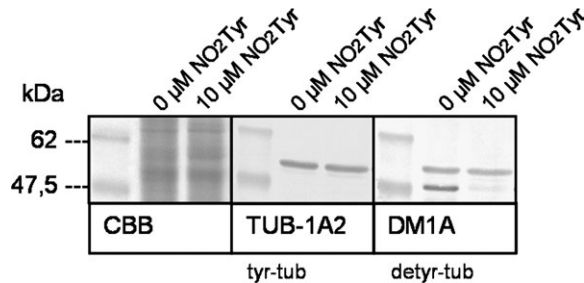


**Fig. 3.** Physiological effects of 24 h treatment with NO<sub>2</sub>Tyr on 4-day-old BY-2 cells. (A) Increase of cell length, given as a percentage of control ( $n=3$  independent experiments with 500 cells each, error bar=SE). (B) Frequency of correct (ratio 1.0–1.09) and incorrect (ratio  $\geq 1.1$ ) cross walls ( $n=2$  independent experiments with 150 ratios each, error bar=SE).

error bar=SE). (D) Inhibition of mitosis (given as a percentage of control) in BY-2 cell culture treated with NO<sub>2</sub>Tyr (filled triangles;  $n=7$  independent experiments with 300 cells each, error bar=SE). (E) Comparison of the inhibition of root growth in rice treated with either Tyr (open diamonds) or NO<sub>2</sub>Tyr (filled diamonds;  $n \geq 11$ , error bar=SE). (F) Comparison of the inhibition of growth (given as a percentage of packed volume) in BY-2 cell culture treated either with tyrosine (open triangles) or NO<sub>2</sub>Tyr (filled triangles;  $n=4$ , error bar=SE). (G) Comparison of the inhibition of mitosis (given as a percentage of control) in rice root tips treated either with tyrosine (open diamonds) or NO<sub>2</sub>Tyr (filled diamonds;  $n=5$  independent experiments with 240 cells each, error bar=SE). (H) Comparison of the inhibition of mitosis (given as a percentage of control) in BY-2 cells treated with either tyrosine (open triangles) or NO<sub>2</sub>Tyr (filled triangles;  $n=5$  independent experiments with 260 cells each, error bar=SE).



**Fig. 4.** Inhibition of growth (given as a percentage of packed volume of control) of BY-2 cell culture. White columns represent growth inhibition with oryzalin only (from 0 nM to 50 nM); black columns represent growth inhibition with additional 0.1  $\mu\text{M}$   $\text{NO}_2\text{Tyr}$  ( $n \geq 3$ , error bar=SE).



**Fig. 5.** Distribution of tyr-tub and detyr-tub in samples derived from total soluble protein extract from 4-day-old BY-2 cells, untreated or treated with 10  $\mu\text{M}$   $\text{NO}_2\text{Tyr}$  for 24 h. The left panel represents the SDS-gel stained with CBB, showing an equal concentration of the applied proteins (left lane protein marker, middle lane untreated cells, right lane cells treated with 10  $\mu\text{M}$   $\text{NO}_2\text{Tyr}$ ). The middle panel shows the reactions of the antibody TUB-1A2 against tyr-tub in the untreated and treated samples. The right panel shows proteins of untreated and treated cells tested with the antibody DM1A against detyr-tub. The upper signal is a cross-reaction with tyr-tub and the lower signal (only abundant in the untreated sample) corresponds to detyr-tub.

such that microtubules are eliminated dependent on their innate turnover) that even in the same organ, coleoptile segments of maize, sensitivity can shift by an order of magnitude when microtubular dynamics were stimulated by auxin (Wiesler *et al.*, 2002). Microtubule dynamics in the rapidly dividing tobacco cell culture is elevated compared with seedlings, where most microtubules are organized in interphase arrays of cortical microtubules.

Incorporation of  $\text{NO}_2\text{Tyr}$  into the C-terminus of  $\alpha$ -tubulin is accomplished by TTL, with  $\text{NO}_2\text{Tyr}$  competing with tyrosine as an alternative substrate of TTL. However, in contrast to the canonical substrate tyrosine,  $\text{NO}_2\text{Tyr}$  cannot be cleaved off by TTC, such that nitrotyrosination of  $\alpha$ -tubulin is irreversible (Eiserich *et al.*, 1999; Kalisz *et al.*, 2000).

In the present work, it is shown that uptake of free  $\text{NO}_2\text{Tyr}$  changes the post-translational modification of  $\alpha$ -tubulin in plant cells (Fig. 5). When tobacco BY-2 cells were treated with 10  $\mu\text{M}$   $\text{NO}_2\text{Tyr}$ , the abundance of tyr-tub (tested with the antibody TUB-1A2) was not affected, suggesting that TTL activity was not affected by  $\text{NO}_2\text{Tyr}$ . In contrast, the abundance of detyr-tub (tested with the antibody DM1A) was strongly reduced. This is evidence for an inhibition of TTC activity by  $\text{NO}_2\text{Tyr}$  and provides an approach to investigate possible functions of detyrosination in plant cells.

The upper band, visualized by the TUB-1A2 antibody, is expected to correspond to a mixture of tyrosinated and nitrotyrosinated  $\alpha$ -tubulin. To verify this, commercially available antibodies against  $\text{NO}_2\text{Tyr}$  have been tested. However, since these antibodies failed to produce specific signals, either in extracts from tobacco or in extracts from rice (data not shown), this prediction could not be tested experimentally. As retyrosination and nitrotyrosination are competing processes, the number of nitrotyrosinated  $\alpha$ -tubulin molecules is expected to increase in close correlation with the decrease of tyr-tub. However, due to the turnover of tubulin (for a review, see Breviaro and Nick, 2000), this correlation can be weakened over time.

It could be observed that the inhibition of mitosis was accompanied by an increase of cell length in BY-2 cells (Figs 2D, 3A). This stimulation is not unique to  $\text{NO}_2\text{Tyr}$ , but was also found in response to the myosin inhibitor 2,3-butanedione monoxime (Holweg *et al.*, 2003). This antagonism between cell division and cell elongation can be ascribed to cross-talk between two auxin signalling pathways that regulate cell expansion and cell division, respectively (Chen, 2001). These pathways are triggered by two receptors that differ with respect to their ligand affinities and the involvement of G-proteins as signal transducers (Campanoni and Nick, 2005). Interestingly, mitosis was more sensitive as compared with cell elongation by almost an order of magnitude. This indicates that the interphasic cortical microtubules driving cell elongation harbour a lower tyrosination activity as compared with the mitotic microtubule arrays. This is consistent with measurements of microtubule dynamics in BY-2 cells using the tip marker EBI for quantification (Dhonukshe *et al.*, 2005), where the assembly activity of mitotic microtubules was roughly twice that of interphasic arrays.

Disorientation of the cell plate emerged as one of the most sensitive and specific symptoms for  $\text{NO}_2\text{Tyr}$  activity. In fact, cell plate formation has been reported to be especially sensitive to perturbations of the microtubular cytoskeleton, for instance in response to experimentally induced doubling of the microtubular pre-prophase band (Murata and Wada, 1991; Yoneda *et al.*, 2005). In BY-2 cells, caffeine applied prior to the onset of mitosis completely inhibited the deposition of callose into the cross wall (Yasuhara, 2005). This was accompanied by reduced depolymerization of phragmoplast microtubules, supporting a model where disassembly of phragmoplast microtubules

in combination with microtubule translocation drives the formation of the cell plate.

Detyrosination of  $\alpha$ -tubulin increases as a consequence of increased microtubule stability (Skoufias and Wilson, 1998), and kinesins have been proposed as targets for the detyrosination signal. In fact, in mammalian cells, kinesin was shown to bind preferentially to detyr-tub (Liao and Gundersen, 1998). In experiments with saturated levels of antibodies against tyr-tub and detyr-tub as competitors for the binding sites of kinesin, it appeared that the antibodies against detyr-tub were more effective in blocking the binding of kinesin to microtubules. The association between detyrosinated microtubules and kinesin binding seems to hold for other systems as well. Recently it could be shown that in the filamentous fungus *Aspergillus nidulans*, UncA, a plus-end-directed kinesin-3 motor, preferentially binds to detyrosinated microtubules (Zekert and Fischer, 2009), although other kinesins (kinesin-1 and kinesin-7) did not show such a preferential binding, suggesting that the detyrosination signal is targeted to specific kinesins.

Evidence has accumulated for an important role for kinesins in the proper organization of the phragmoplast. In the moss *Physcomitrella patens*, it could be shown that the two kinesins KINID1a and KINID1b (kinesin for interdigitated microtubules 1a and 1b) are necessary for the interdigitation of phragmoplast microtubules (Hiwatashi *et al.*, 2008). Double deletion mutants showed that ~20% of the phragmoplasts formed incomplete cell plates, which did not reach the plasma membrane.

The following working hypothesis for the inhibitory effect of NO<sub>2</sub>Tyr on plant mitosis is therefore arrived at: NO<sub>2</sub>Tyr is incorporated into  $\alpha$ -tubulin by a plant homologue of TTL and reduces or inhibits TTC activity, which results in (irreversible) tyrosinated  $\alpha$ -tubulin. The target of the detyrosination signal might be specific kinesins, which play important roles in phragmoplast organization and vesicle transport to the growing cell plate. As a consequence of impaired  $\alpha$ -tubulin detyrosination, the binding of these kinesins is impaired, disturbing phragmoplast function and cell plate formation manifest as oblique cross walls and delayed exit from mitosis. This will lead to pleiotropic secondary effects, such as reduced root growth as a consequence of reduced meristematic activity or elevated cell elongation in BY-2 cells.

To test this model and to gain further insight into the molecular aspects of the detyrosination signal, the function of two homologues of the TTL which have been cloned from rice is presently being analysed.

### Outlook

In this work it could be shown that incorporation of NO<sub>2</sub>Tyr into plant  $\alpha$ -tubulin has some highly specific effects, such as inhibition of cell division, accompanied by stimulation of cell elongation and oblique cell plate formation. Additionally, it could be shown that cells grown with NO<sub>2</sub>Tyr were influenced in terms of the amount of detyrosinated  $\alpha$ -tubulin so that hardly any detyr-tub could

be detected. Besides these findings there are still open questions in the field of the tubulin tyrosination cycle; some aspects are still unclear and need to be determined.

One issue is whether the insertion of NO<sub>2</sub>Tyr into (plant)  $\alpha$ -tubulin is a reversible process or not. To answer this, one might use carboxypeptidase A and test *in vitro* whether decarboxylation of tubulin is impaired when it is purified from cells that have been treated with NO<sub>2</sub>Tyr. However, carboxypeptidase A cleaves a fairly broad range of un-specific substrates, whereas the TTC is specific for tubulin, making it difficult to transfer the results from one experiment to the other.

Thus, the still elusive TTC as well as the putative rice homologues of TTL have to be tested directly. Therefore, an approach to identify, clone, and express tubulin-modifying enzymes from rice has been launched.

Another approach is to run 2D-electrophoresis experiments which could be useful to supply additional data on the different tubulin isotypes, since they can be separated according to their isoelectric point, and their quantity is measurable.

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## ERRATA

### Giampiero Cai

Assembly and disassembly of plant microtubules: tubulin modifications and binding to MAPs  
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