Nitrosative stress triggers microtubule reorganization in Arabidopsis thaliana

Elisabeth Lipka and Sabine Müller*

ZMBP, Developmental Genetics, University of Tübingen, Auf der Morgenstelle 32, D-72076 Tübingen, Germany

* To whom correspondence should be addressed. E-mail: sabine.mueller@zmbp.uni-tuebingen.de

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Abstract

Microtubules (MTs) are essential components of the cytoskeleton and fulfil multiple cellular functions in developmental processes, readily responding to intrinsic and external cues. Nitric oxide signalling is well established in plants, and the MT cytoskeleton is one of its potential targets. To mimic low level nitrosative stress, growth medium was supplemented with 3-nitro-l-tyrosine (NO2-Tyr), a nitrated form of the amino acid tyrosine, and concentration-dependent changes in root growth rate and a reduction in cell division frequencies in Arabidopsis thaliana were observed. In addition, it is reported that exposure to low NO2-Tyr concentrations was not detrimental to plant health and caused subtle and reversible defects. In contrast, growth defects caused by high NO2-Tyr concentrations could not be reversed. Live cell imaging of an MT reporter line revealed that treatment with a low concentration of NO2-Tyr correlated with disorganized cortical MT arrays and associated non-polar cell expansion in the elongation zone. NO2-Tyr treatment antagonized the effects of taxol and oryzalin, further supporting the association of NO2-Tyr with MTs. Furthermore, oblique division plane orientations were observed which were probably induced prior to cytokinesis.

Key words: Cytoskeleton dynamics, microtubule, nitrosative stress, phragmoplast.

Introduction

Nitric oxide (NO) and its reactive derivatives act as intra- and intercellular signalling molecules in a variety of organisms (Besson-Bard et al., 2008). In plants, NO is a key molecule triggering signalling cascades during development and in response to abiotic stresses (Neill et al., 2002, 2008; He et al., 2005; Lombardo et al., 2006; Lombardo and Lamattina, 2012; León et al., 2014). The NO concentrations for signalling during plant development are low, whereas high levels of NO induce programmed cell death (PCD) (Bai et al., 2012). NO signalling acts upon diverse cellular responses including changes in gene expression levels via mitogen-activated protein kinase (MAPK) signalling cascades and regulation of transcription factors, as well as direct post-translational protein modifications (Neill et al., 2002). The nitrated amino acid tyrosine (N-Tyr) is a highly specific product of NO signalling, although post-translational nitration does not affect all proteins to the same extent (Abello et al., 2009). The impacts of N-Tyr incorporation into proteins include changes in enzymatic activity, alterations in proteolytic degradation, effects on protein phosphorylation and immunogenicity, and implications in disease (Abello et al., 2009).

The cytoskeleton is a common downstream target of multiple signalling pathways leading to its extensive reorganization and consequent changes in cell morphology. Tubulin isoforms and many microtubule- (MT) associated proteins (MAPs) are conserved throughout the plant and animal kingdom. Increasing evidence in the literature suggests that the cytoskeleton might be a direct target of NO signalling in mammals as well as in plants. For instance, in mouse brain tissue, α- and β-tubulin isoforms were identified in a protein fraction enriched for N-Tyr (Zhang et al., 2007). Similarly, α- and β-tubulin isoforms from Arabidopsis thaliana were
immunopurified with anti-3-nitroY antibody (Lozano-Juste et al., 2011). Furthermore, N-Tyr-containing proteins were immunoprecipitated with anti-tubulin antibodies, demonstrating the incorporation of N-Tyr into tubulin isoforms (Yemets et al., 2011). In mammalian cells, N-Tyr was incorporated into the extreme C-terminus of α-tubulin by tubulin–tyrosine-ligase (TTL), probably disturbing the tyrosination/detyrosination cycle of α-tubulin (Eiserich et al., 1999). Substitution of the C-terminal tyrosine by N-Tyr correlated with MT disorganization and changes in cell morphology. The modification of the extreme C-terminus of α-tubulin by reversible enzymatically catalysed addition and removal of tyrosine is one of a number of well-characterized post-translational tubulin modifications (PTMs) in mammalian cells and plants, although in plants the respective enzymes have not been identified conclusively (Smertenko et al., 1997; Westermann and Weber, 2003; Verhey and Gaertig, 2007; Giannoutsou et al., 2012).

The significance of PTMs for MT functions, however, is not fully elucidated. Most PTMs occur at the C-terminal tail of tubulins and associate with subpopulations of MTs with distinct functions and subcellular localizations. Furthermore, immunolocalization of PTMs using a variety of PTM-specific antibodies revealed that combinations of different PTMs were present on individual MTs in mammalian and tobacco cells (Smertenko et al., 1997; Janke and Kneussel, 2010; Quinones et al., 2011). Thus, it was suggested that different PTMs might serve as road maps to ensure accurate cargo delivery (Verhey and Hammond, 2009). MAPs and, more specifically, kinesins emerged as possible targets of tyrosine signalling and PTM sensing (Gurdan and Gundersen, 1995).

In plants, the MT cytoskeleton enables diverse cellular functions and is an integral constituent of developmental processes. In directionally expanding interphase cells cortical MTs arrange in parallel bundles, transverse to the axis of expansion, and guide plasma membrane-resident cellulose synthase complexes (Chan et al., 2007; Gutierrez et al., 2009). In mitotically active cells of higher plants, the cytokkeletal preprophase band is indicative of the future site of cell division. During cytokinesis, the phragmoplast facilitates the synthesis and fusion of the cell plate at the site formerly occupied by the preprophase band (Müller, 2012; Rasmussen et al., 2013).

Recently it has been proposed that N-tyrosinylation of the α-tubulin C-terminus might play a role in plant cell division (Jovanović et al., 2010). Rice seedlings and tobacco suspension culture cells were grown in the presence of 3-nitro-L-tyrosine (NO2-Tyr) as an exogenous source of N-Tyr, to add N-Tyr irreversibly to the C-terminus of α-tubulins. Upon treatment, tobacco cell culture cells exhibited mitotic inhibition and obliquely orientated cross walls, supporting the idea of direct impact of NO2-Tyr on prominent MT functions. In a recent report, growth defects of NO2-Tyr were investigated in A. thaliana (Blume et al., 2013). Here growth defects and associated MT reorganization in A. thaliana seedlings grown in the presence of NO2-Tyr are reported. Changes in MT organization were directly monitored, using live cell imaging of MTs, and visualized by a fluorescent MT reporter. In a concentration-dependent manner, NO2-Tyr decreased mitotic activity and caused cell swelling due to re-organized cortical MT arrays; however, the effects of oryzalin or taxol were less pronounced in combination with NO2-Tyr. Thus, in vivo evidence is provided that modulation of NO signalling allows for the reorganization of the MT cytoskeleton that might be relevant for development.

**Materials and methods**

**Plant material and growth conditions**

Throughout this study, transgenic A. thaliana plants, accession Columbia (Col) and Col expressing green fluorescent protein (GFP)–MAP4 (Marc et al., 1998), were used unless indicated otherwise. Seeds were surface sterilized, plated on standard medium plates containing 1× Murashige and Skoog salt mixture (pH 5.7) and 1½ agar, and were stratified for 2–4 d at 4 °C. In the growing chamber, plates were positioned vertically and seedlings grew for 4 d at 22 °C with continuous light (standard conditions). Under sterile conditions, seedlings were then transferred onto nutrient agar plates containing different concentrations of NO2-Tyr (Sigma-Aldrich). Treatment plates were prepared from standard medium supplemented with varying amounts of NO2-Tyr from 1 mM NO2-Tyr stock solution dissolved in 50 μM HCl or dimethylsulphoxide (DMSO). Mock controls contained 0.5 μM HCl and 10 μM tyrosine.

**Construction of XFP–fusion proteins**

TUA6 cDNA was synthesized as described elsewhere (Müller et al., 2006). Primers TUA6 F 5′-GGTACCATGAGAAGTGCAATTCGATCCA-3′ and TUA6 R 5′-CTCGAGTGGATATTCCTCTCTCATC3′ were used to amplify the TUA6 coding sequence flanked by KpnI and XhoI restriction sites (indicated in italics). Subsequently, cTUA6 was cloned into pGEM T (Promega), sequence verified, and transferred into pENTR3C via cloning into the KpnI and XhoI sites. Primers TUA6-Y24A-F 5′-AACATTAGAGACCTACGGCACCA-3′ and TUA6-Y24A-R 5′-TGAGGTGGTGGGCGGTAGGTCTTCAC3′ were used for site-directed mutagenesis to create pENTR-cTUA6 Y24A. To create pENTR cTUA6 Y450A, primer TUA6 F was used with TUA6-Y450A R 5′-CTCGAGTGGATAGGCTCTCTCCATC3′ for amplification of a PCR fragment which was digested with restriction enzymes EcoRI (TU6 internal site) and XhoI to exchange the respective fragment in pENTR cTUA6. Finally, pENTR clones were recombined with pFK-241 pGreenIIS destination vector using LR clonase (Invitrogen).

The MT-binding domain (MBD) contained in the destination vector pEG104-35S::Cherry-MBD (Gutierrez et al., 2009) was recombined into pDONR207 by BP clonase reaction (Invitrogen). Subsequently, an LR clonase reaction (Invitrogen) was performed with the destination vector pUBN:RFP containing the Arabidopsis ubiquitin10 promoter (pUB) (Grefen et al., 2010).

**Imaging and data analysis**

Microscopic images were acquired on a Leica SP2 upright confocal microscope using either a ×40, NA=0.70 water-immersion objective lens or a ×63, NA=1.20, water-immersion objective lens. Imaging of mitotic MT arrays was performed on a Leica SP5 inverted confocal microscope, with a ×40, NA=1.00, water-immersion objective lens, equipped with a resonant scanner. GFP was excited with the 488 nm emitting line of argon lasers. Cell wall patterns were visualized by staining with propidium iodide (10 μg/ml, Sigma-Aldrich) which was excited with a 561 nm HeNe laser. Images were acquired using Leica AF software. Image analysis and processing was performed in ImageJ (http://rsbweb.nih.gov/ij/) and Adobe Photoshop CS5, respectively. Figures were assembled in Adobe Illustrator CS5.
The density of MT bundles per micrometre was determined as described previously in Tanou et al. (2012). In brief, z-projections were assembled in ImageJ and grey values of Plot Profiles were measured and background corrected by subtracting the mean grey values of cortex areas without MTs. Along the plot profile, peaks were defined as grey values which were at least 10 units higher than the grey values of both neighbouring pixels, using Excel. Six intensity classes were deduced and the numbers of peaks were counted for each intensity class. Peaks are indicative of MT bundles.

The intensity of the cytosolic GFP–MBD signal in root cells of the elongation zone was determined in maximum projections of 21 optical sections at 0.5 μm intervals. Fluorescence intensities of areas between cortical MTs, corresponding to unbound cytosolic GFP–MBD, were measured. Averaged grey values per cell were background-subtracted and plotted in Excel.

The Kymograph Plugin (http://www.embl.de/eamnet/html/body_kymograph.html) was used to determine MT plus-end dynamicity, and measurements were used to calculate average growth and shortening velocities in Excel. Pearson’s correlation coefficient was calculated using the Colocalization Finder Plugin in ImageJ (http://rsb.info.nih.gov/ij/plugins/colocalization-finder.html).

Statistical significance of differences in measurements was determined with Student’s t-test.

Results

Effects of nitro-tyrosine on root growth

To mimic nitrosative stress conditions A. thaliana seedlings were exposed to different concentrations of 3-nitro-l-tyrosine [designated NO2-Tyr to distinguish it from intracellular/endogenous (N-Tyr) concentrations] added to the growth medium. Then, the effects of N-Tyr imbalance on seedling development were assessed. Therefore, 4-day-old seedlings expressing the MT reporter GFP–MBD were transferred to growth media containing varying concentrations of NO2-Tyr (0.1, 0.25, 0.5, 1, 5, and 10 μM) and grown for an additional 36 h (1.5 d). Control seedlings were transferred to standard medium, or medium containing either 0.5 μM HCl, 10 μM Tyr, or both (mock controls).

Analysis of root length before and after treatment revealed a dramatic reduction in growth upon NO2-Tyr exposure compared with the non-treated and the mock control seedlings (Fig. 1A). The reduction in root length was significant and decreased further with increasing NO2-Tyr concentrations (Fig. 1A). In contrast, root growth of untreated controls and mock-treated seedlings (0.5 μM HCl, 10 μM Tyr) did not vary (Fig. 1A; Supplementary Fig. S2A available at JXB online). In addition, the use of either HCl or DMSO as the solvent did not make a difference in growth responses.

Statistical significance of differences in measurements was determined with Student’s t-test.
Mitosis is inhibited in nitro-tyrosine-treated seedlings

Observations on rice seedlings and tobacco Bright Yellow (BY)-2 suspension culture cells suggested that NO2-Tyr treatment inhibited cell division (Jovanović et al., 2010). Expression of the MT reporter GFP–MBD allowed the evaluation of mitotic activity in living A. thaliana seedlings (Supplementary Fig. S1B at JXB online). The number of mitotic cells between the quiescent centre and epidermis was counted in image stacks at 2 μm z-intervals (Supplementary Fig. S1B). In control plants, on average 25.5 mitotic MT structures were observed per image stack (Fig. 1C; Supplementary Fig. S1B). In contrast, equivalent image stacks of NO2-Tyr-treated seedlings revealed a significant reduction in mitotic activity. At 0.5 μM NO2-Tyr the mean number of mitotic MT structures dropped to 6.1 and at a concentration of 1 μM NO2-Tyr only 1.3 mitotic cells were observed on average. Mitotic activity was entirely inhibited at 5 μM NO2-Tyr. These results confirmed a concentration-dependent inhibition of cell divisions upon NO2-Tyr treatment in A. thaliana seedlings.

NO2-Tyr-induced growth inhibition effects are reversible at low concentrations

Since high concentrations of NO2-Tyr had dramatic effects on root growth, it was investigated whether low concentrations or extended treatment periods were harming cellular functions to a similar extent and whether NO2-Tyr-induced effects on root growth could be reversed. As described above, seedlings were grown under standard conditions for 4 d and were subsequently transferred onto NO2-Tyr-containing medium for an additional 1.5 d. Then, the root length was measured, and half of the seedlings were retransferred onto standard medium for an extra 4.5 d (Fig. 2, black lines, designated as ‘ret’ for retransfer) to test whether root growth defects were reversed. The remaining half of the seedlings was transferred onto freshly prepared treatment plates of the respective NO2-Tyr concentration (Fig. 2, red lines indicating continuous NO2-Tyr treatment).

The root length of seedlings under different treatment conditions was plotted over time, and regression equations for each treatment were calculated as a reference for growth performance (Fig. 2). Based on similarities of the regression equations, the samples were grouped into four classes (I–IV). Controls (class I) were set to 100%, representing the full growth potential. Class II included low NO2-Tyr samples that were retransferred to standard medium (0.25 μM ret and 0.5 μM ret), attaining 72% recovery of potential growth. In addition, the 1 μM ret sample was grouped with class II, still displaying 55% of the potential growth. In contrast, samples that continuously grew on low NO2-Tyr concentrations (0.25 μM and 0.5 μM) fell into class III possessing only between 30% and 16% of control growth potential. Finally, class IV comprised all high concentration samples (5 μM and 10 μM continuous NO2-Tyr and retransferred 5 μM ret and 10 μM ret), all of which displayed severely reduced growth (1% growth). In addition, class IV also included samples continuously grown on 1 μM NO2-Tyr (7% growth). The clustering of samples into distinct groups indicated that high concentrations of NO2-Tyr irreversibly harmed seedlings even during the initial short-term treatment (1.5 d). On the other hand, low NO2-Tyr concentrations caused mild, non-toxic effects which were droped to 6.1 and at a concentration of 1 μM NO2-Tyr. These results confirmed a concentration-dependent inhibition of cell divisions upon NO2-Tyr treatment in A. thaliana seedlings.

Fig. 2. Growth effects induced by 3-nitro-o-tyrosine (NO2-Tyr) concentrations are reversible. Seedlings were grown on standard medium for 4 d and were subsequently transferred to growth medium containing different NO2-Tyr concentrations as indicated for 1.5 d (time point 1). Then, half of the seedlings were retransferred to standard medium for an additional 4.5 d (time point 2). Root length before (time point 1) and 4.5 d after retransfer (time point 2) is plotted over time. Black curves (continuous line) indicate samples retransferred onto standard medium plates, and red curves (dashed lines) indicate samples that were transferred to freshly prepared NO2-Tyr medium (continuous NO2-Tyr treatment). Equations of linear regression curves are indicative of growth rate performance and are given for each treatment. The equations are grouped into four classes (I–IV) based on the similarity of their slope. Class I contains non-transferred and retransferred control samples and represent 100% of the growth rate potential. Class II comprises samples retransferred from low NO2-Tyr concentrations (0.25, 0.5, and 1 μM ret). Class III contains samples continuously treated with low NO2-Tyr concentrations (0.25 μM and 0.5 μM). Class IV includes high NO2-Tyr concentrations (1, 5, and 10 μM, 5 μM ret and 10 μM ret). Percentage ranges indicate growth performance as achieved in each class as a means to illustrate growth recovery relative to the potential growth as measured for controls (100%).
reversible during the same treatment period. Remarkably, continuous treatment with 0.25 μM NO2-Tyr still allowed 30% growth, while short-term treatment with 1 μM NO2-Tyr could still be reversed to about half (55%) of the potential growth.

Cortical microtubule arrays are disordered upon NO2-Tyr treatment

Low concentrations of NO2-Tyr (0.5 μM) caused non-polar cell expansion in the elongation zone after 1.5 d of treatment, while cell division and cell expansion ceased at concentrations >1 μM NO2-Tyr (Fig. 1B, C). Non-polar cell expansion is typically related to alterations in MT organization. Indeed, in NO2-Tyr-treated plants, the characteristic expansion is typically related to alterations in MT organization. While cell division and cell expansion ceased at concentrations >1 μM NO2-Tyr, growth rate and MT organization in seedlings exposed to the drug treatment, as described before.

Taxol alone had little (11% for 1 μM taxol) or similar effects (54% for 10 μM taxol) on growth inhibition (as a percentage of control) compared with 0.5 μM NO2-Tyr alone (56%) (Fig. 4A, D). Growth inhibition by taxol in combination with NO2-Tyr (55% and 77%) was mostly attributed to NO2-Tyr effects (Fig. 4B–D). At the cellular level, taxol-induced stabilization of MTs (Fig 4G, 4I) was counteracted by NO2-Tyr when both drugs were present in the growth medium (Fig. 4H). High levels of taxol combined with NO2-Tyr caused pronounced cell expansion defects and NO2-Tyr-induced re-organization of MTs (Fig. 4J), indicating that the effects of taxol on MTs were over-ridden by NO2-Tyr.

Oryzalin treatment disrupted MTs, causing irregular expansion of cells and inhibition of mitosis, both contributing to a concentration-dependent reduction in growth rate (Fig. 4E, K, L). The effects of 1 μM oryzalin on growth inhibition (74%) were more severe than those of 0.5 μM NO2-Tyr alone (65%) (Fig. 4E). However, in combination with 0.5 μM NO2-Tyr, the oryzalin effect on growth rate inhibition increased only to 78% (Fig. 4E, M). Consistently, the addition of 0.5 μM NO2-Tyr reduced the oryzalin-dependent destabilization of MTs (Fig. 4M), suggesting that oryzalin binding to tubulin might be less efficient in the presence of NO2-Tyr. Single mutations in helix 7 (H7) of α-tubulins were reported to render plants and protozoa resistant to oryzalin (Anthony et al., 1998; Morrissette et al., 2004; Supplementary Fig. S5 at JXB online). Therefore, tyrosine at the beginning of H7 was mutated to alanine (Y224A) in TUA6 (Fig. 5A), which was identified among nitrotyrosinated proteins in A. thaliana (Lozano-Justo et al., 2011; Supplementary Fig. S5A), and it was expressed as a GFP fusion in A. thaliana protoplasts. Similarly, the C-terminal tyrosine in TUA6, the putative target of the tyrosination/detyrosination cycle, was mutated to alanine (Y450A) (Fig. 5A). Wild-type TUA6 (TUA6 WT) and mutant GFP fusion proteins were co-expressed with red fluorescent protein (RFP–MBD) to visualize MTs (Fig. 5B, D). While TUA6 WT (Fig. 5B, C, F) and the TUA6 Y450A mutant showed co-localization with RFP–MBD (Fig. 5F), TUA6 Y224A was not efficiently incorporated into MTs as determined by Pearsons correlation coefficient (Fig. 5D–F), supporting a critical role for Y224 for the integrity of the tubulin molecule.

Low concentrations of NO2-Tyr counteract responses to taxol and oryzalin
Fig. 3. Organization of cortical microtubule (MT) arrays in epidermal cells at the elongation zone. (A) In controls, interphase cells show characteristic parallel alignment of cortical MTs. Low concentrations of 3-nitro-o-tyrosine (NO2-Tyr, 0.5 μM) disrupt the cortical MT array organization and cells show signs of non-polarized expansion. Representative, inverted images are maximum intensity z-projections of image stacks, taken at 1 μm z-intervals; stacks encompass optical sections from the surface to a median cross-section through the cells. The number of optical sections per stack varies. Scale bar=10 μm. (B–D) Distribution of MT angles and the dynamicity of MT plus (leading) ends in epidermal cells of the elongation zone; (B) MT angles (red angle symbol in A) were determined counter clockwise, relative to the long axis of the cell (indicated by the yellow lines in A). The average angle of MTs (86.8 ± 34.6 °) in NO2-Tyr-treated cells differs significantly from the average angle in control cells (94.6 ± 19.6 °). The total number (n) of evaluated MT angles is given. Results represent the average angle of n=8 cells calculated from eight plants for controls and the average angle calculated from n=7 cells of seven plants treated with 0.5 μM NO2-Tyr. The average angle of cortical MTs upon NO2-Tyr treatment is significantly different from the transverse angle observed in controls (*P=0.0003). It is noteworthy that the standard deviation is rather large for NO2-Tyr. (C) Distribution histogram of MT angles observed in relation to the long axis of the cell in (A); the x-axis represents MT angles in 10 ° bins and the y-axis depicts the frequency of measurements for each bin. (D) Growth (polymerization) and shortening (depolymerization) velocities were determined for MT plus (leading) ends. No significant differences in MT dynamicity were observed between no treatment controls and NO2-Tyr-treated cells. MT growth and shortening velocities were measured in controls (n=23 cells from 10 roots), 0.5 μM NO2-Tyr-treated (n=28 cells from 11 roots), and 1 μM NO2-Tyr-treated (n=21 cells from eight roots) plants. The numbers of analysed plus ends are indicated in the bar diagram. Results represent the average velocities of two independent experiments. Error bars indicate ±standard deviation.
Fig. 4. Effects of NO₂-Tyr in combination with taxol or oryzalin. (A–C) Seedling morphology of (A) control without (−) and with (+) 0.5 μM NO₂-Tyr. (B and C) Taxol treatment as indicated without (−) and with (+) 0.5 μM NO₂-Tyr. (D and E) Growth inhibition is displayed as a percentage of untreated controls. (D) White columns show growth inhibition with taxol only. Grey columns show growth inhibition in the presence of 0.5 μM NO₂-Tyr. Error bars indicate ±standard deviation (n≥24). (E) Normalized growth inhibition as a percentage of control. White columns show growth inhibition with oryzalin only. Grey columns show growth inhibition in the presence of 0.5 μM NO₂-Tyr. Error bars indicate ±standard deviation (n≥27). (F–M) Microtubule (MT) organization in the presence of drugs as indicated. (K and M) Left panel: morphology of the elongation zone and division zone upon drug treatment as indicated.
Long-term, low concentration NO₂-Tyr treatment affects division plane orientation in root meristems

In BY-2 cells, NO₂-Tyr treatment altered the division planes (Jovanović et al., 2010). It was decided to determine whether similar defects occurred in the tissue context of A. thaliana root meristems. In short-term (1.5 d) experiments, cell wall positioning was normal; however, under high NO₂-Tyr concentrations (1–10 μM), mitosis was almost abolished and was already reduced at 0.5 μM NO₂-Tyr (Fig. 1C; Supplementary Fig. S1B). Therefore, cell wall positions in the root meristem were visualized by propidium iodide staining and were analysed in seedlings exposed to 0.5 μM NO₂-Tyr for 6 d. It was reasoned that cell divisions still occurred at this concentration (Fig. 1C) and thus might permit the accumulation of cell wall positioning defects over time. Indeed, a low number of oblique cell walls were observed in primary (Fig. 6B) and lateral root meristems (Fig. 6F) compared with the wild type (Fig. 6A, E).

However, positioning defects in NO₂-Tyr-treated plants were modest compared with mutants exhibiting defects in this pathway, such as the double mutant of PHRAGMOPLAST ORIENTING KINESIN 1 and 2 (POK1 and 2; Müller et al., 2006). While pok single mutants are phenotypically wild type, pok1pok2 double mutants display severely misplaced cell walls due to a failure in phragmoplast guidance (Muller et al., 2006). Thus, it was investigated whether pok1 and pok2 single and pok1pok2 double mutants were hypersensitive to NO₂-Tyr and whether cell wall positioning defects in these plants were induced at lower NO₂-Tyr concentrations than in GFP–MBD plants. As in previous experiments, seedlings were exposed to different NO₂-Tyr concentrations and the growth performance was assessed. Both pok1pok2 double mutants and pok1 single mutants, as well as pok2 single mutants expressing GFP–MBD (pok2;GFP–MBD), showed NO₂-Tyr-induced growth reduction similar to GFP–MBD transgenic plants (Supplementary Fig. S4 at JXB online). Furthermore, cell wall positioning defects were comparable with those of wild-type plants (data not shown). Thus it was concluded that NO₂-Tyr had no effect on either pok1, pok2;GFP–MBD, or pok1pok2 double mutants (Supplementary Fig. S6) and that NO₂-Tyr-induced oblique cell walls were not a result of interference with phragmoplast guidance.

Fig. 5. Arabidopsis thaliana protoplasts co-expressing TUA6 p3SS:GFP-TUA6 (green) and p3SS:RFP-MBD (magenta). (A) Protoplast co-expressing p3SS:GFP-TUA6 WT and p3SS:RFP-MBD. (C) Plot profile of fluorescent signal intensities along the dotted line in (A). (B) Protoplasts co-expressing p3SS:GFP-TUA6 Y450A and p3SS:RFP-MBD. (D) Plot profile of fluorescent signal intensities along the dotted line in (B). (E) Mean Pearsons correlation coefficient determined for GFP and RFP in protoplasts co-expressing p3SS:RFP-MBD together with either p3SS:GFP-TUA6 WT (0.45 ± 0.12, n=14) or mutant p3SS:GFP-TUA6 Y450A (0.46 ± 0.10, n=14) and p3SS:GFP-TUA6 Y224A (0.26 ± 0.12, n=14, *P>0.001).
Discussion

In all organisms investigated so far including plants, the abundance of NO and its derivatives such as free NO$_2$-Tyr is regarded as a reporter of nitrosative stress under pathological conditions (Halliwell et al., 1999; Foissner et al., 2000; Besson-Bard et al., 2008; Moreau et al., 2010). In plants, biotic and abiotic stress conditions lead to NO imbalance and provoke nitrosative responses preceding physiological and developmental changes (Lombardo et al., 2006; Neill et al., 2008; Corpus et al., 2011; Tanou et al., 2012). A solid understanding of NO biosynthesis and the in vivo effects of NO signalling is emerging in the plant field (Besson-Bard et al., 2008; Moreau et al., 2010). However, the detection of low, physiologically and developmentally relevant concentrations of reactive nitrogen species remains challenging (Halliwell et al., 1999; Berton et al., 2012). In the present study, the impacts of different concentrations of exogenously supplied NO$_2$-Tyr on root growth, root tip morphology, MT organization, and phragmoplast guidance in A. thaliana seedlings were evaluated.

A recent study in A. thaliana roots reported that NO stress, induced by sodium nitroprusside (SNP), caused PCD at high SNP levels, but triggered cell cycle arrest in G$_1$ phase at medium SNP levels, which were still above the reported developmentally relevant concentrations (Bai et al., 2012). Consistent with the present findings in response to different NO$_2$-Tyr concentrations, root growth was reduced and meristem cell number decreased upon SNP treatment (Bai et al., 2012). In the experiments reported here, the number of cell divisions decreased significantly at high NO$_2$-Tyr concentrations, indicative of cell cycle arrest. Although parameters such as DNA damage were not analysed, it is plausible that DNA damage induced at high NO$_2$-Tyr concentrations was responsible for the observed irreversible arrest of plant growth. The reversibility experiments are consistent with a recent study reporting reversibility of growth-inhibiting effects (Blume et al., 2013) at NO$_2$-Tyr concentrations similar to those used.
in this study. While stunted and moderately depolarized root hairs were observed here, 10-fold higher NO$_2$-Tyr caused ectopic and distorted root hairs (Blume et al., 2013), reflecting the wide morphological impact of nitrosative stress.

NO is a developmentally important signalling molecule in plants (Moreau et al., 2010). For instance, NO signalling targets root development via signal transduction to a MAPK signalling cascade (Wang et al., 2010). NO is also a significant intermediate of abscisic acid-induced signalling in the control of stomatal aperture (He et al., 2005; Ribeiro et al., 2009). In this context, NO targets the protein phosphatase 2C ABI1, but whether NO directly modifies ABI1, as has been shown for H$_2$O$_2$, is unclear (Meinhard and Grill, 2001; Desikan et al., 2002).

However, direct modification of cytoskeletal proteins might represent one disposition of NO signal propagation. In mammalian cells, N-Tyr is incorporated into the C-terminus of α-tubulin under pathological conditions, strongly suggesting that the cytoskeleton might be one target of NO signalling (Eiserich et al., 1999). Moreover, in BY-2 cells, NO$_2$-Tyr treatment correlated with a decrease in the relative amount of detyrosinated α-tubulin isoforms (Jovanović et al., 2010).

Several recent studies have reported on cytoskeletal targets of NO signalling or nitrosative stress. In protein extracts from BY-2 cells, antibodies against NO$_2$-Tyr preferentially decorated proteins of ~55 kDa molecular weight, similar to anti-tubulin antibodies, and precipitated tubulin cross-reacted with anti-tubulin antibodies, in support of the proposed incorporation of NO$_2$-Tyr into cytoskeletal proteins (Yemets et al., 2011). Indeed, recently, in vivo evidence for extensive nitrotyrosination was provided by immunolocalization of N-Tyr along mitotic MT arrays in BY-2 cells (Blume et al., 2013), indicating that N-Tyr might have specific affinity for highly dynamic MTs. Furthermore, a proteome approach identified the A. thaliana tubulin A6 as a target of nitrogen starvation, which leads to MT depolymerization in transgenic lines overexpressing GFP–TUA6 (Wang et al., 2012). Finally, several tubulin isoforms were identified as putative in vivo targets of nitrotyrosination by immunopurification of proteins from A. thaliana seedlings with anti-nitrotyrosine antibodies and subsequent mass spectrometry (Lozano-Juste et al., 2011).

Recent reports implicate that reactive oxygen species (ROS) signalling targets the MT cytoskeleton causing MT depolymerization, abnormal MT organization, and inhibition of cell cycle progression (Livanos et al., 2012; Yao et al., 2012). Also, depolymerization of MTs was observed following treatment with Verticillium dahlia toxins, which was shown to affect H$_2$O$_2$ and downstream NO homeostasis (Yao et al., 2012). Finally, drug-induced disturbance of ROS homeostasis resulted in the formation of MT paracrystals and abnormally bent MT bundles (Livanos et al., 2012). These reports support the direct modifications of the MT cytoskeleton by nitrosative stresses and the possible incorporation of N-Tyr into tubulin and MTs. However, in contrast to the above-mentioned studies, in the present study subtle and significant changes in the organization of the interphase MT array that were reversible were observed. Thus, evidence is provided that developmentally relevant low concentration NO signalling might act via delicately controlled modulation of MT organization. Consistently, exposure to SNP as an exogenous NO source also caused reorganization of the cortical MT array into randomly distributed MT bundles in A. thaliana epidermis cells of the differentiation zone (Yemets et al., 2009), further correlating NO signalling and cytoskeletal organization. Intriguingly, guard cell function correlated with quantifiable changes in MT clustering or bundling within guard cells (Eisinger et al., 2012a), and abscisic acid-induced guard cell aperture closure was accompanied by the reduction in MT structures (Eisinger et al., 2012b). Although the causal relationship between NO signalling, which acts downstream of abscisic acid, and MT bundling in guard cell function has not been demonstrated, the experimental evidence is suggestive that this relationship indeed exists.

Structural modelling of the interaction between plant α-tubulin and dinitroanilines suggested that slight changes in tubulin primary structure could provoke changes in binding activity of dinitroanilines (Blume et al., 2003). Combined treatment with oryzalin and NO$_2$-Tyr showed multiplicative growth inhibition of BY-2 cells (Jovanovic et al., 2010) and Arabidopsis (this study), supporting the notion that these drugs share a common target. Oryzalin binding to a conserved pocket of α-tubulin containing Thr239, just below the N-loop, might interfere with lateral binding to the M-loop of the adjacent dimer, thereby disrupting MTs and their polymerization (Morissette et al., 2004). Mutation of Thr239 to isoleucine in α-tubulin conferred resistance to oryzalin in goosegras (Mudge et al., 1984), and expression of the respective tubulin mutant Thr239Ile in maize suspension culture also conferred oryzalin resistance (Antony et al., 1998). Oryzalin-induced MT depolymerization is diminished in the presence of NO$_2$-Tyr, implying that NO$_2$-Tyr might interfere with efficient oryzalin binding at its target site. Mutation of Tyr224 to alanine (Y224A) in A. thaliana TUA6, one of the tubulins that precipitated with anti-NO$_2$-Tyr antibody (Lozano-Juste et al., 2011), interferes with efficient incorporation into MTs when expressed in protoplasts, revealing the essential role of this tyrosine. Nevertheless, the observed effects of the Y224A mutant may not be related to failure of N-Tyr incorporation, but rather reflect other structural characteristics of TUA6. A similar observation in transgenic A. thaliana expressing the phosphomimic mutant TUA6$^{T34D}$ (Fujita et al., 2013) further demonstrates how post-translational tubulin modifications contribute to the establishment of the MT cytoskeleton.

Likewise, NO$_2$-Tyr appeared to over-ride taxol effects on growth inhibition and MT organization. Taxol binding stabilizes the β-tubulin M-loop which interacts with N-loops from adjacent tubulin dimers to form lateral contacts (Snyder et al., 2001; Morissette et al., 2004). Since potential nitrotyrosination could interfere with the conformation of the N-loop, lateral binding of dimers might be destabilized even in the presence of taxol, consistent with the present observations. As expected, expression of the GFP–TUA6 Y450A, replacing the C-terminal tyrosine, did not alter MT incorporation efficiency noticeably, consistent with the
detection of detyrosinated tubulin isoforms along MTs in plants (Smertenko et al., 1997).

Computer simulations that modelled the α-tubulin C-terminus in three potential configurations predicted the reorganization of the cortical MT cytoskeleton and changes in cell morphology as a consequence of conformational changes of the C-terminus (Blume et al., 2005, 2013). According to these models, a C-terminal tyrosine would increase dynamicity of the MT polymer. In contrast, removal of the C-terminal tyrosine would cause conformational changes that could contribute to MT polymer stability. The addition of NO2-Tyr at the C-terminus would result in an intermediate MT polymer behaviour in this model. Consistent with the computer predictions, changes in MT organization and corresponding alterations of cell morphology were not observed upon NO2-Tyr treatment. Notably, simulation and modelling of the dynamics of tubulin C-termini also suggested that dynamic interactions between tubulin tails and the MT surface might influence MT polymerization and MT conformation, and thus flexibly alter the interaction of the MT with ligands (Freedman et al., 2011). Indeed, removal of the α-tubulin C-terminal tail altered the efficiency and pH dependency of colchicine binding to tubulin (Chakraborty et al., 2004). Similarly, the anions of glutamate-rich extreme C-termini of tubulin were involved in the regulation of vinblastine-induced tubulin polymerization (Rai and Wolff, 1998). Thus, NO2-Tyr at the C-terminus might lead to conformational changes of the tubulin dimer, and in consequence oryzalin and taxol binding might be inefficient, consistent with the present observations. Taken together, these findings further support the idea that NO signalling targets the MT cytoskeleton via post-translational modification of α-tubulin isoforms by direct incorporation of NO2-Tyr.

In animal systems it is well established that the balance between tyrosinated and detyrosinated α-tubulin in the MT cytoskeleton might reflect the developmental status of cells depending on whether they divide or differentiate (Quinones et al., 2011). Within distinct MT subpopulations, entire filaments may consist of tubulin subunits containing α-tubulins predominantly in their tyrosinated or detyrosinated form. These subpopulations might also be associated with multiple other PTMs at the same time, adding to the level of regulation (Verhey and Gaertig, 2007; Quinones et al., 2011). It has been recognized that stable MTs were associated with an increased degree of detyrosination, while highly dynamic MTs are mostly tyrosinated. Since MTs in most cells are usually very dynamic, the tyrosination cycle provides an effective way to modulate MT organization, for example during transitions between cell cycle phases (Janke and Kneussel, 2010).

In the experiments reported here, cortical MT array organization in the elongation zone appeared less well ordered upon low level NO2-Tyr treatment. MT arrays displayed fewer bundles and oblique angles relative to the longitudinal axis of the cell (Fig. 3) consistent with a recent study reporting the reorganization of cortical and endoplasmic MTs in the root apex, and the transition and elongation zone at concentrations ≥50 μM NO2-Tyr within 2h of treatment (Blume et al., 2013). Although the concentration and treatment period vary, the present results are comparable with those of Blume et al. (2013).

The changes in cortical array organization could not be attributed to changes in MT plus-end dynamicity. However, analysis of a small data set suggested that frequencies of transitions between MT growth, shortening, and/or pausing might be altered upon NO2-Tyr treatment (Supplementary Fig. S4D at JXB online). Nevertheless, the present results are consistent with observations from guard cells, where the organization of MTs changed, despite unaltered MT dynamicity of the MT plus end (Eisinger et al., 2012b). Changes in transition rates were also reported for a mutant of the MT plus-end binding protein AUGMIN subunit8 which is defective in MT reorganization, in addition to an altered MT plus-end shrinkage rate (Cao et al., 2013), implying that changes in transition rates are regulated by MT-associated proteins (MAPs) and contribute to MT reorganization. It is proposed that MAPs might be responsible for the cortical MT array reorganization. Consistent with this idea, changes in cytosolic GFP–MBD were observed upon NO2-Tyr treatment (Supplementary Fig. S4E). Indeed, it was proposed that MAPs might be direct targets of PTMs, that act as road maps to regulate MAP and motor protein trafficking along MTs (Verhey and Gaertig, 2007).

In BY-2 cells, NO2-Tyr treatment readily altered the orientation of the division plane, and it was hypothesized that kinesins required for phragmoplast expansion and vesicle transport were specific targets of detyrosinated α-tubulins (Jovanović et al., 2010). However, defects in phragmoplast expansion and vesicle transport typically result in incomplete cell walls and multinucleated cells (Jurgens, 2005) which were not observed in BY-2 cells (Jovanović et al., 2010), indicating that mechanisms different from phragmoplast expansion and vesicle transport are the targets of NO signalling via incorporation of N-Tyr. Also, division plane orientations were altered in A. thaliana seedling root meristems upon long-term, low concentration NO2-Tyr treatment (Fig. 5). Nevertheless, NO2-Tyr treatment did not produce more pronounced defects in pok single and double mutants and did not induce hypersensitive responses in pok mutants, suggesting that the mechanism of phragmoplast guidance, which is disturbed in pok mutants (Müller et al., 2006), was not specifically affected upon NO2-Tyr treatment. Thus, it is proposed that NO2-Tyr affects the MT cytoskeleton and associated MAPs already during prophase when the division plane is selected and established (Rasmussen et al., 2011; Müller, 2012). Therefore, NO2-Tyr treatment might become a useful tool to induce oblique division planes to study the underlying molecular mechanism.

In summary, the results presented here support the hypothesis that incorporation of N-Tyr into the C-terminus of α-tubulins interferes with the α-tubulin tyrosination/detyrosination cycle, leading to MT reorganization via MAPs that differentially recognize PTMs at the C-terminus of α-tubulins. Thus, it is assumed that MAPs, which might be the prime targets of developmentally relevant NO signalling, transduce the NO signal by reorganizing the MT cytoskeleton.
Supplementary data

Supplementary data are available at JXB online.

Figure S1. (A) Reduction of root tip size. (B) Representative, inverted confocal images depict single optical sections of the root meristem.

Figure S2. Growth response curves of seedling roots under different control conditions.

Figure S3. Growth response of A. thaliana Columbia wild-type (Col wt) roots upon 3-nitro-l-tyrosine (NO2-Tyr) treatment.

Figure S4. Quantification of organization and dynamic of cortical microtubule (MT) arrays in epidermal cells at the elongation zone.

Figure S5. (A) Alignment of TUA6 isoforms. (B) 3D model of the nucleotide-binding site in α-tubulin.

Figure S6. Growth responses to 3-nitro-l-tyrosine (NO2-Tyr) in A. thaliana seedlings grown on standard medium for 4 d and subsequent exposure to different NO2-Tyr treatments for an additional 6 d.

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