Planta (2003) 218: 276–287
DOI 10.1007/s00425-003-1097-1

ORIGINAL ARTICLE

Ravisha R. Weerasinghe · David A. Collings
Eva Johannes · Nina Strömgren Allen

The distributional changes and role of microtubules in Nod factor-challenged *Medicago sativa* root hairs

Received: 10 March 2003 / Accepted: 19 July 2003 / Published online: 27 August 2003

Abstract The normal tip-growing pattern exhibited by root hairs of legumes is disrupted when the hair is exposed to Nod factors generated by compatible bacteria capable of inducing nodule formation. Since microtubules (MTs) play an important role in regulating directionality and stability of apical growth in root hairs [T.N. Bibikova et al. (1999) Plant J 17:657–665], we examined the possibility that Nod factors might affect the MT distribution patterns in root hairs of *Medicago sativa* L. We observed that Nod factor application caused rapid changes in the pattern of MTs starting as early as 3 min after perfusion. Within 3 to 10 min after Nod factor application, first endoplasmic and then cortical MTs depolymerised, initially at the proximal ends of cells. Twenty minutes after exposure to Nod factors, a transverse band of microtubules was seen behind the tip, while almost all other MTs had depolymerised. By 30 min, very few MTs remained in the root hair and yet by 1 h the MT cytoskeleton re-formed. When Nod factors were applied in the presence of 10 μM oryzalin or 5 μM taxol, the MTs appeared disintegrated while the morphological effects, such as bulging and branching, became enhanced. Compared to the treatments with oryzalin or taxol alone, the combinatory treatments exhibited higher growth rates. Since microtubule reorganization is one of the earliest measurable events following Nod factor application we conclude that microtubules have an important role in the early phases of the signalling cascade. Microtubule involvement could be direct or a consequence of Nod factor-induced changes in ion levels.

Keywords Nodulation · Nod factor · Microtubule · *Medicago* · *Rhizobium*

Abbreviations *BNM*: buffered nodulation medium *CLSM*: confocal laser scanning microscopy *MT*: microtubule

Electronic Supplementary Material is available in the online version of this article at http://dx.doi.org/10.1007/s00425-003-1097-1

R. R. Weerasinghe · E. Johannes · N. S. Allen (✉)
Department of Botany, North Carolina State University,
Box 7612, Raleigh, NC 27695-7612, USA
E-mail: nina_allen@ncsu.edu
Fax: +1-919-5153436

D. A. Collings
Plant Cell Biology Group,
Research School of Biological Sciences,
Australian National University, GPO Box 475,
Canberra ACT 2601, Australia

Introduction

Nitrogen-fixing soil bacteria, collectively known as rhizobia, can establish a symbiotic partnership with legumes, which leads to the development of a new plant organ called a root nodule (Heidstra and Bisseling 1996; Downie and Walker 1999). The initiation of a nodule is governed by the molecular dialogue between the correct species or biovar of rhizobia and its appropriate plant host (Long 1996). The cross-talk begins when flavonoids in the plant root exudate activate the transcription of bacterial nodulation genes (*nod* genes), which results in the secretion of specific lipo-chitooligosaccharides (LCOs) called Nod factors (van Brussel et al. 1992; Dénarié et al. 1996). These compounds induce a cascade of signalling events that leads to the eventual formation of a nodule.

The first morphological changes in the root hair are deformations in the shape that occur 2–4 h after perception of the Nod factors (Schultze and Kondorosi 1998). These shape changes, however, are preceded by more rapid physiological responses. In the first minutes after application, Nod factors induce alkalinization of the root hairs (Ehrhardt et al. 1992; Felle et al. 1996), depolarization (Ehrhardt et al. 1992; Kurkdjian 1995; Felle et al. 1995, 1996; Gehring et al. 1997), and ion fluxes (Allen et al. 1994; Felle et al. 1998) across the plasma membrane and calcium spiking over the nuclear

area (Ehrhardt et al. 1996). Subsequently, changes in plant gene expression (Horvath et al. 1993; Journet et al. 1994) occur around 2 h, while cortical cell differentiation and mitosis (Truchet et al. 1991; Timmers et al. 1999) occur after 18–24 h.

The actin cytoskeleton is thought to play an important role in root hair growth and in the process of nodulation (de Ruijter et al. 1999; Emons and de Ruijter 2000). During nodulation, the actin cytoskeleton of *Medicago sativa* and *Phaseolus vulgaris* root hairs changes as early as 5 min after Nod factor application (Allen et al. 1994; Allen and Bennett 1996; Cárdenas et al. 1998). The changes are visualized as a rapid fragmentation of the filamentous actin starting after 5–10 min, at the apical region of the root hair and as actin foci during root hair curling (Allen and Bennett 1996; Cárdenas et al. 1998). In *Vicia*, however, the actin filaments do not fragment but become more dynamic in the presence of Nod factors (de Ruijter et al. 1999). Actin is also involved in the organization of the bacterial symbiosomes in mature root nodules (Whitehead et al. 1998).

Microtubules (MTs) change distribution during nodulation. However, unlike the rapid changes that occurred with actin, changes in MTs have been observed during root hair deformation and infection of the host by bacteria (Timmers et al. 1998, 1999). These changes were seen first in the cortex, around 16–18 h after Nod factor treatments, and later in curling root hairs. However, several lines of evidence suggest that changes might occur in MT organization during the early stages of nodulation, with these changes being potentially significant in the regulation of nodulation. First, the Nod factor-induced calcium spikes in root hairs (Ehrhardt et al. 1996) might be expected to destabilize the MT cytoskeleton, as MTs are traditionally thought to be calcium sensitive (Cyr 1991; Gianì et al. 2002). Second, the disruption of MTs leads to loss of directionality and multiple growth points in growing root hairs of *Arabidopsis* (Bibikova et al. 1999). This loss of directionality is similar to the changes seen in the other tip-growing systems following MT disruption. For example, MT disruption in moss caulonemal cells results in swelling and cell branching (Doonan et al. 1988; Wacker et al. 1988; Schwuchow et al. 1990; Meske et al. 1996), as does MT disruption in fungal hyphae (That et al. 1988) and some pollen tubes (Terasaka and Niitsu 1994). The similarity of the morphological structures that are formed during MT destabilization and early nodulation events such as changes in the direction of tip growth suggests a possible change in MT arrangement as an early event in Nod factor signalling. And third, MTs have been implicated in the regulation of plant calcium-channel recruitment (Thion et al. 1996; Lhuissier et al. 2001). This view is also based on the studies of the *Arabidopsis ton2* mutant, which has constitutively active calcium channels and disorganized MTs (Thion et al. 1998). Since Nod factors cause changes in calcium-channel activation, MTs seem likely candidates involved in the observed growth changes.

Microtubule distribution and function in forming, growing and mature *Medicago truncatula* root hairs has recently been described (Sieberer et al. 2002). The cortical MTs were present in all developmental stages while endoplasmic MTs were reported to form a three-dimensional array in the subapical cytoplasmically dense region in growing root hairs. These endoplasmic MTs, which were sensitive to oryzalin (an MT destabilizer) but not to taxol (an MT stabilizer), are thought to play an important role in tip growth.

In this study, we used immunolocalization of monoclonal anti-α-tubulin to investigate the MT distribution in growing root hairs, and the changes that result from Nod factor application. Nod factors modified the arrangement of MTs as early as 3 min after application. These changes are first observed in initiating root hairs, and at 10 min after Nod factor application almost all initiating root hairs displayed characteristic depolymerization at the base of the root hairs. The depolymerization was transient and root hair MTs recovered 1 h after Nod factor application. When Nod factors were applied to taxol or oryzalin pre-treated root hairs, both endoplasmic and cortical MTs appeared to be very short. We also observed that when MTs are broken down with oryzalin or stabilized with taxol, the growth pattern of control and Nod factor-stimulated root hairs was altered. It would appear that dynamic changes in MT organization are an early response to Nod factors and may form part a of the signal cascade leading to or permitting changes in root hair growth.

Materials and methods

Plant culture

Seeds of alfalfa (*Medicago sativa* L. cv. GT 13R plus) were surface-sterilized in 70% ethanol for 30 min, and then treated with 100% Clorox for 30 min. Seeds were rinsed five times with sterile deionized water and imbibed for 1 h at room temperature. The seeds were germinated overnight on filter paper and then grown on plates containing Buffered Nodulation Medium (BNM; Ehrhardt et al. 1992) with 8 g/l of agar (Fisher Biotech, Fair Lawn, NJ, USA) in the light at 27 °C. Vertically grown 3-day-old seedlings, which reached the size of 15–35 mm, were used in the experiments.

Nod factor additions

Three-day-old roots were immersed for 10 min in 10^{-8} M Nod Factor (NOD RM-IV) (kindly supplied by S. Long and D. Ehrhardt) dissolved in BNM and then rinsed in BNM, except for the two early time points of 5 and 10 min. At selected time points of 5, 10, 15, 20, 30, and 60 min after the Nod factor treatment, the intact plants were fixed. The immunocytochemistry steps to label MTs followed immediately and are described below. Each experiment was carried out with five or six replicate plants per treatment and repeated five times. Control treatments in which plants were treated with BNM instead of Nod factors at the times indicated were performed in parallel.

Pharmacological studies

A stock solution of 10 mM taxol (Sigma) was prepared in 100% dimethyl sulfoxide (DMSO; Sigma) and diluted in BNM to concentrations ranging from 1 to 10 μM, with 5 μM being used for most experiments. Intact roots were treated with these concentrations of taxol and MTs were immunolocalized in their root hairs. A stock solution of 1 mM oryzalin (ChemService, West Chester, PA, USA) was prepared in 100% DMSO and diluted in BNM to concentrations from 0.1 to 50 μM with 10 μM being used for most experiments. Intact roots were treated with these concentrations of oryzalin, fixed, stained and imaged for MT locations. Roots were placed in various combinations of 10^{-8} M Nod Factor, 5 μM taxol, and 10 μM oryzalin to make the treatments as equal as possible. Taxol and oryzalin were applied 20 min prior to Nod factors to ensure their effectiveness. Each root was then rinsed three times in BNM and grown in BNM for 4 h, at which point the growing root hairs were imaged. Universal Imaging Metamorph (UIC, Downingtown, PA, USA) v 4.6 software was used to analyze parallel regions in the obtained images for root hair lengths. The data consist of two independent trials with each treatment carried out five times. The treatment types and duration are shown below.

Control	20 min BNM	4 h BNM		
Nod factor	20 min BNM	10 min Nod	3 h and 50 min BNM	
Oryzalin	30 min oryzalin + BNM		3 h and 50 min BNM	
Oryzalin/Nod	20 min oryzalin + BNM	10 min oryzalin + BNM + Nod	3 h and 50 min BNM	
Taxol	60 min taxol + BNM			3 h BNM
Taxol/Nod	20 min taxol + BNM	10 min taxol + BNM + Nod	30 min taxol + BNM	3 h BNM

Indirect immunofluorescent labelling of microtubules

Intact, Nod factor-treated roots were placed for 1 h in fixative containing PME (150 mM Pipes, 6 mM $MgSO_4$, 15 mM EGTA, pH 6.9), 1% DMSO, 0.01% Triton X-100, 8% formaldehyde, 4% glutaraldehyde. The roots were washed with phosphate-buffered saline (PBS; 2.68 mM KCl, 1.47 mM KH_2PO_4, 13.69 mM NaCl, 0.81 mM Na_2HPO_4, pH 7.4) and treated with 100 μg ml^{-1} lysophosphotidylcholine (Sigma) for 30 min (de Ruijter et al. 1999), acetone at −15 °C (10 min) and 5 mg ml^{-1} $NaBH_4$ (15 min). Three washes of PBS were given after every treatment to remove the residual chemicals. Roots were then incubated in blocking buffer (5% BSA, 0.05% Tween-20 in PBS) for 15 min and labelled overnight with monoclonal anti-α-tubulin (clone N356; Amersham) diluted 7/200 in incubation buffer (1% BSA, 0.05% Tween-20 in PBS). The samples were washed several times with PBS and incubated for 1 h in secondary antibodies [either Cy-3 labelled goat anti-mouse IgG (Jackson, West Grove, PA, USA) diluted1/1,000 in incubation buffer or Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA) diluted 1/2,000 in incubation buffer]. After washing with PBS, whole roots were mounted in PBS on microscope slides, covered with No. 0 cover slips and sealed with melted Valap (Vaseline, lanolin, paraffin, 1:1:1, by vol).

Confocal microscopy

Whole mounted roots were imaged using a confocal microscope (Leica TCS SP) with a 40× numerical aperture (N.A.) 1.25 oil-immersion objective in fluorescent and transmitted light modes. Optical stacks were recorded using sections of approximately 0.1–0.6 μm in thickness, with each plane averaged four to eight times. Cy-3 was excited at 568 nm with emission collected from 580 to 620 nm while Alexa Fluor 488 was excited at 488 nm and emission collected from 500 to 550 nm. Transmitted light DIC (*D*ifferential *I*nterference *C*ontrast) images were recorded concurrently. Images were processed using Leica confocal software, Metamorph and Adobe Photoshop 5.0. Volume rendering of confocal stacks was performed using a maximum projection algorithm.

Light microscopy

Living roots were mounted on microscope slides, covered with No. 1.5 cover slips and observed with 5×, 0.12 N.A. and 20×, 0.60 N.A. Planapo objectives on a Leica DM RXA by DIC microscopy. Images were acquired with an ORCA-ER (Hamamatsu, Hamamatsu, Japan) camera using Metamorph, which was also used for morphometric analysis.

Results

Alfalfa root hairs exhibit net-axial or helical, cortical microtubules

Alfalfa root hairs in the elongation zone show three main stages of growth: (i) bulge formation from epidermal cells, (ii) polar growth and (iii) growth termination. Observation of microtubules was carried out in initiating root hairs and in root hairs exhibiting polar growth. Both respond to Nod factors (Long 1996). Figure 1a–c depicts optical sections of growing root hairs of untreated control plants with MTs immunolabelled after 10 min of buffered nodulation medium

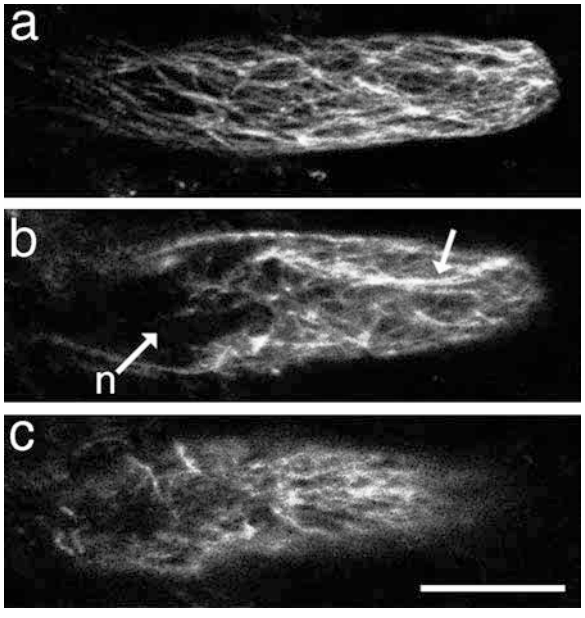

Fig. 1a–c MTs in growing root hairs of alfalfa (*Medicago sativa*) visualized with confocal laser scanning microscopy (CLSM) in z steps of 0.4 μm. Three sections were projected for each fluorescence image. The cell periphery shows predominantly net-axial cortical MTs (**a**, sections 2–4/19; **c**, sections 15–17/19). The endoplasmic MTs are located in the cytoplasm in between the nucleus and the apex (**b**, sections 10–12/19). Some long endoplasmic MTs curl back at the tip (**b**, *arrow*). *n* Nucleus. Bars = 10 μm

(BNM) treatment. The root hairs have cortical MTs arranged in long bundles lying longitudinally along the hair cell. The cortical MTs are predominantly net-axial at the tip area while being more longitudinally arranged at the base (Fig. 1a, c). The endoplasmic MTs of the root hairs are located in the cytoplasm between the nucleus and the tip of the cell (Fig. 1b, arrow). No MTs were observed in the nucleus. The endoplasmic MTs are in a densely organized array, which extends from the vicinity of the nucleus towards the tip. They curl back at the apex of the hair cell (Fig. 1b, arrow).

We observed the MT distribution of three developmental stages of growing root hairs. The initiating root hairs (10–30 μm in length) have predominantly net-axial cortical MT arrays, which are tightly intertwined (Fig. 2a). The MTs are of variable density and degrees of bundling, and they are also present in the tip apex (Fig. 2a, arrow). As the root hairs increase in length (80–100 μm), MTs show a helical pattern at the base. Arrays, which are net-axial just below the tip, extend to the apex of the root hair tip (Fig. 2b). In the longer polar-growing root hairs (150–180 μm), the MT arrays at the base are somewhat loosely arranged (Fig. 2c) when compared to the younger hair cells.

Nod factors cause the disintegration of endoplasmic MTs, 3 min after perfusion

To observe the changes in the MT cytoskeleton associated with Nod factors, roots of intact seedlings were perfused with 10^{-8} M Nod factors for 10 min, and were fixed and immunolabelled at set time intervals either during the Nod factor perfusion (time intervals less than 10 min), or after Nod factor was washed out. The first changes were seen in endoplasmic MTs. Three minutes after Nod factor perfusion cortical MTs appeared unchanged (Fig. 3a, c) whereas, the endoplasmic MTs were shorter in appearance with short MTs seen mainly in areas closer to the nucleus rather than at the tip (Fig. 3b). The longitudinal endoplasmic MTs seen in the control treatments became shorter at the base. No change in the MT pattern was observed in plants that were treated with BNM instead of Nod factors at the time points indicated (data not shown).

MTs depolymerize after Nod factor treatment

Confocal imaging of intact roots demonstrated that the root hair cortical MT pattern changes around 5 min after perfusion of Nod factors (for controls, see Fig. 1). In very young root hairs (10–30 μm), we observed the disappearance of the MTs at the base of the root hair (Fig. 4a, arrow; and see Video 1 in the Electronic Supplementary Material). Concurrent transmitted light images indicated that the MT breakdown originated in the vicinity of the nucleus (Fig. 4b, arrow). The fragmentation of the MTs was more distinct at 10 min following Nod treatment, but the most striking changes were seen at 20 min.

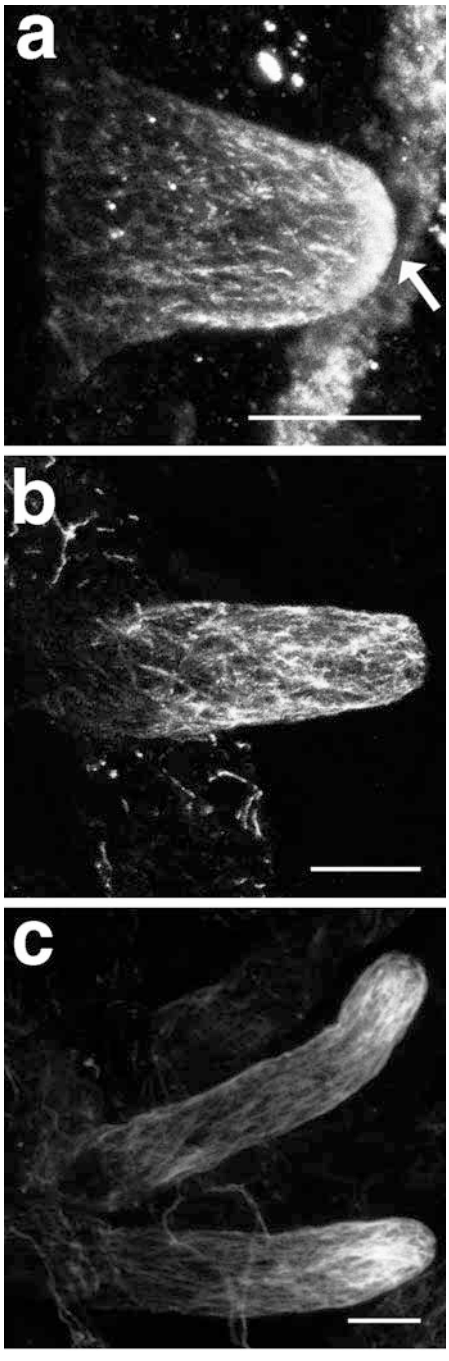

Fig. 2a–c Full-stack projection of CLSM fluorescence images showing MTs of untreated alfalfa root hairs. The bulges (10–30 μm, **a**), medium-sized (80–100 μm, **b**) and older (150–180 μm, **c**) root hairs display net-axial or helical MTs. The MTs extend to the tip of the root hair (**a**, *arrow*). Bars = 10 μm

At 20 min, the bases of the initiating root hairs appeared to be devoid of prominent MTs (Fig. 4c). However, the tips exhibited a ring like structure close to the plasma membrane (Fig. 5a–d; and see Video 2 in the Electronic Supplementary Material). This unusual ring formation occurred in 64.5% of the 206 root hairs observed. The very apex of the majority of the root hairs was devoid of MTs as demonstrated (Fig. 5 and Vi-

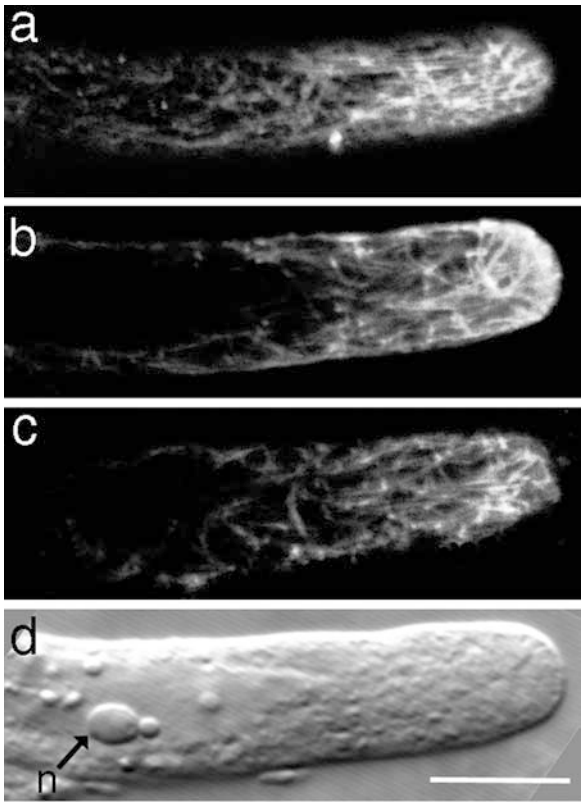

Fig. 3a–d MTs in growing alfalfa root hairs visualized 3 min after Nod factor application. CLSM scanning steps in the z plane were 0.2 μm. Three sections were projected for each fluorescence image. **a, c** The images of the periphery show intact cortical MTs (**a**, sections 6–8/52; **c**, sections 46–48/52). **b** Projection through the middle of the cell (sections 19–21/52). Endoplasmic MTs have begun to disintegrate near the vicinity of the nucleus. The long MTs that curl back are shorter than those of untreated cells. **d** Transmitted light image of the growing root hair. *n* Nucleus, Bars = 10 μm

deo 2). The higher amount of diffuse staining in this region may indicate the presence of higher amounts of heterodimeric tubulin. Some bulges also displayed a ring-like structure along with short longitudinal arrays of MTs leading away from it, parallel to one another. The depolymerization of MTs was complete at 30 min, at which time the young root hairs were primarily devoid of MT labelling (Fig. 4d). A small percentage of the observed root hairs showed some punctate labelling. At 1 h after Nod treatment, the root hairs began to recover longitudinally arranged short MTs, which appeared somewhat diffuse (Fig. 4e).

MTs of 80- to 100-μm root hairs disintegrate 5 min after Nod treatment, but do not show the unusual ring like structure seen in younger root hairs

At 5 min following Nod treatment, MTs in the 80- to 100-μm-sized root hairs displayed an altered pattern similar to that of bulges (Fig. 6a). The depolymerization

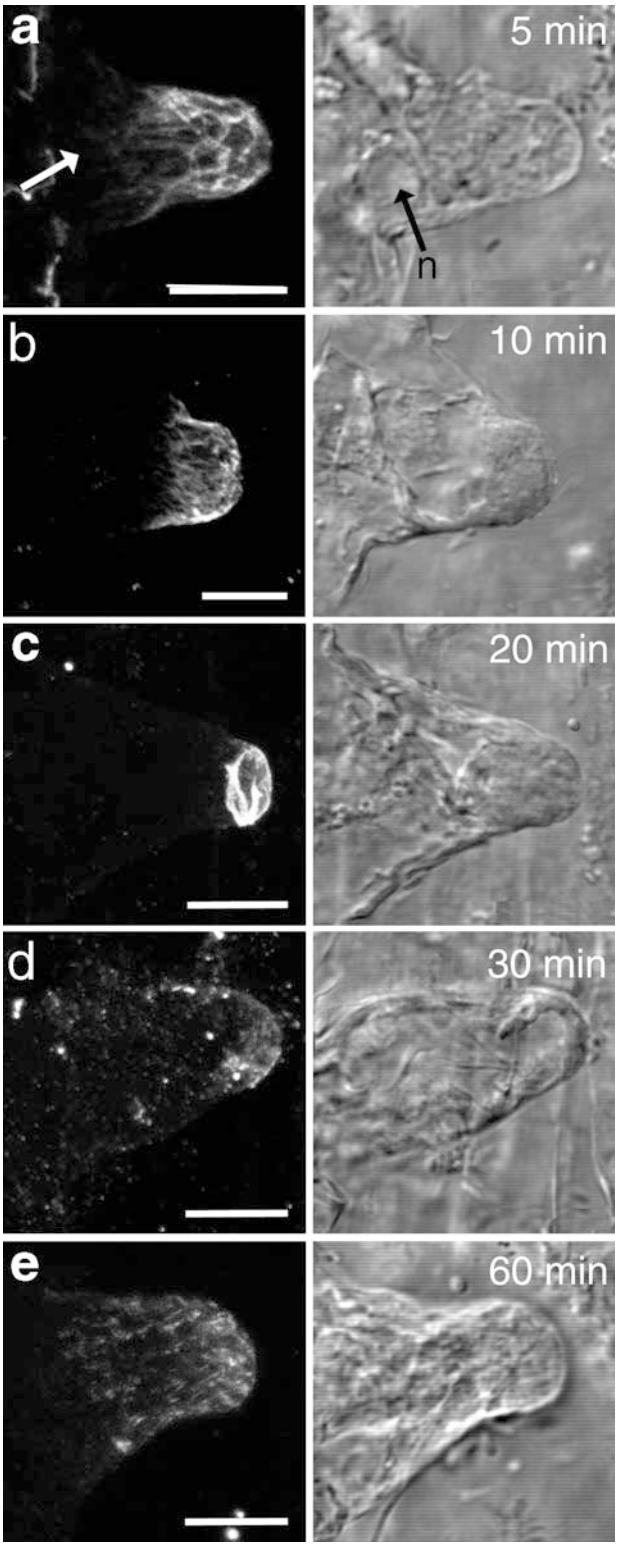

Fig. 4a–e Full-stack projections of fluorescence and individual transmitted light images showing reorganization of MTs in young root hairs (10–30 μm) of alfalfa after perfusion of Nod factors. The disintegration of MTs begins around 5 min (**a**) from the root hair base (**a**, *arrow*) and proceeds towards the tip at 10 min (**b**). At 20 min (**c**) MTs are observed as a ring structure at the tip. The disintegration is complete at 30 min (**d**) and re-formation can be seen at 1 h (**e**). *n* Nucleus. Bar = 10 μm

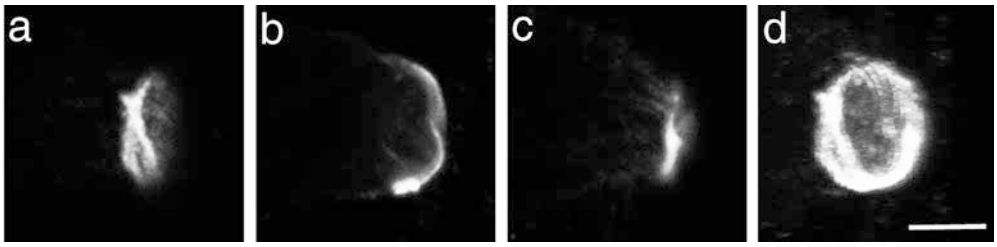

Fig. 5a–d MTs in young alfalfa root hairs 20 min after treatment with Nod factors. CLSM scanning z steps were 0.5 μm. **a–c** Projection of three sections each of the cell periphery (**a, c**) and middle of the cell (**b**) shows that the transverse band of MTs is in the periphery of the cell. **d** The full-stack projection of MTs at an angle of 45° so that the ring of MTs that wraps around the root hair tip is viewed partially end on. Bar = 5 μm

of the MTs began at the base of the root hair and became more prominent at 10 min after Nod treatment (Fig. 6b).

The bases of the medium-sized root hairs were lacking long MTs at 20 min (Fig. 6c). At this stage the root hairs were difficult to fix and image. Unlike the very young hair cells, these root hairs revealed prominent punctate labelling and short MTs at their bases. The tips were brightly labelled with longitudinally arranged short MTs spreading for a short distance from the apex. The very apices lacked MTs. But diffuse staining seen at the apex suggested the possibility of the presence of heterodimeric tubulin. Similar to that of bulges, MTs in the 80- to 100-μm-sized root hairs disintegrated completely at 30 min and started to re-establish themselves at 1 h after the treatment of Nod factors (Fig. 6d, e).

The depolymerization of MTs in longer root hairs occurs at around 10 min following the treatment with Nod factors

The longer root hairs (150–180 μm) did not show an alteration of MT pattern at 5 min after Nod factor treatment (Fig. 6f), unlike the comparatively short younger root hairs described above. At 5 min, 85.5% out of 214 longer root hairs had an MT pattern that was similar to that of the control plants (Fig. 2c). The disruption of MTs in these root hairs was clearly observed at 10 min after Nod factor treatment (Fig. 6g), indicating that the response is somewhat delayed in older hair cells. At 20 min, the pattern of MTs was relatively intact in comparison to the younger root hairs (Fig. 6h). Some displayed short MTs, while the others had very loosely arranged helical MTs with some areas devoid of MTs. Thirty minutes following the Nod treatment, the longer root hairs showed diffused and punctate labelling, indicating disintegrated MTs (Fig. 6i). One hour after treatment, MTs showed some recovery (Fig. 6j). The MTs, however, did not completely recover and were diffuse and short.

MTs are important for the signalling process of Nod-induced root hair growth

Nod factor-induced morphological changes in root hairs are similar to the changes that are seen during the treatment with MT-perturbing agents such as taxol or oryzalin. To determine whether Nod factor-induced MT changes are directly related to Nod factor-induced polar growth changes, we compared the effects exerted by MT-altering drugs with that of Nod factors. Taxol and oryzalin were applied individually and in combination with Nod factors. We expected the stabilization of MTs by taxol to decrease, and the destabilization by oryzalin to enhance the morphological effects induced by Nod factors. The study was conducted in three phases.

First, using immunofluorescence we determined the appropriate concentration of oryzalin and taxol. The cortical MTs of the root hairs depolymerized following a 30-min treatment with 10 μM oryzalin. On the other hand, root hairs treated with 5 μM taxol displayed a similar MT pattern to untreated control root hairs. Neither of these treatments, however, appeared to inhibit root hair growth.

To observe the effects on root hair morphology, the root hair zone of the examined plants was divided into four areas each about 1,500 μm in length: bulges and initiating root hairs (zone I), medium-sized polar growing root hairs (zone II), longer polar-growing root hairs (zone III) and fully grown hairs (zone IV). MT-altering agents were applied for 30 min (oryzalin) and 60 min (taxol), respectively, and then removed. Nod factors were applied as indicated for 10 min following a 20-min pre-treatment with oryzalin or taxol. The treatment schedule is described in Materials and methods.

We recorded the effect of the drug treatment on zone-II and zone-III root hairs 4 h after the respective treatments. For the interpretation of these data it has to be kept in mind that root hairs were growing during the 4-h period between drug/Nod factor treatment and data recording. Thus root hairs sized 10–30 μm at the time of observation were not considered in the analysis since they were not present during treatment. For zones II (medium) and III (old) we measured the length of the root hairs that were clearly in focus using calibrated Metamorph image analysis software. A total of 217 root hairs was examined, of which 37 and 27 were treated with taxol or oryzalin, respectively, and 36 and 39 received combination treatments of taxol/Nod and oryzalin/Nod.

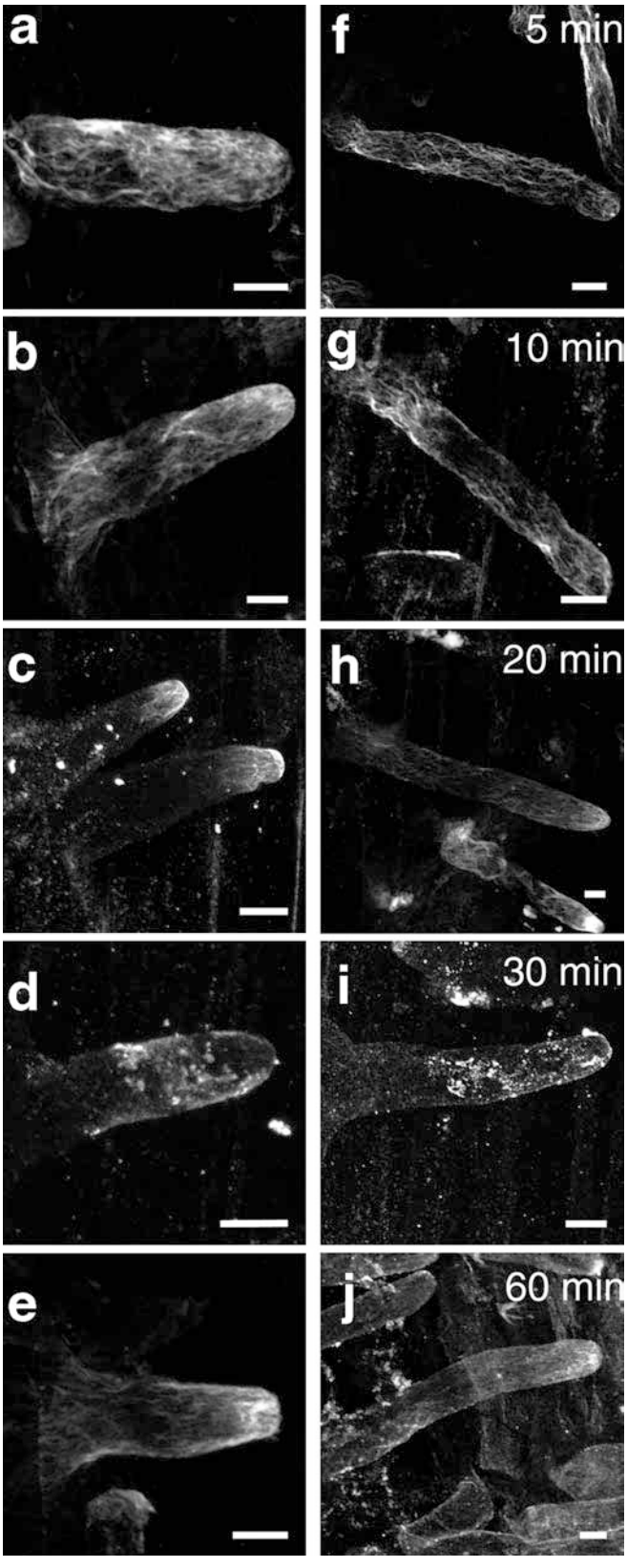

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Fig. 6a–j Time series of alfalfa root hairs (full-stack projections) showing rearrangement of MTs at 5 (**a, f**), 10 (**b, g**), 20 (**c, h**), 30 (**d, i**), 60 (**e, j**) min after treatment with Nod factors. **a–e** For medium-sized (80–100 μm) root hairs, destabilization of MTs begins around 5 min and is complete at 30 min after the Nod factor perfusion. **f–j** The responses of older root hairs (150–180 μm) are delayed in comparison to the younger root hairs. MTs are intact at 5 min (**f**). The reorganization of MTs initiates around 10 min (**g**) after treatment with Nod factors. For roots of both ages, the disintegration is complete at 30 min and MTs starts to recover around 1 h. Bar = 10 μm

Our experiments showed that the taxol treatment alone caused the older root hairs (zone III) to grow at a slower rate than the BNM-treated control (Fig. 7). The average lengths of taxol-treated root hairs (142 ± 18 μm) and BNM-treated control root hairs (187 ± 14 μm) were significantly different at the 0.05 probability level ($P = 6.33E^{-6}$, independent student's *t*-test analysis). When the root hairs were subjected to Nod factors after pre-treatment with taxol, the root hair length increased to 298 ± 55 μm. On the other hand, oryzalin treatment alone caused an increase in growth rate, which also has been observed in root hairs of *Arabidopsis thaliana* (Bibikova et al. 1999). Root hairs pre-treated with oryzalin prior to Nod factor application reached a length of 271 ± 71 μm, which was similar to that observed with either Nod factor (not significantly different at the 0.05 level, $P = 0.606$) or oryzalin treatment by itself (not significantly different at the 0.05 level, $P = 0.779$). Lengths of oryzalin/Nod-treated root hairs were also not significantly different from taxol/Nod treated-root hairs ($P = 0.196$). Thus, for all treatments, except the BNM control (roots not exposed to Nod factors) and taxol, root hairs had grown to a similar length at the time of the measurement, suggesting that they might have reached their maximum length.

The zone-II root hairs displayed an analogous trend to that of zone-III root hairs. However, there were noteworthy differences. Unlike in zone III, the taxol-treated root hairs (95 ± 27 μm) and BNM-treated control root hairs (103 ± 27 μm) exhibited no significant difference at the 0.05 level ($P = 0.352$), indicating similar growth rates. Nevertheless, when the root hairs were treated with Nod factors after pre-treatment with taxol the length increased to 114 ± 26 μm. Similar to zone III, zone-II root hairs treated with oryzalin alone had a higher growth rate than the control root hairs (significantly different at the 0.05 level, $P = 0.019$). The oryzalin-treated root hairs were similar to the Nod factor-treated root hairs, which also showed a higher rate of growth (not significantly different at the 0.05 level, $P = 0.410$). When root hairs were pre-treated with oryzalin prior to Nod factor treatment the length of the root hairs increased even further.

The observed morphological changes showed a similar trend as the growth pattern. As expected, Nod factor-treated root hairs showed characteristic bulging and branching 4 h after Nod factor treatment (Fig. 8b). Oryzalin and taxol also caused bulging and branching of root hairs (Fig. 8c, d). Interestingly, in the presence of Nod factors, oryzalin or taxol pre-treated root hairs displayed a significant increase in the formation of

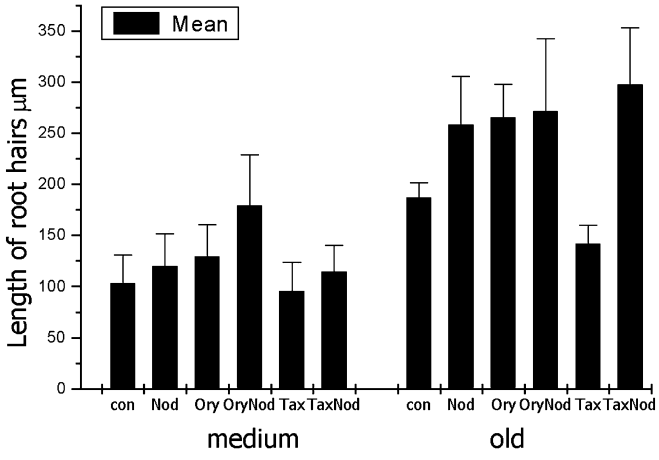

Fig. 7 Four hours after different drug treatments, the lengths of younger (*medium*) and older (*old*) root hairs of alfalfa were measured. Older root hairs are more responsive but medium-sized root hairs show the same trend. Application of Nod factors, oryzalin (*Ory*; $n = 27$) or oryzalin/Nod ($n = 39$) increases the length of root hairs. On the other hand, taxol (*Tax*; $n = 37$) reduces root hair growth. This growth reduction can be overcome by taxol/Nod ($n = 36$) treatment. *con* Control. Mean values ± SD

branches and bulges (Fig. 8e–h, Fig. 9). This observation indicates the possibility of Nod factors enhancing the effects of oryzalin and taxol.

To further investigate the role of MTs in root hair growth and Nod factor signalling, we immunolocalized MTs in roots that were treated with MT-altering agents and/or Nod factors using the same treatment schedule as for the growth studies. As expected, application of the MT-stabilizing drug taxol alone (Fig. 10a) did not appreciably alter the MT arrangement of root hairs when compared to BNM treated controls (Fig. 10b).

When root hairs were treated with both taxol and Nod factors we observed the following changes in the MT pattern. Whereas 20 min following Nod factor treatment the MTs were similar to those of control root hairs (Fig. 10c), they disintegrated into short pieces at 1 h following Nod factor treatment (Fig. 10d). This indicates that Nod factors are capable of overcoming the stabilizing effect of taxol even though the disintegration is somewhat delayed. Oryzalin treatment alone caused a disintegration of MTs (Fig. 10e). In root hairs that were given oryzalin/Nod combination treatment, MT disintegration was observed at both 20 (Fig. 10f) and 60 min (data not shown) after Nod factor application.

Discussion

Our study demonstrates that Nod factor treatment causes rapid and dynamic changes in the pattern of MTs in root hairs. Within 3–10 min after Nod factor treatment, first the endoplasmic and then the cortical MTs disintegrated. In both cases the basal MTs decayed first. The disintegration of cortical MTs was complete at 30 min and the MT network re-formed 1 h after Nod factor exposure. Importantly, many root hairs exhibited a ring-like structure at the tip of the root hair before the complete disintegration of cortical MTs (Fig. 6). Our

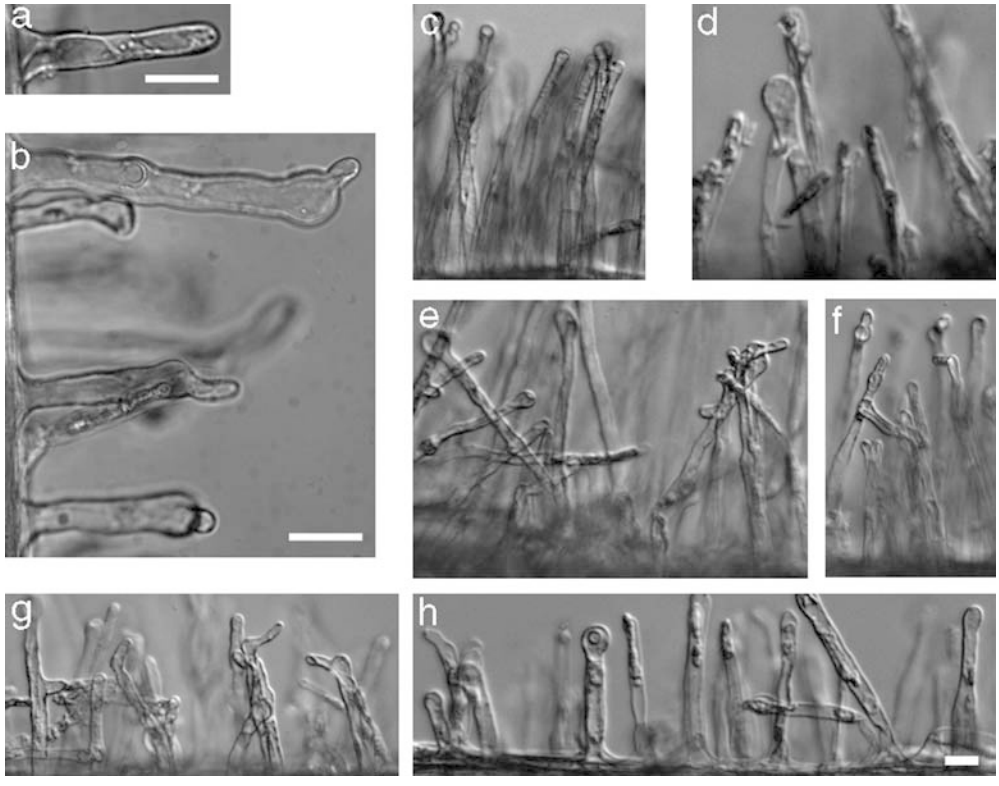

Fig. 8a–h Morphology of alfalfa root hairs. **a** BNM-treated control root hairs appear normal. **b** Root hairs treated with Nod factors show bulbous tips and branches. **c, d** Root hairs treated with 10 µM oryzalin (**c**) and 5 µM taxol (**d**) both display morphologies similar to those treated with Nod factors. **e, g** Longer (**e**) and medium (**g**) sized root hairs treated with oryzalin/Nod show enhanced branching effects. **f, h** Bulbous tips and branching are shown by taxol/Nod-treated older (**f**) and medium-sized (**h**) root hairs. Bar = 20 µm

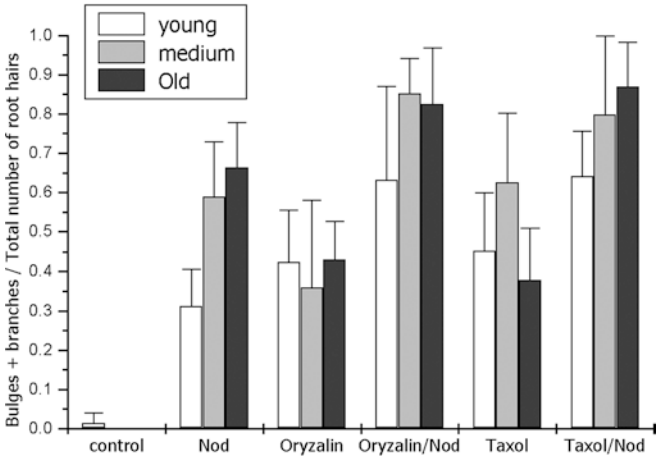

Fig. 9 Ratio of tips that bulged or branched in response to treatments with respect to the total number of alfalfa root hairs. BNM-treated control does not show a significant response. All other treatments show bulging. Root hairs that were treated with either oryzalin/Nod or taxol/Nod show greater responses than treatments with oryzalin or taxol alone

results show that MTs play a role in regulation of the growth machinery of root hairs and strongly influence the growth pattern.

Root hairs develop from root epidermal cells called trichoblasts (Cormack 1949). Before the emergence of root hairs the MTs are arranged perpendicular to the axis of the root (Emons and Derksen 1986; Lloyd et al. 1987). The first step in root hair initiation is bulge formation (Gilroy and Jones 2000) and the bulge can be seen as a triangular protrusion of the trichoblasts. Once initiated, the root hair grows in a polar fashion. Three defined zones of root hairs occur along the root; (I) growing, (II) terminating growth, and (III) full-grown (Long 1996; de Ruijter et al. 1999). The patterns of microtubule distribution were similar in fixed and GFP–MAP4-labelled root hair cells of *M. truncatula* (Sieberer et al. 2002). While immunolabelling of MTs in epidermal cells is comparatively easy, root hairs, especially those that are older and non-growing, are more difficult to label. However, since the root hairs that respond to lipochitooligosaccharides are in zones I (length 10–30 μm) and II (length 30 μm up to 180 μm), the study concentrated on the root hairs in these two zones.

MT visualizations in growing root hairs vary in different plant systems. In *Equisetum*, MTs of the trichoblasts rearrange parallel to the longitudinal axis of the root hair during the formation of the bulge, which becomes the root hair (Emons and Derksen 1986). In *Zea mays*, the bulge is initially depleted of MTs (Baluška et al. 2000), but as it grows in length, helical MTs are seen parallel to the growth axis. In contrast, our study does not show a similar depletion in *M. sativa* and forming bulges appear to have MTs tightly arranged in a helical manner similar to that reported for *M. truncatula* (Sieberer et al. 2002). In polar-growing root hairs of many previously observed systems, the MTs are arranged mainly in the cortical cytoplasm in net-axial or helical orientation (Geitmann and Emons 2000). These cortical arrays are continuous with the endoplasmic MTs located between the nucleus and the tip (Lloyd et al. 1987; Miller et al. 1997; Sieberer et al. 2002). They are also present at the tip of the root hair in a random orientation (Emons 1989). The MT pattern in the polar-growing root hairs of *M. sativa* is consistent with the previously obtained data. However, unlike in *M. truncatula* where the endoplasmic MTs do not curve back at the root hair tip, *M. sativa* has some endoplasmic MTs that do curve back at the tip.

Timmers et al. (1999) proposed that MTs play a significant role during root hair curling and cortical cell division during nodulation of *M. truncatula*. However, they did not observe the early changes in the MT cytoskeleton we report here. In contrast, changes in actin organization have been observed in many systems such as *Vicia* (Ridge 1992; de Ruijter et al. 1998), *M. sativa* (Allen et al. 1994) and *Phaseolus* (Cárdenas et al. 1998) after the application of appropriate Nod factors. These studies established that the effect of Nod factors on the actin cytoskeleton of root hairs is quite consistent in all systems. The response of actin to Nod factors is rapid, and is evident within 5 to 10 min (Cárdenas et al. 1998). Several lines of evidence indicate the possibility that MTs would show dynamic localization changes similar to those seen for actin filaments. Destabilization of actin in the growing protonemata of *Adiantum* leads to the destabilization of MTs (Kadota and Wada 1992). In a contrasting study, in root hairs of *Hydrocharis*, it has been observed that intact MTs are needed for normal functioning of actin (Tominaga et al. 1997). These results, as well as other pharmacological and molecular studies, provide evidence for functional interactions between actin and MTs (Collings and Allen 2000; Igarashi et al. 2000; Staiger 2000). Hence MTs become a potential candidate for regulating a morphological change during the early events of nodulation.

Our study reveals that MT arrays change their distribution during the first minutes of induction for nodulation. We conclude that Nod factors of *R. meliloti* are capable of inducing rapid changes (within 3–10 min) in the MT cytoskeleton of almost all growing root hair cells of alfalfa. Our experiments also demonstrate that taxol treatment causes a slower rate of growth than the control while displaying MTs patterns similar to that of control root hairs. Nod factors overcome the effect of taxol by increasing the length of root hairs and by enhancing bulging and branching. Although the response is delayed the MTs in taxol/Nod-treated root hairs undergo disintegration. These observations appear to indicate that taxol causes a de-acceleration of growth, perhaps because the MTs maintain a stable pattern. It is conceivable that Nod factors override this inhibition by disrupting the stabilized MTs, causing growth to reinitiate. Our observations on oryzalin-treated root hairs show that oryzalin causes disintegration of MTs and increases the rate of growth. When Nod factors are combined with oryzalin, the length of root hairs

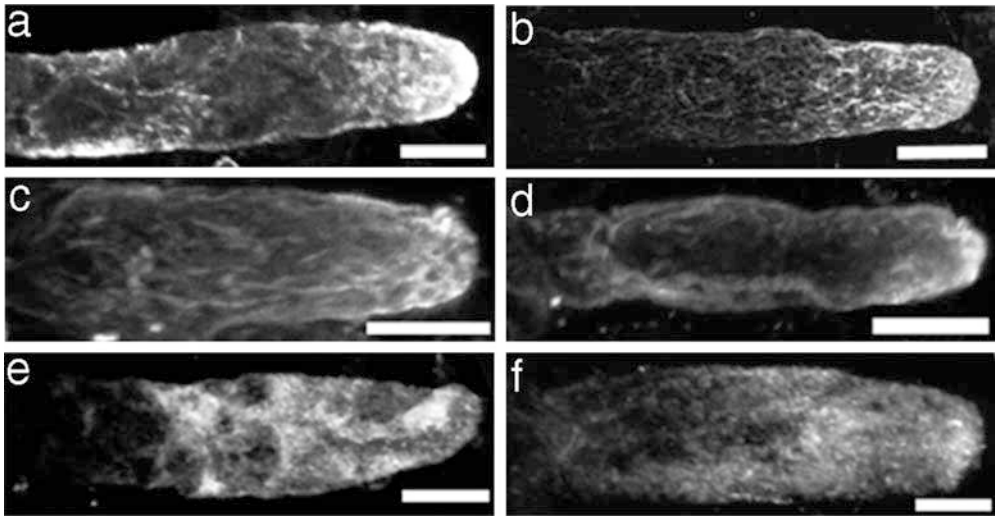

Fig. 10a–f Effects of taxol, oryzalin and Nod factors on MT organization of alfalfa root hairs. MTs of the root hairs were visualized by CLSM (full-stack projection) after 50 min (or 20 min after Nod factor treatment) and 120 min (or 60 min after Nod factor treatment) of each treatment. The MTs of root hairs that were subjected to 10 μm taxol (**a**) for 50 min were intact and comparable to control plants (**b**) treated with BNM for 50 min. A combination of taxol and Nod factor (**c**) did not alter the structure of MTs at 50 min following the treatment and was comparable to treatment with taxol alone. However, at 120 min following taxol/Nod treatment the MTs had disintegrated into short pieces (**d**). Oryzalin alone (**e**) caused disintegration of MTs 50 min after the treatment. Root hairs treated with the oryzalin/Nod factor combination (**f**) displayed disintegrated MTs, exhibiting synergistic effects of oryzalin and Nod factors. Bar = 10 μm

increases further and the MTs appear disintegrated at 1 h after Nod factor application. Thus it seems that oryzalin and Nod factors act synergistically in regulating growth and MTs. Collectively, our results indicate that MT disruption results in increased root hair growth and that Nod factors are capable of changing MT behaviour and thereby directly/indirectly influence the growth pattern of root hairs.

The events that lead to disruption of MTs during Nod factor treatment and subsequent signalling events remain poorly understood. Many studies indicate a link between intracellular calcium levels of the cell and the cytoskeleton. For example, elevated cytoplasmic calcium levels are known to cause fragmentation of actin filaments and depolymerization of MTs (Cyr 1994). In addition, studies on the *Arabidopsis ton* mutant, which has high calcium-channel activity and constitutively disorganized cortical MTs (Thion et al. 1998), suggest that MTs are potential regulatory elements for calcium-channel recruitment. The first response that occurs after Nod factor application is a transient calcium influx at the root hair tip (Felle et al. 1998). In *M. sativa* this occurs a few seconds after treatment. Within 3 min, the cytosolic calcium level increases (Allen et al. 1994; Cárdenas et al. 1999; Felle et al. 1999a, 1999b) and this could activate anion channels in the plasma membrane leading to a depolarization and other downstream events. Although the molecular basis for this activation is unclear, it is possible that Nod factors may influence the MT and actin organization via intracellular calcium levels.

Interestingly, Fu et al. (2001) found that the actin filaments in *Nicotiana tabacum* pollen tubes in which Rop1At, a Rop GTPase, was overexpressed, formed a transverse band behind the tip. This pollen tube tip eventually was seen to have depolarized growth. In root hairs challenged with Nod factors, we see a similar transverse band or ring of MTs (Fig. 4c) just behind the root hair tips and eventually these root hairs also have a changed growth polarity. Thus, it is possible that Rop GTPase in concert with actin and MTs modulates the polarity of root hairs, in a manner similar to pollen tubes.

Our experiments show that the rapid change in both endoplasmic and cortical MTs initiates in the vicinity of the nucleus. Nod factors are also known to cause a subsequent oscillation of cytosolic calcium over the nucleus (Ehrhardt et al. 1996). Recent analyses of non-nodulating mutants (which do not exhibit calcium spiking and have altered root hair deformation patterns) have shown the existence of a strong correlation between calcium spiking and root hair deformation during nodulation (Catoira et al. 2000; Wais et al. 2000). If the MTs are regulatory elements of calcium-channel recruitment, the above evidence suggests the involvement of MTs in tip growth by regulating the inhibition of endogenous root hair growth and/or the initiation of Nod factor-dependent root hair growth (Catoira et al. 2000). The following also supports this view. First, the generation of an artificial calcium gradient at the tip of the root hair gives rise to a reorientation of root hair growth (Bibikova et al. 1997). Second, MT-depolymerizing agents lead to loss of directionality and multiple growth points (Bibikova et al. 1999). However, evidence also indicates that MTs are involved in spatial organization of signal perception and transduction in concert with cell wall molecules, plasma membrane receptors and MT-associated proteins (Kropf et al. 1998; Gundersen and Cook 1999). Thus, it is possible that the rapid

change in MT organization that is seen in our experiments is part of the signal transduction pathway in nodulation or, alternatively, this change may be activated by steps during nodulation. It is clear from our results that Nod factor-caused changes in the normal organization of MTs results in an alteration of polar growth and that Nod factors are capable of causing changes to endogenous polar growth and re-initiating polar growth.

The depolymerization of MTs is an early response seen during nodulation. Hence it would be beneficial to not only assess the role that MTs play in nodulation signal transduction but also to study the possible exact interplay between MTs, actin and calcium. Future studies of Nod factor effects on signal transduction and polar growth of root hairs will aim at dissecting the interaction of calcium and MTs.

Acknowledgements Financial support was provided by the North Carolina Agricultural Research Station (NC AgResStat 407050, NS Allen) and the National Science Foundation (NSF DBI0077503, D Bird).

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