Nod Factors Alter the Microtubule Cytoskeleton in *Medicago truncatula* Root Hairs to Allow Root Hair Reorientation

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The microtubule (MT) cytoskeleton is an important part of the tip-growth machinery in legume root hairs. Here we report the effect of Nod factor (NF) on MTs in root hairs of *Medicago truncatula*. In tip-growing hairs, the ones that typically curl around rhizobia, NF caused a subtle shortening of the endoplasmic MT array, which recovered within 10 min, whereas cortical MTs were not visibly affected. In growth-arresting root hairs, endoplasmic MTs disappeared shortly after NF application, but reformed within 20 min, whereas cortical MTs remained present in a high density. After NF treatment, growth-arresting hairs were swelling at their tips, after which a new outgrowth formed that deviated with a certain angle from the former growth axis. MT depolymerization with oryzalin caused a growth deviation similar to the NF; whereas, combined with NF, oryzalin increased and the MT-stabilizing drug taxol suppressed NF-induced growth deviation. The NF-induced disappearance of the endoplasmic MTs correlated with a loss of polar cytoarchitecture and straight growth directionality, whereas the reappearance of endoplasmic MTs correlated with the new set up of polar cytoarchitecture. Drug studies showed that MTs are involved in determining root hair elongation in a new direction after NF treatment.

The symbiosis between leguminous plants and rhizobia leads to the formation of a new organ, the N₂-fixing root nodule. The different steps involved in the establishment of the symbiosis between these two organisms are tightly controlled through a complex network of signaling cascades (Cullimore and Dénarié 2003; Endre et al. 2002; Geurts 2003; Limpens et al. 2003; Madsen et al. 2003; Parniske and Downie 2003; Radutoiu et al. 2003; Stracke et al. 2002). Nod factors (NFs) are signal molecules secreted by rhizobia which have been shown to be essential for successful symbiosis (Fisher and Long 1992; Lerouge et al. 1990; Roche et al. 1991; Spaink et al. 1991; Truchet et al. 1991). One of the first essential steps in the infection process is the curling of root hairs around the bacteria (Esseling et al. 2004; Hadri and Bisseling 1998; Kijne 1992). Van Batenburg and associates (1986) proposed that root hair tip growth is essential for root hair curling. Recently, hair curling has been described as iterative tip growth reorientation (Esseling et al. 2003).

It has been shown that the actin cytoskeleton is a target of NFs (*Medicago sativa* [Allen et al. 1994], *Phaseolus vulgaris* [Cárdenas et al. 1998, 2003], and *Vicia sativa* [De Ruijter et al. 1998; Miller et al. 1999; Ridge 1992]). During root hair curling of *Medicago* spp. root hairs inoculated with *Sinorhizobium meliloti*, endoplasmic microtubules (EMTs) are present between the nucleus and the root hair tip of the curl, concentrate at the infection site where bacteria enter the root hair, and are present between the nucleus and the tip of the infection thread during infection thread growth in the root hair shank (Timmers 2000). Despite these findings, the responses of the microtubule (MT) cytoskeleton shortly after application of NF and their function in the early infection process remained elusive.

Elongating legume root hairs have EMTs in their subapex (*V. hirsuta* [Lloyd et al. 1987]) that are organized in an extensive array (*M. truncatula* [Sieberer et al. 2002] and *M. sativa* [Weerasinghe et al. 2003]), which they acquire when tip growth begins and retain until growth stops (Sieberer et al. 2002). In contrast to legume root hairs, in growing *Arabidopsis* spp. hairs, the subapical array of EMTs consists of a few bundles of MTs (Van Bruaene et al. 2004).

In the root hair deformation assay, in which NFs are applied globally to roots in a glass microchamber, the so-called Fåhræus slides (Fåhræus 1957), only growth-arresting root hairs respond with root hair deformation (*V. sativa* [De Ruijter et al. 1998; Heidstra et al. 1994] and *M. truncatula* [Sieberer and Emons 2000]), which consists of swelling of the root hair tip (i.e., temporary switch to isodiametric growth) and formation of a new outgrowth from the swelling (i.e., reinitiation of tip-growth) (*V. sativa* [De Ruijter et al. 1998] and *M. truncatula* [Sieberer and Emons 2000]). In *Arabidopsis* spp. root hairs, a pulse application of actin depolymerizing drugs causes swelling, from which tip growth resumes in the original growth orientation (Ketelaar et al. 2003). When the same treatment with actin depolymerizing drugs is performed in *Arabidopsis* hairs where MTs were experimentally ablated, the new axis of cell elongation deviates from the original growth axis by a certain angle (Ketelaar et al. 2003). The newly formed outgrowth after NF-induced root hair swelling also deviates at a certain angle from the original growth axis. This is an indication that MTs are NF effectors. Here, we report the effect of NFs, the MT depolymerizing drug oryzalin, the MT stabilizing drug taxol, and combinations of these agents on MT arrays and cell growth of *M. truncatula* root hairs. MTs were visualized by immunocytochemistry after rapid freeze fixation as well as with a green-fluorescent protein MT binding domain (GFP-MBD) fusion protein decoration in living root hairs.

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Our findings show that the MT cytoskeleton contributes to root hair morphology, determines cytoplasmic polarity in elongating hairs, and, therefore, is an essential part of the tip-growth machinery. It appears to be a direct or indirect target of early NF signaling, and its dynamics a prerequisite for root hair reorientation upon NF perception.

RESULTS

Effects of Nod factor on the MT cytoskeleton and the cytoarchitecture of elongating root hairs.

Within 3 to 5 min after NF application, we observed a subtle and transient shortening of the three-dimensional and dense EMT array in the subapex of tip-growing hairs (Fig. 1B compared with C, F, and H) that was completely restored within 10 to 15 min. The restored EMT array remained present until regular growth termination (data not shown), which was the same as in control root hairs. At 5 min after NF treatment, the EMT array at the basal part of the subapical region had almost completely disappeared (Fig. 1C) or become less dense (Fig. 1F and H) in approximately 76% of the observed hairs (Table

Fig. 1. Microtubules (MTs) in untreated and Nod factor (NF)-treated tip-growing *Medicago truncatula* root hairs. **A**, Full-stack projection; cortical MTs (CMTs) are net-axially oriented and do not reach the very tip of the hair. **B**, Median section of the same hair shows endoplasmic MTs (EMTs) in the subapex of the hair (bracket). **C**, Projection of two median scans shows a short EMT array in the subapex (compare with B). The vacuole has overtaken the nucleus and occupies a large part of the space between nucleus and tip, where the EMT array normally spreads. **D**, Projection of two cortical scans of the hair in C shows that CMTs are not obviously affected shortly after NF treatment. **E**, In a single optical section, the EMT array and the cytoarchitecture appear to be undisturbed. **F**, The next successive section of the hair in E shows that the EMT array has almost completely disappeared. Close to the tip, a few EMTs are present. The vacuole has started to overtake the nucleus. **G**, Projection of two median scans. The EMT array has decreased in density; EMTs in the vicinity of the nucleus are short and fragmented. **H**, The next successive section of the hair in G reveals that a thin, tubular extension of the central vacuole is penetrating the subapical plasma. EMTs are present at a low density. A and B, Five minutes after control treatment. C through H, Five minutes after treatment with 10^{-9} M NF. MTs were decorated with immunocytochemistry and visualized with a confocal laser-scanning microscope in scanning steps of 1 μ m; n = nucleus, v = vacuole. Magnification is the same in all images. Bar = 10 μ m.

1), whereas EMTs closer to the hair tip remained present (Fig. 1C, E through H). The percentage of root hairs with disturbed EMT array 5 min after NF application was higher than 10 min after NF application (significantly different at the 0.05 level, $P = 0.021589$, independent Student's t test analysis). The effect on the EMT array was subtle and could be observed only when a successive sequence of optical sections taken with a confocal laser-scanning microscope (CLSM) was investigated. Although the EMT array or the cytoarchitecture in one section appeared normal (Fig. 1E and G), in the next section, the EMT array was shorter and vacuole extensions penetrated the sub-apex (Fig. 1F and H). To find out whether this was a true effect of NFs and not just medium refreshment or the freeze fixation method, we compared NF-challenged root hairs with control hairs subjected to medium refreshment and freeze fixation. In the latter, only 45% of the hairs responded with a temporarily disturbed EMT array 5 min after treatment with plant growth medium (Table 1), which was lower than 5 min after NF treatment (significantly different at the 0.05 level, $P = 0.010104$). Cortical MTs (CMTs) in a tip-growing hair treated with NF were not obviously affected (Fig. 1D) at any time after NF application, and their over-all patterning was similar to control root hairs treated with plant growth medium (PGM).

The temporary and partial shortening of the EMT array in elongating root hairs after NF application could be observed only in single optical planes taken with a CLSM from immunolabeled root hairs after freeze fixation and freeze substitution, but not in GFP-MBD-expressing root hairs. This could be due to a lack in resolving power (number and optical depth of z scans) of the applied imaging technique to observe GFP-MBD-decorated MTs in living root hairs (Sieberer et al. 2002), and the fact that the MTs in the living hairs, especially the EMTs, are highly dynamic (Sieberer et al. 2002; Vos et al. 2003) (A. C. J. Timmers, *unpublished results*), which makes it difficult to visualize their precise configuration in a still image of the dense, three-dimensional EMT array in a tip-growing root hair.

Together with the temporary shortening of the subapical EMT array after NF treatment went a transient vacuolization of the cytoplasmically dense subapical region in elongating root hairs. Within 5 min after NF application in approximately 80% of the observed hairs, extensions from the central vacuole temporarily entered the dense subapical cytoplasm (Fig. 2B). Tip growth did not stop during the temporary disturbance of the subapical region caused by NFs and the cytoarchitecture recovered within 10 to 15 min (Fig. 2C). Further, we compared the cytoarchitecture of tip-growing *M. truncatula* root hairs treated with NF with those treated with oryzalin, an MT-depolymerizing drug. Also, in tip-growing hairs treated with 1 μ M oryzalin, thin extensions of the central vacuole started

Table 1. Short-term effects of 10^{-9} M Nod factor (NF) and plant growth medium (PGM) refreshment on the endoplasmic microtubule (EMT) array of tip-growing root hairs^a

Time after treatment	% with temporarily disturbed EMT array (SD)	
	NF	PGM
5 min	76.5 (2.12)	45 (2.83)
10 min	33.5 (4.95)	31 (2.83)
30 min	27 (1.41)	23.5 (2.12)

^a Samples were cryofixed 5, 10, and 30 min after NF treatment. EMTs were labeled with immunocytochemistry and visualized with confocal laser scanning microscopy. In all, 40 root hairs per time point (four different roots, 10 hairs per root) for each treatment were observed. The number of root hairs with disturbed EMT arrays was scored. The results are presented as the mean percentages with standard deviation (SD) of two independent repeats of the experiment.

penetrating the dense cytoplasm within 5 min after application (Fig. 2E). In contrast to NF-treated hairs, these vacuolar extensions increased in volume until the dense cytoplasm in the subapex had completely disappeared within 10 to 20 min, except for a cortical layer in the hair tube and a smooth, vesicle-rich region at the very tip of the hair (Fig. 2F). In the presence of oryzalin, the subapical dense cytoplasm and the EMT array never recovered in elongating hairs, and tip-growth proceeded, though at a lower growth rate (Sieberer et al. 2002). Control medium refreshment did not affect, or only slightly affected, the subapical cytoarchitecture of tip-growing hairs (Fig. 2G through I).

Effects of NF on the MT cytoskeleton and the cytoarchitecture of growth-arresting root hairs.

The observed changes in the MT cytoskeleton in elongating root hairs upon NF treatment were subtle and short termed; therefore, we studied the MTs in growth-arresting hairs after NF application as well. In contrast to younger tip-growing hairs, growth-arresting hairs respond to globally applied NF with a distinct change in hair morphology called “root hair deformation.” Several cellular responses of the initial effects of NFs can be elucidated by studying root hair deformation (Lhuissier et al. 2001), allowing the researcher more black and white evaluation of the pronounced cellular responses to NFs, which in our case are NF-induced changes of the MT cytoskeleton.

During growth arrest, the EMT array gradually shortens as the dense cytoplasm in the subapex decreases in length and eventually disappears after growth termination (Sieberer et al. 2002). However, as long as tip growth continues, EMTs are present. We have defined growth arrest as the time between the moment the vacuole permanently overtakes the nucleus and stop of hair elongation (Sieberer and Emons 2000). This process can take as long as 2 to 3 h.

Upon application of 10^{-9} M NF, the short EMT array of a growth-arresting hair disappeared within 5 to 10 min (Fig 3). At 20 to 30 min later, subapical EMTs reappeared inside a single or multiple transvacuolar cytoplasmic strand or strands (Fig. 3C and D). During root hair tip swelling, these cytoplasmic strands were constantly changing shape and volume, and, depending on their volume, contained various

amounts of EMTs (Fig. 4A, B, and E). CMTs were extending to and reaching the very tip of growth-arresting hairs within 5 to 10 min after NF application. This usually is a sign that tip growth is ceasing (Sieberer et al. 2002); however, in

Fig. 3. Time series of endoplasmic microtubules (EMTs) in a growth-arresting *Medicago truncatula* root hair treated with 10^{-9} M Nod factor. Microtubules (MTs) were decorated with green-fluorescent protein MT binding domain and visualized with a confocal laser-scanning microscope in scanning steps of 1 μ m; for each image, three median sections were projected. Magnification is the same in all images. Bar = 10 μ m. **A**, Before treatment; in the subapex there still is a short EMT array present (bracket). **B**, Ten minutes after treatment. The EMT array has disappeared. **C**, Twenty minutes after treatment. Single EMTs have reappeared inside a cytoplasmic strand in the sub-apex of the hair. **D**, Thirty minutes after treatment. EMTs within transvacuolar cytoplasmic strands have increased in density, but a dense EMT array as seen in A has not yet been rebuilt.

Fig. 2. Short-term effect of 10^{-9} M Nod factor (NF), 1 μ M oryzalin, and medium refreshment on the cytoarchitecture of tip-growing *Medicago truncatula* root hairs. Time series of living root hairs obtained with bright-field microscopy. Large bracket indicates the subapical region rich in cytoplasm; small bracket indicates the clear zone (vesicle-rich region); n = nucleus, v = vacuole. Magnification is the same in all images. Bar = 20 μ m. **A**, Before treatment. **B**, Five minutes after NF. The subapical cytoarchitecture is heavily disturbed. **C**, Fifteen minutes after NF. The cytoarchitecture has almost completely recovered. **D**, Before treatment. **E**, Five minutes after oryzalin. The subapical cytoarchitecture is heavily disturbed. **F**, Fifteen minutes after oryzalin. Except for the vesicle-rich region at the very tip, the vacuole almost completely fills the subapex. In the presence of oryzalin, the cytoarchitecture is not recovering. **G**, Before treatment. **H**, Five minutes after medium refreshment. **I**, Fifteen minutes after medium refreshment.

contrast to a fully grown hair, CMT density did not decrease over time (Fig. 4C and D) and CMTs did not converge at the root hair tip (Fig. 5A compared with B) after NF application. As expected, in growth-arresting hairs the prime effect of NFs on MTs was a rapid shortening of EMTs, whereas CMTs were affected to a lesser extent or not at all.

A new root hair outgrowth emerged from the swollen tip between 90 to 120 min after application of NF. The dense three-dimensional EMT array described for a tip-growing root hair progressively reappeared (Fig. 6) and the newly formed outgrowth had a polarized cytoarchitecture (Fig. 7D). Once tip-growth had reinitiated, the very tip of the newly formed outgrowth was devoid of detectable CMTs (Fig. 6A through D), the same situation as in a tip-growing root hair (Sieberer et al. 2002) (Fig. 1A). Cellular responses of growth-arresting hairs to NF application are summarized in Table 2.

Oryzalin in a concentration of 10 μ M has been reported to cause bulging and branching of alfalfa root hairs (Weerasinghe et al. 2003). In growth-arresting root hairs that respond to NF with root hair deformation, we found that 1 μ M oryzalin triggered swelling and outgrowth of root hair tips in a similar fashion as do NFs (see below). Therefore, we compared the effect of 1 μ M oryzalin on the MT cytoskeleton in hairs of this developmental stage with the effect of NF. In growth-arresting hairs treated with 1 μ M oryzalin, EMTs did not reappear during root hair swelling at any time after treatment (Fig. 5D and F). Further, no EMTs were found in the subapex of the outgrowth that emerged from the oryzalin-induced swelling (data not shown). CMTs did not converge at the very tip of the hair, but appeared to be fragmented (Fig. 5C and E). CMTs in the shank of the hairs were not obviously affected by 1 μ M oryzalin (Fig. 5C and E). They also remained present in the

swelling, but were completely absent in the newly formed outgrowth (data not shown). Thus, although NF and oryzalin both induced swelling and outgrowth, the behavior of the MT cytoskeleton was different.

MTs determine the angle with which the new outgrowth deviates from the former growth axis during root hair deformation.

After application of 10⁻⁹ M NF, growth-arresting hairs responded within minutes and their tips started to swell. Between 90 and 120 min after NF application, a tip-growing tube reinitiated from the swelling. It is important to note that NF-treated growth-arresting root hairs can become longer than control root hairs (Sieberer and Emons 2000). The newly formed outgrowth had a fully recovered polarized cytoarchitecture similar to a young tip-growing hair with a smooth, vesicle-rich region at the very tip, a tip-nucleus distance of 30 to 40 μ m, and accumulated cytoplasm in the subapex (Fig. 7A compared with D). On average, the growth axis of the newly formed outgrowth deviated from the original growth axis of the hair by 23.3° (Table 3).

The tips of elongating *M. truncatula* root hairs swelled after treatment with low concentrations of actin-depolymerizing drugs and subsequent recovery of tip-growth occurred in the original growth orientation (Fig. 7B and C; Table 3) which, in the case of cytochalasin D, was significantly different at the 0.05 level from NF ($P < 0.01$) or oryzalin-induced growth reorientation ($P < 0.01$) (independent Student's *t* test analysis).

Oryzalin had an effect on the morphogenesis of a growth-arresting hair similar to NF. After root hair swelling, a new outgrowth formed between 90 and 120 min after application of 1 μ M oryzalin. The newly formed outgrowth deviated by 29.5°, on average, from the original growth orientation of the hair (Fig. 7E; Table 3), which was similar to NF-induced growth deviation (not significantly different at the 0.05 level, $P = 0.102237184$). However, in oryzalin-treated hairs, the newly formed outgrowth did not regain the same cytoarchitecture as in NF-treated hairs. The tip region contained a thin layer of cytoplasm (Fig. 7E, small bracket), no dense cytoplasm accumulated in the subapex, and the nucleus did not obtain a distance to the tip of 30 to 40 μ m (Fig. 7A compared with D and E). Indeed, oryzalin-treated growth-arresting hairs did not get new growth potential but, instead, stopped elongating at, or before, the time and length normal for the species (data not shown).

When a combination of 10⁻⁹ M NF and 1 μ M oryzalin was applied to growth-arresting hairs, the hair tips formed a swelling. A new outgrowth emerged from the swelling between 90 and 120 min after application (Fig. 7F). However, the polarized cytoarchitecture was not restored, which was the same after oryzalin treatment alone. The subapex in such hairs was devoid of dense cytoplasm (Fig. 7A compared with D and F). Interestingly, the newly formed outgrowth deviated, with a steeper angle of 68.5° (Table 3) on average, from the original growth axis of the hair compared with the outgrowth of hairs treated with either 1 μ M oryzalin (significantly different at the 0.05 level, $P < 0.01$) or NF alone (significantly different at the 0.05 level, $P < 0.01$) (Table 3). The effects of NF and 1 μ M oryzalin were additive, showing that the (partial) impairment of MTs leads to an increase of the angle at which the newly formed outgrowth deviates from the original growth axis.

When oryzalin was applied within 60 min to hairs initially treated with NF only, the deviation of the outgrowth was similar to that of hairs exposed to a combination of NF and oryzalin, whereas an application of oryzalin later than 60 min after NF treatment caused a deviation similar to that of NF alone (data not shown).

Fig. 4. Microtubules (MTs) in the swellings of growth-arresting root hairs treated with 10⁻⁹ M Nod factor (NF). MTs were visualized with confocal laser-scanning microscope in scanning steps of 1 μ m. Magnification is the same in all images. Bar = 20 μ m. **A**, Sixty minutes after NF; projection of two median sections shows endoplasmic MTs (EMTs) inside the swelling. **B**, Seventy minutes after NF; projection of two median sections shows EMTs inside the swelling. EMTs are located in cytoplasmic strands that constantly change shape, size, and orientation. **C**, Seventy minutes after NF; full-stack projection shows CMTs remain present in a high density. **D**, Seventy minutes after NF; full-stack projection shows CMTs in a high density and a net-axial orientation in the shank of the hair and in a random pattern in the swelling. **E**, A projection of three median sections of the hair in D shows that EMTs appear in different densities within cytoplasmic strands inside the swelling. A through C, Immunocytochemistry; D and E, green-fluorescent protein MT binding domain.

These findings indicate that MTs serve in determining growth directionality of *M. truncatula* hairs. This was exemplified further by the approximately 12% of root hairs that showed problems with restricting tip growth to only one site (Fig. 7G). To learn whether MTs have to be dynamic for NF to alter them such that the directionality of root hair elongation changes, we applied NF to root hairs in which the MTs were stabilized with taxol.

In growth-arresting root hairs treated with a combination of 10^{-9} M NF and 1 μ M taxol, tip growth ceased within minutes of application and hair tips started to swell. Tip growth reinitiated between 90 to 120 min after application. The newly formed outgrowth deviated with a smaller angle of approximately 16.4° C from the original growth axis (Fig. 7H; Table 3) when compared with NF (significantly different at the 0.05 level, $P < 0.01$), oryzalin ($P < 0.01$), or a combination of NF and oryzalin ($P < 0.01$). Taxol alone did not cause changes in root hair morphology, but did slow elongation speed (Sieberer et al. 2002).

Fig. 5. Microtubules (MTs) in a growth-arresting hair treated with 10^{-9} M Nod factor (NF), in a fully grown hair, and in a growth-arresting hair treated with 1 μ M oryzalin. Magnification is the same in all images. Bar = 20 μ m. **A**, Twenty-five minutes after NF; full-stack projection. Cortical MTs (CMTs) appear in the very tip (arrowheads), but do not converge. **B**, Fully grown hair; full-stack projection. CMTs have decreased in density. The subapex is devoid of endoplasmic MTs (EMTs). **C**, Thirty minutes after oryzalin. In a full-stack projection, CMTs appear fragmented in the hair tip. **D**, Projection of two median sections of the hair in **C** shows that EMTs have completely disappeared. **E**, Sixty minutes after oryzalin. Full-stack projection shows that, in the swelling, the tip region still is devoid of a CMT array; CMTs in the shank remain present. **F**, Projection of two median sections of the hair in **E** shows that EMTs are completely absent in the swelling. **A** and **C** through **F**, Green-fluorescent protein MT binding domain; **B**, immunocytochemistry.

DISCUSSION

Endoplasmic MTs are a target for NF signaling and involved in NF-induced root hair reorientation.

Our study shows that treatment with NF causes a rapid, subtle, and transient shortening of the subapical EMT array in tip-growing *M. truncatula* root hairs. The effect of NF on the EMTs is more severe than on CMTs. The short EMT array of growth-arresting hairs completely disappeared after NF application, but started to reappear after 20 to 30 min, after which growth resumed, albeit in a different orientation. This deviation of the new outgrowth from the original growth axis was larger when NF and the MT-depolymerizing drug oryzalin were applied together and smaller when NF was applied in combination with taxol, a MT-stabilizing drug. In addition, the oryzalin-induced absence of EMTs, as well as their stabilization by taxol, suppressed the NF-induced new growth potential in growth-arresting hairs. Together, these findings demonstrate that the MT cytoskeleton contributes to the tip-growth process and to the determination of growth direction of elongating hairs, and that it is a direct or indirect target of early NF signaling. The latter two features are crucial for root hair curling around rhizobia.

Our results on changes of the MT cytoskeleton in root hairs after NF challenge differ substantially from those reported by Weerasinghe and associates (2003). These authors describe a complete disintegration of the MT cytoskeleton in elongating *M. sativa* root hairs after NF perfusion, starting at the base of the hair within 3 min with the EMTs and within 10 min with the CMTs. The disintegration of the CMTs was completed within 30 min. The MT network reformed 60 min after NF exposure although, in hairs longer than 150 to 180 μ m, the recovery was incomplete. In *M. truncatula* root hairs, we never observed such a dramatic change in MT organization at any time

Fig. 6. Microtubules (MTs) in the newly formed outgrowth after application of 10^{-9} M Nod factor (NF) to growth-arresting *Medicago truncatula* root hairs. **A**, Immunocytochemistry; **B** through **D**, green-fluorescent protein MT binding domain. Magnification is the same in all images. Bar = 10 μ m. **A**, At 120 min after NF; projection of two median sections shows endoplasmic MTs (EMTs) in the emerging outgrowth. **B**, At 150 min after NF; projection of two median sections shows EMTs in the subapex of the newly formed outgrowth. EMTs are located inside cytoplasmic strands. **C**, At 120 min after NF; full-stack projection shows a dense EMT array in the subapex of the outgrowth. In the shank and in the swelling, CMTs are net-axially oriented. The very tip of the outgrowth is devoid of detectable MTs. **D**, At 150 min after NF; full-stack projection. The MT array has been almost completely rebuilt.

after NF application. Species-specific differences in the NF response of MTs may explain the different observations made in *M. sativa* and *M. truncatula*, but they are not very likely, because the organization of the MT cytoskeleton in elongating root hairs and the pattern of root hair deformation after NF application appear to be similar in the two species (Weerasinghe et al. 2003; this article). The different method of NF application used by Weerasinghe and associates (2003) or a not fully optimized chemical fixation protocol could be the reason for the dissimilar results. Gentle application of NF was described to be crucial for an undisturbed cytoarchitecture of root hairs (Esseling et al. 2004) and, thus, the cytoskeleton. Further, it has been shown that a not-optimized chemical fixation can cause severe artifacts of the actin cytoskeleton—and, hence, the

cytoarchitecture—in root hairs (Esseling et al. 2000; Ketelaar and Emons 2001) and pollen tubes (Lovy-Wheeler et al. 2005), reasons why we used freeze fixation and GFP technology.

The subtle changes in the MT pattern after NF treatment that we report here could point toward a transient change in the dynamic instability parameters of EMTs. In tip-growing *M. truncatula* root hairs, EMTs are highly dynamic (Vos et al. 2003) and more sensitive to MT depolymerizing drugs than CMTs (Sieberer et al. 2002), which could explain why EMTs are more severely affected by NF than CMTs. An NF-induced increase in MT dynamic instability parameters could be a prerequisite for the changes in the MT cytoskeleton that are needed to redirect root hair growth for obtaining root hair curling around rhizobia. Measurements of MT dynamic instability parameters have been performed successfully in the plant cell cortex (Donukshe and Gadella 2003; Shaw et al. 2003; Vos et al. 2004), including the cortex of *Arabidopsis* root hairs (Van Bruaene et al. 2004). In the large volume of the subapex of *M. truncatula* root hairs with its dense EMT array, analysis of the behavior of individual MTs was impossible to carry out with current methods. As often is the case in cell biology, new instrumentation should bring further answers.

The question arises of how the dynamic instability could be altered by NF. MT depolymerization can be triggered, for instance, by increased cytoplasmic calcium levels (Cyr 1991, 1994). Elongating root hairs have a tip-focused Ca^{2+} gradient, which increases in legumes within minutes after application of NF (Allen et al. 1994; Cárdenas et al. 1998; De Ruijter et al. 1998; Felle et al. 1998, 1999a,b), leading to several downstream events affecting the cytoskeleton (Lhuissier et al. 2001). The *Arabidopsis* ton mutant has high calcium-channel activity and constitutively disorganized CMTs (Thion et al. 1998). These findings could imply that NF influences the MT organization in the root hair tip of legumes via transiently increased calcium levels.

The effect of NF on the MT cytoskeleton differs from that of oryzalin.

The effect of oryzalin on MTs partly resembles the effect of NF: in both cases, the EMT array shortens. Further, NF and oryzalin cause a similar deformation phenotype in growth-arresting hairs and, when applied together, they have an addi-

Fig. 7. Root hair deformation of growth-arresting root hairs after treatment with actin inhibitors, Nod factor (NF), or microtubule inhibitors. Bright-field images of living root hairs. Large bracket indicates the subapical region that is rich in cytoplasm; small bracket indicates the smooth (vesicle-rich) region; n = nucleus, v = vacuole. Magnification is the same in all images. Bar = 20 μm . **A**, At 60 min after growth medium refreshment, showing undisturbed tip-growth pattern **B**, At 60 min after recovery from a 45-min pulse treatment with 1 μM cytochalasin **D**, At 60 min after recovery from a 45-min pulse treatment with 10 nM latrunculin; the newly formed outgrowth shows no deviation from the original growth axis. **D**, At 120 min after 10^{-9} M NF; the newly formed outgrowth deviates by approximately 35° from the original growth axis and has a similar cytoarchitecture as a tip-growing hair. **E**, At 180 min after 1 μM oryzalin; the newly formed outgrowth deviates by approximately 36° from the original growth axis and has not regained the cytoarchitecture of a tip-growing root hair. **F**, At 140 min after a combination of 10^{-9} M NF and 1 μM oryzalin; the newly formed outgrowth deviates by approximately 100° from the original growth axis and has not regained the polar cytoarchitecture of a tip-growing root hair. **G**, Same treatment as in **F**; some of the hairs had problems with restricting tip growth to one site (upper hair) or with maintaining one growth directionality (lower hair). Root hairs have not regained the polar cytoarchitecture of tip-growing hairs. **H**, At 140 min after a combination of 10^{-9} M NF and 1 μM taxol; the newly formed outgrowth shows no deviation from the original growth axis and has not regained the polar cytoarchitecture of a tip-growing hair.

tive effect on root hair deformation. This indicates that, although both NF and oryzalin cause depolymerization of EMTs, their mode of action is different. If NF had the same mode of action on MTs as oryzalin, a higher concentration of NF or oryzalin alone should lead to a similar deformation phenotype as a combination of NF and oryzalin. This is not the case (data not shown). Oryzalin binds to tubulin heterodimers, preventing further growth of MTs, which eventually leads to MT depolymerization as long as oryzalin is present (Anthony and Hussey 1999). The action mechanism of NF on MTs is unknown. The most important difference between NF and oryzalin is the fact that EMTs start to reappear in an NF-treated hair, eventually forming a dense three-dimensional EMT array in the subapex of the new outgrowth.

MTs and actin filaments are early targets of NF signaling.

Recovery of tip growth in *M. truncatula* root hairs from actin-depolymerizing drugs after tip swelling takes place in the original orientation of hair elongation (Fig. 7B and C). The same has been observed in *Arabidopsis thaliana* root hairs that were treated with F-actin-depolymerizing drugs (Ketelaar et al. 2003). In terms of root hair morphogenesis, NF-induced root hair deformation resembles hair deformation after MT depolymerization by oryzalin and not after fine F-actin depolymerization. The NF-induced deformation phenotype in *M. truncatula* root hairs can be mimicked in *A. thaliana* by a pulse treatment with F-actin-depolymerizing drugs in root hairs where MTs were experimentally ablated (Ketelaar et al. 2003). Thus, it can be concluded that, in addition to a signaling pathway toward the actin cytoskeleton (*V. sativa* [De Ruijter et al. 1999; Ridge 1992], *M. sativa* [Allen et al. 1994] and *P. vulgaris* [Cárdenas et al. 1998, 2003]), NF induces a signal transduction pathway toward MTs. If NF would signal only toward the actin cytoskeleton, the newly formed outgrowth in an NF-challenged hair should occur in the same direction as after treatment with actin-depolymerizing drugs; that is, without a deviation from the original growth axis. This clearly is not the case.

From our results, it cannot be concluded whether either MTs or actin filaments are the prime target of NF signaling, whether one of the two cytoskeleton compounds influences the other, or whether they respond simultaneously to NF. Even though the importance of the actin cytoskeleton in NF signaling is paramount, we have demonstrated that the MT component of the cytoskeleton also is essential for setting up the interaction between rhizobia bacteria and legumes.

MATERIALS AND METHODS

Plant transformation with GFP-MBD.

The GFP-MBD fusion gene, linked to an upstream 35S promoter, was provided in a pUC18 vector by R. Cyr (Marc et al.

1998). The complete insert from this plasmid was transferred to pCambia1390 (CAMBIA, Canberra, Australia; provided by T. W. J. Gadella, Jr. (University of Amsterdam, The Netherlands), by using the *HindIII-EcoRI* sites. Transformed roots of *M. truncatula* cv. Jemalong were obtained by using *Agrobacterium rhizogenes* according to the protocol described by Boisson-Dernier and associates (2001).

Plant culture.

Seed of *M. truncatula* cv. Jemalong (*Fabaceae*) were germinated as described previously (Sieberer et al. 2002). For live observation and immunocytochemistry of wild-type root hairs after NF or drug treatment, seedlings were grown at an angle of approximately 25°C for 24 to 30 h between a cover slip and a glass slide (Fåhræus slides) (Fåhræus 1957; Heidstra et al. 1994) in sterile conditions at 24°C with a 16-h day length. The space between cover slip and glass slide was 1.2 mm. Each of these microchambers contained two seedlings and 2.4 ml of PGM (1.36 mM CaCl₂, 0.97 mM MgSO₄, 1.12 mM Na₂PO₄, 1.36 mM KH₂PO₄, and 20 µM Fe-citrate, pH 6.5). The plants with transgenic roots were transferred 3 to 4 weeks after transformation into square, 12-cm plastic dishes (Greiner Labortechnik, Kremsmünster, Austria) with a round perforation of approximately 5 mm in diameter in the middle of one of the four sides. Each individual plant was put in the perforation in such a way that the root was inside on PGM containing 0.8% agar and the stem part was outside the plate. Plates were put vertically in a culture room at 25°C with an 18-h day length.

NF treatment and drug studies.

Before any treatment, the Fåhræus slides were placed gently on the microscope stage and plants were left to recover for at least half an hour.

NF (NodRm-IV[C16:2, Ac, S]) was used in a concentration range of 10⁻⁸ to 10⁻¹¹ M in PGM. All concentrations used gave similar results. Presented are results obtained with 10⁻⁹ M NF, because this concentration was used by us in previous work on *M. truncatula* root hairs (Esseling et al. 2004; Sieberer and Emons 2000). NF application was done on the stage of the mi-

Table 3. Deviations from the original growth axis of root hairs after 180 min of Nod factor (NF) or cytoskeleton inhibitor treatment^a

Treatment	Deviation in degrees (SD)
1 µM cytochalasin D	15.8 (14.9)
10 ⁻⁹ M NF	23.3 (18.4)
1 µM oryzalin	29.5 (14.2)
10 ⁻⁹ M NF+ 1 µM oryzalin	68.5 (20.8)
10 ⁻⁹ M NF+ 1 µM taxol	16.4 (13.1)

^a Values are averages of 40 hairs with their standard deviation (SD). Three independent replicates for each treatment were performed.

Table 2. Nod factor (NF) effects on growth-arresting *Medicago truncatula* root hairs

Effects ^a	Growth-arresting root hairs	Fully grown root hairs	Effects of treatment on growth-arresting root hairs ^b
Tip growth	Gradually decreasing growth rate until growth stops	No cell elongation	Tip growth switches to isodiametric growth within 5 min; reinitiates within 90 to 120 min
Dense cytoplasm in subapex	Gradually shortening until disappearance at growth-arrest	Absent	Disappearance within 5 min; reappearance within 90 to 120 min
EMT array	Gradually shortening until disappearance at growth-termination	Absent	Disappears within 5 min; reappears in transvacuolar cytoplasmic strands within 20 to 30 min
CMTs	Start reaching the hair tip; net-axially aligned.	Converge at hair tip; low density; net-axially aligned	Single CMTs reach the hair tip; high density; net-axially aligned in hair tube; randomly aligned in swelling
Distance nucleus tip	Gradually increasing	Random position in root hair	Larger than 30 to 40 µm for 60 min; distance of 30 to 40 µm from tip in new outgrowth

^a EMT = endoplasmic microtubule, CMT = cortical microtubule.

^b Effects of treatment with 10⁻⁹ M NF.

croscope at room temperature in Fåhræus slides with a constant flow of 800 $\mu\text{l min}^{-1}$, gradually replacing the PGM in the slides. The final volume in each slide was 2.4 ml. For rapid freeze fixation, roots were taken out of the Fåhræus slides 5, 10, 20, 30, 70, and 120 min after NF application and within 2 to 3 s directly plunged into liquid propane.

Oryzalin (Greyhound Chromatography, Birkenhead, U.K.) was dissolved in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) as 10 mM stock solution and used at 1 μM in PGM. Taxol (paclitaxel; Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) was dissolved in DMSO as 10 mM stock solution and used at 1 μM in PGM.

For combination treatments of NF and MT inhibitors the final concentrations were 10^{-9} M NF and 1 μM oryzalin, or 10^{-9} M NF and 1 μM taxol in PGM.

In Fåhræus slides, drug solutions or a combination of NF and drugs were applied on the microscope stage with a constant flow of 800 $\mu\text{l min}^{-1}$, gradually replacing the PGM in the slides. The total volume washed through each slide was 2.4 ml. The drug- or NF-induced deviations from the original growth axis were measured in growth-arresting hairs with a length of at least 400 μm by the time treatment started.

Drugs were applied to GFP-MBD-expressing root hairs by submerging the roots growing on agar in solutions of 10^{-9} M NF or 1 μM oryzalin in PGM. After submerging, roots were covered with a gas-permeable plastic foil (bioFOLIE 25; Sartorius AG, Vivascience Support Center, Göttingen, Germany) to prevent them from drying, and observed as described below.

Bright-field microscopy.

Root hairs were observed with a $\times 20$ 0.4 n.a. (numerical aperture) or a $\times 40$ 0.55 n.a. objective (Nikon, Kawasaki, Japan) on an inverted microscope with Hoffman modulation contrast system (DIAPHOT 200; Nikon), equipped with a CCD camera (DXC-950P; Sony, Tokyo). Image recording and processing were done with a real-time digital contrast and low-light enhancement image processor (ARGUS-20; Hamamatsu Photonics, Hamamatsu City, Japan). Quantitative measurements of root hair growth directionality were done with ImageJ. Only root hairs with the swelling and the newly formed outgrowth in one optical plane have been taken to determine the deviation of the new outgrowth from the original growth axis. To prevent light-induced stress, low light and green filters were used during quantitative live measurements and related image recording.

Whole-mount immunocytochemistry of MTs.

For rapid freeze fixation, seedlings were taken from the Fåhræus slides and directly plunged into liquid propane and kept there at least for 20 s. Roots were excised and transferred into cryogenic vials, containing water-free methanol with 0.05% (vol/vol) glutaraldehyde. In this solution, roots were freeze substituted for 48 h at -90°C and allowed to warm to room temperature over a 24-h period. Samples were rehydrated in a graded series of methanol in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 KCl, 1.5 mM KH_2PO_4 , 8.1 mM Na_2HPO_4 , pH 7.4) containing fixative (0.1% glutaraldehyde [vol/vol] and 4% [wt/vol] paraformaldehyde). After rehydration, a partial cell wall digestion was carried out in a saturated suspension of driselase (Fluka, Buchs, Switzerland) and macerocyme R10 (Serva, Heidelberg, Germany) in 100 mM 2-morpholinoethanesulfonic acid (pH 6.15) for 40 min at 35°C . The specimens then were washed two times for 5 min in PBS and, to block unspecific sites, incubated for 5 min in PBS containing 0.1% (wt/vol) acetylated bovine serum albumin (BSA_{ac} ; Aurion, Wageningen, The Netherlands) and 0.05% (vol/vol) Triton X-100 (BDH Laboratory Supplies, Poole, U.K.). The samples were incubated in monoclonal anti- α -tubulin clone

DM 1 α (Sigma-Aldrich, St. Louis) diluted 1:300 (vol/vol) in PBS containing 0.1% (wt/vol) BSA_{ac} and 0.05% (vol/vol) Triton X-100 for 12 h at 4°C . After washing three times for 5 min in PBS, the samples were incubated in anti-mouse/immunoglobulin G/Alexa 488, raised in goat (Molecular Probes, Eugene, OR, U.S.A.), and diluted 1:300 (vol/vol) in PBS containing 0.1% (wt/vol) BSA_{ac} and 0.05% (vol/vol) Triton X-100 for 12 h at 4°C . Specimens were washed twice for 5 min with PBS and for 5 min with CITIFLUOR PBS solution (Citifluor Ltd., London), after which they were mounted in an antifading medium (PROLONG ANTIFADE, Molecular Probes).

Microscopic observation of MTs after immunocytochemistry.

Antibody-labeled MTs were visualized with a BIO-RAD MCR 600 CLSM (Bio-Rad Laboratories Ltd., Hertfordshire, U.K.) with an argon-krypton ion laser attached to a NIKON DIAPHOT 300 (Nikon) inverted microscope equipped with a $\times 60$ FL 1.4 n.a. oil immersion objective (Nikon) or with a $\times 100$ FL 1.2 n.a. water immersion objective (Leitz Wetzlar, Wetzlar, Germany). Z-stacks were collected with Z-steps of 1 μm . Images were acquired and projected with Confocal Assistant (version 4.02; Bio-Rad) and processed with Scion Image (beta 4.0.2; Scion Corporation, Frederick, MD, U.S.A.) and Adobe Photoshop 5.5 (Adobe Systems Incorporated, Mountain View, CA, U.S.A.).

To compare early effects of NF versus PGM on the MT cytoskeleton, for each time point per treatment the EMTs and CMTs of 40 root hairs were compared with the MT cytoskeleton of untreated young tip-growing *M. truncatula* hairs. Only root hairs with similar fluorescent signal intensity and evenly distributed fluorescent signal were imaged. The experiment was repeated twice.

Microscopic observations of GFP-MBD-expressing root hairs.

For observation, the roots growing on agar were submerged in sterile water and covered with a gas-permeable plastic foil (bioFOLIE 25; Sartorius AG, Vivascience Support Center, Göttingen, Germany) to allow direct observation and to prevent them from drying. The opened dish was put on the microscope stage of a ZEISS LSM510 (Carl Zeiss SA, Le Pecq, France) and observations were carried out either with a $\times 40/0.8$ WPH2 Achroplan or $\times 63/0.9$ WPH3 Achroplan objective. In general, Z-stacks were made of whole root hairs with a Z-step of 1 μm between subsequent optical sections. Image projections were made with ZEISS LSM Image Examiner and images were processed with Image-Pro plus (Media Cybernetics, L.P., Silver Spring, MD, U.S.A.).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

National Institutes of Health, Image J webpage:
rsb.info.nih.gov/ij