Research Note

Lipo-chito-Oligosaccharide Nodulation Factors Stimulate Cytoplasmic Polarity with Longitudinal Endoplasmic Reticulum and Vesicles at the Tip in Vetch Root Hairs

Deborah D. Miller, Hetty B. Leferink-ten Klooster, and Anne Mie C. Emons

Laboratory of Plant Cell Biology, Department of Plant Sciences, Wageningen University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands.

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Vetch root hair development has four stages: bulge, growing, growth terminating, and full-grown hair. In the assay we used, the nodulation factor induced swellings and outgrowths in growth-terminating hairs. Bulges, swellings, and full-grown hairs have transverse endoplasmic reticulum (ER) and no tip-accumulated vesicles. Growing hairs and outgrowths show vesicle accumulation in the tip and longitudinal subapical ER. Bulge walls and walls of swellings appear mottled.

During *Rhizobium*-legume interaction, rhizobial nodulation (Nod) factor is sensed by root hairs. In an assay where Nod factors are added to roots growing between slide and cover slip, tips of growth-terminating *Vicia sativa* spp. *nigra* L. (vetch) root hairs will swell, and a new tip-growing outgrowth emerges from each swelling (Heidstra et al. 1994). Changes in the $[Ca^{2+}]_c$ accompany this deformation process (Cárdenas et al.

Fig. 1. Development of one living root hair, growing at 0.8 $\mu\text{m}/\text{min}$. **A**, Bulge containing cytoplasm at the periphery and a large vacuole in the center emerges from an epidermal cell. **B**, Young root hair acquiring a polarized cytoplasm. The tip where the vesicle-rich region is located (arrow) appears as a smooth area. **C and D**, Growing root hairs that have acquired organelle zonation. The tip contains a cytoplasmic-dense area. **V** is a large vacuole in the basal part of the hair. **E**, Root hair terminating growth. These hairs have a much smaller cytoplasmic-dense region and vesicle-rich region and more small vacuoles close to the tip. **N** = nucleus. **F**, Full-grown hair that contains a thin layer of cytoplasm located at the periphery and a large vacuole in the center. Bar = 15 μm . After surface sterilization, *Vicia sativa* spp. *nigra* L. (vetch) seeds were imbibed in sterile water and placed on 0.8% agarose. The plates were set at 4°C for 3 days in order to synchronize germination at 20°C. Seedlings were grown between glass slides containing plant growth medium consisting of 1.36 mM CaCl_2 ; 0.97 mM MgSO_4 ; 1.12 mM Na_2PO_4 ; 1.36 mM KH_2PO_4 ; and 20 μM Fe-citrate, pH 6.5 (De Ruijter et al. 1998). Root hairs were observed, and light micrographs of the roots in slides were taken with a Nikon 20 $\times$ DIC 0.5 NA or 40 $\times$ Plan DIC 0.7 NA objective on a Nikon Optiphot microscope. Images were recorded on a Panasonic wv-E550 3-CCD camera with a Prisms framegrabber and AcQuis 2.0 software (Synoptics Ltd., Cambridge, U.K.).

Corresponding author: A. M. C. Emons; Telephone: +31 317 484329; Fax: +31 317 485005; E-mail: annemie.emons@pcb.dbw.wau.nl

Table 1. Ultrastructural features found with electron microscopy related to the cytoarchitecture seen with differential interference contrast (DIC) microscopy

	Electron microscopy					DIC light microscopy				
	N	Hairs with accumulated vesicles in tip	Endoplasmic reticulum orientation to hair axis		Mottled cell wall	N	Nonrandom nucleus location (%)	Smooth-tip cytoplasm (%)	Dense subapical cytoplasm (%)	Reverse fountain streaming (%)
			Axial	Transverse						
Epidermal cell	9	0	0	9	0	>100	0	0	0	0
Bulge	11	0	0	11	11	>100	100	0	0	0
Growing hair	18	18	18	0	0	>100	100	100	100	100
Growth-terminating hair	5	5	0	5	0	>100	50	100	0	100
Full-grown hair	2	0	0	2	0	>100	0	0	0	0
Swelling	7	0	0	7	7	>100	100	0	0	0
Outgrowth	4	4	4	0	0	>100	100	100	100	100

Fig. 2. Ultrastructure of the stages of root hair development. **A**, Longitudinal section of a bulge emerging from the surface of the epidermal cell. V = vacuole, N = nucleus, GB = Golgi body, ER = endoplasmic reticulum. Bar = 1 μ m. **B**, High magnification of bulge tip. Organelles are seen throughout the cytoplasm. ER is parallel to the long axis of the epidermal cell. No accumulation of vesicles is seen at the bulge tip. The cell wall of the bulge appears mottled (W), but the rest of the epidermal cell wall does not. Bar = 300 nm. **C**, Transition stage from bulge to root hair. The cytoplasm is polarly organized, with a vesicle-rich region (GV) located at the apex of the root hair. The other organelles, including GB, are located subapically. ER is becoming longitudinally oriented in the subapical region. N is close to the base of the root hair. Bar = 1 μ m. Roots with hairs were rapidly frozen, freeze-substituted (Miller et al. 1999), and embedded in Spurr's resin. The seedling was removed from the slide and the root tip was excised, placed on a Formvar-coated loop, plunge frozen in liquid propane, and substituted for 36 to 40 h in 3.7% paraformaldehyde in pure methanol or pure acetone. The infiltration took place at 4-h intervals with increasing amounts of Spurr's in acetone (1:5; 1:2; 1:1; 2:1; 5:1; pure; pure). Samples were polymerized at 60°C for 24 h and sectioned on a Reichert Ultramicrotome. Sections were stained with uranyl acetate and lead citrate in an automatic electron microscopy stainer (LEICA EMstain) and observed in a JEOL EM 1200EXII.

Fig. 3. Ultrastructure of root hair development stages. **A**, Young, growing root hair. The vesicle-rich region (GV) can be seen at the very apex of the hair. Subapically, all other organelles are observed. The endoplasmic reticulum (ER) is parallel to the long axis of the hair. M = mitochondrion, N = nucleus, V = vacuole. Bar = 500 nm. **B**, Root hair in which growth is terminating. Few vesicles are located at the hair tip, and all organelles, including Golgi bodies (GB), are close to the apex. The ER is transverse to the long axis of the hair. Bar = 200 nm. **C**, Full-grown hair. The cytoplasm is located around the periphery of the cell and no organelle zonation is observed. W = wall. Bar = 200 nm.

Fig. 4. Nodulation (Nod) factor-induced deformation in a living root hair. **A**, Swelling of root hair tip 1 h 20 min after Nod factor addition. Swelling lacks cytoplasmic zonation. Arrow points to a cytoplasmic strand that traverses swelling. V = vacuole. **B**, Initiation of new root hair tip 1 h 41 min after Nod factor addition. At one side of the swelling a new growth region initiates where the cytoplasm takes on a polarized cytoarchitecture. Arrow indicates a vesicle-rich region building up at one site of the swelling. **C–E**, New outgrowth from swelling 2 h 29 min after Nod factor addition. N = nucleus. **D**, Outgrowth has the same cytoarchitectural features as a growing root hair (**E** and **F**). Growth speed of the outgrowth (0.8 $\mu\text{m}/\text{min}$) is comparable to that of a growing root hair. Bar = 15 μm . Seedlings were treated with 10^{-10} M *Rhizobium leguminosarum* bv. *viciae* Nod factors NodRlv V [Ac, C18:4] (Spaink et al. 1992) diluted in plant growth medium.

1999; De Ruijter et al. 1998) as well as occur in the actin cytoskeleton (Cárdenas et al. 1998; Miller et al. 1999) in all developmental stages of the root hairs within 3 min (De Ruijter et al. 1999).

Here we report on the ultrastructural changes that occur during root hair development and Nod factor-induced deformation, focusing on Golgi vesicles and endoplasmic reticulum (ER). Since electron microscopy provides a limited data set, we used differential interference light microscopy of living cells to statistically confirm the relevance of the results (Table 1).

Root hairs are tip-growing extensions of epidermal cells (Derksen and Emons 1990; Miller et al. 1997), and like all plant cells grow by exocytosis, incorporating Golgi vesicle membranes into plasma membranes and their content delivery into an expandable wall. The first stage of root hair development (Figs. 1A to F) is bulge formation (Figs. 1A and 2A, and B) (Dolan et al. 1994), which was reported by Haberlandt in 1887 as “Ausstülpung.” The vacuole occupies most of the cell, the nucleus is opposite the bulge, the ER is transverse to the future hair axis (Fig. 2B), and the Golgi bodies are dispersed without any vesicle accumulation (Fig. 2A). The cell wall has a mottled appearance (Fig. 2B), which is never seen before bulge formation. Bibikova et al. (1998) reported wall acidification at the bulge site. The prevention of acidification arrested the process, whereas upon pH restoration, cell growth resumed. Acid-induced growth has been implied as the basis for root wall extensibility (Pritchard 1994), and acidic proteins (McQueen-Mason et al. 1992) that mediate wall relaxation have been isolated (Wu et al. 1996). It is possible that the expansins are exerting this effect (Link and Cosgrove 1998; Reinhardt et al. 1998).

Dolan et al. (1994) discerned on the basis of growth speed that there is a stage between bulge and tip-growing hair. At this stage, vesicles are delivered to the hair tip for the first time (Fig. 2C), resulting in a 1- to 2 μm -long apical vesicle-rich region (Figs. 1D and 3A). Vesicles at the growing root hair apex also were reported for freeze-substituted hairs of *Equisetum hyemale* (Emons 1987), *V. hirsuta* (Ridge 1988; Ridge 1993), *V. villosa* (Sherrier and VandenBosch, 1994), and *Arabidopsis thaliana* (Galway et al. 1997). The subapical region that contains ER, mitochondria, Golgi bodies, and plastids extends from the vesicle-rich region to the nucleus, the tip of which is 20 to 40 μm from the hair tip. The formation of the vesicle-rich region accompanies a reorientation of the ER directed to the hair axis (Figs. 2C and 3A). Thus, bulge formation and tip-growth are two distinct processes (Ridge 1995). Wymer et al. (1997) found no $[\text{Ca}^{2+}]_c$ increase preceding bulge formation, but a tip-directed $[\text{Ca}^{2+}]_c$ gradient was sustained until growth stopped. Furthermore, elevated $[\text{Ca}^{2+}]_c$ in the tip was absent in the *rhd-2 Arabidopsis* mutant that forms bulges but no hairs (Wymer et al. 1997). The high $[\text{Ca}^{2+}]_c$ is probably involved in exocytosis (Carroll et al. 1998).

Cytoarchitecture changes as hair growth ceases (Figs. 1E and 3B). The vesicle-rich region becomes thin, the subapical cytoplasmic-dense region becomes short, and vacuoles move close to the tip. Most of the subapical ER is transverse to the hair axis, but vesicles are present at the tip (Fig. 3B). In a full-grown hair (Figs. 1F and 3C), the vesicle-rich and cytoplasmic-dense regions are absent, the cytoplasm is a thin layer between the plasma membrane and vacuole, and the nucleus can be anywhere in the cell.

When the Nod factor is added (Figs. 4A to F), cytoplasmic strands become more numerous in the tip of the growth-terminating hairs (Miller et al. 1999; Sieberer and Emons *in press*) and these tips swell (Fig. 4A). Figure 5A shows a swollen tip. Hardly any vesicles are present at the plasma

membrane (Fig. 5B), and the ER is transverse to the hair axis. The wall of swellings stains in a mottled pattern that resembles the wall of bulges, which suggests that the Nod factor alters it (Figs. 5B and C). When the swelling stops, a normal wall is formed next to the mottled wall (Fig. 5C). However,

Fig. 5. Ultrastructure of root hairs treated with Nod factor at growth-termination. **A**, 1h 24 min after treatment with Nod factor. Hair tip is swollen and organelle zonation is not observed. Most of the swelling is occupied by a large vacuole (V). At the lower side, the section is oblique through the cell wall. Bar = 2.5 μ m **B**, Cross-section at high magnification of the distal portion of the swelling shown in **A**, illustrating a small number of vesicles at the edge of the swelling next to the plasma membrane (GV). Note the mottled texture of the wall (W). Bar = 200 nm. **C**, When swelling stops, a new normal cell wall (NW) is deposited along the plasma membrane of the swelling. (1h 10 min after Nod factor application; lateral side of swelling is shown). **C** is a later stage than **D**; swelling and outgrowth are not synchronous in all hairs of a root. Bar = 200 nm. **D**, A new outgrowth has emerged from the swelling with the same organelle distribution as a growing root hair. The vesicles are enriched at the apex. The endoplasmic reticulum (ER) is longitudinal to the hair's growth axis. The adjacent root hair in the figure is at the same developmental stage. M = mitochondrion. Bar = 250 nm. **E**, In growing root hairs and outgrowths from swellings, Golgi vesicles are often found between the ER strands. P = plastid. Bar = 250 nm.

the method used is not a special cell wall method (Emons 1988). Young, thin walls appear electron transparent (Fig. 3) and may be detached from the plasma membrane (Fig. 2). Figure 5C illustrates the difference between a mottled pattern and a normal wall. The wall composition, however, needs future attention.

Felle et al. (1996) have shown that Nod factors elevate the cytoplasmic pH in alfalfa root hairs, which may change the wall. Additionally, Nod factor-induced wall modification was reported for root cortical cells (Van Spronsen et al. 1994).

In a swollen hair tip, a spot of elevated $[Ca^{2+}]_c$ moves toward the plasma membrane until it anchors somewhere in the swelling. At this site, the new vesicle-rich region forms (Fig. 4B) and tip-growth resumes (Figs. 4C and D) (De Ruijter et al. 1998). Vesicles accumulate in the outgrowth apex, whereas the other organelles are subapical with longitudinal ER (Fig. 5D). Figure 5E shows vesicles in an outgrowth between the ER strands, similar to that in a growing hair.

We see parallels between outgrowths from Nod factor-induced swellings and the formation of tip-growing hairs from bulges. In both, two different events occur. The first is bulge formation and swelling, which is undirected growth accompanied by wall loosening. The second is polar growth. No ultrastructural differences were observed between outgrowth and normally growing hair. Though indirect, the Nod factor stimulates cytoplasmic polarity with vesicles at the tip and longitudinal ER. Without the Nod factor, these hairs would not have acquired a new polarity nor would they have become longer than normal for their species. Extrapolation of these results to *Rhizobium*-induced root hair curling suggests that curling can be achieved by a local, progressive induction of polarity away from the existing tip (Batenburg et al. 1986; Emons and Mulder 2000).

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