Research Note

***Rhizobium* Nod Factors Induce an Increase in Sub-apical Fine Bundles of Actin Filaments in *Vicia sativa* Root Hairs within Minutes**

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Accepted 11 May 1999.

We studied the response of the actin cytoskeleton in vetch root hairs after application of host-specific Nod factor. Within 3 to 15 min, the number of sub-apical fine bundles of actin filaments (FB-actin) increased in all developmental stages. Tip growth resumed only in hairs in which the FB-actin density and the length of the region with FB-actin exceeded a minimal value.

Lipochito-oligosaccharides (LCOs, Nod factors) purified from *Rhizobium* spp. induce root hair deformation (Mylona et al. 1995), which is swelling of the tip followed by an outgrowth from that swelling within 3 h (Heidstra et al. 1994). In an assay in which LCOs are applied to vetch roots growing between coverslip and slide, the root hair response is restricted to those hairs that are terminating growth (Heidstra et al. 1994). A certain cyto-architecture of the hairs is mandatory for the ability to deform in response to LCOs (Heidstra et al. 1997; Miller et al. 1997) and the response is a re-initiation of tip growth (de Ruijter et al. 1998).

In root hairs, the presence of dense, sub-apical, net-axial fine bundles of actin filaments, which we have called FB-actin, correlates with tip growth (Miller et al. 1999). However, the actin cytoskeleton in root hairs that deform by LCOs contains bundled filaments up into, and transversely looping through, the hair tip (Miller et al. 1999). Thus, in order to obtain the FB-actin, the cytoskeleton must change. Breakdown and rearrangements of actin filaments have been reported for root hairs of alfalfa after incubation with appropriate Nod factors (Allen et al. 1996). Cárdenas et al. (1998) have reported a diffuse fluorescence of injected fluorescent phalloidin in root hair tips within minutes after exposure of LCOs from *Rhizobium etli* to roots of *Phaseolus vulgaris*, which they interpreted as fragmented filaments. Paradoxically, when we observe vetch root hairs after LCO application in the differential interference contrast (DIC) microscope, cytoplasmic strands remain present at any time (Miller et al. 1999). Cyto-

plasmic strands disappear when actin filaments are depolymerized, which was shown by microinjection of the monomeric, actin-binding protein profilin into *Tradescantia* sp. stamen hair cells (full-grown cells, Staiger et al. 1994; growing cells, Valster et al. 1997). From these experiments it was concluded that bundles of filamentous actin are required for the presence of cytoplasmic strands. Thus, there is an apparent contradiction between our observation of intact cytoplasmic strands in living root hairs shortly after exposure to LCOs, and the observations of Cárdenas et al. (1998) of a fragmented actin cytoskeleton.

We optimized a chemical fixation and staining method to visualize the actin cytoskeleton in intact vetch root hairs and obtained results similar to the results obtained after freeze fixation and freeze substitution (Miller et al. 1999), the most reliable method. The actin cytoskeleton of 80 to 90% of all root hairs stained with fluorescein-phalloidin. This procedure thus allowed the study of a population of hairs in each developmental stage at various times after LCO application, instead of a few injected hairs. For all times after LCO application (3, 6, 9, 12, 15, and 30 min) at least 12 different roots were examined in at least six different experiments, and the same number of roots were used for controls. To exclude recording any effects on the actin cytoskeleton of a change of medium itself, in controls new growth medium was applied instead of LCOs. In all roots, 70 to 80% of the root hairs of all developmental stages responded to LCO application within 3 to 15 min. This response was an increase of the number of FB-actin (Fig. 1A). The hairs that were terminating growth obtained a density of bundles of actin filaments that is similar to the density seen in growing control hairs (Fig. 1B).

For a detailed analysis, we compared densities of actin filament bundles in control and LCO-treated root hairs over a 50- μ m distance from the tip. Fluorescent bundles of actin filaments were imaged in a confocal laser scanning microscope at 1 μ m z-steps. z-Series were flat projected and all actin bundles were counted at distinct distances from the tip, on a grid with lines placed perpendicular to the root hair. During imaging and processing similar settings were used for all treatments and developmental stages. Numbers of actin filament bundles were corrected for width, and their mean values were plot ver-

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sus distance from the tip for each developmental stage (Figs. 2A, 3A, 4A). These profiles of mean actin filament bundle density reflect relative values, since the procedure does not record the thinnest bundles of actin filaments or actin filament bundles present in between the optical sections of a z-series. However, similar settings during imaging and processing provided reproducibility and allowed data comparison.

In growing hairs, which keep on growing after application of LCOs, an increase in FB-actin occurred over 5 to 40 μm from the hair tip (Fig. 2A). Mean actin filament bundle densities were determined from randomly chosen growing root hairs that had been imaged at 3 to 15 min after application of LCOs (Fig. 2A). Figure 2B and C are representative for the

actin cytoskeleton of a control and an LCO-treated hair. The number of actin filament bundles increased significantly, while their orientation remained net-axial and the root hair tip remained free of filamentous actin.

In root hairs that respond to LCOs with root hair deformation, i.e., those that are terminating growth, the mean number of actin filament bundles almost doubled over a distance of at least 50 μm from the root hair tip, within 3 to 15 min (Figs.

Fig. 1. Effect of lipochito-oligosaccharides (LCOs) on sub-apical actin filaments in root hairs. **A**, Percentage of vetch root hairs with an increase in the number of sub-apical actin filaments at 15 min after application of 10^{-10} M *Rhizobium leguminosarum* bv. *viciae* Nod Rlv V [Ac, C_{18:4}] (LCOs) (y axis) is displayed for each developmental stage (x axis). Root hairs in each developmental stage respond to LCOs. Increase is relative to bundle densities in control root hairs, to which fresh growth medium without LCOs was applied. A random population of root hairs from at least 12 different roots was examined in at least six different experiments; the same number were used for controls. **B**, Percentage of vetch root hairs with a density of sub-apical actin filament bundles similar to or above density in growing hairs (y axis) is displayed for each developmental stage (x axis) at 15 min after application of fresh medium with or without Nod factor. Hairs terminating growth obtain a new actin filament bundle density that correlates with growth. **A** and **B**, Error bars indicate SE.

Figs. 2. Growing root hairs. **A**, Mean densities of actin filament bundles (y axis) are displayed vs distance from tip (x axis). For each developmental stage of vetch root hairs, density profiles show an increase in actin filament bundles after application of lipochito-oligosaccharides (LCOs). Position on root and differential interference contrast (DIC) image prevent sampling errors. Random sampling was done within each zone at 3 to 15 min after treatment, z-series were processed, and means of numbers of actin filaments displayed. Error bars indicate SE. Typical distribution and density of actin filament bundles (**B**) after replacing growth medium (control at $t = 3$ min) and (**C**) after application of LCOs ($t = 15$ min). Shown is a loss of thick bundles and increase of fine bundles of actin filaments. Bar = 10 μm . **B–C**, Staining of actin filaments in root hairs. Root hairs were prefixed for 3 min in 200 μM m-maleimido benzoyl *N*-hydroxysuccinimide ester in medium. Cytoplasmic streaming stopped within 1 min. Roots were fixed for 20 min in a final concentration of 4% p-formaldehyde and 0.1% glutaraldehyde in actin stabilizing buffer (ASB: 100 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid) pH 6.8, 1 mM MgCl_2 , 1 mM CaCl_2 , 75 mM KCl] with 1 mM 4-2-aminoethylbenzene sulfonyl fluoride. Root hairs were permeabilized for 12 min with 100 μg of L- α -lyso-phosphatidylcholine per ml in ASB. Actin filaments were stained with 0.33 μM fluorescein-phalloidin in ASB supplemented with 0.05% acetylated bovine serum albumin (BSA). Roots were mounted in CITIFLUOR-glycerol.

1A, 3A). The mean filament bundle density became almost similar to the density in a growing hair (compare Figures 2A and 3A). Figure 3B and C are examples of hairs that are terminating growth. The control hair (Fig. 3B) has actin filament bundles up to the tip, and only a few FB-actin. After LCO application dense sub-apical FB-actin appeared up to the hair tip (Fig. 3C). These fine bundles of actin filaments often had a wider range of orientations in relation to the long axis of the hair than growing hairs after LCO application (compare Figures 3C and 2C). This difference is reflected in the number and orientation of cytoplasmic strands (not shown).

Also, full-grown root hairs responded to the application of LCOs with an increase of actin filament bundles. However, most full-grown hairs responded after a longer time span of 9 to 15 min after LCO application, compared with a 3- to 9-min interval wherein most other hairs responded. In Figure 4A the comparison of control and LCO-treated, full-grown hairs shows a 30 to 40% increase in density of actin filament bundles over 10 to 50 μm from the tip. The final densities attained never reached those of control growing hairs (compare density profile of control in Figure 2A with LCO profile in Figure 4A). Figure 4B and C are micrographs of a control and an LCO-treated, full-grown hair, showing an increase of FB-actin after the application of the signal.

In an earlier report, we defined FB-actin as dense actin filaments in the sub-apical area of a growing root hair (Miller et al. 1999). Our quantitative approach allows us now to define FB-actin more precisely. By the method used, FB-actin is characterized by a minimal density of 7 to 8 actin filament bundles present over a minimal length of 10 μm behind the tip region devoid of actin filaments. These minimal values in the sub-apical area are typical for hairs terminating growth (control in Figure 3A). In these cells, LCO application leads to a rise above the minimal values for FB-actin and indeed to a re-initiation of tip growth. Tip growth is exocytosis at the tip, which implies a localized insertion of Golgi vesicles into the plasma membrane, and the delivery of their content into the expanding cell wall. Therefore, the functional significance of the LCO-induced increase of actin filament bundles seems to be the preparation for the delivery of new Golgi vesicles. The density of actin filaments bundles in full-grown hairs that had responded to LCOs never exceeded the minimal values for FB-actin, which may be the reason why these hairs do not respond with root hair deformation.

Figure 5 shows details of actin filament bundles after LCO application. Mechanisms that may explain the increase are (i) unbundling of thick actin filament bundles into finer bundles, (ii) increased polymerization in new directions, or (iii) new

Fig. 3A–C. See Figure 2 caption. Root hairs that are terminating growth. Typical distribution and density of actin filament bundles (B) after replacing growth medium ($t = 6$ min) and (C) after application of lipochito-oligosaccharides (LCOs; $t = 3$ min). Bar = 10 μm .

Fig. 4A–C. See Figure 2 caption. Full-grown root hairs. Typical distribution and density of actin filament bundles (B) after replacing growth medium ($t = 15$ min) and (C) after application of lipochito-oligosaccharides (LCOs; $t = 15$ min). Bar = 10 μm .

Fig. 5. Details showing examples of diverging fine bundles of actin filaments continuous with thicker bundles, in lipochito-oligosaccharide (LCO)-treated vetch root hairs. In all images, root hair tip is at left. Bar = 5 μ m. **A**, Projected z-series of sub-apex of a growing root hair after LCO application ($t = 12$ min). Shown is an increase in number of actin filament bundles closer to the tip. **B**, Projected z-series of a root hair terminating growth after LCO application ($t = 5$ min) at 30 to 40 μ m from the tip. Shown are fine filaments deviating from a thick bundle of actin filaments (arrows). **C**, Partial z-projection over 5 μ m depth within a growing root hair after LCO application ($t = 3$ min) at 50 to 60 μ m from the tip. Arrowheads indicate sites of apparent local unbundling in a thick bundle of actin filaments. **D**, One single optical section showing a site 25 μ m from tip where a bundle of actin filaments splits into two thinner bundles in a hair that was terminating growth when LCOs were applied ($t = 30$ min).

nucleation and polymerization of fine bundles of actin filaments. From the present experiments, one is not able to choose between these possibilities.

Our observations show, in a quantitative manner, how LCOs change the configuration of the actin cytoskeleton. The changes are too fast to be caused by new gene transcription and de novo protein synthesis. This implies that LCOs trigger a signal transduction cascade that leads to molecules that influence the actin cytoskeleton. After perception of the LCO signal molecule, an influx of calcium ions into the root hairs takes place in *Medicago sativa* (Felle et al. 1998). Gehring et al. (1997) have measured an increase of the cytoplasmic calcium concentration ($[Ca^{2+}]_c$) in root hairs of *Vigna unguiculata* after application of LCOs for at least 10 min. This high $[Ca^{2+}]_c$ in the root hair tip may modify the equilibrium between monomeric actin, actin binding proteins, and actin filaments. Acting in synergy, actin-binding proteins such as profilin (Staiger et al. 1997) and actin depolymerizing factor (ADF; Rozycka et al. 1995), present in tips of growing hairs (Jiang et al. 1997), may cause the formation of FB-actin.

The determination of the changes in the actin cytoskeleton provides a new assay for deciphering the signaling cascade after LCO application to roots.

ACKNOWLEDGMENTS

We gratefully acknowledge M. T. M. Willemse for comments on the manuscript and D. R. Foreman for correcting the English text.

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