From signal to form:

Nod factor as a morphogenetic signal molecule to induce symbiotic responses in legume root hairs

John Esseling

Promotor: Prof. Dr. A.M.C. Emons
Hoogleraar plantencelbiologie, Wageningen Universiteit.

Samenstelling promotiecommissie:

Dr. D.G. Barker, Laboratory of Plant-Microbe Interactions, Toulouse, France.
Prof. Dr. J.W. Kijne, Universiteit Leiden.
Prof. Dr. H. van den Ende, Universiteit van Amsterdam.
Prof. Dr. T. Bisseling, Wageningen Universiteit.

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Outline

The Haber-Bosch procedure fixes the almost inert atmospheric nitrogen into ammonia or nitrate, ions that organisms can use to build nitrogen into DNA, RNA, amino acids and therefore proteins. This is a controlled industrial process that requires pressures ranging from 200 to 400 atmospheres, temperatures from 400° to 650° Celsius and an iron catalyst. A completely different, but equally productive process that does not require such extreme pressures and temperatures – it even takes place in our own gardens – is via the symbiosis between soil living *Rhizobium* bacteria and legume plants. The rhizobia fix the atmospheric nitrogen for the plant, and in exchange the plant provides the bacteria with sugar and a comfortable home, the root nodule. To establish such a symbiosis – the plants need to know that the bacteria are there and *vice versa* – complex signal transduction pathways in the plant and in the bacteria are activated. In response to flavonoids that are excreted by the legume roots, the bacteria produce specific lipochito-oligosaccharide signal molecules, the nodulation factors or nod factors (NFs), which in turn activate symbiotic responses in the legume root.

The primary targets of the rhizobia, and therefore also of the NFs, are the legume root hairs. When the legume root hairs interact with the bacteria, they curl around the bacteria and thus entrap them in a root hair pocket. From this pocket, a plant-derived infection thread starts to grow which brings the bacteria into the root cortex, where cell divisions already took place to start nodule development. When situated in this nodule, the bacteria differentiate into bacterioids, which then start to fix atmospheric nitrogen.

To understand the earliest interactions between the bacteria and the root hairs, first you should understand the root hair itself, how it develops, how it grows, what is involved in root hair growth and what happens to the root hair after NF application. Therefore, we describe in the first chapter the root hair actin cytoskeleton as a backbone of cytoplasmic strands, the cellular highway, its role in root hair growth and the target of NF signalling.

After global application of NF to legume root hairs, the growth terminating root hairs respond with deformation which is a swelling of the root hair tip, followed by a reinitiation of tip growth. Also early nodulin genes, the so-called ENODs, are expressed in NF treated root hairs. However, in the presence of bacteria, growing root hairs are the hairs that curl and entrap the bacteria. Therefore it has been hypothesized that bacteria are needed for curling and that root hair curling is a constant reorientation of the root hair tip growth towards the inducing principle, the NF excreting bacteria. To test these hypotheses, we developed a new experimental assay that is described in chapter 2. With this so called spot application assay, we were able to deposit a small droplet of purified NF on the side of the tip of growing root hairs. The result of this is that

after the spot application the root hair reorientated its growth axis toward the site of application, and that repeated spot applications lead to repeated reorientations, reminiscent of hair curling. This led us to conclude that bacteria are not needed for curling and that isolated NF alone is sufficient to induce this process. Since upon NF spot application, the root hairs also express the *ENOD11-GUS* reporter gene, the root hair reorientation and root hair branching responses are physiological responses.
Recent mutagenesis and genetic approaches in the model legumes *Lotus japonicus* and *Medicago truncatula* have resulted in the identification of a small set of genes coding for proteins that are involved in the recognition of the NF and the transduction of the signals leading to the symbiotic responses. Transmembrane serine/threonine receptor-like kinases with extracellular Lys-M domains have been shown to be involved in NF recognition and bacterial infection and are hypothesized to form heterodimeric NF receptors. In addition, they probably constitute the earlier proposed signalling and bacterial entry receptor. Three more downstream genes, *DMI1*, *DMI2/NORK* and *DMI3* have recently been cloned in *M. truncatula*. The *DMI1* gene is a novel gene that might code for a ligand-gated cation channel, and the *DMI3* gene likely codes for a calcium and calmodulin-dependent protein kinase. Therefore, DMI3 might be involved in transducing the information from NF-induced calcium spiking to nodulation. *DMI2/NORK* (Nodulation Receptor-like Kinase), encodes a LRR receptor-like protein kinase. Besides having a role in setting up a successful symbiosis, we describe in chapter 3 that DMI2 is also involved in non-symbiosis-related cellular processes in root hairs. In other words, NF induced signal transduction taps into the already existing cellular signalling pathways. The non-symbiotic phenotype of *M. truncatula dmi2-1* manifests itself as an enhanced sensitivity to touch. We showed that NF induced morphological responses are not dependent on DMI2, but that in setting up a symbiosis, DMI2 has a role in transducing the signal from the NF to $Pro_{MtENOD11}$-GUS gene expression.
Pea plants can be successfully nodulated by certain strains of rhizobia that produce hardly detectable amounts of NF. In addition, very low concentrations of purified NF elicit changes in root hair morphology and gene expression in *Vicia sativa* and *Medicago sativa*. Therefore, we tested with the spot application assay what is the lowest NF concentration that is still able to activate the signalling cascade leading to root hair reorientation and $Pro_{MtENOD11}$-*GUS* expression. In chapter 4, we show the exciting result that one single NF molecule is sufficient to induce root hair reorientation and $Pro_{MtENOD11}$-*GUS* expression.
In chapter 5 we describe the results that we obtained after spot application or microinjection of pharmacological agonists or antagonists of signal transduction pathways. As such, we show that the NF-induced calcium influx is involved in root hair reorientation, and that a putative heterotrimeric G-protein coupled phosphoinositide pathway is involved in $Pro_{MtENOD11}$-*GUS* expression.

Chapter 6 is a review article in which the focus is on the current state of the art concerning the unravelling of NF induced signal transduction. It combines the knowledge from known NF induced responses in legume root hairs with several mutagenesis screens, pharmacological approaches and cell biological analyses. It ends with the words that probably all the tools are there – the mutants, the drugs, and the cell biological knowledge – and that it is now up to the scientists to find the perfect symbiosis between each other and their different research disciplines, to get the different pathways together, to understand the system.

Finally, in chapter 7 we describe that lithium-ion-induced root hair deformation in Arabidopsis can be caused by inhibition of SHAGGY-like kinases.

Chapter 1

The root hair actin cytoskeleton as backbone, highway, morphogenetic instrument and target for signalling.

John Esseling, Norbert de Ruijter and Anne Mie Emons

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

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1 Introduction

The plant actin cytoskeleton is a structural cell element that connects cytoplasmic components with each other, with the cell surface and with neighbouring cells through plasmodesmata. It is the dynamic backbone of cytoplasmic strands. Along this backbone, cell components move and are targeted to specific sites in the cell. The specific targeting of cell organelles is instrumental in cell morphogenesis. Moreover, the actin cytoskeleton may connect to receptors, transduce signals and mediate cell responses. Actin monomers and filaments are bound to actin binding proteins that determine actin's function in place and time. Since root hairs are accessible for the application of signal molecules and drugs, and can be visualized and manipulated in the living state, these tip growing cells are ideal for the study of the functions of the actin cytoskeleton.
In this review we will discuss what is known, mainly from fixed cells, about the actin cytoskeleton of root hairs. The first topic is a summary of the methods that have been used to visualize actin in root hairs. As a second topic, the actin cytoskeleton's function as intracellular backbone of cytoplasmic strands will be discussed. Third, the typical types of cytoplasmic streaming in the different developmental stages of root hairs and the way in which these patterns change after treatment with actin perturbing drugs will be covered. The streaming patterns of organelles will be related to their transport system, that is, the configuration of actin filaments found during these stages. The fourth topic is the role of actin in morphogenesis. Alone, the actin filaments can not exert all these functions. Apart from polymerisation kinetics, the function of the actin cytoskeleton is determined by actin binding proteins (ABPs). In the fifth section we will discuss the ABPs known to occur in root hairs. The last section of this review will address the way nodulation signals, well-characterized lipochito-oligosaccharides (LCOs) excreted by *Rhizobium* bacteria, affect the actin cytoskeleton of root hairs.

2 Actin visualization

To study the cytoskeleton, a good visualization method is a prerequisite. Over time, different techniques have been developed for the visualization of actin filaments. Most techniques include fixation and several cell wall degradation steps. Recent achievements however, allow the study of the actin cytoskeleton and its dynamics in living cells.
At the ultrastructural level, actin filaments have been visualized with heavy meromyosin fragments (radish root hairs: Seagull and Heath 1979) and immunogold labelling (*Nicotiana* pollen tubes: Lancelle and Hepler 1991). Heavy meromyosin fragments are very useful in determining the polarity of the actin filaments. In fact, the terminology barbed and pointed end for the growing

and non-growing ends respectively, is based on the fishbone-like pattern of heavy meromyosins on individual actin filaments in electron micrographs. Microfilaments, which probably are actin filaments, can also be observed in the electron microscope after glutaraldehyde osmium tetroxide fixation and uranyl acetate lead citrate staining (*Equisetum hyemale* root hairs: Emons 1987). Such contrast staining is a good method to show the orientation of microfilaments in the electron microscope, only if the nature of the filaments is known to be actin. The disadvantage of these methods is that they all need fixation steps, which may more or less disturb the cytoarchitecture and probably the arrangement of the cytoskeleton. Cryofixation followed by freeze substitution is preferred over chemical fixation with aldehydes, because it preserves the cytoarchitecture much better (root hairs: Emons and Derksen 1986; Emons 1987; Emons 1989; Ridge 1988).

In the light microscope, actin filaments can be visualized with fluorescently labelled phalloidin (Wieland and Faulstich 1978) and with anti-actin antibodies (for plant actin: Andersland et al 1994). A common disadvantage of anti-actin antibodies is that they also label actin monomers. This may mask labelling of filamentous actin. For root hairs, anti-actin labelling has been performed in vetch (Miller et al 1999).

Phalloidin is a toxic bicyclic heptapeptide from the poisonous mushroom *Amanita phalloides*. It is a relatively small molecule, with an approximate diameter of 12-15 Å and a molecular weight < 1500 Dalton. From in vitro experiments it is known that phalloidin only binds to the actin monomers within an actin filament, and, if present in a high concentration, shifts actin's monomer/polymer equilibrium towards the polymer, lowering the critical concentration for polymerization up to 30-fold (Cooper 1987; Miki et al 1987). Also with these methods, the fixation step is the most critical; the cytoarchitecture of the fixed cell has to resemble that of a living cell. Phalloidin may not bind to all filaments present in a cell, since it shares its actin-binding site with the actin binding protein cofilin / actin depolymerizing factor (ADF) (Nishida et al 1987; Ressad et al 1998).

Recently, freeze substitution was adapted for a light microscope study of the actin cytoskeleton of vetch root hairs, and a quicker aldehyde fixation method followed by a mild plasma membrane permeabilization was developed, which gave similar results (Miller et al 1999). In the last method, stabilization of the actin cytoskeleton with m-maleimidobenzoyl N-hydroxysuccinimide ester (MBS ester) (Sonobe and Shibaboka 1989) was followed by fixation with a mixture of paraformaldehyde and glutaraldehyde. For plasma membrane permeabilization, the fixed root hairs were treated in increasing concentrations of L-α-lysophosphatidylcholine, after which the actin cytoskeleton was labelled with fluorescein phalloidin. The advantages of this method are the facts that en bloc staining of all hairs along the root is possible, and that the method is fast. However, it has to be optimized for every cell type and compared to actin configurations obtained after freeze substitution. Fig. 1a shows the actin

cytoskeleton in a root hair of *Vicia sativa*, chemically fixed without a pre-treatment with MBS ester and with enzymatic wall degradation. Fig. 1b shows the actin cytoskeleton in a root hair prepared with the optimized method in which the roots were pre-treated with MBS ester before chemical fixation and no enzymes for wall digestion were used.

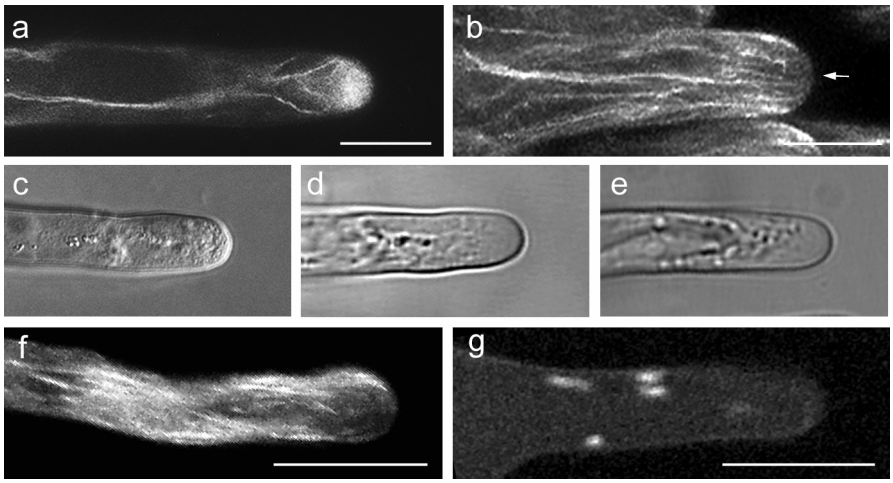

Fig. 1. Visualization of the actin cytoskeleton in *Vicia sativa* and *Arabidopsis thaliana* root hairs, and the effects of fixation on the root hair cytoarchitecture.

a The actin cytoskeleton visualized with rhodamine phalloidin after conventional fixation with 3% paraformaldehyde and 0.05% glutaraldehyde without stabilization with MBS ester, followed by enzymatic wall digestion. Note the accumulation of actin filaments in the tip. **b** Actin cytoskeleton visualized with fluorescein phalloidin after an optimized fixing-staining procedure. F-Actin was stabilized with 300 μM m-maleimido benzoyl N-hydroxysuccinimide ester (MBS ester) in growth medium, and fixed by stepwise increasing the aldehyde concentration to 4% paraformaldehyde and 0.1% glutaraldehyde in Actin Stabilizing Buffer (ASB: 100 mM Pipes pH 6.8, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 75 mM KCl) with 1 mM 4-2 aminoethylbenzene sulfonyl fluoride (AEBSF). Root hairs were permeabilized with 100 µg/ml L-α-lyso-phosphatidylcholine in ASB and actin filaments were stained with 0.33 µM fluorescein phalloidin in ASB supplemented with 0.05% acetylated BSA (ester-aldehyde-choline procedure).

Actin filament bundles are net-axially aligned, are denser in the subapical area and the apex is devoid of actin filament bundles (arrow). **c** Differential interference contrast (DIC) image of **(a)**. Note the poor preservation of the cytoarchitecture in the tip. **d** DIC image after the ester-aldehyde-choline procedure (as in **(b)**). Note the presence of a smooth area in the tip. **e** DIC image of living, growing root hair showing the "reference" cytoarchitecture with subapical dense cytoplasm and a smooth area at the tip. **f** *Arabidopsis thaliana:* Actin filament bundles in living, growing root hair at 3 min after microinjection with Alexa-488 phalloidin **g** *Arabidopsis thaliana:* Fluorescence in living root hair at 30 min after microinjection with Alexa-488 phalloidin, showing accumulation of signal in aggregates in the cytoplasm. Magnification is the same for **a**, **c**, **d**, **e**. Bar in (**a**, **b**, **f**, **g**) **=** 20 μm.

The optimized method preserves the cytoarchitecture better (Fig. 1d). This becomes clearly seen when one compares root hairs prepared with the two types of fixation (Fig. 1c and 1d respectively) with living root hairs (Fig. 1e), using the differential interference contrast (DIC) microscope.

For the study of cytoskeletal dynamics in response to internal and external stimuli, living cells are the most interesting. One approach to visualize actin dynamics in plant cells is microinjection of fluorescent phalloidin. For *Tradescantia* stamen hair cells two methods were developed. When injected in high concentrations (20 µM needle concentration), phalloidin immediately stabilizes the actin cytoskeleton (Valster and Hepler, 1997). A disadvantage of this 'snap shot method' is that the cell dies. Actin remains functional when injection is done at lower concentrations of phalloidin (8-9 µM needle concentration), as shown by Staiger et al. (1994) and Valster et al. (1997). Also this method has to be optimized for each cell type. Cárdenas et al. (1998) injected 20 µM needle concentration FITC phalloidin in living *Phaseolus* root hairs (the 'snap shot method') and reported stabilization of actin filament bundles followed by root hair death, generally 10 minutes after the injection. Microinjection of 0.66 µM needle concentration Alexa-488 phalloidin in *Medicago truncatula* and *Arabidopsis thaliana* root hairs is not lethal to the root hairs and shows an actin staining pattern in the first 10 minutes after injection. However, within 30 minutes after injection, this pattern is broken down and fluorescence accumulates in aggregates, which move along with the cytoplasmic streaming (J. Esseling, unpublished results). Fig. 1f shows the actin cytoskeleton 3 minutes after Alexa-488 phalloidin injection in a growing *Arabidopsis thaliana* root hair. Fig. 1g shows a growing *Arabidopsis thaliana* root hair, 30 minutes after Alexa-488 phalloidin injection.

The most promising method is the use of GFP-fusion proteins. GFP (Green Fluorescent Protein) is a protein isolated from the jellyfish *Aequorea victoria* (Chalfie et al 1994). Its gene can be coupled to any gene of interest. In a number of studies, GFP-actin fusion proteins have been used to visualize actin localization in mammalian cells (Ballestrem et al 1998), yeast (Doyle and Botstein 1996) and *Dictyostelium discoideum* (Aizawa et al 1997b; Neujahr et al 1997; Westphal et al 1997). However, also GFP-actin fusion proteins have their problems. Expression of GFP-actin fusion proteins will show both actin filaments and monomers, which could mask actin filaments. Also labelling of only a subpopulation of GFP-actin filaments has been reported (Doyle and Botstein 1996). Moreover, expression of GFP-actin can severely affect morphology and function of cells (Aizawa et al 1997b).

Another approach is the use of a fusion between GFP and full length actin binding proteins or actin binding domains, which was used in *Drosophila melanogaster* (Edwards et al 1997), *Dictyostelium discoideum* (Aizawa et al 1997a; Pang et al 1998), yeast (Doyle and Botstein 1996) and, most recently, in plants (Kost et al 1998). Kost and colleagues used the actin binding domain of a mouse talin gene coupled to GFP, which they introduced in tobacco pollen

tubes and tobacco BY-2 cells via particle bombardment and in *Arabidopsis thaliana* via *Agrobacterium* transformation (Kost et al 1998). However, they report that pollen tubes with a high level of expression, terminate growth and die.

3 Actin: the backbone

Plant cells contain vacuoles. Mature cells have one large vacuole, which fills most of the cell. So-called cytoplasmic strands traverse this vacuole. The cytoplasmic strands are continuous with the cortical cytoplasm, which covers the whole plasma membrane. Together, cortical cytoplasm and cytoplasmic strands are one connected system. Immunocytochemistry shows that cytoplasmic strands contain actin filament bundles. Moreover, when the actin filaments are depolymerized by, for instance, microinjection of the actin monomer binding protein profilin, the cytoplasmic strands become thinner and snap back into the cortical cytoplasm. This has been shown in mature (Staiger et al 1994) and growing (Valster et al 1997) *Tradescantia* stamen hair cells.

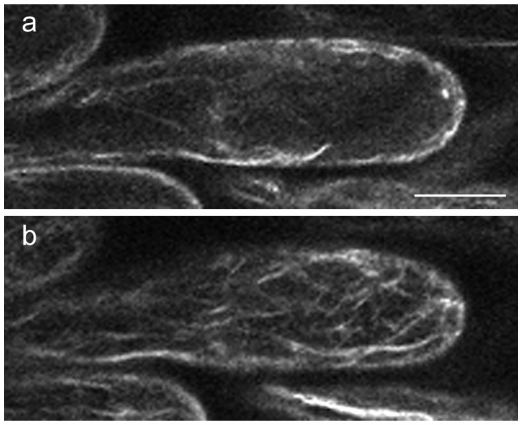

Fig. 2. Effect of cytochalasin D on the actin cytoskeleton of *Vicia sativa* root hairs.
a,b Confocal optical planes through a growing root hair that was treated with 2.0 μM cytochalasin D for 30 min, stained after the ester-aldehyde-choline procedure with fluorescein phalloidin and imaged at the mid plane **(a)** and cortical plane **(b)**. Filamentous actin is not present in the mid plane of the cell, but actin filament bundles have remained in the cortical cytoplasm. The magnification is the same for **a,b**. Bar in (**a**) = 15 μm.

Likewise, in root hairs, partial depolymerization of the actin filaments, in this case performed by use of cytochalasin D at relatively high concentrations (1-5 μM), causes the formation of one large vacuole in growing root hairs of *Vicia sativa*, which had cytoplasmic strands before application of the drug. However, bundles of actin filaments remain present in the cortical cytoplasm, after profilin injection in *Tradescantia* stamen hair cells (Valster et al 1997), as well as after

treatment of root hairs with high concentrations of cytochalasin D (Fig. 2a,b). Actin filament bundles appear to be the backbones of cytoplasmic strands.

4 Actin: the highway

When living plant cells are studied with the light microscope, an obvious feature is the movement of the organelles inside cytoplasmic strands, also known as cytoplasmic streaming. In rotational streaming (Kamiya 1981), or cyclosis, all organelles follow one direction. In the more common circulation streaming, the pattern is less organized; tracks in which organelles move in opposite directions may be located next to each other in the same cytoplasmic strand (Lichtscheidl and Url, 1987; Lichtscheidl and Weiss, 1988). In fountain streaming, a central track of organelles is running upward, splitting up and then reversing its orientation. Tip growing cells have reverse-fountain streaming. Iwanami (1956) introduced this term for pollen tubes. In wide pollen tubes (*Lilium*) or root hairs (*Hydrocharis*, Tominaga et al 1997; Tominaga et al 1998) an upward flow of cytoplasm to the tip at the cell borders and downward flow in a transvacuolar cytoplasmic strand in the cell centre characterize this type of streaming. The upward flow never reaches the plasma membrane at the tip of a growing root hair or pollen tube, but reverses just below the so-called vesicle rich region. In thinner root hairs (such as from *Arabidopsis*, *Medicago* and *Vicia*) and pollen tubes (*Nicotiana tabacum*, De Win et al 1999; *Papaver rhoeas*, A. Geitmann, personal communication 1999), the downward streaming may be in the centre or at the side of the hair or tube. However, the most outstanding feature of reverse fountain streaming remains: the reversal of the upward streaming before the hair tip is reached.

The speed of cytoplasmic streaming has been measured in different regions of growing and full- grown root hairs of *Equisetum hyemale* (Emons 1987). In the tip of a 2 days old *Equisetum hyemale* root hair the speed of cytoplasmic streaming is 0 μm/s. Below the tip, the speed of streaming increases to a more steady level of about 3 μm/s. The cytoplasmic streaming pattern and speed in full-grown hairs is completely different compared to growing hairs. The flow does not reverse before reaching the tip of the hair as it does in growing hairs, but streams through the very tip in thin strands of cytoplasm between the large central vacuole and the plasma membrane. This type of cytoplasmic streaming is of the circulation type (Tominaga et al 1998). In full-grown root hairs of *Equisetum hyemale*, the speed of cytoplasmic streaming is different when compared to the speed in growing hairs. Throughout the whole full-grown root hair, the speed of cytoplasmic streaming is between 4 and 6 μm/s (Emons 1987). So, an organelle of 1 μm moves about 5 times its own length per second. For comparison, if a 2 m tall sprinter runs 5 times his/her own length per second, his/her speed would be 36 km/h. Another type of organelle movement has been described as saltatory movement. It is observed when single

organelles move in a non co-ordinated individual way over only short distances (review: Emons et al 1991). This also occurs in root hairs (unpublished results).
In plant cells, cytoplasmic movement is actin based. After injection of the G-actin ABP profilin into *Tradescantia* stamen hair cells, cytoplasmic streaming ceases (full-grown cells: Staiger et al 1994; growing cells: Valster et al 1997). In these experiments, a second injection with fluorescent phalloidin confirmed that the stop of cytoplasmic streaming was caused by depolymerization of actin filaments. Moreover, actin perturbing drugs, such as the actin filament capping fungal toxin cytochalasin D, stops cytoplasmic streaming in *Vicia sativa* root hairs at concentrations above 5 μM (De Ruijter et al personal communication 1999).

5 Actin, the morphogenetic instrument

In higher plants, cell morphogenesis is attained by local cell growth. One can discriminate between three types of growth: isodiametric -, intercalary - and tip growth. Insertion of new plasma membrane and exocytosis of cell wall material through Golgi vesicles (Roberts 1994) determine plant cell growth. Thus, in case of growing cells, the exocytotic vesicle is the unit of plant cell growth. The location of exocytosis determines where and when growth will take place. The most common type of plant cell growth is intercalary growth of elongating cells such as cells of the root elongation zone. In tip growing cells, exocytosis occurs at one side of the cell. Well-known examples of tip growing cells in higher plants are pollen tubes (Schnepf 1986) and root hairs (Derksen and Emons 1990).
Root hairs develop as tubular extensions of specialized root epidermal cells, the trichoblasts (Dolan et al 1994). The onset of root hair growth from a trichoblast can be seen as a local expansion of the trichoblast, the bulge. The bulge contains a peripheral layer of cytoplasm with cytoplasmic strands around a large vacuole (Fig. 3a) (Miller et al 1999). The actin cytoskeleton in bulges is characterized by filament bundles running in the cytoplasm along the plasma membrane of the bulge, longitudinal to the root axis (Fig. 3b) in the same orientation as in the trichoblasts that have not formed a bulge yet (Miller et al 1999).
Growing root hairs have a polar cytoarchitecture with a central vacuole in the basal part of the cell and a cytoplasmic dense region at the cell apex (Fig. 3c). Electron microscopic studies have shown that the first 1-3 μm from the tip contains almost exclusively secretory vesicles (chemical fixation: Bonnett and Newcomb 1966, freeze substitution: Emons 1987; Ridge 1988; Sherrier and Van den Bosch 1994; Galway et al 1997). We call this the vesicle rich region. In the differential interference microscope it appears as a very smooth thin layer of cytoplasm at the tip (Fig. 3c). During growth, the cytoplasmic dense region remains at the tip, while the vacuole increases in size. In these growing hairs, bundles of actin filaments are present in cytoplasmic strands around and

traversing the central vacuole (Miller et al 1999). These bundles lie longitudinally and helical in the root hair, perpendicular to their orientation in the epidermal part of the trichoblast (Fig. 3a). In the cytoplasmic dense region, the filament bundles flare out into thinner and thinner bundles and maybe even into single filaments (Fig. 3d). We call these subapical net-axially aligned fine bundles of actin filaments, FB-actin. The very apex of the growing hair is devoid of actin filament bundles (arrow Fig. 3d). In vetch, this region is approximately 2-6 µm deep and 5-8 µm wide (Miller et al 1999) and coincides with the vesicle rich region.

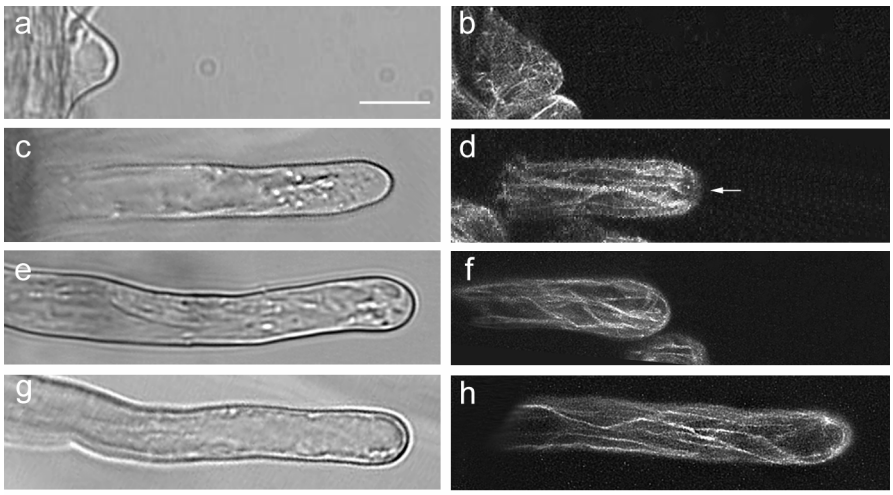

Fig. 3. The cytoarchitecture and actin filament configuration of subsequent developmental stages of *Vicia sativa* root hairs.
(**a,c,e,g)** DIC images of living root hairs, representative for each developmental stage. **(b,d,f,h)** Representative fluorescence images showing the actin filament distribution in each developmental stage; actin was visualized with fluorescein phalloidin after the ester-aldehyde-choline procedure. The images are projections of confocal z-series. **a,b** Bulge containing a major central vacuole. Actin filament bundles are randomly oriented and pass through the peripheral cytoplasm at the tip. **c,d** Growing root hair with a smooth area at the very tip, and a subapical cytoplasmic dense region. The actin filament bundles are longitudinally oriented in the hair. In the subapical region, dense fine actin filament bundles are present (FB-actin), while the tip is devoid of actin filament bundles (arrow). **e,f** Root hair in which growth is terminating. Small vacuoles are present close to the tip with small cytoplasmic strands between them. The area devoid of actin is absent, and fine actin filament bundles are present in the tip. **g,h** Full-grown root hair with one large vacuole and peripheral cytoplasm. Thick actin filament bundles are present in strands looping through the extreme tip. The magnification is the same in all images. Bar in (**a**) = 20 µm.

The cytoarchitecture of root hairs that terminate growth is characterized by a shortening of the cytoplasmic dense region with an enlargement of the main vacuole further into the tip-region (Fig. 3e). The area with FB-actin is very short

and a region devoid of actin in the extreme tip is not present any more (Fig. 3f) (Miller et al 1999, De Ruijter et al 1999).
In full- grown hairs, cytoplasmic polarity has clearly been lost; the central vacuole extends into the tip, and is surrounded with only a thin layer of peripheral cytoplasm (Fig. 3g). Actin filament bundles are found in this thin peripheral layer of cytoplasm just as elsewhere in the cell cortex, with some bundles looping through the tip and other bundles turning in the cortical cytoplasm before they reach the tip (Fig. 3h).

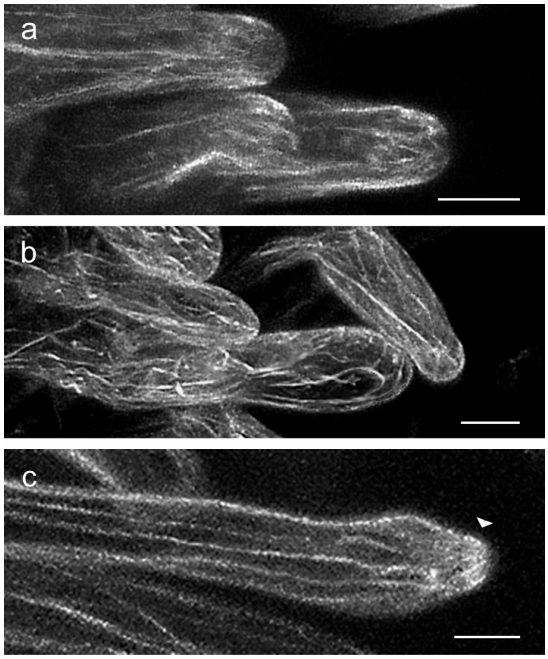

Fig. 4. The presence of dense subapical fine actin filament bundles (FB-actin) correlates with tip growth in root hairs of *Vicia sativa*.
a Growing root hairs with FB actin in the subapical region, and the very tip devoid of actin filament bundles. **b** Growing root hairs treated with 1.0 μM cytochalasin D for 30 min with an actin cytoskeleton typical for hairs terminating growth. Note the disappearance of FB-actin and the presence of actin filament bundles in the very tip. **c** Root hair that was terminating growth treated with 10^{-10} M LCO for 75 min, with a new actin cytoskeleton typical for growing hairs, FB-actin (arrowhead), and a small area devoid of actin at the extreme tip. Bar = 15 μm.

FB-actin is not present in the subapex and there is no apical area devoid of filamentous actin. From the actin cytoskeleton configuration during root hair development, we hypothesized that the role of FB-actin is to retain the Golgi vesicles in the subapical area and to deliver them to the base of the Golgi vesicle rich region (Miller et al 1999).
We had two ways to test this. First, the inhibition of actin filament elongation by the F-actin capping drug cytochalasin-D should change the cytoarchitecture of a growing root hair into that of a hair that is terminating growth, and, after longer treatment, into that of a full-grown hair. On the other hand, a signal molecule

that reinitiates growth in hairs that are terminating growth, should induce FB-actin. When cytochalasin-D is used at low concentrations (<1 μM), the bundles of actin filaments in the hair tube remain present and are still functional, as shown by the fact that cytoplasmic streaming continues. However, indeed, as

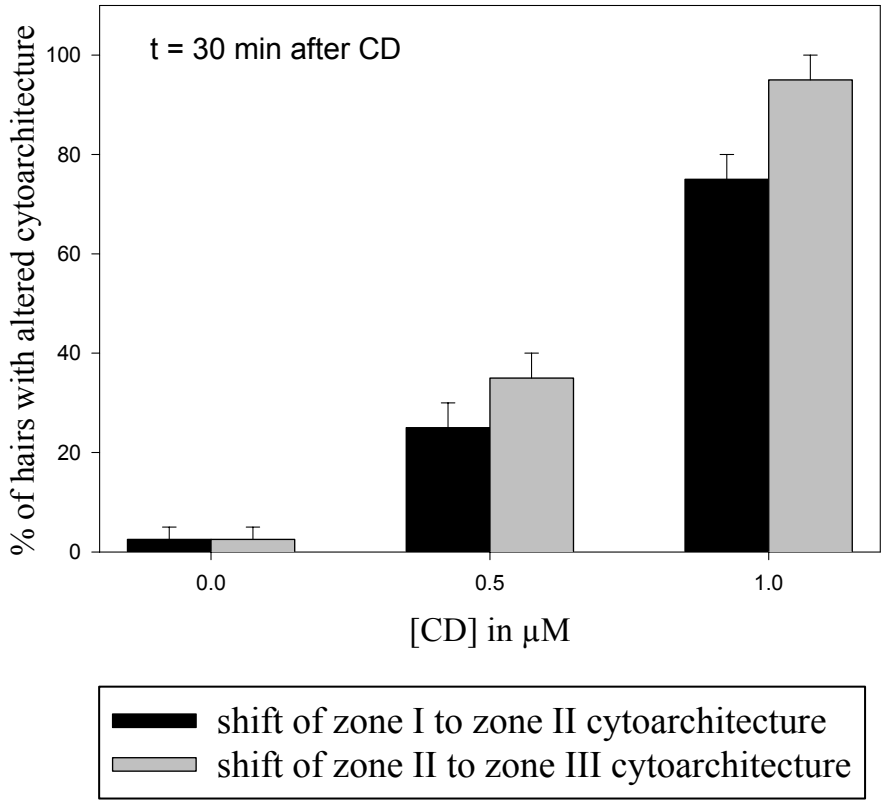

Fig. 5. Cytochalasin D effects on the cytoarchitecture of *Vicia sativa* root hairs.
Histogram showing the percentage of root hairs in zone I and II that had obtained the cytoarchitecture of respectively zone II and III after 30 min (y-axis), related to the concentration of CD in μM (x-axis). The shift to a cytoarchitecture of the next developmental stage increases with the concentration of CD; zone II hairs are more sensitive than zone I hairs. Zone I: Growing root hairs; Zone II: Root hairs terminating growth; Zone III: full-grown root hairs.

predicted by our hypothesis, when FB-actin disappears (compare Fig. 4a before and 4b after cytochalasin-D application to growing hairs), hairs obtain the cytoarchitecture of hairs that are terminating growth (Fig. 5).
To reinitiate growth in legume root hairs, host specific lipochito-oligosaccharides (LCOs) can be used. LCOs, also known as Nod factors, are well-characterized signal molecules excreted by *Rhizobium* bacteria that induce reinitiation of tip growth in legume root hairs that are terminating growth (Fig. 6).

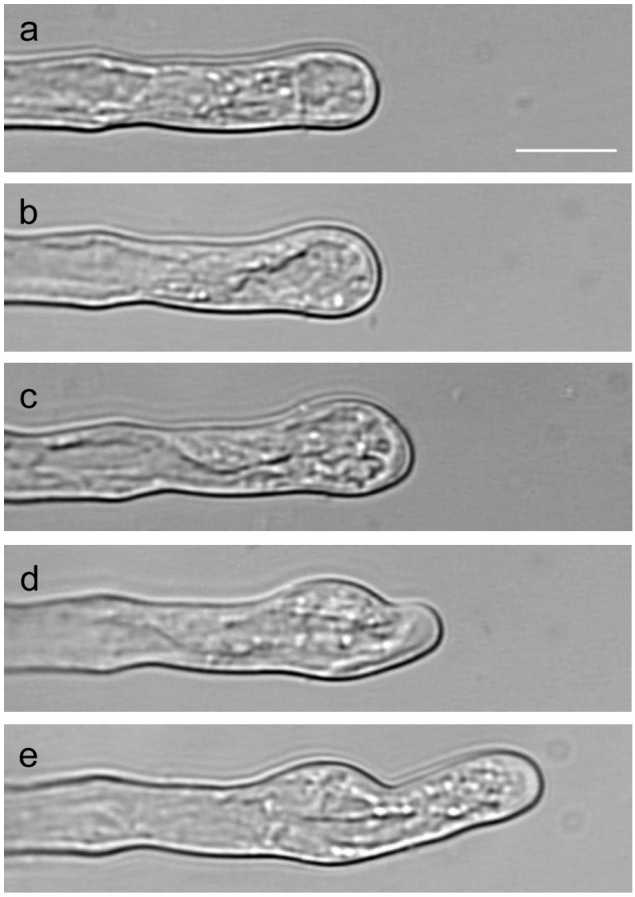

Fig. 6. Root hair deformation after LCO application to roots of *Vicia sativa*.
a-e A time-series of DIC images from one living root hair that was terminating growth, showing a swelling and reinitiation of a new outgrowth from that swelling after application of 10^{-10} M LCO.
a Typical cytoarchitecture of a hair terminating growth. **b** An initial swelling is visible at the tip at 1 h after LCO application. **c** At one side of the swelling cytoplasm accumulates at 1 h 15 min after LCO application, a smooth area becomes visible, and tip-polarized growth is initiated. **d** Cytoplasmic strands orient towards the new tip at 1 h 30 min after LCO application, and renewed tip growth is apparent. **e** The cytoarchitecture of the outgrowth (at t = 2 h 30 min after LCO) has similar characteristics as a normal growing hair (see **Fig. 1e, 3c**). Magnification is the same for **a-e**. Bar (in **a)** = 20 μm.

Indeed, the application of LCOs induces the formation of FB-actin, prior to new tip growth (Fig. 3f before and 4c after LCO application; Miller et al 1999).
Since a growing root hair elongates in one direction, and actin filaments are continuous in the whole hair, actin polymerization in the direction of growth has to take place during root hair elongation. During root hair elongation, the area with FB-actin has a more or less constant length. The most logical explanation for this phenomenon is that filaments elongate at the tip of the FB-actin zone and bundle at the base of this zone. Then, actin monomers have to be incorporated into filaments at the tip of the zone with FB-actin. The situation is clearly different from that at the leading edge of motile animal cells. There, it is widely accepted that polymerization of new filaments and membrane protrusion are tightly coupled (Welch et al 1997). Here, in the root hair, polymerization of new filaments would be coupled to Golgi vesicle retention and/or Golgi vesicle delivery to the vesicle rich area at the extreme tip of the root hair.
In plant cells, growth itself is the incorporation of Golgi vesicles. The study of actin in root hairs shows that FB-actin is one of the factors that determine where and when Golgi vesicles are incorporated into the plasma membrane. Therefore, FB-actin can be seen as one of the morphogenic instruments of plant cells. It could well be that the same morphogenic instrument is involved in other types of growth, including bulge formation prior to tip growth. Results from studies by Foissner et al. (1996), in which the actin cytoskeleton after cell wounding was investigated, demonstrated the involvement of fine filaments of actin in recruiting vesicles for cell wall repair. Because vesicle incorporation in isodiametrically and intercalary growing cells is far less localized, and therefore less abundant, than in tip growing cells, the area with fine bundles of actin filaments should be a thin layer along the plasma membrane. Such a thin peripheral network of actin filaments is difficult to image, but has been reported for, among other cells, potato suspension cells (Collings and Emons 1999).

6 The regulation of the actin cytoskeleton

The changing configurations of the actin cytoskeleton during root hair development and correlated patterns of cytoplasmic streaming are indications for a highly dynamic actin cytoskeleton. The outstanding question in understanding actin dynamics, is the role of actin polymerization and the regulation of polymerization. The fuel for actin polymerization has to be a large cellular pool of unpolymerized actin, which should be maintained at concentrations well above the critical concentration for actin polymerization. To maintain this pool, cells employ actin binding proteins (ABPs) (review plant ABPs: Ayscough 1998; plant cells: De Ruijter and Emons 1999). Much of our understanding about the function of ABPs comes from in vitro research (Ruhlandt et al 1994; Carlier et al 1997) and work on ABPs from animal cells (Puius et al 1998).

In plants, profilin is the best-characterized ABP (reviewed by Staiger et al 1997). Originally, it was identified in plants as a pollen allergen (Valenta et al 1991) and has now been isolated from a wide variety of plant species (Staiger et al 1993; Mittermann et al 1995; Darnowski et al 1996; Huang et al 1996; Clarke et al 1998). In *Arabidopsis thaliana*, thus far 10 gene members have been isolated (Huang et al 1996) and maize is predicted to have at least six profilin genes (Staiger et al 1993). Profilin was originally identified as an actin monomer sequestering protein that can inhibit actin filament growth in vitro (Carlsson et al 1977) and can be placed in the group of ABPs with assembly/disassembly function. Indeed, when microinjected into plant cells, i.e. *Tradescantia* stamen hair cells, profilin causes depolymerization of actin filaments in a dose dependent manner (Staiger et al 1994; Valster et al 1997), consistent with an actin monomer binding function of profilin. Another actin cytoskeleton regulating function of profilin is the shift in equilibrium from ADP-actin to ATP-actin at low profilin concentrations. Since ATP-actin binds more efficiently to the barbed end of actin filaments, lowering of the profilin concentration promotes actin polymerization (Hartwig and Kwiatkowski 1991; Aderem 1992; Goldschmidt-Clermont et al 1992). Like many other ABPs, profilin is a multifunctional protein. It can reversibly bind to poly-L-proline and to the phospholipid phosphatidyl-inositol 4,5-biphosphate (PIP_2) (Goldschmidt-Clermont et al 1990; Goldschmidt-Clermont et al 1991; Sohn and Goldschmidt-Clermont 1994). The binding to PIP_2 provides a link between the phosphatidyl-inositol signal transduction pathway and the actin cytoskeleton (Aderem 1992). In vitro, human platelet-derived profilin binds to PIP_2 and inhibits its hydrolysis by unphosphorylated PLC (Goldschmidt-Clermont et al 1991). When phosphorylated by a tyrosine kinase, PLC is activated and is able to hydrolyze the PIP_2-profilin complex, resulting in the release of profilin (Goldschmidt-Clermont et al 1991). In plants, experiments in *Papaver rhoeas* pollen tubes indicate a possible role for profilin in signal transduction. In these cells, profilin regulates protein kinase and protein phosphatase activity and actin based cytoskeletal protein assembly (Clarke et al 1998). Also a controlling role of profilin in phospho-inositide turnover in plant cells has been demonstrated (Drøbak et al 1994), and recently it has been shown that in microspores and mature pollen, profilin is associated with the plasmamembrane (Von Witsch et al 1998). All these data indicate that profilin is an interesting ABP in plant cells, since it links signal transduction directly to the actin cytoskeleton. Unfortunately, there are no publications yet on the role of profilin in root hairs. In growing pollen tubes (Vidali and Hepler 1997) and root hairs (Braun et al 1999), profilin is localized throughout the cytoplasmic dense region.

To maintain continuous polymerization at one end, actin must be depolymerized at the other end, and the subunits recycled. ADF / cofilin (Maciver et al 1998) could be an important protein for the regulation of actin depolymerization. Plant-ADF was first identified in *Lilium longiflorum* pollen tubes (Kim et al 1993), after which Rozycka et al. (1995) and Lopez et al. (1996) found ADF in *Zea mays*

pollen tubes. Plant ADF appears to be modulated by pH (Lopez et al 1996), interacts with phosphoinositides (Gungabissoon et al 1998) and promotes actin dynamics in vitro (Jiang et al 1997b). When pH increases (to about 8.0), the ability of ADF to sever and depolymerize actin bundles increases (Staiger et al 1997; Maciver et al 1998). In vitro experiments with maize ADF indicate that it binds to actin monomers in a 1:1 complex (Staiger et al 1997). It also promotes treadmilling by accelerating the association rate at barbed ends and the dissociation rate at pointed ends of filaments (Carlier et al 1997). In *Dictyostelium*, data suggest that ADF (here named cofilin) has a role in remodelling the cortical actin meshwork into actin filament bundles (Aizawa et al 1997a). ADF localization in maize root hairs, in the apex and subapex (Jiang et al 1997a), where FB-actin is present (Miller et al 1999) suggests that ADF has a role in remodelling the actin cytoskeleton in growing root hairs. In bulging trichoblasts, which do not have visible FB-actin (Miller et al 1999), ADF is localized in the cytoplasm without specific distribution (Jiang et al 1997a).
A third possible actin binding protein known to occur in root hairs, is a protein that labels with anti-spectrins from human and chicken origin (De Ruijter et al 1998). Although the gene has not been isolated yet, the anti-spectrin antibodies recognize an interesting protein product, since it marks tip growth very efficiently. The epitope is not accumulated in the trichoblast, present in the apex of a growing root hair, disappears from the tip when growth stops, re-appears at the plasma membrane of the swelling after application of LCOs, and is again present at the tip of the outgrowth (De Ruijter et al 1998). It has a punctuate pattern in the vesicle rich region at the root hair tip (Miller et al 1997) and, therefore, may be a membrane protein that is inserted into the plasma membrane by Golgi vesicles. Spectrin-like proteins may indeed be involved in membrane sorting and Golgi dynamics (Lippincott-Schwartz 1998). The same antibodies label tips of pollen tubes (Derksen et al 1995) and hyphae (Kaminsky and Heath 1995) and localize to the plasma membrane of growing cells (De Ruijter and Emons 1993). Since a plant spectrin gene has not been reported yet, we can only speculate about the function of this spectrin-like plant protein. Recently, a gene of the spectrin family has been found in *Arabidopsis* (McCurdy and Kim 1998).

7 Actin as target for Nod factor signalling

Nitrogen fixing *Rhizobium* bacteria can live in symbiosis with legume plants. In the legume root, *Rhizobia* induce the formation of so-called root nodules, where the nitrogen fixation takes place (Mylona et al 1995). For this root nodule formation, the bacteria grow into the root via an infection thread, which is first formed inside the root hair (Kijne 1992). During infection thread formation, the root hair curls around the bacteria. This root hair curling means modification of root hair growth. Application of very low concentrations (as low as 10^{-12} M,

Heidstra et al 1994) of purified host-specific LCOs to liquid grown legume roots induce several responses in a time course. In the first minutes after application, LCOs trigger physiological responses, such as alkalinization of the root hair (Ehrhardt et al 1992; Felle et al 1996), depolarization of the root hair plasmamembrane (Ehrhardt et al 1992; Felle et al 1996; Kurkdjian 1995) and calcium spiking (Ehrhardt et al 1996). It induces the expression of early nodulin genes, such as ENOD5, ENOD12, VsLb1 and Mtrip1 (Horvath et al 1993; Journet et al 1994; Cook et al 1995; Heidstra et al 1997a), and causes root hair deformation in 80-90% of growth terminating root hairs (Heidstra et al 1997b; De Ruijter et al 1998) about 1-2 hours after application and, finally, induces mitotic activity in cells of the root cortex (Mylona et al 1995).
Root hair deformation is characterized by a swelling of the root hair tip, followed by an outgrowth from that swelling (Heidstra et al 1994). In fact, outgrowth is reinitiation of tip growth from the swelling, with all characteristics of normal root hair tip growth (De Ruijter et al 1998; Fig. 6a-e).
The earliest cytoskeleton responses to LCOs reported so far are fragmentation of the actin cytoskeleton within the first 15 minutes after Nod factor application (*Medicago sativa*: Allen and Bennet 1996; *Phaseolus vulgaris*: Cárdenas et al 1998). This fragmentation was reported as discontinuities in the longitudinal actin bundles and formation of discrete foci of phalloidin staining at the root hair tip. FITC phalloidin microinjected *Phaseolus* root hairs show a fluorescent glow at the tip of the hairs within 5 minutes after Nod factor treatment (Cárdenas et al 1998). This glow has been interpreted as actin filament fragmentation. As discussed above, actin filament breakdown causes the disappearance of cytoplasmic strands (full-grown cells: Staiger et al 1994; growing cells: Valster et al 1997; vetch root hairs Fig. 2a,b). Thus, the results of Cárdenas et al. (1998) would suggest that actin filament fragmentation by Nod factor should cause the breakdown of cytoplasmic strands in root hairs.
For *Vicia sativa* root hairs, it has been shown that, consistent with the idea that cytoplasmic strands can not exist without filamentous actin, actin filaments remain intact after Nod factor application (Miller et al 1997; De Ruijter et al 1999). However, the number of fine bundles of actin filaments increased in the subapical area of the root hair within 3-15 minutes after Nod factor application, in 80% of the all root hairs (Fig. 7). This increase in the number of fine bundles of actin filaments occurred in all stages of root hair development.

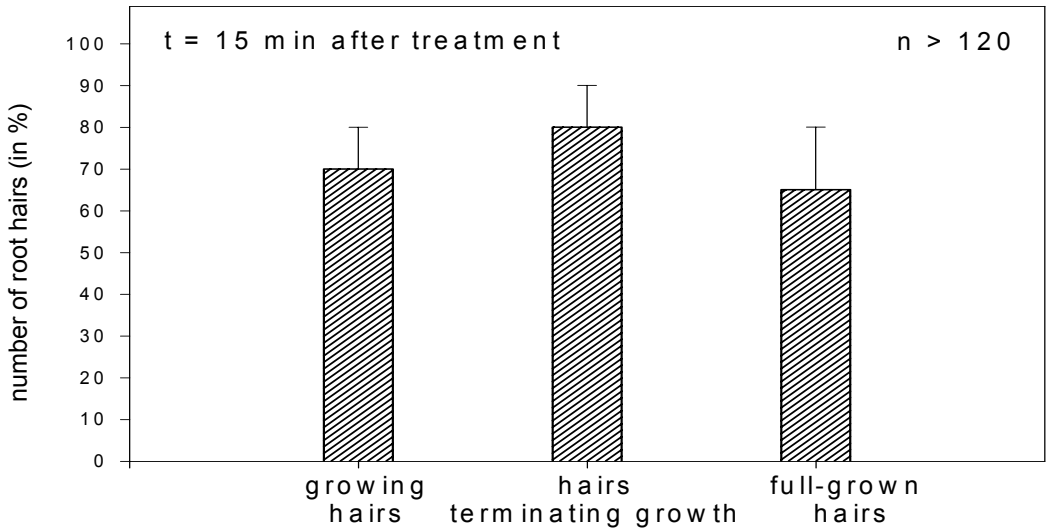

Fig. 7. Percentage of root hairs of *Vicia sativa* with a LCO-induced increase in the density of sub-apical actin filament bundles.

The percentage of *Vicia sativa* root hairs with an increase in the number of sub-apical actin filament bundles at 15 minutes after application of 10^{-10} M *Rhizobium leguminosarum* bv *viciae* Nod RIv V [Ac, C18:4] (LCOs) (y-axis) is displayed for each developmental stage (x-axis). Root hairs in each developmental stage respond to LCOs. The density of actin filament bundles is compared to the density of actin filament bundles 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 6 different experiments, and the same amount was used for the controls. Error bars indicate standard error (SE).

Thus, root hairs that do and do not respond to Nod factor by root hair deformation, show a uniform response in the actin cytoskeleton, which is an increase in the number of subapical fine bundles of actin filaments (Fig. 8).

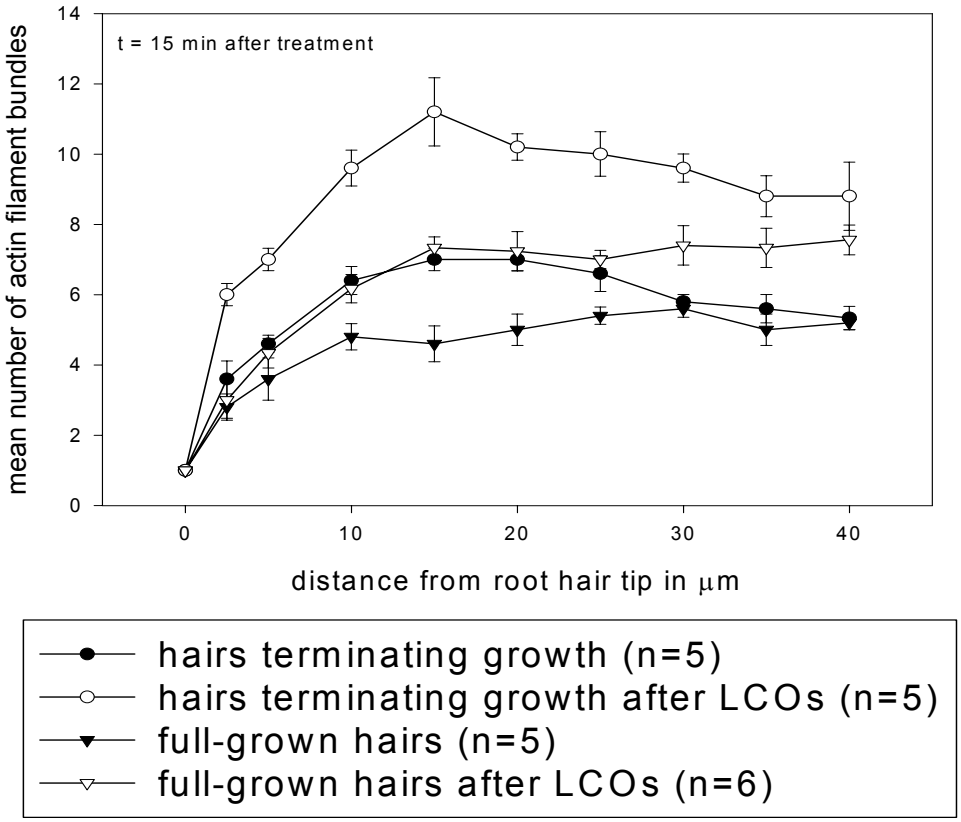

Fig. 8. Mean densities of actin filament bundles in the subapical area of control and LCO treated *Vicia sativa* root hairs.

Mean densities of actin filament bundles (y-axis) are displayed versus the distance from the tip (x-axis). In all root hairs, the density profile shows an increase in actin filament bundles after application of LCOs (Shown for root hairs terminating growth and root hairs terminating growth). Note that the increase in full-grown hairs does not exceed the critical FB-actin density that correlates with growth. Random sampling was done within each zone at 3-15 minutes after treatment, z-series were processed, and means of numbers of actin filaments displayed. Error bars indicate SE.

Figure 9a is an example of hairs in the developmental stage of growth termination taken before, and figure 9b taken 3 min after Nod factor application.

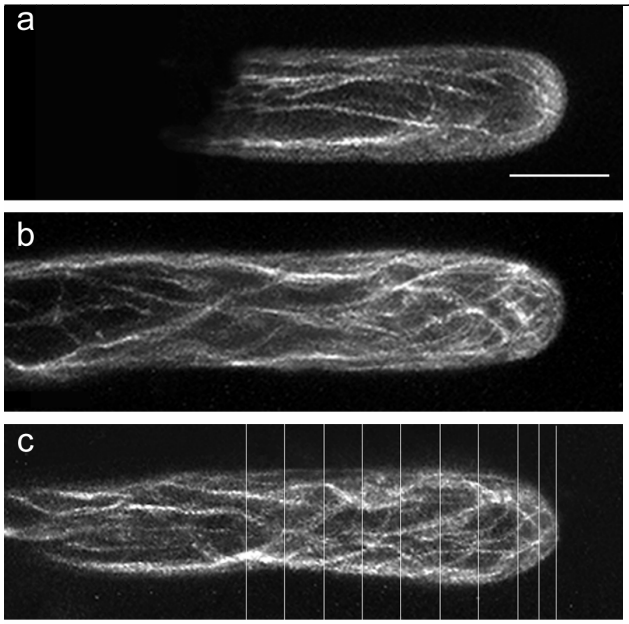

Fig. 9. Increase in the density of bundles of actin filaments in *Vicia sativa* root hairs that were terminating growth at the moment of LCO application.

a Typical distribution and density of actin filament bundles in a control hair after replacing growth medium (t = 6 min), with actin filament bundles up to the tip, and only few sub-apical fine bundles of actin filaments. **b,c** Typical distribution and density of actin filament bundles after application of LCOs (t = 3 min respectively t = 6 min). Note that dense sub-apical FB-actin has appeared. 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. **c** To compare data, all settings during imaging and processing were similar. Confocal z-series were made at 1 μm steps. To quantify the number of actin filament bundles, a grid with equally spaced lines was superimposed onto the projected image of a root hair. The grid was perpendicular to the long axis of the hair and the zero-line was always positioned at the very tip. The number of all fine and thick actin filament bundles hit by these cross-lines was counted on a high-resolution screen. All figures have the same magnification. Bar = 15 μm.

The density of subapical net-axial fine bundles of actin filaments has clearly increased. After quantification (for procedure see legend of Fig. 9c) of this increase in fine bundles of actin filaments, an interesting difference appears (De Ruijter et al 1999). After Nod factor treatment, the number of fine bundles of actin filaments in full-grown hairs did not exceed the threshold level of that seen in control growth terminating hairs (Fig. 8), which are the cells that still have some capacity for growth and which do respond with root hair deformation. Among other factors, like an already stiff secondary cell wall at tips of full-grown vetch root hairs (still to be proven), the limited increase of FB actin in full-grown hairs may be the reason why those hairs do not respond with root hair deformation. In figure 10 we have mapped the occurrence of the reaction of the actin cytoskeleton to Nod factor in relation to the changes in calcium in the root hair (Ehrhardt et al 1996; De Ruijter et al 1998; Felle et al 1998).

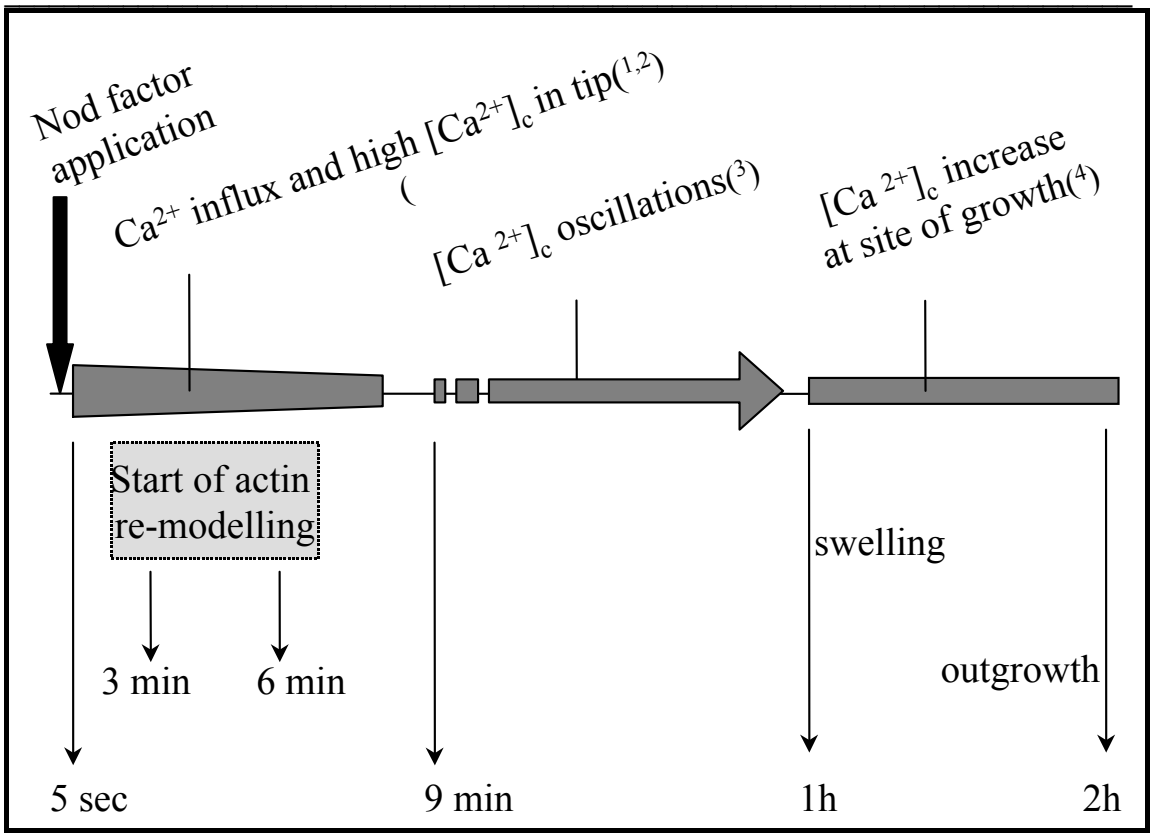

Fig. 10. Time scale correlating the increase in FB actin with the changes in cytoplasmic free calcium ion concentration in root hairs responding to LCOs. [1]Felle et al (1998); [2]Gehring et al (1997); [3]Ehrhardt et al (1996); [4]De Ruijter et al (1998).

The increase in the number of fine bundles of actin filaments in the subapical area of the root hair suggest a signal transduction pathway involving the activation of several ABPs. Since the in vitro binding of profilin and ADF / cofilin to PIP_2 provides a link between the phosphatidyl-inositol signal transduction pathway and the actin cytoskeleton (Yonezawa et al 1990; Aderem 1992; Staiger et al 1997), it can be hypothesized that this signal transduction pathway is involved in Nod factor signalling. Recent experiments support this hypothesis (Pingret et al 1998). Inhibitors of phospholipase C (PLC) applied simultaneously with Nod factor to *Medicago truncatula* roots inhibit the expression of a MtENOD12-GUS construct in the root epidermis, indicating that the activity of PLC increases after LCO treatment (Pingret et al 1998). In vitro, PLC cleaves PIP_2 into inositol (1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG) (Dicheva and Irvine 1995), suggesting a rapid increase in IP_3 after Nod factor application.
The increase of cytoplasmic pH after LCO application to *Medicago sativa* root hairs (Ehrhardt et al 1992; Felle et al 1995; Felle et al 1996) suggests another correlation between ADF regulated changes in the actin cytoskeleton and Nod factor treatment. In LCO treated *Medicago sativa* root hairs, the pH increases to levels (Ehrhardt et al 1992; Felle et al 1995; Felle et al 1996) at which plant-ADF is able to sever and depolymerize actin filaments (Staiger et al 1997;

Maciver et al 1998). Moreover, in growing maize root hairs, the localization of maize ADF3 coincides more or less (Jiang et al 1997a) with the region of *Vicia sativa* root hairs in which an increase in fine bundles of actin filaments is seen shortly after Nod factor treatment (De Ruijter et al 1999). Up till now, the distribution of ADF in legume root hairs is unknown, but it can be hypothesized that Nod factor induced cytoplasmic pH changes influence the severing/depolymerizing activity of ADF, which may result in a contribution to the increase of subapical fine bundles of actin filaments.
Profilin and ADF are, based on their activities, distribution and involvement in signal transduction pathways, good candidates for ABPs involved in the short term actin rearrangements after Nod factor treatment. In analogy to knowledge about ABP function in animal cells and yeast, we expect that more ABPs are involved in the rearrangement of the actin cytoskeleton by Nod factor.

8 Future prospects

To investigate the complex signal transduction pathways leading from a signal molecule like the Nod factor to changes in cell morphogenesis, a bioassay is a useful tool. We have used the occurrence of root hair deformation by Nod factor as a bioassay to study the influence of external factors on this process (Heidstra et al 1997b, Miller et al 1999). Full root hair deformation is swelling of the root hair tip followed by root hair outgrowth from that swelling. In vetch, this whole process takes 2-3 h (Fig. 6). The discovery of the rapid, and quantifiable increase of FB-actin in root hairs after Nod factor application (Fig. 8, 9), provides an additional bioassay, like the measurement of calcium dynamics does. Instead of waiting for 2 h to score root hair deformation, one can score the rapid effect Nod factor has on the actin cytoskeleton and on calcium dynamics (See Fig. 10). By using drugs that affect the cytoskeleton and signal transduction cascades in combination with Nod factor, one will be able to discriminate between the early factors in the signal transduction cascade that change the cytoskeleton and later factors that need for instance calcium spiking and induction of new gene transcription. Ultimately, this has to be done in living cells. Therefore, GFP-technology and microinjection techniques will be necessary, and should be applied in combination with molecular genetic and biochemical approaches.

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Chapter 2

Nod Factor induced root hair curling: Continuous polar growth towards the point of Nod Factor application

John J. Esseling, Franck G.P. Lhuissier, 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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ABSTRACT

A critical step in establishing a successful nitrogen fixing symbiosis between *Rhizobium* bacteria and legume plants, is the entrapment of the bacteria between root hair cell walls, usually in characteristic 180º-360º curls, shepherd's crooks, which are formed by the host's root hairs. Purified bacterial signal molecules, the nodulation factors (NF), which are lipochito-oligosaccharides, induce root hair deformation in the appropriate host legume (Heidstra et al., 1994; de Ruijter et al., 1998; Sieberer and Emons, 2000), and have been proposed to be a key player in eliciting root hair curling (Emons and Mulder, 2000). However, for curling to occur, the presence of intact bacteria is thought to be essential (Catoira et al., 2001). Here we show that, when spot-applied to one side of the growing *Medicago truncatula* root hair tip, purified NF alone is sufficient to induce reorientation of the root hair growth direction, or a full curl. Using wild type *Medicago truncatula* containing the p*MtENOD11::GUS* construct (Vernoud et al., 1999), we demonstrate that *MtENOD11::GUS* is expressed after spot application. The data have been incorporated into a cell biological model, which explains the formation of shepherd's crook curls around NF-secreting *Rhizobia* by continuous tip growth reorientation.

INTRODUCTION

Bacteria of the genera *Rhizobium, Bradyrhizobium, Azorhizobium, Mesorhizobium* and *Sinorhizobium*, collectively referred to as rhizobia, can establish a symbiosis with legume plants. The bacteria induce the development of a new plant organ, the root nodule, in which nitrogen fixation takes place. Located in this nodule, the bacteria are provided with photosynthate. In return, the symbiont converts atmospheric nitrogen into ammonia, a form that can be readily assimilated by the host plant. A requirement for successful infection in many legumes is the entrapment of bacteria between root hair cell walls. Usually, this is accomplished by the formation of a tight curl, a shepherd's crook, of the host plant root hairs to which bacteria have become attached (Hadri and Bisseling, 1998; Kijne, 1992). The subsequent formation of the host-produced infection thread (Nutman, 1956) is initiated within this curl where bacteria have been enclosed between root hair cell walls. In experimental conditions, infection threads can also originate from bacteria which are entrapped between the tips of two uncurled root hairs (Haack, 1964).

Nodulation factors (NF) are molecules synthesized and excreted by rhizobia in response to plant flavonoids (Fisher and Long, 1992). Application of purified NF to legume roots induces the formation of polarized cytoplasmic bridges (pre-infection threads) in the outer cortical cells (Van Brussel et al., 1992), and cell divisions in the inner cortex (Van Brussel et al., 1992; review: Kijne, 1992). Moreover, various early-nodulin genes (ENODs) are expressed in response to NF(Compaan et al., 2001; Journet et al., 2001; Pichon et al., 1992; Pingret et al., 1998; Scheres et al., 1990; Yang et al., 1993).

One of the best characterized biological activities of purified NF is to induce root hair deformation in the appropriate host. Root hair deformation assays are therefore widely used to evaluate the specificity of NF towards a given legume host (Lerouge et al., 1990; Ardourel et al., 1994; Catoira et al., 2000). In the classical assay, deformation affects growth terminating root hairs and starts with a swelling of the cell apex from which an outgrowth emerges (Heidstra et al., 1994). This outgrowth exhibits all the characteristics of a growing root hair, with a vesicle rich area at the extreme tip, followed by a subapical cytoplasmic dense region with the nucleus at its base (*Vicia sativa*: de Ruijter et al., 1998; Miller et al., 2000; *Medicago truncatula*: Sieberer and Emons, 2000). Initial swelling of the root hair tip starts within minutes after NF application, whereas outgrowth generally initiates at least one hour later. Based on such observations, it has been proposed that NF (Emons and Mulder, 2000) might be the inducing principle in bacteria-associated root hair curling (van Batenburg et al., 1986). However, until now, no direct evidence for this hypothesis has been provided. It is generally thought that for root hair curling to occur, the presence of bacteria is essential (Catoira et al., 2001). We now show that NF alone, when spot applied,

can induce root hair tip growth reorientation and root hair branching towards NF in *M. truncatula*.

RESULTS

Spot application of host specific Nod Factor onto the tip of growing wild-type air-grown root hairs induces root hair growth axis reorientation

In all previous reported assays to study the effects of purified NF on legume root hairs (Lerouge et al., 1990; Ardourel et al., 1994; Catoira et al., 2000; Heidstra et al., 1994; de Ruijter et al., 1998; Miller et al., 1999; 2000; Sieberer and Emons, 2000), a liquid medium containing NF is globally applied to the root hairs. In these assays, growth-terminating root hairs respond to the NF application with root hair deformation (Heidstra et al., 1994; Miller et al., 1999; 2000; de Ruijter et al., 1998; Sieberer and Emons, 2000) or root hair branching (Catoira et al., 2000), depending on the assay. However, in nodulation assays in which NF excreting *Rhizobium* bacteria are applied to legume roots (Ardourel et al., 1994), growing root hairs curl around the bacteria (Kijne, 1992). Therefore, we hypothesized that in the case of root hair curling, the local presence of NF, excreted by the bacterial colony, is causing the root hair curling (Emons and Mulder, 2000). To test this hypothesis, we developed the spot application assay, in which a droplet of purified NF is applied to one side of the apical dome of a growing root hair. Since it is technically impossible to locally apply a droplet of NF solution on a root hair growing in liquid or agar-based medium, experiments were carried out on air growing root hairs from seedlings that were grown along vertical agar plates. These root hairs are further referred to as air-grown root hairs.

At a concentration of 10^{-9} M, NF spot application resulted in a reorientation of the growth axis of air-grown root hairs towards the site of application (Figure 1). Already within minutes after application, a change in cell morphology is visible, as the extreme tip of the root hair shifts towards the site of application. Fifteen minutes after application, the reorientation is clearly visible and becomes more pronounced with time (Figure 1). Provided that the root hair under study maintains growth after the initial reorientation, spot-application can be repeated several times, thus giving rise to multiple growth axis reorientations of the same root hair (Figure 2a). When spot application is performed repeatedly on the same side of the air-grown root hair with short time intervals, partial curls can be obtained (Figure 2b). Complete curls can not easily be obtained experimentally,

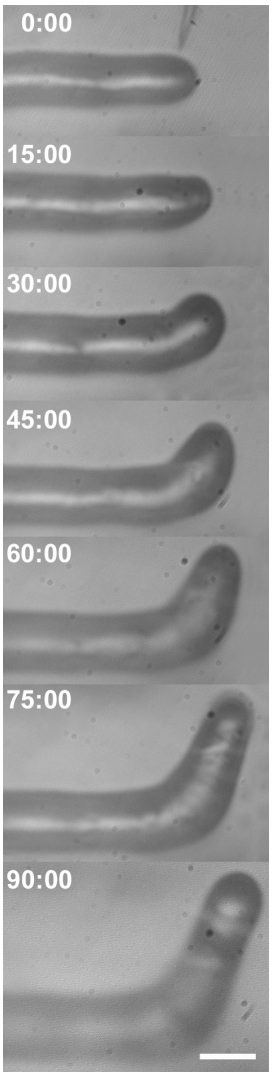

Figure 1. The response of a growing *M. truncatula* root hair to a single spot application of 10^{-9} M purified Nod Factor (NF).

Fifteen minutes after NF application, the reorientation of the root hair growth axis towards the site of application is already visible, and becomes more pronounced at 30 minutes. As can be seen, root hair growth is continuous during and after reorientation, and the root hair diameter does not change. Bar = 15 µm.

because the inner side of the curl becomes less and less accessible for the micropipette with increasing numbers of spot applications. However, single spot applications can sometimes lead to complete curls within 50 minutes after NF application (N=6; Figure 2c-f).

Root hair reorientation upon NF spot application is NF type specific

Nod factors are complex molecules, consisting of β-1,4-linked tetramers or pentamers of D-glucosamine, which are mono-N-acylated on the terminal nonreducing residue, and N-acetylated on the other residues. Moreover, the

chitin oligomer backbone can be decorated with O-acetyl- and O-sulphate groups, which determines the specifity of a bacterium for its host (Lerouge et al., 1990; Roche et al., 1991; Spaink et al., 1991; Truchet et al., 1991; Ardourel et al., 1994). It has been shown that for *Medicago* the sulphate

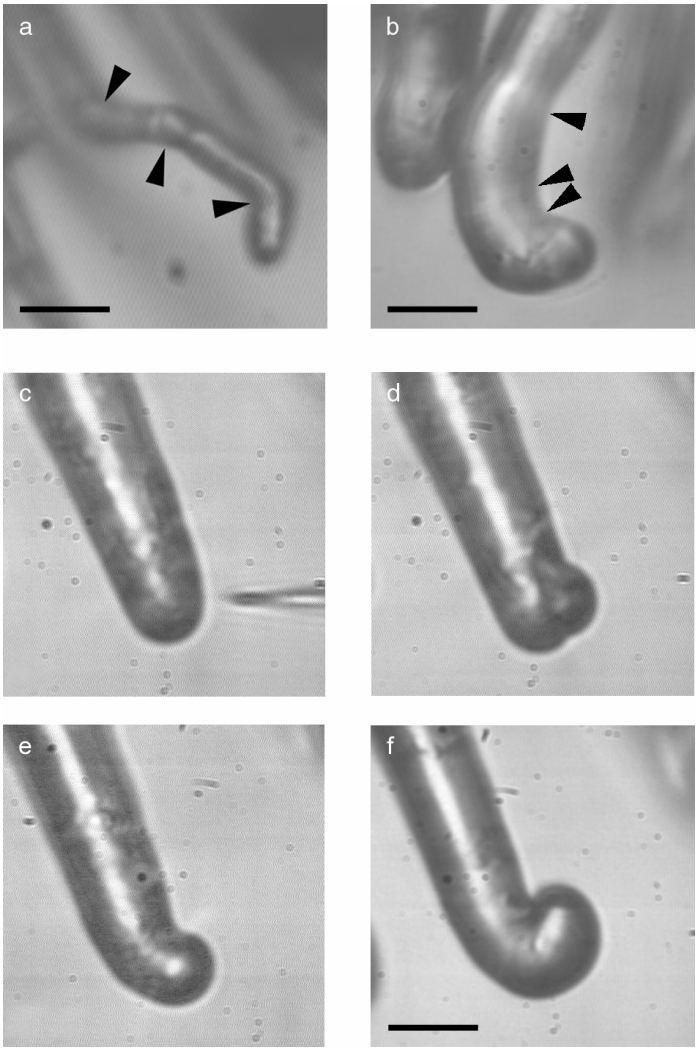

Figure 2. Iterative spot application of NF leads to iterative root hair growth axis reorientation, leading to root hair curling.

a. Growing root hair after 3 successive spot applications of NF on different sides, showing that multiple applications lead to multiple growth axis reorientations. Bar: 30 µm. **b.** Growing root hair after 3 successive spot applications of NF on one side of the root hair tip, showing multiple reorientations of the root hair growth axis, leading to a partial root hair curl. Arrowheads point to the position of successive NF spot applications. **c-f.** Timeseries of root hair curling after single spot application. With a micropipette (**c**) a micro-droplet of 10^{-9}M Nod factor is applied to one side of the apical dome of a growing root hair (**d**). After 25 minutes, a clear reorientation of the root hair growth axis towards the side of application is visible (**e**), and in 50 minutes, a shepherds crook is formed (**f**). Bar in **b-f**: 18µm.

decoration is essential to establish a successful infection (Ardourel et al., 1994), and that purified non-sulphated NF fails to induce root hair deformation in *M. truncatula* (Catoira et al., 2000). Therefore, we used spot application of the non-sulphated NF as a control to test the specificity of the root hair reorientation response (Table I).

Table I

Responses of growing and full grown root hairs to spot application of different NF molecules

	Growing root hairs			Full grown root hairs		
NF application on the tip	N treated	N reoriented	%	N treated	N reoriented	%
NF NodRm-IV (C16:2,Ac,S)	34	34	100	16	0[b]	0
NonSulphated NF	16	0[a]	0	nd		
Chitotetraose (GlcNac)4	15	0[a]	0	nd		
Sulphated Chitotetraose	18	0[a]	0	nd		
Water	7	0[a]	0	nd		
	N treated	N branched	%			
NF 30µm below the tip	8	8	100	4	0[b]	0
NF 60µm below the tip	3	3	100	3	0[b]	0

[a]Hairs were growing straight after application. [b]No morphological changes were observed. nd: Not done

In figure 3a we show that upon spot application of 10^{-9} M non-sulfated NF, root hairs did not respond with root hair reorientation. Also, when 10^{-6} M chitotetraose [$(GlcNac)_4$], the β-1,4-linked tetramer backbone of all NF (Figure 3b), 10^{-9} M sulfated chitotetraose (Figure 3c), or the NF solvent H_2O (Figure 3d) were spot applied, root hair reorientation was not observed in all applied hairs (Table I). This indicates that growth axis reorientation of air-grown root hairs upon NF spot application is a NF-specific response and not triggered by mechanical stimuli.

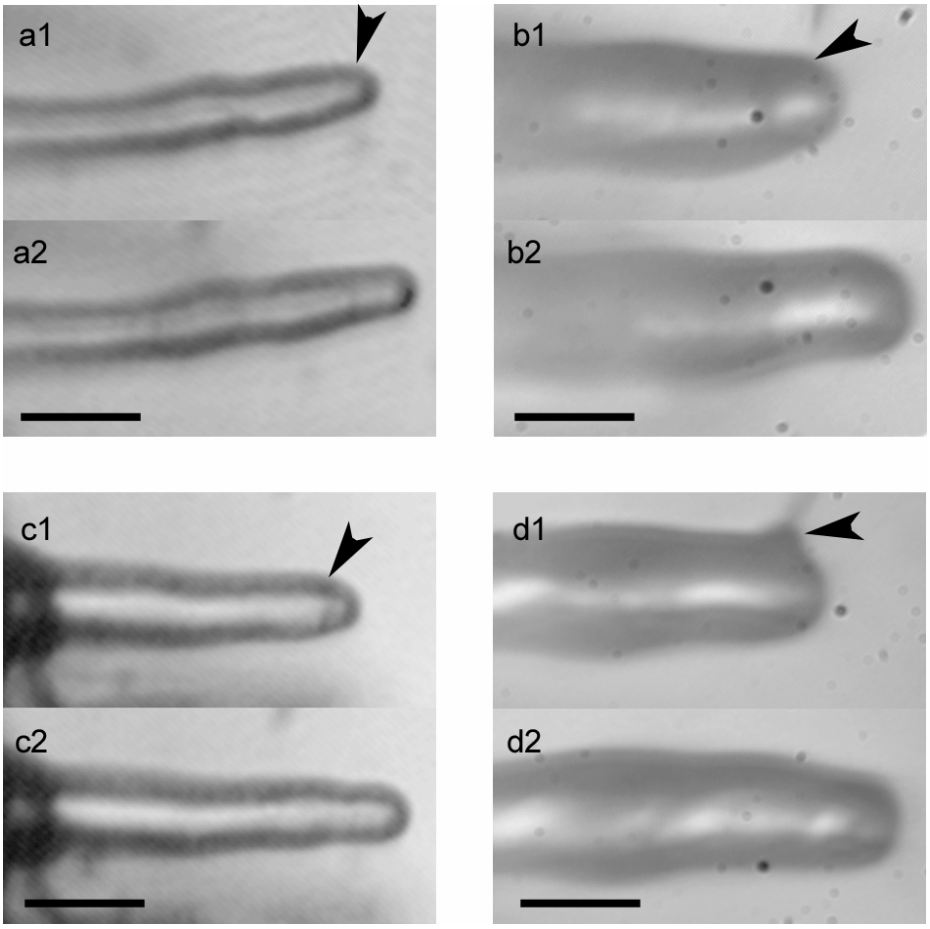

Figure 3. Spot application controls showing that root hair reorientation is a NF specific response.

a. Spot application of 10^{-9} M non-sulphated NF (from the *Sinorhizobium meliloti* NodH mutant) does not lead to growth reorientation. Bar: 30 µm. **a1.** At the moment of application. **a2.** 25 min. after application. **b.** Growing *M. truncatula* wild-type root hair after spot application with 10^{-6} M chitotetraose showing no growth axis reorientation. Bar: 18 µm. **b1.** At the moment of application. **b2.** 45 min. after application. **c.** Spot application of 10^{-9} M sulphated chitotetraose does not lead to growth reorientation. Bar: 30 µm. **c1.** Prior to application. **c2.** 30 min. after application. **d**. Growing *M. truncatula* wild type root hair after spot application of millipore water showing no growth axis reorientation. Bar: 18 µm. **d1.** At the moment of application. **d2.** 55 min. after application. Arrowheads point to the site of application

Expression of the NF-induced early nodulin gene *MtENOD11* is maintained after NF spot application

We now demonstrated that root hair reorientation after NF spot application is a NF specific response. In addition, we wanted to test if early nodulin gene (ENOD) expression is induced by NF spot application. Therefore, we performed single spot applications of 10^{-9} M NF on the side of the tip of growing root hairs of transgenic *M. truncatula* plants. These transgenics are transformed with the p*MtENOD11::GUS* reporter construct. *MtENOD11::GUS* is not expressed during

normal root hair development but is strongly expressed after global NF application (Vernoud et al., 1999; Journet et al., 2001). As can be seen in Figure 4a, NF spot application on these transgenic root hairs resulted in root hair reorientation and *MtENOD11::GUS* expression, indicating that this NF specific response is induced by NF spot application.

Neither spot application of the non-sulphated NF, nor the chitotetraose backbone or the sulphated chitotetraose resulted in *MtENOD11::GUS* expression (results not shown).

Root hair branching after Nod Factor spot application at 30 or 60 µM below the tip of a growing root hair

A bacteria-entrapping curl not necessarily has to develop at the root hair tip. In the presence of bacteria, lateral root hair branches can develop which subsequently curl around the bacteria (Dart, 1974). Moreover, depending on the assay, root hairs can branch after global NF application (Catoira et al., 2000). Since we now show that the local presence of NF at the side of the dome of the growing root hair tip is sufficient to induce reorientation of the root hair growth

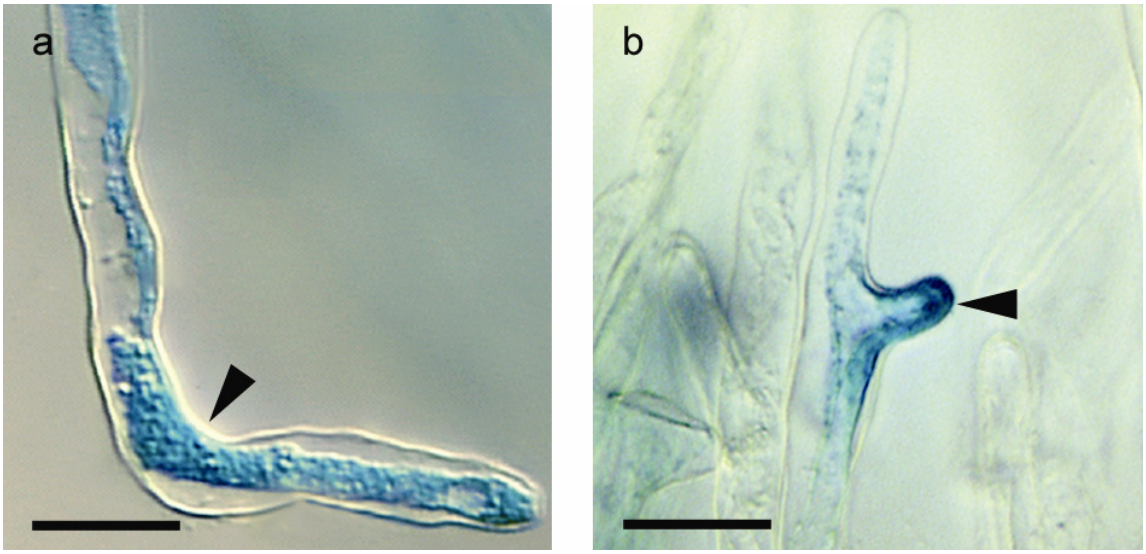

Figure 4. p*MtENOD11::GUS* expression is maintained in reorienting and branching wildtype *M. truncatula* root hairs after NF spot application.

a. Transgenic *M. truncatula* wild type root hair carrying the p*MtENOD11::GUS* fusion construct stained for *GUS* expression 120 min. after spot application of 10^{-9} M NF showing positive GUS staining of the cytoplasm. Bar: 30 µm. **b**. Transgenic *M. truncatula* wild type root hair carrying the p*MtENOD11::GUS* fusion construct stained for *GUS* expression 75 min. after spot application of 10^{-9} M NF 60 µm below the growing tip, showing root hair branching at the site of application and GUS staining of the cytoplasm. Bar: 30 µm.

axis, we hypothesized that local presence of NF on the shank of a growing root hair would result in a new growth axis at the site of application, i.e. root hair branching. When NF was applied 30 (n=8) to 60 µm (n=3) distally from the growing tip, the existing tip stopped growing, and within 20 minutes, a branch started to grow at the site of NF spot application. Moreover, branching hairs express *MtENOD11::GUS* after NF spot application (Figure 4b), indicating that this branching response is a physiological response.

DISCUSSION

Root hair curling is continuous tip growth reorientation

Root hair curling is a critical step in the establishment of a successful invasion of the root hair by *Rhizobium* bacteria, but the mechanisms underlying this curling process remain largely unknown. In this work, we show that spot application of purified NF is sufficient to induce (partial) root hair curling, and therefore that the presence of bacteria is not required, which was thought before (Catoira et al., 2001). These results strongly support the model of van Batenburg et al. (1986), that root hair curling is continuous reorientation of tip growth. In this model it was proposed that root hair curling can only occur when at least the following conditions are fulfilled (van Batenburg et al., 1986): a) the attachment of one inducing principle (e.g. the NF droplet), b) within the growth area of the root hair; c) translocation of the inductor along the growing root hair tip (e.g. iterative spot application); and d) redirection of the original plant-driven tip growth. An alternative hypothesis for root hair curling could be that reorientation of the root hair growth axis is achieved by differential stimulation of wall expansion on the opposing site of NF presence. However, this is inconsistent with 1) the observation that spot application of NF on the shank of a growing root hair results in a growing branch at the site of application and 2) with the computer simulations by van Batenburg et al. (1986).

The Cell Biology of Root Hair Curling – A hypothesis

Based on what we know about root hair tip growth and how root hairs react to global NF application at the cell biological level, we would like to propose a hypothesis to explain the formation of a tight curl, the shepherd's crook, around a colony of bacteria. In a growing root hair (Figure 5a), the dense sub-apical fine bundles of actin filaments deliver Golgi-derived vesicles to the root hair tip (Miller et al., 1999; 2000).

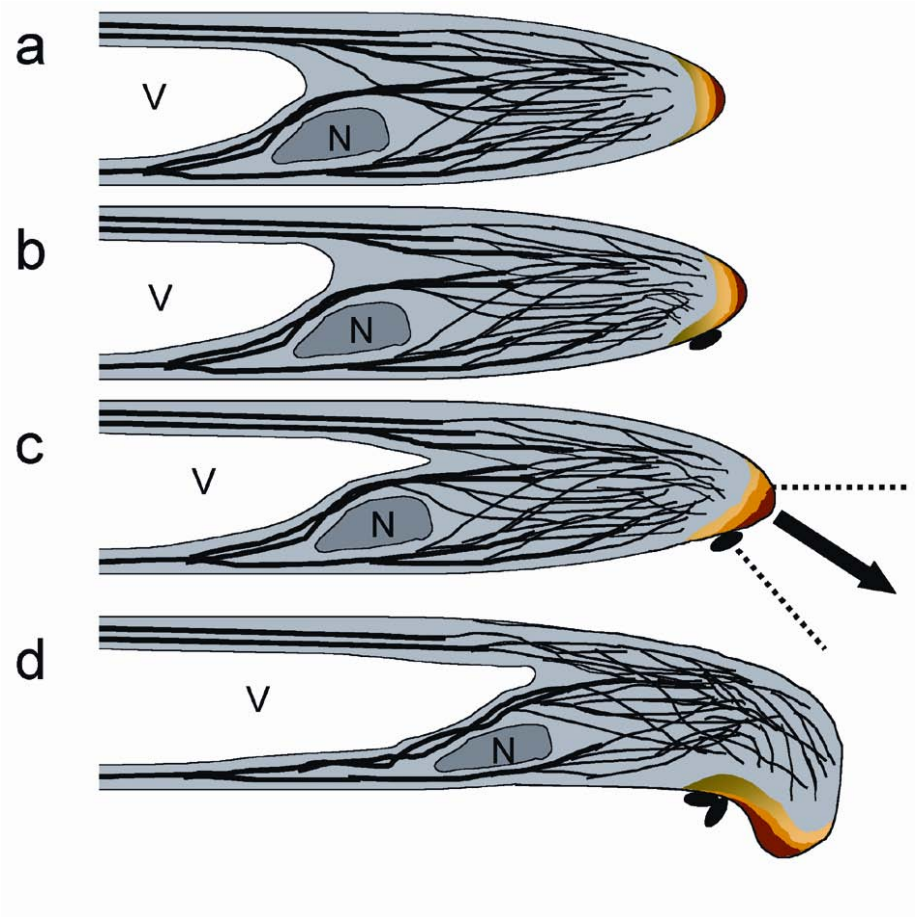

Figure 5. Cartoon of the putative mechanism involved in root hair growth axis reorientation during curl formation around *Rhizobium* bacteria.

a. Growing root hair with a tip-focused calcium gradient in an area devoid of detectable actin filaments, a subapical fine F-actin area (black lines), the nucleus (N) positioned at the base of the subapical fine F-actin area and the vacuole (V). **b.** A bacterium attaches to the root hair tip and locally excretes NF which induces a local calcium influx, leading to a gradual increase in $[Ca^{2+}]_c$. **c.** High $[Ca^{2+}]_c$, and thus the growth area, shifts towards the attached bacteria thus re-directing the growth. Note that the new growth direction is the resultante of the NF induced direction and the original growth axis. **d.** The enlarging bacterial colony also produces NF thus shifting again the growth area towards itself. In the end, these continuously repeated growth axis reorientations give rise to a tight curl, entrapping the bacteria.

Aided by a tip localized cytosolic calcium gradient (Wymer et al., 1997; de Ruijter et al., 1998; Cárdenas et al., 1999), the membrane of these vesicles inserts into the plasma membrane, thus delivering the vesicle content into the existing cell wall via exocytosis (Battey and Blackbourn, 1993; Battey et al., 1996). Since the newly inserted cell wall is flexible (Cosgrove, 1993; Roberts, 1994), and the cell is under turgor pressure (Passioura and Fry, 1992), the tip expands. When a host-specific bacterium attaches to the root hair dome, the bacterium locally excretes NF, which is then immobilized within the cell wall (Goedhart et al., 2000). Since NF increases the cytosolic calcium ion

concentration, $[Ca^{2+}]_c$ (Felle et al., 1998), at the plasma membrane (Cárdenas et al., 1999), the local presence of bound NF in the cell wall induces a local $[Ca^{2+}]_c$ increase. Therefore, the region of high $[Ca^{2+}]_c$ at the plasma membrane, i.e. the region where exocytosis occurs, will gradually shift from the tip towards the cell wall area with bound NF (Figure 5b). Furthermore, the density of subapical fine bundles of actin filaments, which deliver the Golgi-derived vesicles to the tip area, increases upon global NF application (de Ruijter et al., 1999). Since NF is immobilized locally within the cell wall (Goedhart et al., 2000), NF spot application will only locally increase the subapical fine bundles of actin filaments. This results in a shift in the region where vesicles are being delivered and inserted into the plasma membrane from the existing vesicle rich area to the side of the hair that has become the new center of activity under the influence of NF. Thus, a new growth axis which is the resultante of the original root hair growth axis and the NF induced growth direction, is initiated towards the attachment side, as previously proposed Emons and Mulder (2000) (Figure 5c). The attached bacterial colony creates a new center of growth activity, thus redirecting tip growth in the direction of the colony. The multiplication of the present bacteria enlarges the area of surface contact between the two organisms. Thus, the new cell tip contacts new bacteria, which also excrete NF, and the process described above is repeated (Figure 5d). The continuous reorientation of tip growth results in a continuous rotation of the tip in a single direction and can give rise to the tight curl (van Batenburg et al., 1986, Emons and Mulder 2000) within which the bacteria are entrapped, the shepherd's crook.

MtENOD11::GUS expression after NF spot application will be a valuable tool for deciphering NF-induced signalling pathways. Spot application of NF in combination with pharmacological agents on these transgenics and early symbiosis mutants of *M. truncatula*, will give new insights in the role of certain proteins in NF induced signaling.

MATERIAL AND METHODS

Medicago truncatula seed preparation and seedling growth

Seeds of *M. truncatula* jemalong A17 and *M. truncatula* jemalong carrying a p*MtENOD11::GUS* fusion construct (Journet et al., 2001) were scarified in concentrated sulphuric acid for 10 minutes and thoroughly washed with running demineralized water. Seeds were then sterilized in a mixture of 30% hydrogen peroxide and 96% ethanol (1:1) for 2 minutes and extensively rinsed with sterile demineralized water. Seeds were subsequently imbibed overnight in sterile demi water at 4ºC. To synchronize germination, the imbibed seeds were allowed to vernalize in the refrigerator for 4 days at 4ºC on plates containing 0.8% agar in sterile demi water, wrapped in aluminium foil. For germination, the agar plates containing the vernalized seeds were transferred to the plant growth room at 25°C. After germination, 24hrs later, about 1 to 1.5 cm long seedlings were transferred to fresh agar plates and allowed to grow for 8 days at 25ºC, with a 16/8 hours light/dark rhythm in a slightly oblique position from the vertical. In such growth conditions, the root hairs that develop in air above the root are suitable for spot application.

Spot Application Assay

A water pressure microinjection device (water pressure device: Gilmont, Barrington, IL, USA; needle holder: Eppendorf, Merck Eurolab BV, Amsterdam, The Netherlands) was used to apply micro-droplets (0.2 nl) of purified NF [NodRm-IV(C16:2, Ac, S)] diluted with Millipore water to a final NF concentration of 10^{-9} M, 10^{-9} M non-sulphated NF, 10^{-6} M chitotetraose [$(GlcNac)_4$], 10^{-9} M sulphated chitotetraose backbone, or Millipore water to one side of growing root hair tips. Subsequent growth axis reorientations were recorded every 15 minutes with a video camera linked to an inverted Nikon Diaphot microscope.

p*MtENOD11::GUS* Expression

p*MtENOD11::GUS* expression was assessed by incubating the seedlings for 24 hrs in the β-glucuronidase substrate X-Gluc (2 mM 5-bromo-4-chloro-3-indolylglucuronide, 1% [w/v] dimethylformamide, 0.1 mM $K_3[Fe(CN)_6]$, 0.1 mM, $K_4[Fe(CN_6)] \cdot 3H_2O$, 1 mM EDTA, and 50 mM KH_2PO_4, pH 7.0) at 37ºC (Journet et al., 1994). Images were recorded with a Panasonic CCD camera linked to Nikon Optiphot upright DIC microscope.

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Chapter 3

A non-symbiotic root hair tip growth phenotype in *NORK*-mutated legumes: Implications for Nod Factor-induced signaling, and formation of a multifaceted root hair pocket for bacteria

John J. Esseling, Franck G.P. Lhuissier 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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ABSTRACT

The *Medicago truncatula DMI2* mutant is mutated in the nodulation receptor-like kinase, NORK. Here, we report that *NORK*-mutated legumes of three species show an enhanced touch-response to experimental handling, which results in a non-symbiotic root hair phenotype. When care is taken to not induce this response, *DMI2* root hairs respond morphologically like wild-type to Nod Factor (NF). Global NF application results in root hair deformation, and NF spot application induces root hair reorientation or - branching, depending on the position of application. In the presence of *Sinorhizobium meliloti, DMI2* root hairs make 2-D 180° curls, but do not entrap bacteria in a 3-D pocket, since curling stops when the root hair tip touches its own shank. Since *DMI2* does not express *Pro$_{MtENOD11}$:GUS* upon NF application, we propose a split in NF-induced signaling, with one branch to root hair curling, the other to *ENOD11* expression.

INTRODUCTION

The symbiosis between legume plants and soil living rhizobia provides by far the largest amount of organic nitrogen in the global nitrogen cycle. Therefore, in agriculture, systems of crop rotation are widely used, in which legumes are used as a valuable green fertilizer. The processes leading to symbiotic nitrogen fixation have attracted the interest of researchers, not only for its environmental and economical relevance, but also for a better understanding of plant development and signal transduction (reviewed in: Lhuissier et al., 2000; D'Haeze and Holsters, 2002; Geurts and Bisseling, 2002). One of the research 'milestones' in rhizobia-legume symbiosis was the discovery and characterisation of the Nodulation Factors (NF) as the key signal molecules excreted by rhizobia that lead to a successful symbiosis (Lerouge et al., 1990; Roche et al., 1991; Spaink et al., 1991; Truchet et al., 1991; Fisher and Long, 1992). Global application of purified NF to legume roots leads to various responses, such as changes in ion fluxes (Ehrhardt et al., 1992; Felle et al., 1995, 1996, 1998, 1999a, 1999b; Cárdenas et al., 1999; Shaw and Long, 2003; review: Cárdenas et al., 2000), changes in the root hair actin cytoskeleton (Cárdenas et al., 1998; Miller et al. 1999; de Ruijter et al., 1999; review: Esseling et al., 2000), calcium (Ca^{2+})- spiking (Ehrhardt et al., 1996; Wais et al., 2000; Walker et al., 2000; Shaw and Long, 2003), root hair deformation (Heidstra et al., 1994; review: Miller et al. 1997), increase of Ca^{2+} prior to root hair deformation (de Ruijter et al., 1998), expression of various early nodulin genes (ENODs; Scheres et al., 1990; Pichon et al., 1992; Yang et al., 1993; Pingret et al., 1998; Compaan et al., 2001; Journet et al., 2001) and cell divisions in the root cortex (Review: Kijne, 1992; Timmers et al., 1999).

Mutagenesis screens in *Drosophila* (Nusslein-Volhard and Wieschaus, 1980), zebrafish (Driever et al., 1996; Haffter et al., 1996) and *Arabidopsis* (Bechtold et al., 1993; reviews: Meinke et al., 1998; Somerville and Somerville, 1999) have proven to be a successful tool to dissect the genetic basis of signal perception and transduction in those organisms. Therefore, extensive screens in the model legumes *Lotus japonicus* and *Medicago truncatula* have been performed (Penmetsa and Cook, 1997; 2000; Sagan et al., 1995; Szczygłowski et al., 1998). From these screens, mutants that are blocked in the early steps of symbiosis have been isolated (*M. truncatula*: Sagan et al., 1995; Penmetsa and Cook, 1997; *L. japonicus*: Szczygłowski et al., 1998). The most recent addition to the NF signal transduction pathway is the cloning and description of LysM domain containing proteins, which could be NF receptor proteins involved in signaling and initiation of infection (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003), as has been proposed before (Ardourel et al., 1994). NFR1 and NFR5 from *L. japonicus* (Madsen et al., 2003; Radutoiu et al., 2003) most probably constitute a (or the) signaling receptor, since *nfr1* and *nfr5* mutants lack all early NF induced responses (Madsen et al., 2003; Radutoiu et al., 2003), such as the earlier described *nfp* mutant of *M. truncatula* (Ben Amor et al., 2003). The other protein, LYK3 from the *M. truncatula* SYM2 region

(Limpens et al., 2003), most likely codes for the NF-structure specific entry receptor.

Earlier, the two non-nodulating mutant genes *NORK* and *SymRK* have been positionally cloned and sequenced in *M. sativa* and *L. japonicus*, respectively (Endre et al., 2002; Stracke et al., 2002). The two genes turned out to encode homologous receptor-like kinases, and to have homologues in various other legumes (Endre et al., 2002; Stracke et al., 2002). Complementation studies show that targeted expression of the *M. truncatula NORK* homolog in the roots of the *DMI2* TR25/*dmi2-1* mutant (Sagan et al., 1995) rescues the non-nodulation phenotype (Endre et al., 2002), indicating that *DMI2* codes for a receptor-like kinase. The TR25/*dmi2-1 M. truncatula* mutant is one of the eight nodulation defective mutants from the three complementation groups *DMI1*, *DMI2* and *DMI3* (doesn't make infections 1, 2 and 3), which have been described by Catoira et al. (2000) and Wais et al. (2000). In general, upon global application of purified NF, all the mutants show altered root hair deformation (Catoira et al., 2000), indicating a disruption of root hair tip growth, and *DMI1* and *DMI2* are defective for, or show aberrant Ca^{2+}-spiking (Wais et al., 2000).

We monitored the effects NF application elicits in *M. truncatula* wild-type and TR25/*dmi2-1* root hairs. The results show that medium refreshment induces a 'touch response', which is enhanced in the mutant, thus unveiling a non-symbiotic root hair phenotype. This enhanced touch response is correlated with the *NORK* mutation, also in *M. sativa* and *L. japonicus* (MNNN-1008, and SYMRK respectively). When care is taken to not induce this touch response, *M. truncatula* TR25/*dmi2-1* root hairs respond in a wild type fashion to NF, and curl around rhizobia, but do not make a closed root hair pocket. Together with the absence of Ca^{2+} spiking (Wais et al., 2000) and NF-induced *MtENOD11* expression (Vernoud et al., 1999; Catoira et al., 2000; this work), our results point to an early split in the NF-induced signaling cascade upstream of *DMI2/NORK*.

RESULTS

Growing *M. truncatula dmi2-1* root hairs are more sensitive to medium refreshment than wild type root hairs

Immediately upon draining and replacing the growth medium of *M. truncatula* roots in the Fåhraeus slide (Fåhraeus, 1957) we observed the appearance of vacuoles in the cytoplasmic dense region (Fig 1A)

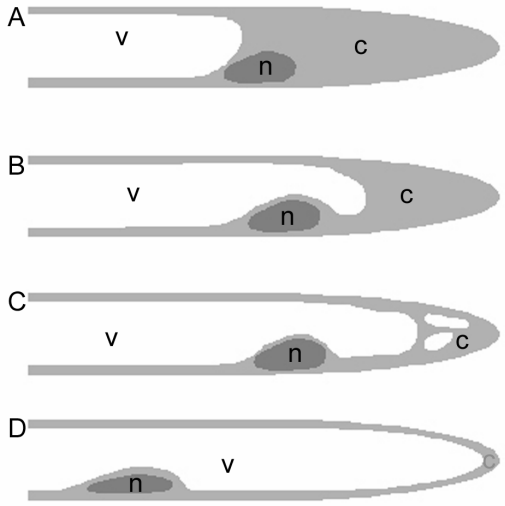

Figure 1. Cartoon of root hair cytoarchitecture

Simple representations of the cytoarchitecture of developmental stages of root hairs **(A)** Growing root hair. The subapex of the root hair is filled with cytoplasm (c) and the nucleus (n) is at the base of this area. The shank of the root hair is filled with the central vacuole (v), and cortical cytoplasm. **(B)** Early growth terminating root hair. The first sign of growth termination is that the central vacuole overtakes the nucleus, and, therefore, that the subapical region with dense cytoplasm is getting shorter. **(C)** Late growth terminating root hair. The central vacuole expands more and more into the subapex, and smaller vacuoles, or extensions of the central vacuole appear into the remaining cytoplasm. **(D)** Full grown root hair. The nucleus has lost its fixed position in the root hair. The vacuole completely fills the root hair, and is surrounded by a thin layer of cytoplasm.

at the tip of growing root hairs, which resembles the cytoarchitecture of late growth-terminating root hairs (Fig 1B,C). Medium refreshment was done by draining away the medium by placing the Fåhraeus slide (Fåhraeus, 1957) vertically on top of filter paper and pipetting new medium, a method which works well for global NF application to *Vicia sativa* root hairs (Heidstra et al., 1994; de Ruijter et al., 1998). We scored cytoarchitecture of root hairs of wild type and *dmi2-1* seedlings growing in the same slide after using this draining and replacement method (Figure 2). Before medium change, 98% of the wild-type and *dmi2-1* root hairs in the root cone with growing hairs had the appropriate cytoarchitecture of a growing root hair (Figure 1A). Immediately after medium change, 25% of the wild-type root hairs had kept this cytoarchitecture. In *dmi2-1*, the effect of medium change was more drastic: only 2.7% of the root hairs had kept the original cytoarchitecture of a growing root hair. The remaining 75% (wild-type) and 97.3% (*dmi2-1*) had obtained a (late) growth-termination type of cytoarchitecture (Fig 1C). Approximately 50 to 60% of the wild-type root hairs recovered the growing root hair cytoarchitecture, while in *dmi2-1* the percentage of root hairs with the proper cytoarchitecture remained at a level of 2 to 5 %. This shows that the mutant does not show any recovery of the cytoarchitecture (Figure 2).

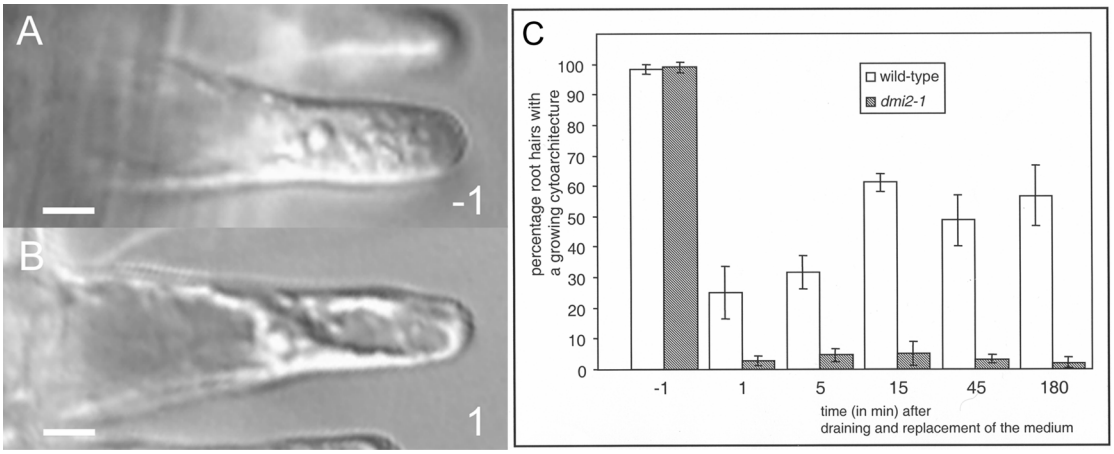

Figure 2. Medium change-induced touch response in *M. truncatula*.
(A) Young growing *M. truncatula dmi2-1* root hair just before medium change. **(B)** *M. truncatula dmi2-1* root hair in the same region on the root as the one in **(A)**, just after medium change. In just one minute, the cytoplasm has reorganized such that it evenly surrounds the central vacuole creating a cytoarchitecture of a full grown root hair. **(C)** Percentage of *M. truncatula* root hairs with a cytoarchitecture of a growing root hair before - and after – medium change. Before medium change, this percentage in wild-type (closed diamonds) and *dmi2-1* (open circles) is about 100%. After medium change, both in wild-type and in *dmi2-1* this percentage decreases. In time, wild-type shows recovery, whereas in *dmi2-1*, the typical cytoarchitecture does not recover. Monitored were 6 roots per wild-type and *dmi2-1* respectively, >50 root hairs per root. Bar in **(A)** and **(B)** = 10 µm.

To prevent the disruption of the cytoarchitecture by changing the medium, we developed a much gentler way of refreshing the medium. Prior to medium refreshment, the Fåhraeus slides with seedlings were carefully placed on the microscope stage, and were left to recover for at least half an hour from their transfer from the culture room to the microscope stage. For medium replacement, the slide was left on the microscope stage, and the medium in the slides replaced by adding fresh medium on one side of the horizontally placed slide, and slowly pipetting away on the other side. This was done in such a way that the approximately 800 µl medium in the slide was completely refreshed in approximately 1 minute. After this gentle medium perfusion, root hair growth continued in wild type and *dmi2-1*, no disruption of root hair cytoarchitecture occured, and no subsequent morphological response was observed (Figure 3).

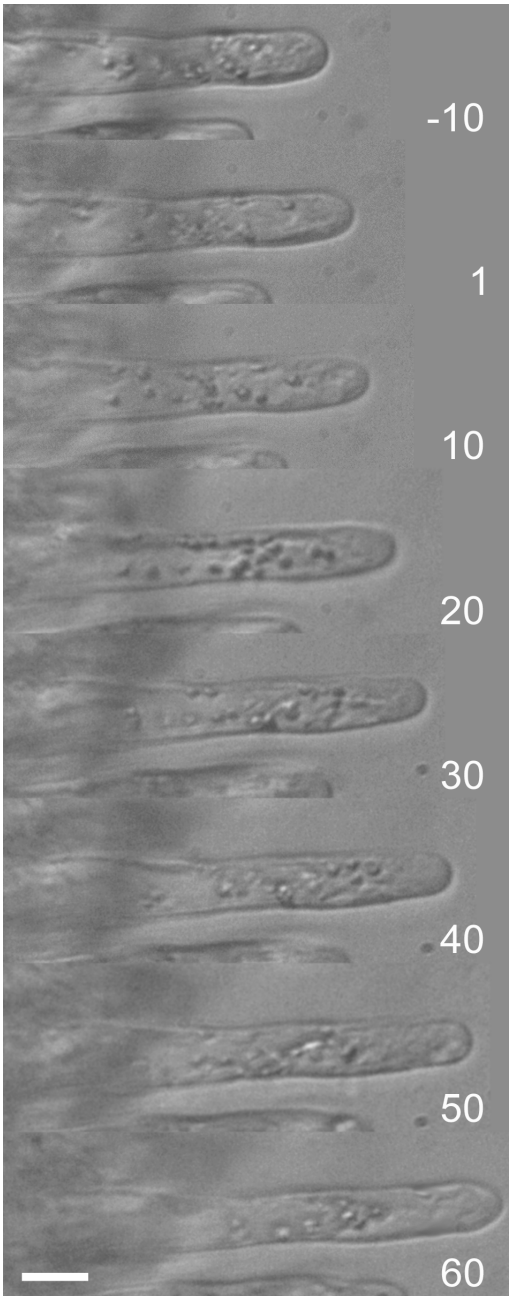

Figure 3. Time series of a growing *M.truncatula dmi2-1* root hair before and after medium refreshment via gentle perfusion.

The gentle way of refreshing the growth medium does not disturb root hair cytoarchitecture and growth, and therefore, root hair morphology. Images were taken every 10 minutes, except of the one 1 min after medium refreshment. Bar = 15 µm.

From the above described experiment, it seems as if *dmi2-1* root hairs have a non-symbiotic phenotype, which is caused by an increased susceptibility to mechanical manipulation. Both wild-type and *dmi2-1* root hairs reacted identically to application of an osmotic shock or cold treatment (data not shown). However, in an experiment in which we applied touch to single growing root hairs, they reacted differently. A microinjection needle was positioned below a root hair growing in agarose, and the needle was moved up and down again, such that the root hair was bent and returned to its original position.

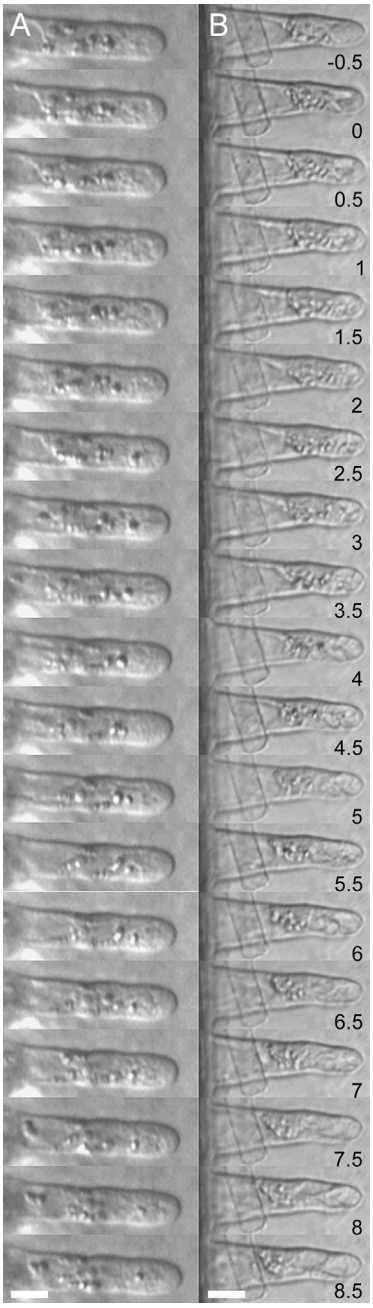

Figure 4. Time series of a *M.truncatula* wild-type and *dmi2-1* root hair before and after touch with a needle.

(A) Wild-type root hair. Time 0 is the root hair just after needle touch. Needle touch does not affect the cytoarchitecture and/or root hair growth. **(B)** *dmi2-1* root hair. Immediately after touch with the needle, vacuoles appear which increase in time, and the root hair stops growing. Images were taken every 30 seconds. Bars are 15 µm.

After this touch, the root hair cytoarchitecture was followed in time. In wild-type (Figure 4a), this touch did not result in any change in cytoarchitecture (n = 10 out of11 root hairs from 3 plants), whereas in *dmi2-1*, the cytoarchitecture changed from a growing into that of a late growth terminating root hair (n = 15 out of 16 root hairs from 4 plants; Figure 4b). We conclude that *dmi2-1* root hairs are more sensitive to touch than root hairs of wild-type plants.

Root hair cytoarchitecture as determinant for root hair deformation by Nod Factor

We replaced the culture medium with medium containing 10^{-9} M NF using the method that caused maximal disturbance. The immediate response to this draining and replacement, i.e. disruption of the growing root hair cytoarchitecture, followed by a recovery in wild-type and not in *dmi2-1*, was similar as described above. Forty-five minutes after NF addition, 27% of the *dmi2-1* root hairs that had a growing root hair cytoarchitecture just before draining and replacement, showed root hair swelling (Has), whereas in wild-type this was only 6% (Figure 5). Three hours after NF treatment, 61% of *dmi2-1* root hairs, which had a growing root hair cytoarchitecture at the time of application, showed complete root hair deformation with swelling and outgrowth, whereas in wild-type, only 12% showed root hair deformations in this area of the root (Figure 5). From these experiments, we conclude that mechanical manipulation via draining and replacement of the medium induces growth termination in the root hairs that were short and growing at the time of NF application. This made them respond to the NF with complete root hair deformation (RHD), i.e. swelling and outgrowth, like root hairs of that cyto-architecture normally do.

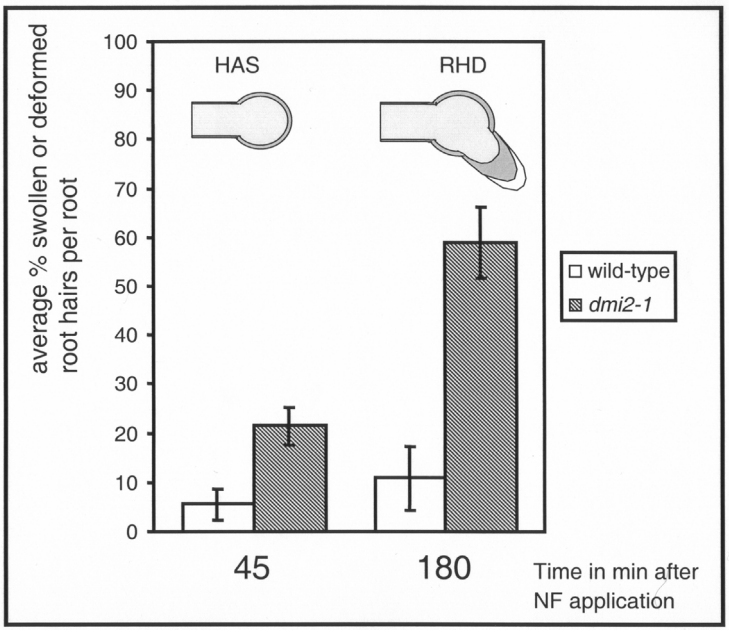

Figure 5. Percentage of wild type and *dmi2-1* root hairs showing root hair swelling (HAS) and root hair deformation (RHD) in hairs that were growing at the time of rough NF application.

45 minutes after application, there is already a clear difference between wild-type and *dmi2-1*. A significant higher percentage *dmi2-1* root hairs shows swelling of the root hair tip. 180 minutes after application, 59% of the *dmi2-1* root hairs which were growing at the time of NF application (but became growth terminating because of the medium change), show NF-induced root hair deformation. In wildtype, only 11% of the root hairs which were growing at the time of NF application show NF-induced root hair deformation. Monitored were 6 roots for wild-type and *dmi2-1* respectively, >50 root hairs per root.

Alfalfa wild type and MNNN-1008 and *L. japonicus* wild type and SYMRK root hairs respond in a similar fashion to medium change as *M. truncatula* wild type and *dmi2-1* root hairs

Since we observed the increased sensitivity to medium change in *M. truncatula dmi2-1* root hairs, we performed similar medium draining and replacement experiments on the alfalfa MNNN-1008 (Dudley and Long, 1989) and the *L. japonicus* cac41.5 SYMRK (Stracke et al., 2002) mutants, orthologues of *M. truncatula DMI2* (Endre et al., 2002; Stracke et al., 2002). For each species, two wild type and two mutant seedlings were grown together in the same Fåhraeus slide, and growing root hair cytoarchitecture (Figure 1A) was scored before and after medium change (Monitored were 6 roots for *M. sativa* wild-type and MNNN1008, and 4 roots for *L. japonicus* wild-type and cac41.5 SYMRK respectively, >50 root hairs per root). As can be seen in figure 6A, the effect of draining and replacement of the medium in the alfalfa wild type and MNNN-1008 mutant was comparable to *M. truncatula* wild type and *dmi2-1*. Immediately after medium change, the percentage of root hairs with the proper cytoarchitecture dropped from 59 to 2 procent (Figure 6A), and did not recover. In the wild-type a decline in the percentage of growing root hairs can be seen at the moment of medium change, followed by an immediate recovery. For *L. japonicus* roots, a difference between the wild type and the mutant root hairs was already visible before medium refreshment. In the mutant, the root hairs were swollen and branched, while wild type root hairs looked normal (Figure 7). To score the effect of medium change, only those root hairs with a growing cyto-architecture were followed in time. The effect of medium change for these *L. japonicus* root hairs was similar to the comparable root hairs in *M. truncatula* and *M. sativa*: wild type root hairs showed recovery, while in the mutant the percentage of root hairs with the growing cytoarchitecture decreased in time (Figure 6B).

We conclude that the NORK mutation in the described receptor kinase (Endre et al., 2002) produces a consistent non-symbiotic phenotype of enhanced susceptibility to medium change in the 3 legumes mutated in this protein.

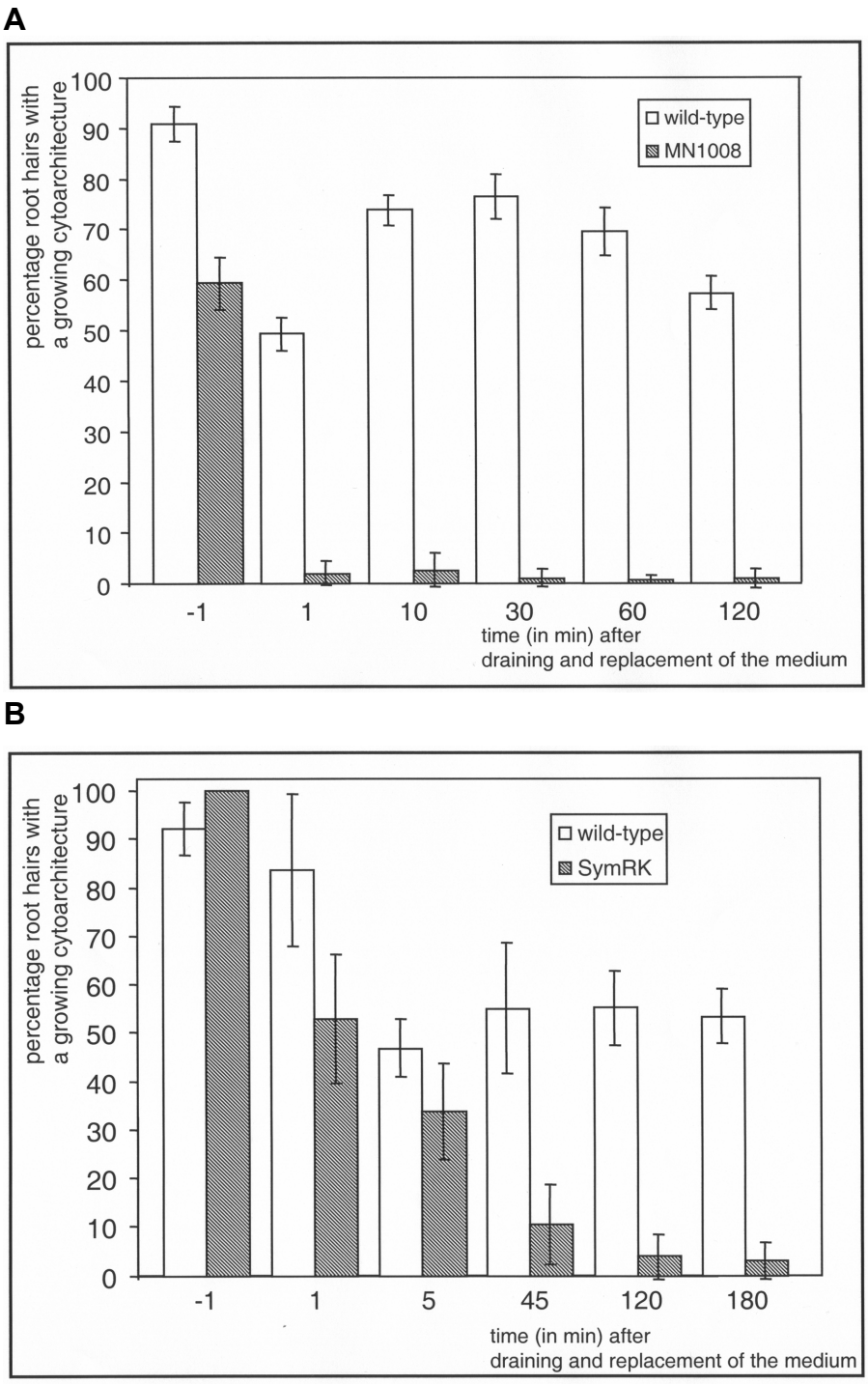

Figure 6. Medium change induced touch response in *M. sativa* - and *L. japonicus NORK* mutants. Medium change experiment as described in Figure 2, but with other species and their respective *NORK* mutant. Both for *M. sativa* **(A)** and *L. japonicus* **(B)** the same trend is visible: Medium change induces a decrease in the percentage of root hairs with a cytoarchitecture of a growing root hair from which the wild-types are able to recover, and the *NORK* mutants not. Monitored were 6 roots for *M. sativa* wild-type and MNNN1008, and 4 roots for *L. japonicus* wild-type and Cac41.5 respectively, >50 root hairs per root.

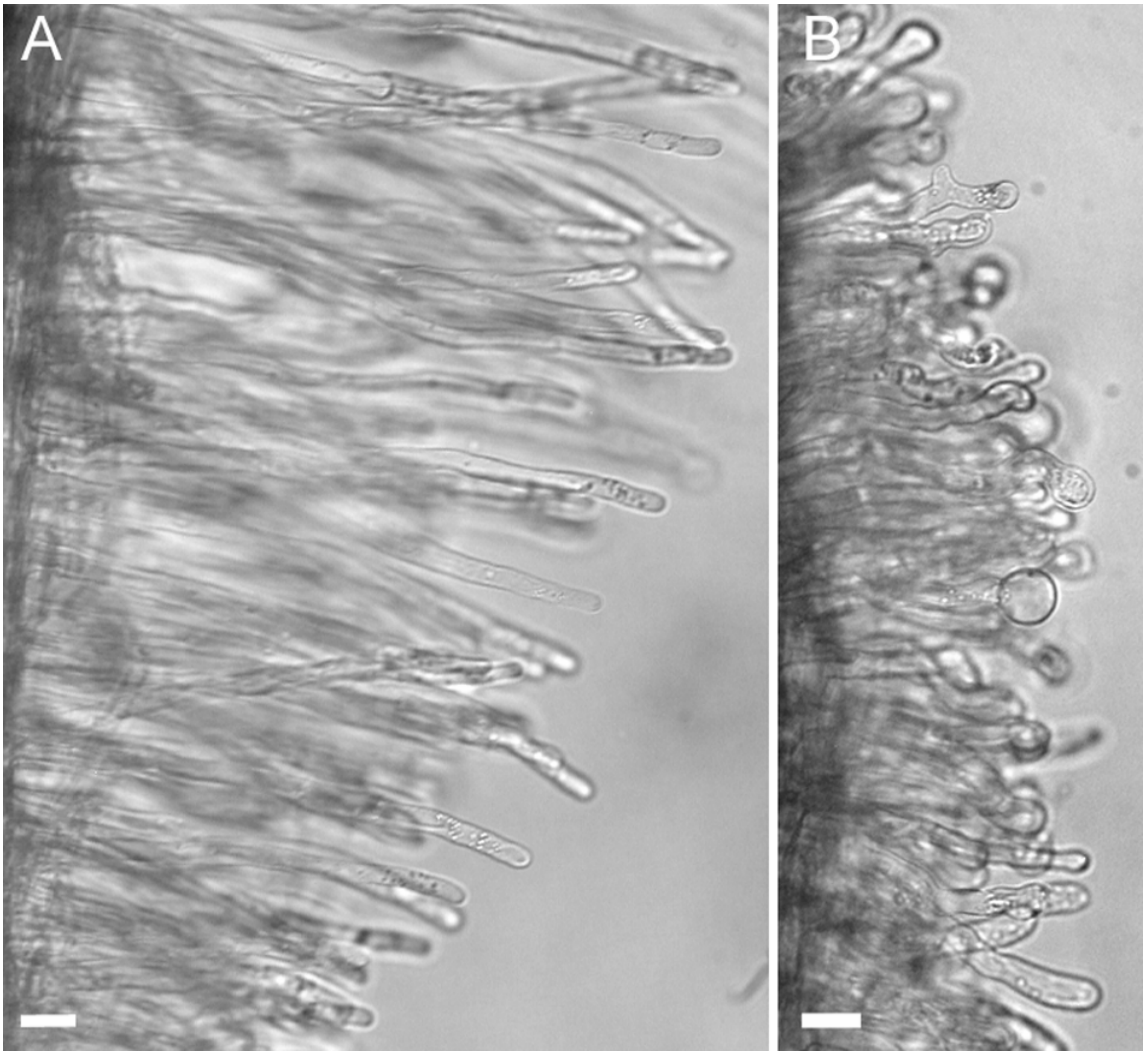

Figure 7. Difference of *L. japonicus* wild-type and Cac41.5 when grown in liquid medium between glass slides.

(A) Wildtype root. The root hairs have a normal diameter and show a normal elongation pattern. **(B)** *L. japonicus* Cac41.5 root before mediumchange. Now already, most of the root hairs are short, swollen, and/or branched. Only root hairs with a proper shape and cytoarchitecture like the *M truncatula* root hair in figure 2A, were followed during the medium change experiments. Bars are 30 µm.

Growing *M. truncatula dmi2-1* Root Hairs show wild-type responses to Nod Factor spot application

Since the responses of wild type and *M. truncatula dmi2-1* root hairs to gentle global application of NF are identical, we hypothesized that growing *dmi2-1* root hairs should respond to spot application of purified NF (Esseling et al., 2003) like wild type, with reorientation of the root hair growth axis towards the site of application, and root hair branching when applied 30 µm below the root hair tip. In addition, to better understand the position of *DMI2* in relation to *DMI1* and *DMI3*, we also used the C71 (*dmi1-1*) and TRV25 (*dmi3-1*) mutants in our spot application experiments.

Spot application of NF at the side of the tip of growing root hairs resulted for all mutant root hairs (*dmi2-1*, N=13/13, Figure 8a; *dmi1-1*, N=16/16, Figure 8f; and *dmi3-1*, N=12/12, Figure 8g) in reorientation of the growth axis towards the site of application in a time-course similar to that for the wild-type (Esseling et al., 2003).

Spot application 30 µm below the tip of the growing *dmi2-1* root hair (N=5/5) resulted in the formation of a root hair branch at the site of application (Figure 8c). In accordance with what has been reported for the *dmi2-1* mutant (Vernoud et al., 1999; Catoira et al., 2000), $Pro_{MtENOD11}$*:GUS* expression could not be detected in reoriented (Figure 8b) and branched *dmi2-1* root hairs (Figure 8d), whereas the wild-type root hairs showed $Pro_{MtENOD11}$*:GUS* expression (Figure 8e), as has been reported before (Esseling et al., 2003). The endogenous $Pro_{MtENOD11}$*:GUS* expression in the root cap of wild-type and *dmi2-1* roots was sustained (not shown).

We conclude that *dmi2-1* root hairs still have the property to reorient their growth axis when challenged with NF, which we have shown to be a prerequisite for root hair curling (Esseling et al., 2003), and ask the question whether the *NORK* mutated root hairs indeed can curl around bacteria.

M. truncatula dmi2-1 root hairs curl, but are unable to entrap *Sinorhizobium meliloti*

To test whether *dmi2-1* root hairs have the ability to curl around, and entrap rhizobia, we inoculated 8 day-old wild-type and *dmi2-1* plants with their symbiotic partner *S. meliloti*. Five days after inoculation, the inoculated parts of the root were cut off. The parts that were not in contact with the agar were microscopically examined for curled root hairs. Interestingly, for wild-type and *dmi2-1* roots, a comparable amount of curled root hairs per cm of inoculated root was counted (28.9 +/- 4.2 SD for wild-type, and 31.4 +/- 2.3 SD for *dmi2-1*; Figure 9). For curl evaluation, we excluded reoriented (“deformed”) root hairs such as that in Figure 10A, and made two categories of curls: single-faceted 2-D 180° curls, and multi-faceted 3-D >180° curls. The latter arise because the tip of the hair continues growing after it touches upon its own shank at 180° curling (see figure 10C, arrowhead). Upon examination with Hoffman modulation contrast microscopy and CLSM, it appeared that 14.5% of the curled wild-type root hairs was of the multi-faceted 3-D type, and that only in these curls bacteria were entrapped (Figure 10D) and infection threads had grown (Figure 10B, E). From these data, we conclude that a multi-faceted root hair pocket is a requirement for infection thread formation in wild-type. Multi-faceted 3-D curls in which bacteria were entrapped, as well as infection threads, were absent in *dmi2-1* root hairs (Figure 10C and F). The root hairs stopped curling (growing) when the tip of the root hair touched its own shank.

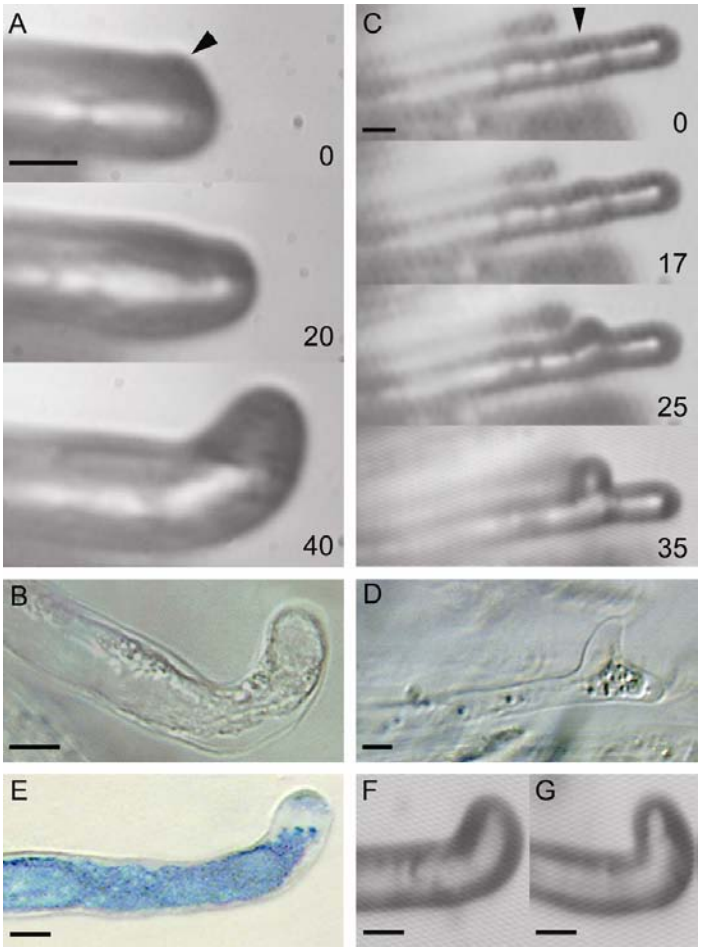

Figure 8. Responses of *M. truncatula dmi2-1* $Pro_{MtENOD11}$*:GUS* root hairs to spot application of 10^{-9} M NF.

(A) Time series of reorientation of root hair growth after spot application of 10^{-9} M NF on the side of the tip of a growing *M. truncatula dmi2-1* root hair. At 20 minutes, the reorientation is visible, and at 40 minutes, it is pronounced. **(B)** Reoriented *M. truncatula dmi2-1* root hair carrying the $Pro_{MtENOD11}$*:GUS* reporter gene, 60 minutes after spot application and 24 hrs after GUS reaction. In all of 13 root hairs, no GUS was detected, showing that the root hair does not express *MtENOD11* after NF spot application. **(C)** Time series of the formation of a root hair branch after spot application of 10^{-9} M NF 30 µm below the tip of a growing *M. truncatula dmi2-1* root hair. At 17-min after application, the first sign of the formation of a branch is visible, which becomes more pronounced later in time. **(D)** The same root hair as in **(C)**, 60 minutes after spot application and 24 hrs after GUS reaction. In all of 5 root hairs, no GUS was detected, showing that the root hairs do not express $Pro_{MtENOD11}$*:GUS* after NF spot application. **(E)** $Pro_{MtENOD11}$*:GUS* expression in a wild type root hair, 60 minutes after NF spot application and 24 hrs after GUS reaction. Clearly the reorientation towards the site of application and the GUS expression are visible. **(F)** Growing C71 (*dmi1-1)* root hair, 45 minutes after NF spot application. **(G)** Growing TRV25 (*dmi3-1*) root hair, 55 minutes after NF spot application. Both the C71 and TRV25 root hairs show root hair reorientation towards the site of application in a time-frame comparable to wild-type root hairs. Arrowheads point to the site of application, and bars are 10µm.

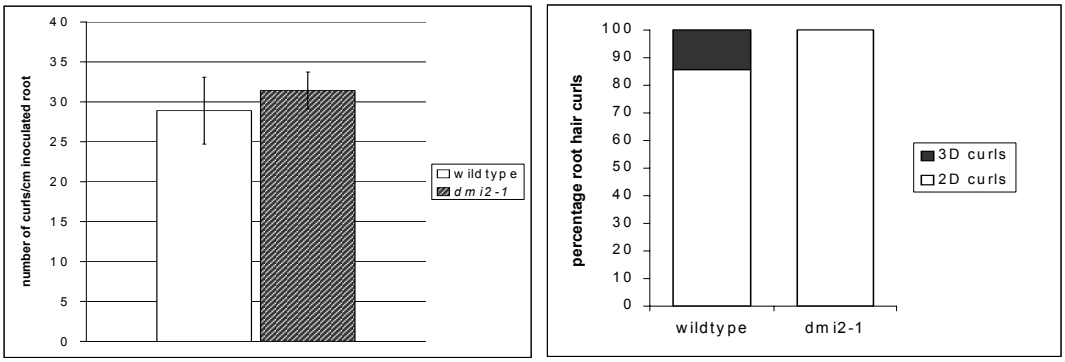

Figure 9. Number of root hair curls per cm inoculated root.
(A) For wild-type and *dmi2-1* a similar number of curled root hairs per cm inoculated root were counted. Error bars are standard deviations. **(B)** Of the wild-type curled root hairs, 14.5% had a complex 3-dimensional bacteria entrapping structure. The other 85.5% were single-faceted 2-D 180° curls without entrapped bacteria. On *dmi2-1*, all root hair curls were single-faceted 2-D 180° curls without entrapped bacteria.

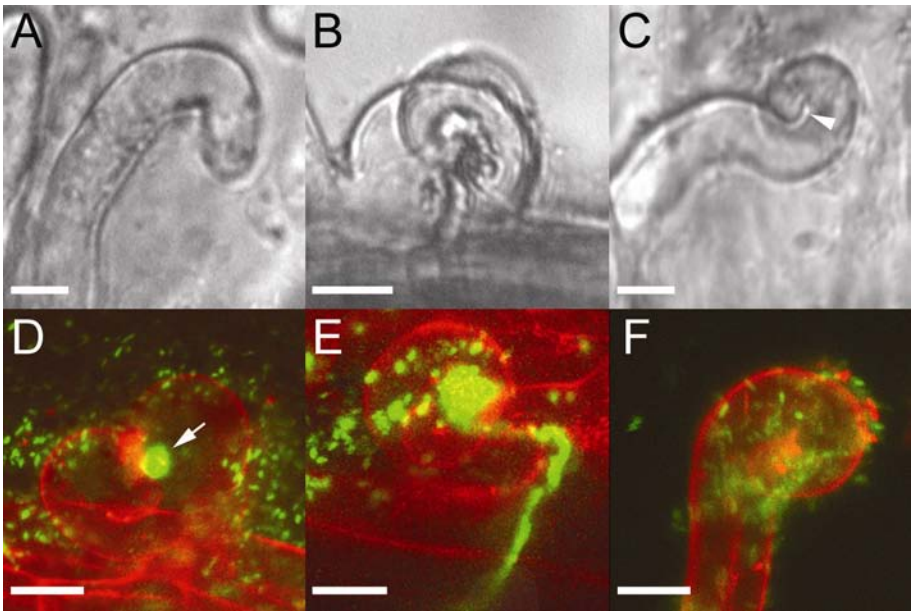

Figure 10. *M. truncatula* wild-type and *dmi2-1* root hairs curl around *Sinorhizobium meliloti* 2011-GFP.
(A) Reoriented wild-type root hair in the presence of *S. meliloti*. **(B)** Brightfield image of a wild-type root hair curl with an infection thread. Note the complex multifaceted 3-D structure of the curl. **(C)** Brightfield image of a curled *dmi2-1* root hair. Arrowhead points to the furrow at 180° curling. **(D)** Projection of 30 images from a CLSM Z-stack of a wild-type curled root hair, entrapping a GFP-expressing bacterial colony (arrow). The cell wall (red) was counter-stained with 0.1% propidium iodine. Note the multifaceted 3D structure of the root hair curl. **(E)** Projection of 35 images of a Z-stack from a wild-type curled root hair with a bacterial colony in an 'closed' pocket. **(F)** Projection of 20 images of a Z-stack from a *dmi2-1* root hair, curling in the presence of bacteria, but unable to entrap them. Note the bacteria on the outside of the root hair. Monitored were 4 roots per wild-type and *dmi2-1*. Bars are 15 µm.

Since *dmi2-1* root hairs lack NF-induced $Pro_{MtENOD11}$*:GUS* expression (Vernoud et al., 1999; Catoira et al., 2000; this work), but have the ability to curl in the presence of rhizobia, we conclude that there is an early branch in the signaling cascade upstream of *DMI2/NORK*. One branch leads to root hair curling, the other toward $Pro_{MtENOD11}$*:GUS* expression.

DISCUSSION

Cytoarchitecture as determinant of the morphological response of legume root hairs to Nod Factor

Typical for a root hair-bearing root is the 'up-side-down Christmas tree', the cone which the root hairs form along the root. Along this cone, one can find bulges, growing root hairs, growth terminating root hairs and full grown root hairs (*M. truncatula*: Sieberer and Emons, 2000). To better describe the effects of NF on *V. sativa* root hairs, Heidstra et al. (1994) suggested a zonation of root hairs in this cone. According to this zonation, zone I corresponds to young growing hairs which do not deform upon NF application, zone II root hairs are those responding to NF with root hair deformation, and zone III are the full-grown root hairs. From this zonation, one would easily expect a correlation between position of the root hair on the root and the ability to deform upon NF application. However, pretreatment of *V. sativa* root hairs with the ethylene inhibitors AVG or Ag^+ changed the susceptibility of the zone I hairs to NF, in that they were able to deform upon NF application (Heidstra et al., 1997). Pretreatment with AVG or Ag^+ induced growth termination: the cytoarchitecture of zone I hairs changed into that of zone II hairs (see Figure 1 in: Heidstra et al., 1997), indicating that the ability to deform is correlated with the cytoarchitecture and not with the position of the root hair on the root. Further analysis has shown that for *V. sativa* (de Ruijter et al., 1998) and for *M. truncatula* (Sieberer and Emons, 2000; this paper) the cytoarchitecture i.e. its developmental stage, is the only microscopic characteristic determining whether a root hair will respond to NF with root hair deformation, or not. Therefore, position of the root hair along the root should not be used as an absolute determinant to predict NF-induced root hair behavior.

Enhanced touch-response in DMI2/NORK mutated legumes

In order to cope with the environment in which they are growing, plants need to be able to respond to a myriad of external stimuli, such as temperature, drought, touch, and gravity. The sensitivity to external stimuli is extremely high, which is nicely shown by Fasano et al. (2002). A transient touch stimulation by a 2-second-short pulse of growth medium, expelled from a micropipette positioned adjacent to an *Arabidopsis* root, is already enough to induce a rapid signaling, leading to a cytosolic Ca^{2+} wave in the root (See Figure 3D in: Fasano et al., 2002). Related to this, we now show that direct touch with a needle has a dramatic effect on the cytoarchitecture of growing *M. truncatula dmi2-1* root hairs, and that draining and replacement of the growth medium induces a quick change of cytoarchitecture in legume root hairs, a change which could be

prevented with a very careful medium replacement. The difference between wild type and *NORK*-mutated legumes is the ability to recover from these touch responses. The sensitivity of the polarly organised cytoplasm to mechanical stress may be causal to the tendency to show spikes and exaggerated oscillations in the level of cytoplasmic Ca^{2+} in untreated *dmi2* root hairs (Shaw and Long, 2003), and might indicate where to search for the mechanism of NORK activity in the root hair. From our results, we conclude that NORK is a component that is not essential for tip growth itself, but protects the tip growth process against environmental disturbance. Whether or not related to this, at the same time, NORK is essential for NF signal transduction toward $Pro_{MtENOD11}$*:GUS* expression. The differences in the responses between the three *NORK* mutants could depend on the nature of the mutation (Endre et al., 2002; Stracke et al., 2002).

The absence of multi-faceted curls in *M. truncatula dmi2-1* and their requirement for infection in wild type

NF spot application onto the hemisphere of *dmi2-1* root hairs induces reorientation of the root hair growth axis towards the site of application, or, when applied 30 µm below the tip, it induces the formation of a root hair branch. For wild-type root hairs, these responses have been shown to mimic root hair curling (Esseling et al., 2003).

Inoculation of *M. truncatula* wild-type and *dmi2-1* roots with *S. meliloti* 2011 resulted in a similar amount of root hairs per centimeter of root, which curled at least 180°. A noticeable difference was that in *dmi2-1* root hairs, the hairs did not curl further and, therefore, did not form multi-faceted 3-dimensional structures. Apparently, as soon as the tip of the root hair touched its own shank, the root hair stopped growing, and therefore curling. Since the *dmi2* root hairs have been shown to be very responsive to touch (this study), we think that the touch of the root hair tip to its own shank is enough to induce growth arrest and, therefore, also arrests curling before a tight pocket has been formed around the bacteria.

In addition, this experiment shows that in wild type the formation of a 3D multi-faceted curl is necessary for infection thread formation. A closed root hair pocket is formed in which the growing colony of bacteria is entrapped, and because it grows, it most probably exerts force. This has been suggested a long time ago (Dart, 1974). Inhibition of the production of this multi-faceted 3D curl in the mutant does not seem to be a sufficient cause for inhibition of infection thread formation, because the mutant also lacks NF-induced *ENOD-11* expression.

A split in NF-induced signal transduction

Analysis of *M. truncatula* nodulation-defective mutants resulted in genetic dissections of NF-induced signaling (Catoira et al., 2000; Wais et al., 2000; Reviewed in: Geurts and Bisseling, 2002). Based on the observed morphological responses of the root hairs to global NF application, a linear pathway was suggested (Fig 11A), in which *DMI1, DMI2/NORK* and *DMI3* were placed between root hair swelling (Has) and root hair branching (Hab), followed by *NSP*, subsequent ENODs expression, cortical cell division (ccd) and nodulation (Catoira et al., 2000). Studies on the behavior of NF-induced Ca^{2+}-spiking in these and other nodulation defective mutants, led to a refinement of this linear NF pathway (Wais et al., 2000). In this scheme (Fig 11B), Has was left out, and *DMI1/DMI2* preceded Ca^{2+}-spiking, followed by *DMI3*, root hair deformation (RHD), i.e. swelling and outgrowth, *NSP*, expression of ENODs and cortical cell division, *HCL*, and nodulation.

We now show that NORK/DMI2 and its downstream products are not required in the root hair for the early NF-induced morphological responses. Gentle global NF application results in complete root hair deformation of growth terminating *dmi2-1* root hairs, and NF spot application results in root hair reorientation, or root hair branching, depending on the position of application. Moreover, *dmi2-1* root hairs can curl in the presence of rhizobia. Therefore, we propose an early split in NF-induced signal transduction in the root hair (Figure 11C). One branch of the pathway leads to the morphological responses: root hair deformation, root hair branching, or root hair reorientation and curling, depending on the assay. The other branch includes DMI2/NORK and $Pro_{MtENOD11}$*:GUS* expression. Basically, it follows the pathways that have been proposed by Catoira et al. (2000) and Wais et al. (2000), with the important difference that the morphological responses branch off soon after NF perception. Since growing *dmi1-1* and *dmi3-1* root hairs also show root hair reorientation upon NF spot application, we would like to conclude that DMI1 and DMI3 are also not required for NF induced morphological responses, and we include them in the branch which leads to $Pro_{MtENOD11}$*:GUS* expression.

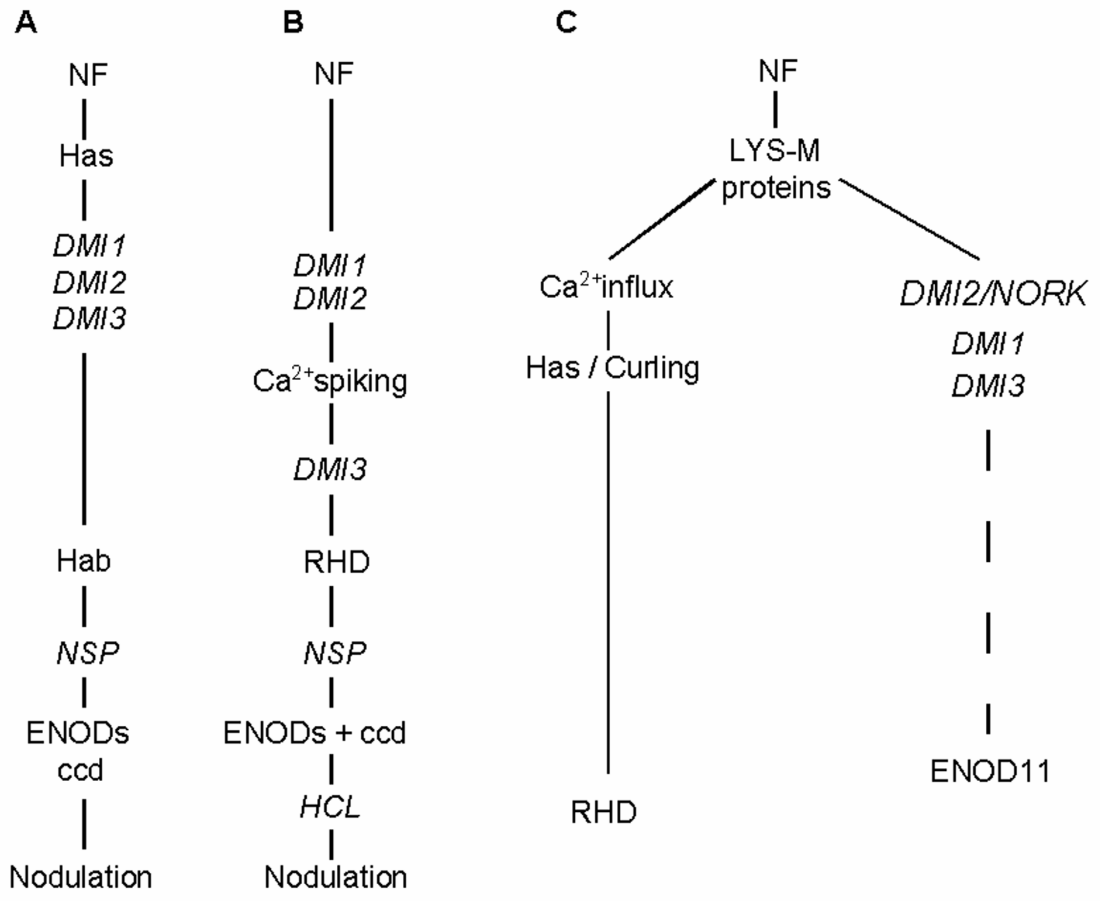

Figure 11. Putative NF-induced signal transduction pathways.
(A) Pathway proposed by Catoira et al. (2000). **(B)** Pathway proposed by Wais et al. (2000). The difference between these two pathways is the positioning of Ca^{2+}-spiking between *DMI1-DMI2* and *DMI3*, and the combination of Has and Hab into RHD. **(C)** New pathway in which our results are integrated into the already published results. From our results we can conclude that DMI2/NORK, DMI1, DMI3 and its downstream $Pro_{MtENOD11}$*:GUS* expression are not required for the early NF-induced morphological responses. Therefore, we propose that very soon after NF perception via LYS-M proteins, and before DMI2/NORK, DMI1 and DMI3, the pathway forms a split. One part, including the rapid NF-induced Ca^{2+}-influx, branches off to root hair reorientation (RHR)/Has and RHD, and the other part via DMI2/NORK to $Pro_{MtENOD11}$*:GUS* expression. Since we only used $Pro_{MtENOD11}$*:GUS* as a NF reporter gene, we can only make a statement about this gene, and we do not want to exclude the possibilty that in DMI2/NORK mutants other NF-induced genes are expressed.

Interestingly, work has been published recently which also points in the direction of a dual signaling pathway (Shaw and Long, 2003a,b). By measuring Ca^{2+} levels in growing *M. truncatula* root hairs before and after NF application, these authors found an uncoupling between the rapid NF-induced Ca^{2+} influx (Felle et al., 1998) at the root hair tip (Cárdenas et al., 1999; reviewed in Cárdenas et al., 2000) and NF-induced Ca^{2+}-spiking (Ehrhardt et al., 1996; Wais et al., 2000; Walker et al., 2000). They show that in about 50% of the tested *DMI2* root hairs, the rapid NF-induced Ca^{2+} influx at the tip is sustained. In addition, the basal H_2O_2 production which is thought to activate Ca^{2+}-channels in the root hair tip during normal root hair tip growth (Foreman et al., 2003), goes down after NF

application in *M. truncatula* wild-type and *DMI2* root hairs (Shaw and Long, 2003b). As we discussed before (Esseling et al., 2003), this NF-induced Ca^{2+} influx at the tip is expected to be required for sustained root hair elongation needed for root hair curling, a process that is not impaired in *DMI2* root hairs (this work). NF-induced lowering of the endogenous H_2O_2-induced Ca^{2+} influx at the existing root hair tip, combined with the NF-induced local Ca^{2+} influx, could give the directional information for reorientation of root hair tip growth (Figure 11C).

We now show that physical manipulation/touch of growing *M. truncatula* root hairs induces the non-symbiotic phenotype in *dmi2-1* root hairs. Therefore, we would like to leave the absence of NF-induced Ca^{2+}-spiking in *dmi2-1* root hairs (Wais et al., 2001; Shaw and Long, 2003) as unresolved. The initial Ca^{2+}-spiking experiments on *dmi2* mutants showed that *dmi2-3* was leaky for Ca^{2+}-spiking, and the experiments were performed on excised root segments (Wais et al., 2001). Excision of the root could be enough to induce the non-symbiotic phenotype in *dmi2-1* root hairs. Although the later Ca^{2+}-spiking experiments were performed on intact seedlings, and the cytoarchitecture of the injected wild-type root hairs seems normal (Shaw and Long, 2003a), it might be that the iontophoresis is enough to reveal the (mild form of the) non-symbiotic phenotype in the *dmi2-1* root hairs, which could result in the absence of NF-induced Ca^{2+}-spiking.

The described combination of cell biology and mutant analysis has contributed to a better understanding of the signaling processes required for the symbiosis between rhizobia and legumes. The challenge is now to find out if, and how, the enhanced sensitivity to touch is related to infection thread formation.

MATERIALS AND METHODS

Seed preparation

M. truncatula wild-type and TR25 with $Pro_{MtENOD11}$*:GUS* seeds were removed from the pods and scarified in concentrated sulphuric acid for 10 minutes. *M. sativa* wild-type and MNNN-1008 seeds were scarified for 2 minutes, and *L. japonicus* wild-type Gifu and Cac 41.5 (SYMRK) seeds for 10 minutes in concentrated sulphuric acid. After scarification, all seeds were extensively washed with running demineralized water and sterilised in a mixture of 30% (w/v) hydrogen peroxide and 96% (w/v) ethanol (1:1 [w/v]) for 2 minutes. Subsequently, seeds were washed with sterile demineralized water (3 x 2 minutes, 3 x 30 minutes) and transferred to a small glass jar containing fresh sterile demineralized water. The capped jar was stored at 4ºC overnight. Imbibed seeds were then transferred onto 0.8% (w/v) agar plates prepared in sterile demineralized water. The plates were sealed with parafilm, wrapped in aluminium foil and stored at 4ºC until root protrusion.

Spot Application Assay

Spot applications were performed as described before (Esseling et al., 2003). In short, a water pressure microinjection device (water pressure device: Gilmont, Barrington, IL, USA; needle holder: Eppendorf, Merck Eurolab BV, Amsterdam, The Netherlands) was used to apply micro-droplets of purified NF [NodRm-IV(C16:2, Ac, S)] diluted with Millipore water to a final NF concentration of 10^{-9} M to one side of growing root hair tips. Subsequent growth axis reorientation was recorded with a video camera (Hitachi, Tokyo) linked to an inverted Nikon Diaphot TMD microscope (Nikon, Tokyo).

Global NF application procedure

One- to 1.5-cm-long seedlings were transferred to sterile Fåhraeus slides containing sterile plant growth medium (PGM) and grown for 36 hours in a climate chamber (25ºC, 16-h-light/8-h-dark). Prior to observation, the Fåhraeus slides were gently placed on the microscope stage, and were left to recover for at least half an hour. For this purpose, the microscope was placed into the culture room. For gentle NF application, the PGM in the Fåhraeus slides was replaced by PGM containing 10^{-9}M NF, by adding PGM with NF on one side of the slide, and slowly pipetting away on the other side. For NF application via draining and replacement, the PGM in the Fåhraeus slides was drained away by placing the bottom of the slide on a piece of tissue filter paper, and PGM with NF was pipetted in the slide, using a blue-tip 1000 µl pipette (Socorex). Images were taken from the area on the root hair cone which had root hairs with the appropriate cyto-architecture before medium change, and were recorded with a Sony CCD camera (Sony, Tokyo) linked to an upright Nikon Optiphot DIC microscope.

Needle touch experiment

After germination, *M. truncatula* wildtype and TR25 seedlings were placed on 0.3% (w/v) agarose in PGM. The square petridishes were closed with parafilm, and left horizontal in a climate chamber (25ºC, 16-h-light/8-h-dark). One day later, the roots had

grown into the agarose, and root hairs were formed. To prepare for the touch experiment, the parafilm was removed from the plates, and with the lid still on, the plates with the seedlings were gently placed on a Nikon Diaphot 200 inverted microscope, with Hoffman modulation optics. After half an hour of acclimatisation, the lid of the plate was removed, and the plate was again left for half an hour. Thereafter, a microinjection needle usually used for spot application or microinjection, was gently positioned under the root hair of interest. For another 10 minutes, the root hair of interest was followed to make sure that this needle positioning did not disturb root hair cytoarchitecture and/or growth. Touch was applied by moving the needle up and down, in such a way that the root hair was bent in both directions. Time lapse recording of the root hair was performed with a Hamamatsu CCD camera, coupled to an Argus-20 low-light enhancement image processor (Hamamatsu Photonics, Hamamatsu City, Japan). Images were recorded every 30 seconds. To prevent an ambiguous approach, the experiment was performed "blind" without knowing which plate contained the wild-type or *dmi2-1* seedlings, before and at the moment of the experiment.

Sinorhizobium meliloti inoculation procedure

After germination, seedlings of about 1- to 1.5-cm long were transferred to agar plates in buffered Nod medium (BNM; Ehrhardt et al., 1992) with 1µM aminoethoxyvinyl glycine (AVG). The plates were sealed with parafilm, leaving the top of the plates opened for ventilation, and stored vertically in a climate chamber (20°C, 50% relative humidity, 16-h-light/8-h-dark). After eight days, the root of each seedling was inoculated at the bulge-area with a GFP-expressing *Sinorhizobium meliloti* 2011 (pHC60; Limpens et al., 2003) culture, diluted to an $OD_{600\ nm}$ of 0.01 with sterile Millipore water. After sealing with parafilm, the plates were placed back into the climate chamber until observation. Prior to observation, the roots were cut off just above the inoculation spot, and placed into 0.1% (w/v) propidium-iodide for 15 minutes to stain the cell wall. Fluorescence images were acquired with 488 and 568 nm laser light on a Zeiss LSM510 coupled to a Zeiss axiovert microscope (Carl Zeiss, Jena, Germany). Bright field images were obtained on a Nikon Diaphot 200, equipped with Hoffman modulation contrast, and acquired with a Hamamatsu CCD camera, coupled to an Argus-20 low-light enhancement image processor (Hamamatsu Photonics, Hamamatsu City, Japan).

$Pro_{MtENOD11}$:*GUS* Expression

$Pro_{MtENOD11}$:*GUS* expression was assessed by incubating the seedlings for 24-h in the β-glucuronidase substrate X-Gluc (2 mM 5-bromo-4-chloro-3-indolylglucuronide, 1% [w/v] dimethylformamide, 0.1 mM $K_3[Fe(CN)_6]$, 0.1 mM, $K_4[Fe(CN_6)]\cdot 3H_2O$, 1 mM EDTA, and 50 mM KH_2PO_4, pH 7.0) at 37ºC (Journet et al., 1994). Images were recorded with a CCD camera (Sony, Tokyo) linked to an upright Nikon Optiphot DIC microscope.

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Chapter 4

One single Nod Factor molecule is sufficient to initiate symbiotic responses in a legume

John J. Esseling[1], Bela M. Mulder[1,2] and Anne Mie C. Emons[1]

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

[2]FOM Institute for Atomic and Molecular Physics, Kruislaan 407, 1098 SJ Amsterdam, The Netherlands

Legumes can live in symbiosis with nitrogen fixing bacteria, the rhizobia. To establish such a symbiosis, rhizobia excrete signal molecules, the Nod Factors (NF), which induce symbiotic responses in the host's root hairs, like root hair curling and early nodulin gene expression. We found that upon spot application of a concentration range of purified nod factors, growing *Medicago truncatula* root hairs still reacted with root hair reorientation and $Pro_{MtENOD11}$-*GUS* expression at very low concentrations. We calculated the probability that an applied droplet of a certain concentration contains one molecule NF and show that one NF molecule is sufficient to induce root hair reorientation and $Pro_{MtENOD11}$-*GUS* expression.

Annually, rhizobia living in symbiosis with legume plants fix approximately the same amount of atmospheric nitrogen as the chemical fertilizer industry. This natural nitrogen fixation process takes place in root nodules. The nodule is a plant organ that develops under the influence of the rhizobia and hosts them. To reach the developing nodule, first, bacteria are entrapped into a root hair curl, which they initiate. Then, from this bacterial microcolony, a plant derived infection thread grows through the root hair into the root cortex, where cell divisions take place for nodule development. To initiate these symbiotic responses, the bacteria excrete specific signal molecules, the nodulation factors or Nod factors (NFs), which are lipochito-oligosaccharides (*1,2*). The common *nodABC* genes in rhizobia synthesize the chitin backbone of the NF molecules, whereas species-specific nod gene products decorate this backbone. These decorations confer the host specificity of the rhizobia to the legumes (*3*). The fact that the *Rhizobium leguminosarum* bv. *viciae* strains PRE and PRE-X are able to infect certain pea cultivars efficiently, although NF production by these bacteria is too low to be detected by thin layer chromatography (TLC; *4*), shows the high specificity of the NF as the bacterial signal molecule for setting up a successful symbiosis. Introduction of the NF overproduction *nodD* gene into the PRE strain did not result in an increased amount of pea cultivars that can be nodulated by this strain (*4*), which means that the amount of produced NF molecules is not the rate-limiting step for setting up an effective symbiosis, only a few molecules might be sufficient.

In purified form, NFs induce a variety of responses in the host's root hairs, like ion fluxes and Ca^{2+} spiking, changes in the root hair cytoskeleton and subsequent root hair deformation and gene expression (*5-11*). Also for these responses to occur, only low NF concentrations are required. Concentrations as low as 10^{-12} M have been reported to be effective for induction of root hair deformation in *Vicia sativa* (*8*) and *ENOD12-GUS* expression in *Medicago sativa* (*10*).

In such a classical global application assay, these concentrations still contain many molecules. In the spot application assay that we developed, 1 picoliter droplets of purified NF are applied on the side of the hemisphere of a growing root hair. The effect on root hairs is a reorientation of the root hair growth axis in the direction of the site of application. Also the $Pro_{MtENOD11}$-GUS reporter gene is expressed (*12*). To determine the lowest concentration at which NF induces root hair reorientation and $Pro_{MtENOD11}$-GUS expression, we calculated for several NF concentrations the probability that one NF molecule is present in a 1 pl droplet, applied droplets of these concentrations onto *M. truncatula* root hairs and scored root hair reorientation and $Pro_{MtENOD11}$-GUS expression.

The concentration of our initial NF stock was determined to be 1.70 ± 0.22 x 10^{-5} M using the modified Kean partitioning assay (*13*). From this stock a dilution series was prepared, with dilution factors ranging from 10^3 to 10^{11} in steps of a single decade, yielding approximately 1 ml solution volume at each dilution. Using a micropipette, spot applications from a single solution where

then performed on several growing root hairs. To prevent any observer bias, these experiments were performed “blind” without knowing which specific solution was being applied. Assuming that the deposited droplets were hemispherical in shape, their volume could be estimated from the microscopic images to be 1 ± 0.5 pl. The treated root hairs were then observed in a time course to determine whether or not reorientation of the growth axis occurred. The results of these experiments are summarized in Table 1. The spot application assay offers a clear-cut yes-or-no answer: the root hairs continue their straight growth direction, or change their growth direction toward the spot with NF. In addition, *Pro$_{MtENOD11}$-GUS* expression was detected in all root hairs that responded with root hair reorientation, whereas the hairs that were growing straight after application were negative for *Pro$_{MtENOD11}$-GUS* expression (Figure 1, Table 1).

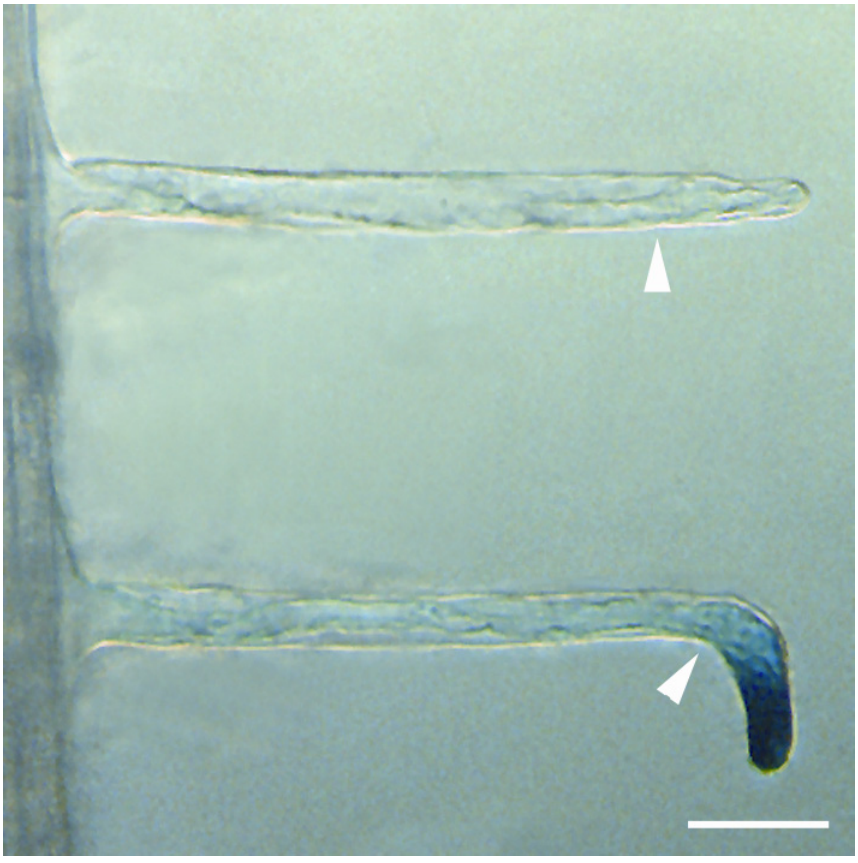

Figure 1.

The figure shows two root hairs from wild-type *M. truncatula* with the Pro$_{MtENOD11}$-GUS reporter gene, 1.5 h after spot application and 24 h after GUS staining. Both root hairs were subsequently spot applied with the same needle, which contained 1.7 10^{-12} M NF. The upper root hair did not respond with either root hair reorientation or Pro$_{MtENOD11}$-GUS expression, whereas the lower root hair shows both. Arrowheads point to the site of spot application. Bar, 30µm.

Table 1. Results from spot applications of different dilutions of NF stock.

NF concentration	Number of Repeats	Number of reorientations	% of reorientations	Number of Gus positive root hairs
$1.7\ 10^{-8}$	25	25	100	25[a]
$1.7\ 10^{-9}$	13	13	100	nd*
$1.7\ 10^{-10}$	15	15	100	nd*
$1.7\ 10^{-11}$	10	10	100	10 [a]
$1.7\ 10^{-12}$	13	9	69	9 [a]
$1.7\ 10^{-13}$	12	2	17	2 [a]
$1.7\ 10^{-14}$	11	0	0	nd*
$1.7\ 10^{-15}$	12	0	0	nd*
$1.7\ 10^{-16}$	10	1	10	nd*

[a] Only the reorientated root hairs showed *Pro$_{MtENOD11}$-GUS* expression.

* Spot application experiments were performed on wild type plants without the Pro$_{MtENOD11}$-GUS reporter gene.

We observe that in our experiments at dilutions of 10^7 and higher not all spot applications lead to root hair growth axis reorientation. In fact at dilutions of 10^9 and higher only a single reorientation event is logged out of a total of approximately 30 applications. These observations allow us to assess how many NF molecules are actually necessary to elicit the reorientation response. This question is brought into focus when one realizes that a 1 pl sample from a 10^7 dilution of our original stock *on average* only contains a single molecule.

In order to quantify this inherently statistical question we develop a Bayesian approach. The details of this approach are discussed in the Supplementary Material. In the first step we estimate the probability that an applied droplet from a given solution contains a specified number of molecules. Note that this can be estimated solely on the basis of measured parameters. In the next step we assume that the root hair presented with a droplet containing a specified number of molecules, will respond to this stimulus with an efficiency depending on this number. This efficiency is of course unknown, but we choose to model it by a smooth two-parameter function of molecule number that interpolates between the obvious limits of zero (= no response) when no molecules are

present and unity (= sure response) when the number of molecules tends to infinity. The two parameters of the efficiency function are then extracted from the data employing a maximum likelihood analysis. The results from this step indicate that the efficiency is optimally modeled by a single step function with the value zero for no molecules and unity for all numbers of molecules larger than one. This implies that on the basis of our data it is highly likely that a *single* NF molecule is sufficient to trigger the reorientation response. As likelihoods are intrinsically unnormalized we can only quantify this likelihood by considering its ratio to an alternative hypothesis. We therefore choose to compare the hypothesis that a single molecule is sufficient to the hypothesis that two molecules are sufficient. The ratio of these two likelihoods is in the order of 10^8, and this result is very robust under variations of the measured parameters that are input into our model. This result lends very strong evidence to the hypothesis that indeed a single NF molecule can be detected and its presence signaled by the putative NF-receptor and its associated signaling pathway. But of course does *not* constitute a proof! For comparison: likelihood ratios in the order of 10^8 are considered relevant in criminal trials involving forensic DNA evidence (*14*). As a graphical illustration of our results we plot in Figure 2 the estimated probability of a positive response as a function of dilution on the basis of the single molecule compared to the observed frequencies in our trials.

Shaw and Long tested two known different NF-induced Ca^{2+} responses in growing *M. truncatula* root hairs at two different NF concentrations (*15*). At final bath concentrations of $\leq 10^{-9}$M NF, 13 out of 37 growing root hairs (~35%) responded to NF application with Ca^{2+} influx as well as Ca^{2+} spiking. The other 24 root hairs reacted with Ca^{2+} spiking, but no Ca^{2+} influx was detected. At a final bath concentration of 10^{-8}M NF, all root hairs responded with Ca^{2+} influx and subsequent spiking. For this global application assay, a small volume of NF stock solution was pipetted into the medium in which the root hairs were growing, until the required final bath concentration was reached. Since NFs become entrapped inside the cell wall of the whole epidermal cell (*16*), at a certain low concentration only part of the cell wall will be imbibed with NF. At such a NF concentration, Ca^{2+} spiking will be observed in every cell that has perceived NF molecules. However, Ca^{2+} influx into root hair tips will only be observed if that cell has entrapped NF in the observed tip area. This area is smaller than the whole wall surface. Therefore, if Ca^{2+} influx and Ca^{2+} spiking require the same amount of NF, the method used by Shaw and Long should show a higher incidence of Ca^{2+} spiking than of Ca^{2+} influx at root hair tips. When using spot application, we apply the droplet with NF at the site where we know that it will result into an instantaneous root hair reorientation (*12*). We find that all reoriented root hairs are blue from $Pro_{MtENOD11}$-*GUS* expression and conclude that the concentration needed for root hair reorientation equals

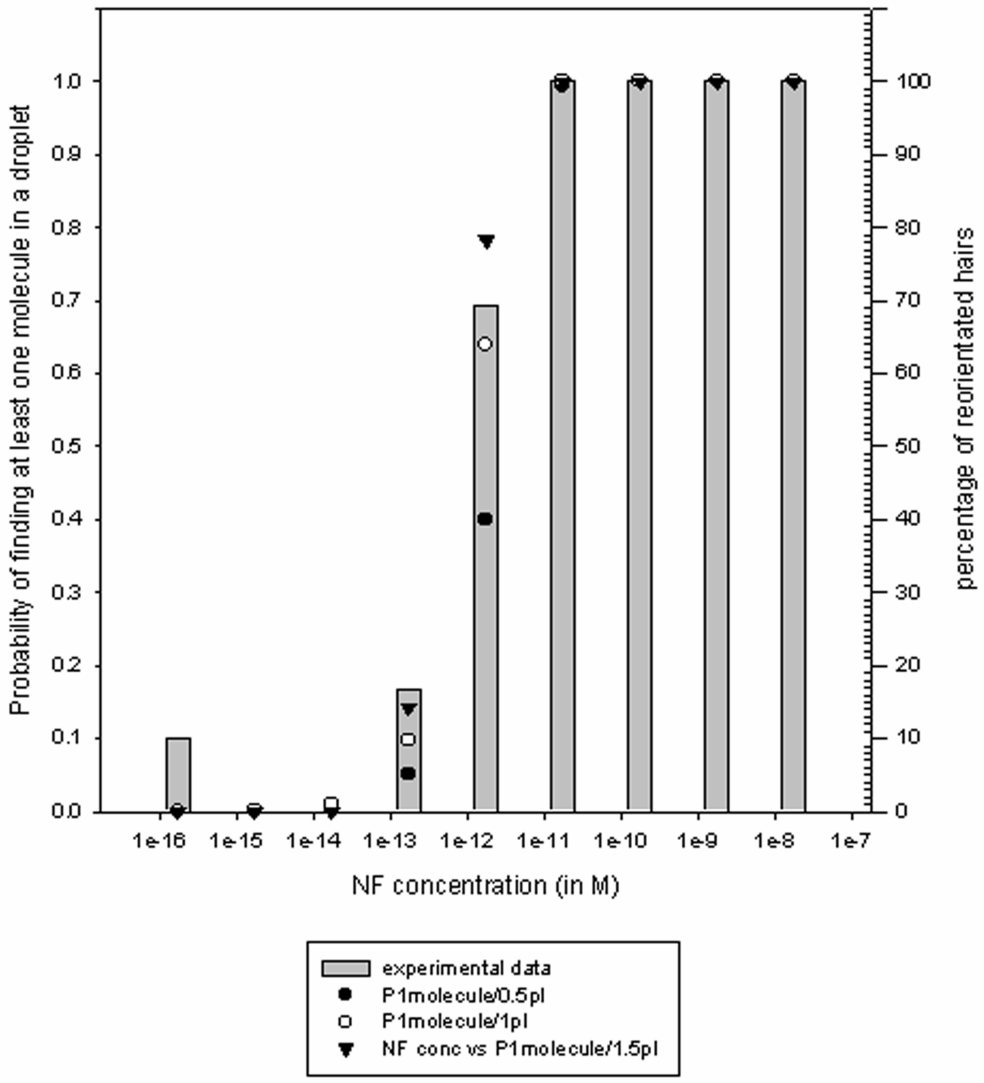

Figure 2.
In this figure, the probabilities of finding at least one NF molecule in a droplet of 0.5, 1 and 1.5 pl are plotted against the NF concentration in the needle. In the same figure, the experimental data are presented in percentages.

the concentration required for *Pro*$_{MtENOD11}$*-GUS* expression. Though this has not been visualized directly for the spot application, all data about Ca^{2+} gradients in growing and NF-stimulated root hairs suggest that NF stimulates this local Ca^{2+} influx (*5, 9, 15, 17*). Whether Ca^{2+} spiking relates to gene expression is an as yet unresolved question.

Can one molecule be enough to set off a whole signalling cascade? Activation of a signal transduction cascade by a single molecule has been

reported for the heterodimeric Epidermal Growth Factor (EGF) receptor from animal cells. In A431 carcinoma cells, activation of EGF-signalling by a single EGF molecule involves receptor dimerisation and its subsequent autophosphorylation (*18*). The putative NF receptor consists of a heterodimer of transmembrane serine/threonine receptor-like kinases with extracellular Lys-M domains (*19-21*), and therefore may behave in a similar way. Future characterization of the interaction between NF and the putative NF receptor molecules will reveal if such a dimerisation mechanism is the basis of NF-induced signalling.

How does a NF molecule reach the receptor through a 100 nm thick cell wall?

We know that NF molecules do not diffuse laterally in the cell wall, because after global application, the outgrowth of deformed root hairs is not labelled with radioactive (*8*) or fluorescent NFs (*16*). Fluorescence correlation microscopy (FCM) has revealed a >1000-fold reduction of the molecular mobility of fluorescently labelled NF molecules in the cell wall of a growing root hair (*16*). If the receptor is mobile in the plane of the plasma membrane, this immobility of the NF may seem a first condition inferring specificity, which has resemblances with ligand recognition by animal T cells that express the CD4 antigen. When mixed with antigen presenting cells (APCs), these T-cells first form a tight interface with an APC. Within this place of tight contact agonist peptide-MHC ligands do not move laterally and when there is only one ligand present, the T-cell responds with transient intracellular Ca^{2+} signalling (*22*). Still, however, the question remains how a NF molecule can breach the cell wall and find this mobile receptor embedded in the plasma membrane. A first possibility, supported by the work with fluorescently labelled NF (*16*) is that the NF molecules do move perpendicularly through the cell wall. Another possibility is that the NF binds to an extracellular glyco-domain of the transmembrane receptor proteins, but the putative receptor kinase does not have a glycosylation site. A third possibility is the interaction of the NF with a specific cell wall molecule that in its turn transduces the signal to the Lys-M dimer.

The fact that one single NF molecule is sufficient to initiate the symbiotic responses, root hair reorientation and *Pro$_{MtENOD11}$-GUS* expression in a legume, provides insight into activation of this signalling mechanism, and directs further research questions to be asked in respect to this system and the activation of other non symbiosis-related signal transduction pathways in plant cells.

Supplementary Material

We analyse the following experimental situation: a growing root hair is supplied with a small number of NF molecules and subsequently the observation is made whether the root hair responds by changing its growth direction (on) or no effect on the growth direction is observed (off). The small number of NF molecules is obtained by taking a small sample volume V from a (dilute) solution at concentration c (measured in number of molecules per unit volume). The average number of molecules present in such a volume is given by

(1.1) $$\langle n \rangle = cV$$

The probability of finding exactly k molecules in a known sample volume V is given by

(1.2) $$P_k(c \mid V) = \frac{(cV)^k}{k!} e^{-cV}$$

To model the uncertainties in the size of the actual volume delivered to the system we assume that it is drawn from a Gamma distribution with mean $\langle V \rangle$ and standard deviation $\sigma = \varepsilon \langle V \rangle$

(1.3) $$p(V) = \frac{1}{\langle V \rangle} \frac{\left(\frac{1}{\varepsilon}\right)^{\frac{1}{\varepsilon}}}{\Gamma\left(\frac{1}{\varepsilon}\right)} \left(\frac{V}{\langle V \rangle}\right)^{\frac{1}{\varepsilon}-1} e^{-\frac{V}{\varepsilon \langle V \rangle}}$$

The measurements of the droplet diameter indicate that the volume was $V = 1 \pm 0.5$ pl ($\langle V \rangle = 1$ pl, $\varepsilon = 0.5$). The probability of finding exactly l molecules in a sample drawn from the solution is thus

(1.4)
$$\begin{aligned} P_l(c) &= \int_0^\infty dV P_l(c \mid V)\, p(V) \\ &= \frac{(c\langle V \rangle)^l}{l!} \frac{\left(\frac{1}{\varepsilon}\right)^{\frac{1}{\varepsilon}}}{\Gamma\left(\frac{1}{\varepsilon}\right)} \frac{\Gamma\left(l + \frac{1}{\varepsilon}\right)}{\left(\frac{1}{\varepsilon}\right)^{l+\frac{1}{\varepsilon}}} \left(1 + \varepsilon c \langle V \rangle\right)^{-\left(l+\frac{1}{\varepsilon}\right)} \end{aligned}$$

We assume that there is finite detection efficiency $1 \geq \eta(k) \geq 0$ with which the root hair is able detect and respond to the presence of k molecules in the applied droplet. The probabilities of response and non-response can thus be expressed as

(1.5)
$$\begin{aligned} P_{on}(c) &= \sum_{k=0}^{\infty} \eta(k) P_k(c) \\ P_{off}(c) &= 1 - P_{on}(c) \end{aligned}$$

A useful two-parameter representation for the detection efficiency is

(1.6) $$\eta(k \mid k_*, \lambda) = \frac{\left(\frac{k}{k_*}\right)^{\lambda}}{1 + \left(\frac{k}{k_*}\right)^{\lambda}}$$

where $k_*, \lambda > 0$. This can accommodate a wide variety of behaviours between the obvious limits $\eta(0 \mid k_*, \lambda) = 0$ (assuming no false positives exists) and $\lim_{k\to\infty} \eta(k \mid k_*, \lambda) = 1$ (response is certain if a sufficiently large number of molecules is present), ranging from smooth quasi-linear for $1 > \lambda > 0$ to sigmoidal for $\lambda > 1$. The parameter k_* sets the crossover-point between the low and high efficiency regimes and λ determines the steepness of this crossover. We now want to estimate these parameters from the data supplied by our experiments, for which we employ the maximum likelihood method. The likelihood of observing M_{on} responses out of M trials performed at a given concentration c is given by

$$P(M_{on}, M, c \mid k_*, \lambda) = \frac{M!}{M_{on}!(M - M_{on})!} P_{on}(c \mid k_*, \lambda)^{M_{on}} P_{off}(c \mid k_*, \lambda)^{M - M_{on}} \tag{1.7}$$

The likelihood over all trials performed at a series of concentrations is then simply

$$L(k_*, \lambda) \propto \prod_i P\left(M^{(i)}{}_{on}, M^{(i)}, c^{(i)} \mid k_*, \lambda\right) \tag{1.8}$$

Analysing this likelihood on our data shows that at fixed k_* the likelihood increases as $\lambda \to \infty$, while at fixed λ the likelihood increases as $k_* \downarrow 0$. This is consistent with a step function with the step located at $k_* < 1$. We thus deduce the following likely form for the detection efficiency

$$\begin{aligned} \overline{\eta}(0) &= 0 \\ \overline{\eta}(k) &= 1 \quad k \geq 1 \end{aligned} \tag{1.9}$$

This already suggests that on the basis of our data a single molecule could suffice to trigger a response. To further test this idea we compare the hypothesis that a single molecule is sufficient to the hypothesis that at least two molecules are needed
To this end define the probability that there are more than k molecules in our sample volume

$$P_{\geq k}(c) = \sum_{l=k}^{\infty} P_l(c) \tag{1.10}$$

The likelihood of the data at a given concentration under the assumption that k *or more* molecules are necessary for a response is the given by

$$P(M_{on}, M, c \mid k) = \frac{M!}{M_{on}!(M - M_{on})!} P_{\geq k}(c)^{M_{on}} \left(1 - P_{\geq k}(c)\right)^{M - M_{on}} \tag{1.11}$$

and for the whole concentration series

$$L(k) \propto \prod_i P\left(M^{(i)}{}_{on}, M^{(i)}, c \mid k\right) \tag{1.12}$$

We now calculate the likelihood ratio

$$\frac{L(1)}{L(2)} \approx 1.7 \text{ x } 10^8 \tag{1.13}$$

showing that indeed the data support the hypothesis that a single molecule is sufficient to achieve a response.

Materials and methods

Seed preparation

M. truncatula wild-type A17 and wild-type with *Pro$_{MtENOD11}$:GUS* seeds were removed from the pods and scarified in concentrated sulphuric acid for 10 minutes. After scarification, all seeds were extensively washed with running demineralized water and sterilised in a mixture of 30% (w/v) hydrogen peroxide and 96% (w/v) ethanol (1:1 [w/v]) for 2 minutes. Subsequently, seeds were washed with sterile demineralized water (3 x 2 minutes, 3 x 30 minutes) and transferred to a small glass jar containing fresh sterile demineralized water. The capped jar was stored at 4ºC overnight. Imbibed seeds were then transferred onto 0.8% (w/v) agar plates prepared in sterile demineralized water. The plates were sealed with parafilm, wrapped in aluminium foil and stored at 4ºC until root protrusion.

Spot application

Spot applications were performed as described before on 8 to 10 days old plants (*12*). In short, a water pressure microinjection device (water pressure device: Gilmont, Barrington, IL, USA; needle holder: Eppendorf, Merck Eurolab BV, Amsterdam, The Netherlands) was used to apply micro-droplets of purified NF [NodRm-IV(C16:2, Ac, S)] diluted with Millipore water to one side of growing root hair tips. Subsequent growth axis reorientation was recorded with a video camera (Hitachi, Tokyo) linked to an inverted Nikon Diaphot TMD microscope (Nikon, Tokyo).

Droplet volume

From images of the droplets just after application on the root hair, we infer that the shape of the droplet after spot application is approximately a half sphere. Subsequently, we measured the diameter from 33 applied droplets in µm, and calculated their volume, which appeared to be 1 pl (+/- 0.5 SD).

NF concentration

The concentration of the NF-stock was measured as described (*13*). In short, the measurement is based on the Kean partitioning assay, in which sulphated hydrophobic molecules, SDS for calibration or the NF molecules, form a complex with methylene blue. This hydrophobic complex is subsequently partitioned from the methylene blue solution with a 40% n-butanol 60% heptane (v/v) mixture, whereafter the absorbance of the methylene blue in the organic phase is measured at 655nm.

Pro$_{MtENOD11}$:GUS Expression

Pro$_{MtENOD11}$:GUS expression was assessed by incubating the seedlings for 24-h in the β-glucuronidase substrate X-Gluc (2 mM 5-bromo-4-chloro-3-indolylglucuronide, 1% [w/v] dimethylformamide, 0.1 mM $K_3[Fe(CN)_6]$, 0.1 mM, $K_4[Fe(CN_6)]\cdot 3H_2O$, 1 mM

EDTA, and 50 mM KH_2PO_4, pH 7.0) at 37ºC. Images were recorded with a CCD camera (Sony, Tokyo) linked to an upright Nikon Optiphot DIC microscope.

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Chapter 5

Nod factor signalling: A rapid Ca^{2+} influx required for root hair curling and a putative G-protein coupled phosphoinositide pathway leading to *Pro$_{MtENOD11}$-Gus* expression

John J. Esseling and Anne Mie C. Emons

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

ABSTRACT

Nodulation factors (NFs) are specific signal molecules excreted by rhizobia in order to establish a nitrogen fixing symbiosis with legume plants. When applied in purified form, these NFs elicit a range of symbiotic responses in the host's root hairs. To test which signalling processes these NFs elicit in the root hairs, we used drugs interfering with calcium ions and G-protein coupled phosphoinositide signalling, and used them in combination with NF spot application. The results that we obtained again show an early split in NF induced signalling in the root hairs. One branch involves a Ca^{2+} influx and leads to root hair curling, the other branch includes a putative G-protein induced phosphoinositide pathway leading to $Pro_{MtENOD11}$-*GUS* expression.

INTRODUCTION

Legume plants can live in symbiosis with bacteria of the genus *Rhizobium*. In response to flavonoids that are excreted by the legume root, the rhizobia start to make specific signalling molecules. These so-called nodulation factors, or nod factors (NFs), induce in their turn symbiotic responses in the legume root (Fisher and Long, 1992). In response to the NFs, the root hairs entrap bacteria in a root hair curl, from which an infection thread starts to grow which delivers the bacteria into the root cortex, the place where the nitrogen fixing nodule develops. To better understand the effects of NF on legume root hairs, various studies have been carried out in which purified NF is applied to legume roots. From such studies, we know that NF induce ion fluxes in and out the root hairs (Ehrhardt et al., 1992; Felle et al., 1995, 1996, 1998, 1999a, 1999b; Cárdenas et al., 1999; Shaw and Long, 2003; review: Cárdenas et al., 2000), changes in the root hair actin cytoskeleton (Cárdenas et al., 1998; Miller et al. 1999; de Ruijter et al., 1999; review: Esseling et al., 2000), calcium (Ca^{2+})- spiking (Ehrhardt et al., 1996; Wais et al., 2000; Walker et al., 2000; Shaw and Long, 2003), root hair deformation (Heidstra et al., 1994; review: Miller et al. 1997), increase of Ca^{2+} during to root hair deformation (de Ruijter et al., 1998), expression of various early nodulin genes (ENODs; Scheres et al., 1990; Pichon et al., 1992; Yang et al., 1993; Pingret et al., 1998; Compaan et al., 2001; Journet et al., 1994; 2001) and cell divisions in the root cortex (Review: Kijne, 1992; Timmers et al., 1999).

Two commonly used possibilities to unravel signalling pathways in animal and plant cells are mutagenesis screens, in which point mutations are induced in the genome, followed by a study of the effects of these mutations, and pharmacological approaches, in which pharmacological agonists and antagonists with known functions are applied to the organisms or cells of interest, whereafter the effects of these drugs are scored. Here we report experiments in which we used drugs interfering with calcium ions and cellular signalling. The results show, like we have shown before (Esseling et al., 2004) that an early split in NF-induced signalling is likely, one branch leading to root hair curling and involving calcium ions, the other to *Pro$_{MtENOD11}$-GUS* expression, possibly via a G-protein coupled phosphoinositide pathway, the latter in line with the work of Pingret et al. (1998) and den Hartog et al. (2001).

RESULTS AND DISCUSSION

Drugs were applied at physiological conditions

Signal transduction utilizes the molecules and proteins that are already present in the cells. Thus, the inherent danger of using pharmacological agents to study signal transduction pathways is that these drugs interfere with physiological processes that are going on in the cell. Therefore, we tested whether the drugs had an effect on the not activated cells by globally applying concentration ranges of each drug to root hairs growing in liquid medium, and scoring disturbance of the cytoarchitecture of growing root hairs, cytoplasmic

streaming and root hair growth in. We used the highest concentration which just did not the cells by the above mentioned criteria.

A NF induced elevated level of cytoplasmic calcium ions, $[Ca^{2+}]_c$, on one side of the root hair tip induces root hair reorientation.

One of the fastest physiological responses of *M. sativa* root hairs to global NF application is a rapid decrease of the Ca^{2+} concentration in the medium surrounding root hairs within seconds after NF application (Felle et al., 1998). Later, Felle et al. (1999) showed that this decrease might be caused by an influx of Ca^{2+} in the root hairs. More evidence for this has been shown by Cárdenas et al. (1999). These authors show that within 5 minutes after NF application the Ca^{2+} influx into the root hair tip increases two to four times compared with untreated root hairs, leading to an elevated level of Ca^{2+} in the root hair tip. Moreover, Shaw and Long (2003) showed that the NF-induced Ca^{2+} influx is independent of NF-induced Ca^{2+}-spiking, and that this Ca^{2+} influx is maintained in the non-nodulating mutants *dmi1*, *dmi2* and *dmi3*, which show root hair reorientation upon NF spot application (Esseling et al., 2004).

Gd^{3+} ions are known to block stretch activated/voltage operated Ca^{2+} channels (Yang and Sachs, 1989) and they block the Ca^{2+} influx in maize egg cells after gamete fusion (Antoine et al., 2000, 2001). When Gd^{3+} was applied in a gradient, pollen tubes grow away from the highest Gd^{3+} concentration (Malhó and Trewavas, 1996), and these ions block growth pulsations in *Petunia* pollen tubes (Geitmann and Cresti, 1998). When globally applied onto *M. truncatula* root hairs growing in between glass slides, we observed concentration dependent effects. The highest concentration Gd^{3+} at which root hair growth continues with an undisturbed cytoarchitecture is 25µM, whereas higher concentrations disturb the cytoarchitecture, and consequently, inhibit root hair growth.

Spot application of 25µM Gd^{3+} on the side (n=11) or on the extreme tip (n=6) of the root hair had no observable effects on root hair growth. This is in line with the results obtained with global application of Gd^{3+}. Interestingly, spot application of a 25µM Gd^{3+} mixed with 10^{-9}M NF on the side of the tip of growing *M. truncatula* root hairs did not result in root hair reorientation (n=16; Table 1, Figure 1A).

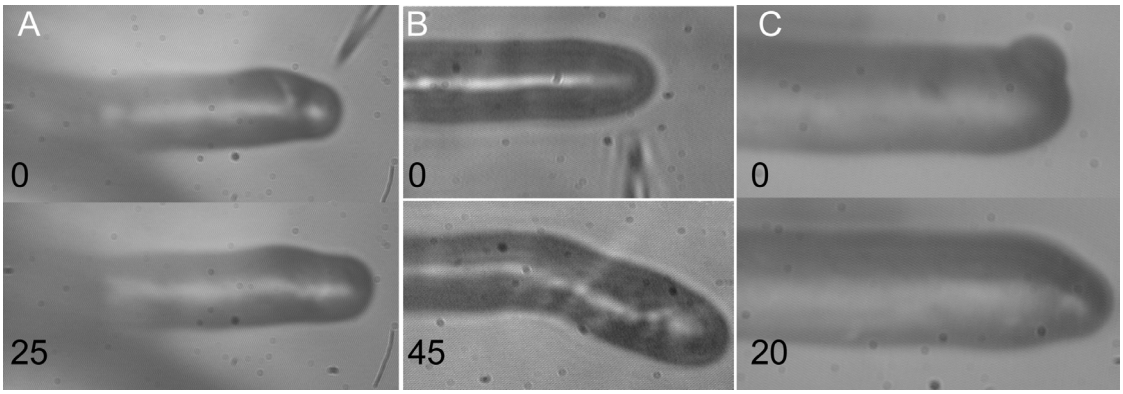

Figure 1. Time series after spot application of Ca^{2+} inhibiting or enhancing molecules. A**. Spot application of 2µM Gd^{3+} in combination with 10^{-9}M NF. Root hair growth is continuous, and in its original growth direction.** B**. Spot application of the Ca^{2+} ionophore A23187 in combination with 2µM Ca^{2+}. Six out of 38 root hairs reoriented towards the site of application.** C**. Spot application of the Ca^{2+} chelator EGTA results in a slight reorientation of the root hair tip away from the spot of application. Time is in minutes.**

After the application, the root hairs continued to grow in their original growth direction. Therefore, we can conclude that a local NF-induced Ca^{2+}-influx in a legume root hair is required for root hair reorientation, and, because NF-induced Ca^{2+} spiking is not inhibited by 100µM Gd^{3+} (Engstrom et al., 2001), that there is an early split in NF-induced signalling. Most likely, at the site of NF application, the $[Ca^{2+}]_c$ at the plasma membrane increases and the growth direction of the root hair shifts toward this high $[Ca^{2+}]_c$. This has been observed in pollen tubes that reorient their growth direction towards high Ca^{2+}, when microinjected caged calcium is uncaged in one side of the growing pollen tube tip (Malhó and Trewavas, 1996). In addition, when the caged ionophore A-23187 is uncaged in one side of the tip of growing Arabidopsis root hairs, the $[Ca^{2+}]_c$ in that side of the root hair tip increases, and the root hair reorients its growth direction towards this high $[Ca^{2+}]_c$. By using the free form of this ionophore, Felle et al. (1998) showed that application of this molecule to *M. sativa* root hairs mimics the NF induced Ca^{2+} influx and that it induces membrane depolarization. We hypothesized that for spot application we could use this ionophore to locally increase $[Ca^{2+}]_c$ on one side of the root hair tip, thus mimicking a Ca^{2+} influx. To make sure that calcium was present in the applied droplet, we mixed in some $CaCl_2$. Upon spot application of this 2µM A-23187/ 2µM $CaCl_2$ mixture, we observed that some root hairs reorient their growth axis toward the site of application (n=6 out of 38 root hairs; Table 1, Figure 1B), whereas the other stopped growing. This indicates that a Ca^{2+} influx can lead to growth reorientation in *M. truncatula* root hairs. The fact that the other 32 root hairs stopped growing might indicate a complete disruption of Ca^{2+} at the root hair tip, since uncaging of caged A-23187 sometimes resulted in burting of the root hair tip (Bibikova et al., 1997)

Because in pollen tubes, local uncaging of an injected caged Ca^{2+} chelator in one side of the pollen tube tip gives reorientation away from the chelator (Malhó and Trewavas, 1996), we hypothesized that spot application of a Ca^{2+} chelator would result in a reorientation away from the application. This further would show that an elevated level of $[Ca^{2+}]_c$ on one side of the tip results in reorientation towards that higher calcium. Therefore we spot applied the calcium chelator EGTA. We observed a slight reorientation of the root hair tip away from the application site in the first 15 à 20 minutes after application (n=13 out of 13, Table 1, Figure 1C), but thereafter, root hair growth rapidly continued in its original direction. Repeated spot applications 15 à 20 minutes after the first application (n=19) did not enhance the original reorientation (not shown). All root hairs under study stopped growing after the second application. This shows that, at least for a short moment, the EGTA has an effect on root hair tip growth, which is in accordance with earlier results obtained for Arabidopsis root hairs (Bibikova et al., 1997). The observation that an additional application of EGTA leads to growth arrest is not unexpected, since without Ca^{2+} tip growth is not possible (de Ruijter et al., 1998).
The question remains however, what the source of the Ca^{2+} is in the experiments in which Ca^{2+} was not mixed with the spot application liquid. Since the root hairs are growing in air, they cannot take up Ca^{2+} up from the surrounding medium. Most likely, the Ca^{2+} ions come from the cell wall, in which the Ca^{2+} cross link the pectins (Carpita and Gibeaut, 1993). Another possibility is that the applied droplet contains enough Ca^{2+} ions. Although we use demineralised water to dilute the NF stock and to dissolve the ions, it can be that it takes up Ca^{2+} ions from the glass of the micropipette.

Role of G-proteins in Nod Factor induced root hair reorientation and *Pro$_{Mt\text{-}ENOD11}$-GUS* expression: the use of mastoparan and pertussis toxin to study NF signalling.

Mastoparan is a cationic peptide of 14 amino acids which was isolated from wasp venom (Ross and Higashijima, 1994). In animal systems, it mimics the activated intracellular domain of G protein-coupled receptors, thus activating heterotrimeric G proteins (Ross and Higashijima, 1994). It has been shown that application of mastoparan to legume roots mimics NF. In *V. sativa*, application of mastoparan induces root hair deformation and activation of phospholipase C (PLC) and phospholipase D (PLD), resulting in an increased concentration of phosphatidic acid (PA) and diacylglycerol pyrophosphate (DGPP; den Hartog et al., 2001). In *M. sativa* root hairs, it induces *Pro$_{MtENOD12}$-GUS* expression at a concentration of 1µM (Pingret et al., 1998). We used the same concentration for spot application on the side of the tip of growing root hairs. None of the root hairs after spot application (n=46/46; Table 1, Figure 2B) showed reorientation of the growth axis. However, upon spot application on *Pro$_{MtENOD11}$-GUS* root hairs (n=6/6; Table 1, Figure 2B), *Pro$_{MtENOD11}$-GUS* expression was detected, showing that spot application of

mastoparan partially mimics NF application. What we can conclude from these results is that NF-induced root hair reorientation and *Pro$_{MtENOD11}$-Gus* expression are not coupled to each other, the one can occur without the other. This is in agreement with our earlier work (Esseling et al., 2004), in which we demonstrated that growing root hairs of the nodulation defective *M. truncatula dmi2/NORK* mutant also show root hair reorientation toward spot applied NF, without *Pro$_{MtENOD11}$-Gus* expression.

When compared with animal cells, heterotrimeric G proteins appear not to be very abundant in plant cells; so one might doubt the involvement of these proteins in NF induced signalling. The Arabidopsis genome contains a single G_α gene (Ma et al., 1990), in polyploid species like soybean, two different G_α copies have been identified, and a possible second class of G_α genes has been found in wild oat (reviewed in Assmann, 2002). Also, the G_β gene has only a single copy in Arabidopsis (reviewed in Assmann, 2002) and from G_γ, two copies have been found in Arabidopsis (Mason and Botella, 2000, 2001). It can be that there are just no more classes of heterotrimeric G proteins in plant cells, or it can be that splicing varieties exist, that have not been found yet. Another possibility which we can not exclude is that, like in animal cells, mastoparan activates the so called small G-proteins (Koch et al., 1991), proteins from which it is known that they exist in plants (Valster et al., 2000) and that they are involved in root hair and pollen tube tip growth (Li et al., 1999; Molendijk et al 2001). However, we can also use pertussis toxin (ptx). Ptx, a toxin from *Bordetella pertussis* bacteria is a well-characterized specific inhibitor of animal heterotrimeric G-proteins (Bokoch et al., 1983). By catalyzing the ADP ribosylation of a cysteine residue on the 41kD G_α subunit, it interferes with the interaction with the receptor, what results in a block of heterotrimeric G-protein activation. Also in plant cells, it has been shown to ribosylate a 41kD G_α protein that cross reacts with antibodies against the animal G_α subunit (Ma et al., 1999), and it inhibits a variety of putative heterotrimeric G-protein mediated processes (Wu and Assmann, 1994; Ritchie and Gilroy, 2000), including inhibition of NF induced *Pro$_{MtENOD12}$-GUS* expression (Pingret et al., 1998). To determine the involvement of heterotrimeric G-proteins in NF induced root hair reorientation, ptx was spot applied in combination with 10^{-9}M NF. As a control, 1 µg/ml ptx alone was applied on the side of growing root hairs, which did not affect root hair growth (n=23/23; Table 1). In combination with 10^{-9} M NF, ptx does not inhibit root hair reorientation (n=37/37; Table 1, Figure 2A), but inhibits *Pro$_{MtENOD11}$-GUS* expression (n=9/9; Table 1, Figure 2A). This shows that, maybe besides mastoparan activated small G-proteins, pertussis toxin inhibited heterotrimeric proteins are involved in NF induced signalling.

Role of PLC in Nod Factor induced root hair reorientation and *Pro$_{MtENOD11}$-GUS* expression

In plant guard cells, phosphoinositide-specific phospholipase C activity is inhibited by U-73122 (1-(6-{[17ß-3-methoxyestra-1,3,5(10)-trien-17-

yl]amino}hexyl)-1H-pyrrole-2,5-dione; Staxén et al., 1999). Moreover, it has been shown that in *Medicago* sp. this drug inhibits NF induced Ca^{2+} spiking (Engstrom et al., 2002) and inhibits *Pro$_{MtENOD12}$-GUS* expression (Pingret et al., 1998). Spot application of 1µM U-73122 on the side of the tip of growing root hairs had no visible effect on root hair growth (n=18; Table 1). In combination with 10^{-9} M NF, spot application of 1µM U-73122 resulted in root hair reorientation toward the application site in all 24 applied root hairs (Table 1, Figure 2B), indicating that U-73122 does not inhibit NF-induced root hair reorientation. In all these root hairs, *Pro$_{MtENOD11}$-GUS* expression is blocked completely (Table 1, Figure 2D), indicating that U-73122 inhibits NF induced *Pro$_{MtENOD11}$-GUS* expression. A nice control for U-73122 is the inactive analog U-73343 (1-(6-{[17ß-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl)-2,5-pyrrolidinedione; Staxén et al., 1999, Engstrom et al., 2002).

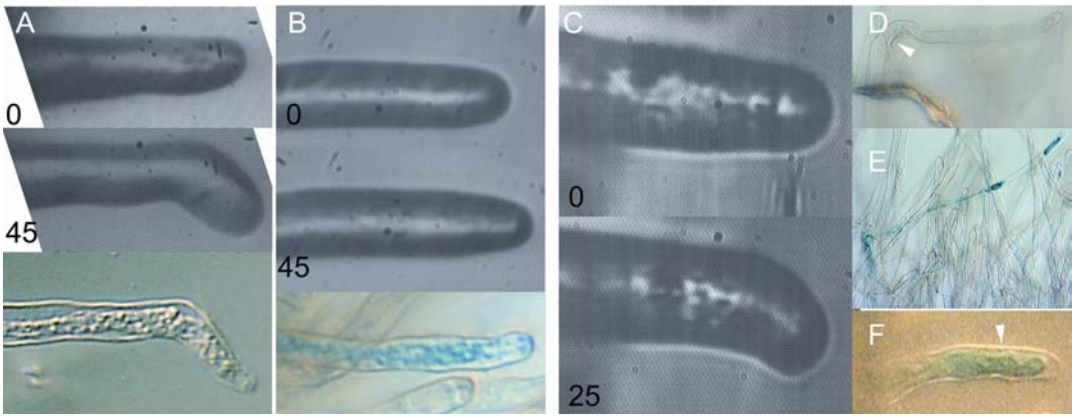

Figure 2. Spot application of agonists and antagonists of a heterotrimeric G-protein coupled phosphoinositide signalling pathway, and uncaging of microinjected caged IP_3.

A. Application of the heterotrimeric G-protein inhibitor pertussis toxin, mixed with 10^{-9}M NF does not inhibit NF-induced root hair reorientation, but results in the absence of *Pro$_{MtENOD11}$-GUS* expression. **B**. Spot application of 1µM mastoparan does not result in root hair reorientation, but the *Pro$_{MtENOD11}$-GUS* gene is expressed. **C**. Spot application of the PLC inhibitor U-73122, in combination with 10^{-9}M NF does not inhibit root hair reorientation, but inhibits expression of the *Pro$_{MtENOD11}$-GUS* gene. Arrowhead points to the site of application (**D**). **E** shows a picture of a root hair after spot application of U-73343, the inactive control molecule for U-73122, mixed with 10^{-9}M NF. The root hair shows reorientation of its growth axis, and *Pro$_{MtENOD11}$-GUS* gene is expressed. **F**. *Pro$_{MtENOD11}$-GUS* expression after global uncaging of microinjected caged IP_3. Arrowhead points to the site of injection.

Spot application of U-73343 alone had no observable effects on root hair growth (n=19; Table 1), and when combined with 10^{-9} M NF, it did not inhibit NF-induced root hair reorientation or *Pro$_{MtENOD11}$-GUS* expression (n=18 out of 18 root hairs; Table 1, Figure 2E).

Neomycin is another PLC inhibitor which has been successfully used in NF studies (Pingret et al., 1998, Den Hartog et al., 2001). In *V. sativa*, it inhibits

NF induced root hair deformation and formation of PA (Den Hartog et al., 2001), and in *M. sativa* it inhibits NF induced $Pro_{MtENOD12}$-*GUS* expression (Pingret et al., 1998). Spot application of 100µM Neomycin alone did not affect root hair growth (n=14; Table 1), and in combination with 10^{-9} M NF, root hair reorientation was not inhibited (n=17; Table 1). However, when spot applied on $Pro_{MtENOD11}$-*GUS* root hairs, $Pro_{MtENOD11}$-*GUS* expression was completely inhibited (n=9; Table 1). These results show that NF-induced gene expression might be regulated by PLC activity, possibly via calcium spiking.

Table 1

Drug	Concentration[a]	Number of applied hairs	Number of reorientations	N GUS expression
Ptx	2.5 µg/ml	23	0*	nd
Ptx + NF (10^{-9}M)	2.5 µg/ml	37	37	9 out of 9
Mastoparan	1µM	46	0*	6 out of 6
U73122	1µM	18	0*	0
U73122 + NF (10^{-9}M)	1µM	24	24	0
U73343	1µM	19	0*	0
U73343 + NF (10^{-9}M)	1µM	18	18	18
Neomycin	100µM	14	0	0
Neomycin + NF (10^{-9}M)	100µM	17	17	9 out of 9
Gd^{3+}	25µM	11	0	nd
Gd^{3+} + NF (10^{-9}M)	25µM	16	0	nd
A23187 (+ 2µM $CaCl_2$)	2µM	38	6	nd
EGTA	2mM	13	13[#]	nd
IP_3 injection	100µM	8	--	3

[a] All concentrations are concentrations in the needle.
* Root hair growth was not affected, root hairs were growing straight after application.
[#] Root hairs slightly reoriented away from the application, but root hair growth resumed its original direction after 15 to 20 minutes.

Microinjection and subsequent uncaging of caged IP_3 induces $Pro_{MtENOD11}$-*GUS* expression

A mode of action of PLC is hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into the second messenger inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Munnik et al., 1998). IP_3 releases Ca^{2+} from internal stores. Since we now show that inhibition of PLC via neomycin or U-73122 inhibits NF-induced $Pro_{MtENOD11}$-*GUS* expression, and not root hair reorientation, we hypothesized that application of NF induces an increase of IP_3, which then would result in gene expression. To test this, we microinjected caged IP_3 (needle concentration 100µM) into $Pro_{MtENOD11}$-*GUS* root hairs, which was subsequently uncaged with a UV laser when the root hair recovered from the microinjection. Six hours

after the uncaging, the roots were put in the GUS reagent, and were checked for *Pro$_{MtENOD11}$-GUS* expression. Some of the injected hairs showed GUS expression (n=3 root hairs out of 8 injected; Table 1, Figure 3), indicating that release of IP_3 via uncaging may induce *Pro$_{MtENOD11}$-GUS* expression, thus mimicking NF. Since it was shown that inhibition of PLC inhibits Ca^{2+} spiking (Engstrom et al., 2001), it might be that NF-induced IP_3 release is triggering Ca^{2+} spiking. In addition, modelling predicted that repeated IP_3 release is involved in activation of Ca^{2+} oscillations (de Young and Keizer, 1992). This might explain why the GUS staining in the IP_3 injected root hairs was not as strong as it would be after NF application. We uncaged the injected IP_3 by scanning the whole root hair once with the UV laser, thus releasing the IP_3 from its cage at once. Possibly, repeated pulses of the UV laser around the nucleus, where the NF induced calcium spikes originate (Ehrhardt et al., 1996), might result in a stronger GUS expression.

NF induced signalling.

Recent genetic approaches in the model legumes *Medicago truncatula* and *Lotus japonicus* have resulted in the identification of eight to ten genes coding for proteins that are involved in the recognition of the NF and the transduction of the signals, leading to the symbiotic responses. Transmembrane serine-threonine receptor-like kinases with extracellular Lys-M domains have been shown to be involved in specification of bacterial infection (Limpens et al., 2003) and in NF recognition (Madsen et al., 2003; Radutoiu et al., 2003), and it is hypothesized that they form heterodimeric NF receptors. Three more downstream genes, *DMI1*, *DMI2/NORK* and *DMI3* have recently been cloned in *M. truncatula* (Ané et al., 2004; Lévy et al., 2004; Endre et al., 2002). The *DMI1* gene is a novel gene that might code for a ligand-gated cation channel (Ané et al., 2004) and the *DMI3* gene likely codes for a calcium and calmodulin-dependent protein kinase (Lévy et al., 2004). Therefore, DMI3 might be involved in NF-induced calcium spiking. *DMI2/NORK* (Nodulation Receptor-like Kinase), encodes a leucine rich repeat (LRR) receptor-like protein kinase (Endre et al., 2002). Besides having a role in setting up a successful symbiosis, we found that DMI2/NORK is also involved in non-symbiosis-related cellular processes in root hairs (Esseling et al., 2004 Symbiosis-specific signalling uses the already present signalling proteins in the root hairs – one might say that NF signalling taps into already existing pathways. Therefore it is not surprising that besides the 'genetic' pathway (Lys-M proteins, Dmi1, 2 and 3, and other genes), also the phosphoinositide signal pathway, a general signalling pathway in plant cells (Munnik et al., 1998), is involved in NF signal transduction. Now it will become exciting to study how these pathways are interconnected with each other. A way how to study this could be via RNA_i studies in which enzymes of heterotrimeric G-protein coupled phosphoinositide signalling are knocked down in mutants that are found in mutagenesis screens.

METHODS

Plant Preparation

Seeds of *M. truncatula* jemalong A17 and *M. truncatula* jemalong carrying a p*MtENOD11-GUS* fusion construct were scarified in concentrated sulphuric acid for 10 min and thoroughly washed with running demineralised water. Seeds were then sterilized in a mixture of 30% hydrogen peroxide and 96% ethanol (1:1) for 2 minutes and extensively rinsed with sterile demineralised water. Seeds were subsequently imbibed overnight in sterile demineralised water and allowed to synchronize for 4 days at 4ºC on plates containing 0.8% (w/v) agar in sterile demineralised water. After germination at 25ºC, seedlings were transferred to fresh agar plates (0.8% (w/v) agar in sterile demineralised water) and allowed to grow for 8 to 10 days at 25ºC with a 16/8 hours light/dark rhythm in a slightly oblique position from the vertical. In such growth conditions, the root hairs that develop in air above the agar are suitable for spot application.

Spot Application Assay

A water pressure microinjection device (water pressure device: Gilmont, Barrington, IL, USA; needle holder: Eppendorf, Merck Eurolab BV, Amsterdam, The Netherlands) was used to apply micro-droplets of purified NF [NodRm-IV(C16:2, Ac, S)] diluted with Millipore water to a final NF concentration of 10^{-9} M. Root hair growth was followed with a video camera linked to an inverted Nikon Diaphot microscope.

Pro$_{MtENOD11}$-GUS Expression

Pro$_{MtENOD11}$-GUS expression was assessed by incubating the seedlings for 24 h in the β-glucuronidase substrate X-Gluc (2 mM 5-bromo-4-chloro-3-indolylglucuronide, 1% [w/v] dimethylformamide, 0.1 mM $K_3[Fe(CN)_6]$, 0.1 mM $K_4[Fe(CN_6)] \cdot 3H_2O$, 1 mM EDTA, and 50 mM KH_2PO_4, pH 7.0) at 37ºC (Journet et al., 1994). Images were recorded with a Panasonic CCD camera linked to Nikon Optiphot upright DIC microscope.

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Chapter 6

Dissection of Nod factor signalling in legumes: cell biology, mutants, and pharmacological approaches

John J. Esseling 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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ABSTRACT

Nodulation factors (NFs) are lipochitooligosaccharide signal molecules which are excreted by soil-living rhizobia. These molecules elicit a range of responses in the legume roots, with which the bacteria can live in symbiosis. In this review we will focus on the genetic, pharmacological and cell biological approaches which have been, and are, undertaken to decipher the signalling pathways which lead to the symbiotic responses in the plant.

I INTRODUCTION

Nitrogen. Without realising it, we all breathe it in and out. But our body is unable to use this gas for production of amino acids and other organic nitrogen containing organic molecules. For this, we highly depend on the nitrogen fixing symbiosis between legume plants and soil living rhizobia (bacteria of the genera *Rhizobium, Bradyrhizobium, Azorhizobium, Mesorhizobium* and *Sinorhizobium*). A first requirement for establishing a symbiosis is the entrapment of the bacteria between root hair cell walls, usually via root hair curling. Then, the bacteria invade the host root via a plant derived infection thread through the root hair(s) and the cortex cells (Review: Nutman, 1956; Gage, 2002). Bacteria induced cell divisions in the cortex result in the development of a new plant organ, the root nodule. Located in this nodule, the bacteria are provided with photosynthate. In return, and tightly controlled by an amino acid cycling between host and symbiont (Lodwig *et al*., 2003), the bacteria convert atmospheric nitrogen into ammonia, a form that can be readily assimilated by the host plant.

In this review we focus on the signalling events between bacteria and host root hairs, a complex process in which a lot of genes, and molecular signalling, is involved.

II Lipochito-oligosaccharides, the signal molecules

Genetic analysis of *Rhizobium* has lead to the identification of a set of genes, the *nod* (nodulation) genes, as the key bacterial genes required for symbiosis. Mutations in the *nodABC* genes, which are common in all rhizobia, resulted in a complete abolition of all plant responses to the bacteria (Dénarié *et al*., 1992; Fisher and Long, 1992). Moreover, other *nod* genes, like *nodH, nodPQ, nodFE* and *nodL* for *Sinorhizobium meliloti*, determine the host specificity of the bacteria to the legumes. It was found that the *nod* genes are involved in the synthesis of lipochito-oligosaccharide molecules (Dénarié *et al*., 1992; Fisher and Long, 1992). In general, these lipochito-oligosaccharides consist of a backbone of β-1,4-linked tetramers or pentamers of D-glucosamine, synthesised by the common *nodA,B,C* gene products (Lerouge *et al*., 1990; Roche *et al*., 1991a; 1991b; Spaink *et al*., 1991; Schultze *et al*., 1992). The species specific nod genes decorate this backbone. In *S. meliloti*, the *nodFE* genes code for acyl carrier proteins and β-ketoacyl synthases (Spaink *et al*., 1991; Demont *et al*., 1993; Ardourel *et al*., 1994). These proteins synthesise the poly-unsaturated fatty acid chains which mono-N-acylate the terminal non-reducing residue of the chito-oligosaccharide backbone. NodL, homologous to acetyl transferases, is required for the O-acetylation of the terminal non-reducing glucosamine residue (Spaink *et al*., 1991). The NodPQ and NodH proteins are homologues to ATP sulphurylase, adenosine 5' phosphosulphate kinase, and sulphotransferases. They control the O-sulphation of the reducing glucosamine residue of the lipochito-oligosaccharide molecule, and, by this, determine the specificity of *S. meliloti* for the legumes *Medicago sativa* and *Medicago truncatula* (Roche *et al*., 1991a; Truchet *et al*., 1991; Schwedock and Long, 1990). Without sulphate on the lipochito-oligosaccharide, no symbiosis is possible between *S. meliloti* and

M. sativa or *M. truncatula*. Thus, the lipochito-oligosaccharides, the so-called nodulation factors, or Nod Factors (NFs), are the bacterial signal molecules required for symbiosis.

III Root hair cytoarchitecture and development

Root hairs are tip-growing extensions of epidermal cells, the trichoblasts. Like all plant cells, they grow by exocytosis, incorporating the membranes of Golgi vesicles into plasma membranes, and their content into a flexible wall. The first stage of root hair development is bulge formation (Haberlandt, 1887; Dolan *et al*., 1994). Most of the cell is still completely vacuolated, the nucleus is opposite to the bulge (Sieberer and Emons, 2000; Miller *et al*., 2000), there is no apparent vesicle accumulation in the dome of the bulge, and the ER is transverse to the future root hair axis (Miller *et al*., 2000). Also, the levels of calcium are equally distributed within the cytoplasm of these cells (de Ruijter *et al*., 1998). The actin cytoskeleton in these bulges is characterised by filament bundles in the cytoplasm along the plasma membrane, longitudinal to the root axis. This is in the same orientation as in the trichoblasts that have not formed a bulge yet (Miller *et al*., 1999; review: Esseling *et al*., 2000). In living trichoblasts of *M. truncatula*, cortical microtubules (CMTs) are oriented transverse to the long axis of the root, while toward both ends of the cell, with respect to the bulge, the CMTs are more oblique (Sieberer *et al*., 2002). CMTs in the bulge are continuous with the CMTs of the epidermal part of the cell, and loop through the dome of the bulge (Sieberer *et al*., 2002).

At the onset of root hair tip growth, a steep calcium gradient develops in the tip (de Ruijter *et al*., 1998) and cytoplasm accumulates at the apex of the bulge. In the light microscope a specific polar cytoarchitecture becomes visible. This cytoarchitecture ischaracterised by a smooth, homogeneous appearance of the cytoplasmat the root hair tip, followed by an accumulation of more granular cytoplasm in the subapex, with the nucleus at its base. This is called the tip growth unit (Miller *et al*., 1997; de Ruijter *et al*., 1998; Miller *et al*; 1999; Sieberer and Emons, 2000). Electron microscopy shows that the smooth area in the tip contains primarily (exo- and endocytotic) vesicles, while the subapex contains the organelles, with the ER longitudinally oriented along the root hair growth axis (Miller *et al*., 2000). Below the subapical cytoplasmic dense region, the tube of the root hair is almost completely filled with the central vacuole, surrounded by a thin layer of cytoplasm Sieberer and Emons, 2000; Miller *et al*., 2000; review: Ketelaar and Emons, 2001). In these growing hairs, bundles of actin filaments (F-actin) are present in cytoplasmic strands that lay around or traverse the central vacuole (Miller *et al*., 1999). In the cytoplasmic dense area, the thicker F-actin bundles flare out into thinner and thinner bundles, the fine F-actin (Ketelaar *et al*. 2002, 2003; previously called FB-actin: Miller *et al*. 1999 and de Ruijter *et al*. 1999). The extreme tip of the root hair, which coincides with the vesicle rich area, is devoid of detectable F-actin (Miller *et al*., 1999). In growing *M. truncatula* root hairs, CMTs are longitudinal in the lower part of the root hair tube, and net-axial in the subapex (Sieberer *et al*., 2002). In GFP-Microtubule

Binding Domain of mouse Map4 (GFP-MBD) labeled living root hairs as well as in immunolabeled freeze-fixed/freeze-substituted root hairs, no CMTs could be detected in the very root hair tip. In addition, *M. truncatula* root hairs have an array of endoplasmic microtubules (EMTs; Sieberer *et al*., 2002), which has not been described yet in non-legume root hairs. This dense EMT array is exclusively located in the cytoplasmic dense region between the nucleus and the tip of the root hair. From drug studies, it can be concluded that these EMTs are involved in maintaining the cytoplasmic dense cytoarchitecture, including the position of the nucleus at 30 to 40 µm below the tip (Sieberer *et al*., 2002). In *Arabidopsis* root hairs this process in which the nucleus follows the growing root hair tip has been shown to be at least in part actin based (Ketelaar *et al*., 2002).
A first visible sign of root hair growth termination is the phenomenon that the vacuole overtakes the nucleus, preceded by extensions of the vacuole invading the subapical cytoplasmic dense area (Sieberer and Emons, 2000). At this moment, the root hair still has the growth potential to increase its length by approximately 10%. When extensions of the vacuole appear in the subapical cytoplasm, the nucleus starts moving away from the tip, which is a characteristic of root hair growth termination (Ketelaar *et al*., 2002; Sieberer *et al*., 2002), and the calcium gradient in the tip slowly dissipates (de Ruijter *et al*., 1998). In these growth terminating root hairs, the area with fine F-actin becomes shorter and shorter, and F-actin bundles can be detected in the extreme tip of the hair (Miller *et al*., 1999; de Ruijter *et al*., 1999). CMTs are net-axially oriented all along the root hair tube, and start reaching the very tip (Sieberer *et al*., 2002). As the cytoplasmic dense area gets shorter in these hairs, also the EMT array gets shorter, but as long as growth has not completely stopped, the extreme root hair tip remains devoid of EMTs (Sieberer *et al*., 2002).
In full-grown root hairs, cytoplasmic polarity is completely lost. The root hair is completely filled with the central vacuole, which is surrounded by only a thin layer of peripheral cytoplasm, and the nucleus can be found at any random position (Sieberer and Emons, 2000; Ketelaar *et al*., 2002; review: Ketelaar and Emons, 2001). The steep calcium gradient at the tip has completely disappeared, and the levels of calcium are equally distributed within the cytoplasm (de Ruijter *et al*., 1998). Thick bundles of actin filaments are found in the thin layer of cortical cytoplasm within the whole cell. Some bundles can loop through the tip, whereas other bundles turn before they even reach the tip (Miller *et al*., 1999; de Ruijter *et al*., 1999; review: Esseling *et al*., 2000). No detectable fine F-actin is present any more and there is no apical area devoid of F-actin. CMTs, in a lower density, converge at the tip, and EMTs are not present any more (Sieberer *et al*., 2002).

IV Observed Nod factor induced root hair responses

1 Root hair morphology

a) Root hair deformation and branching

The so-called root hair deformation is probably one of the best-characterised biological activities of global application of purified NF. Two slightly different root

hair deformation assays are therefore widely used to evaluate the specificity of NF towards a given legume host (Lerouge *et al*., 1990; Ardourel *et al*., 1994; Catoira *et al*., 2000), and to study the processes of tip growth (Heidstra *et al*., 1994; de Ruijter *et al*., 1998; Miller *et al*., 1999; 2000; Sieberer and Emons, 2000). The hair branching (Hab) response is the deformation which occurs after flooding an air grown root system with a NF solution (Ardourel *et al*., 1994). The other assay is based on the clover infection assay described by Fåhraeus (1957). Roots and root hairs are grown in liquid plant growth medium (PGM) between glass slides, and NF is applied by replacing the PGM for PGM with a low concentration of purified NF. In this assay, growth terminating root hairs deform. The process starts almost immediately after NF application with an increase of cytoplasmic strands in the tip of the growth terminating root hairs (Sieberer and Emons, 2000) and a gradual swelling of the root hair tip, from which approximately one hour later, an outgrowth emerges (Heidstra *et al*., 1994), like in the branching assay (Ardourel *et al*., 1994). This outgrowth exhibits all the characteristics of a growing root hair, with high calcium (de Ruijter *et al*., 1998), and a vesicle rich area at the extreme tip, followed by a subapical cytoplasmic dense region with the nucleus at its base (de Ruijter *et al*., 1998; Miller *et al*., 2000; Sieberer and Emons, 2000). Also the configuration of the actin cytoskeleton in the outgrowth is like in a growing root hair (Miller *et al*., 1999).

b) root hair curling

Global application of purified NF results in root hair deformation in growth terminating hairs (Heidstra *et al*., 1994; de Ruijter *et al*., 1998; Sieberer and Emons, 2000), whereas in the soil, young growing root hairs are the ones which entrap the bacteria (Kijne, 1992). Therefore, it has been thought that the presence of bacteria is essential for root hair curling to occur (Catoira *et al*., 2001). However, since NF is known to induce cell polarity (van Brussel *et al*., 1992), and to reinitiate tip growth (Heidstra *et al*., 1994; de Ruijter *et al*., 1998; Sieberer and Emons., 2000), it has been hypothesised that NF might be the inducing principle in bacteria-associated root hair curling (van Batenburg *et al*., 1986; Emons and Mulder, 2000). According to this model, root hair curling can only occur when at least the following conditions are fulfilled (van Batenburg *et al*., 1986): the attachment of one inducing principle (NF) within the growth area of the root hair, translocation of the inductor along the growing root hair tip, and redirection of the original plant-driven tip growth. To test this, a new assay was designed in which a little droplet of purified host specific NF is applied to the side of the tip of growing *M. truncatula* root hairs (Esseling *et al*., 2003). As a result of this, we observed an immediate reorientation of the root hair growth axis towards the site of application (Figure 1; Esseling *et al*., 2003). Multiple applications on the still growing root hair resulted in multiple reorientations, and when the applications were performed on the same side of the hair (= translocation of the inductor along the growing root hair tip), partial curls could be formed. Even, in rare cases, one single application resulted in 180° curls. When the droplet was applied 30 to 60 µm below the tip of the growing root hair,

the root hair tip ceased growth and, within 20 minutes, a root hair branch developed at the site of application (Esseling *et al*., 2003), as predicted in the computer simulations by van Batenburg *et al*. (1986). These results show that under the influence of the local presence of NF, a new growth axis is set up, and thus that root hair curling can be explained as a gradual continuous shift of the original growth axis towards the NF induced growth axis.

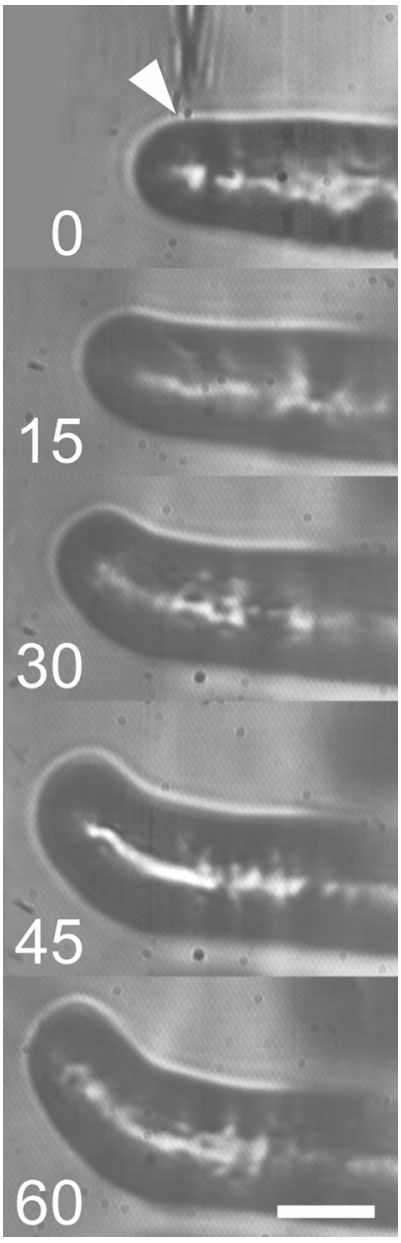

Figure 1. Root hair reorienation after spot application of 10^{-9} M purified Nod Factor (NF) on the side of the tip of a growing *M. truncatula* root hair.

Spot application of 10^{-9} M purified NF on a growing *M. truncatula* root hair results in root hair reorientation. 15 minutes after NF application, the reorientation of the root hair growth axis towards the site of application is already visible, and becomes more pronounced at 30 minutes. As can be seen, root hair growth is continuous during and after reorientation, and the root hair diameter does not change. Arrowhead indicates the site of application. Bar = 10 µm.

2) Ions

Upon application of purified NF to legume roots, a whole range of responses has been described (for reviews: Lhuissier *et al*., 2001; Geurts and Bisseling, 2002; D'Haeze and Holsters, 2002), varying from ion fluxes, changes in the actin cytoskeleton which lead to changes in root hair morphology and expression of ENODs, the early nodulin genes. Using non-invasive ion-selective microelectrodes, Felle *et al*. (1998) observed the fastest response described: an almost immediate decrease of Ca^{2+} in the growth medium surrounding alfalfa root hairs. This decrease could be blocked with the general calcium-channel blocker nifedipine, which indicates an influx of Ca^{2+} into the root hairs. With invasive ion-selective microelectrodes, Felle *et al*. (1999a; 1999b) really demonstrated an influx of Ca^{2+} in the alfalfa root hairs (Felle *et al*., 1999a; 1999b). This influx was visualised by Cárdenas *et al*. (1999; Review: Cárdenas *et al*., 2000) in early growth terminating *Phaseolus vulgaris* root hairs, and by Shaw and Long (2003) in growing *M. truncatula* root hairs as an increase of cytosolic Ca^{2+} in the root hair tip. Moreover, Shaw and Long (2003) showed that the calcium flux is biphasic in nature, since the initial Ca^{2+} flux was followed by a sustained elevated level of cytoplasmic Ca^{2+}.

Felle *et al*. (1998) also measured an increase of $[Cl^-]$ in the medium, which followed the rapid $[Ca^{2+}]$ influx within a few seconds. This is caused by a chloride efflux out of the root hairs, which leads to the earlier described NF induced membrane depolarisation (Ehrhardt *et al*., 1992; Felle *et al*., 1995; Kurkdjian, 1995). To rebalance the charges in the root hairs, this depolarisation is counteracted and subsequently stopped by a K^+ efflux, and a membrane repolarisation through activity of H^+ pumps (Felle *et al*., 1998).

Visualisation of NF-induced Ca^{2+} responses in root hairs has lead to the discovery of NF-induced calcium spiking. Initially in *M. sativa* root hairs (Ehrhardt *et al*., 1996), and later in more legume species, like *M. truncatula*, *Pisum sativum*, *Vicia sativa* and *Lotus japonicus* (Wais *et al*., 2000; Walker *et al*., 2000; Harris *et al*., 2003; Shaw and Long, 2003), Ca^{2+} sensitive fluorescent dyes were injected in root hairs, which were subsequently treated with NF. Interestingly, regular oscillations of cytoplasmic calcium were observed in the perinuclear area, starting about 9 minutes after NF application and with a frequency of one every minute (Ehrhardt *et al*., 1996). It appeared that the oscillations occurred as asymmetric spikes, with the initial phase of the spike faster than the falling phase. Once initiated, Ca^{2+}-spiking has been observed to continue as long as the period of experimental observations (20 to 60 minutes), and sometimes up to 3 hours after NF treatment.

Since the calcium spikes are perinuclear of origin, the authors suggest that the calcium stores or the channels that mediate the calcium release are localised in this region (Ehrhardt *et al*., 1996). From electron microscopy studies we know that ER is concentrated in this perinuclear area (Miller *et al*., 2000) which makes the ER the most probable source of the calcium, like in animal cells (review: Verkhratsky, 2002). A clue about the function of these regular oscillations in the level of cellular calcium may come from results obtained in animal systems

(Dolmetsch *et al*., 1998). Dolmetsch *et al*. (1998) developed a method to induce calcium spikes with various frequencies in T-lymphocytes, and found that in this way, they could regulate gene expression.

3) Actin cytoskeleton

The actin cytoskeleton is also a target of NF signalling. As soon as root hair tip growth reinitiates from a swelling, one hour or so after NF treatment, fine F-actin builds up again. Therefore, it can be postulated that NF induces fine F-actin. However, F-actin in root hairs responds much earlier to NF application. The first publications on this, report NF-induced fragmentation of the actin cytoskeleton. For *M. sativa*, Allen and Bennet (1996) describe discontinuities in the longitudinal actin bundles and the formation of discrete foci of phalloidin staining at the root hair tip. Microinjection of FITC-phalloidin and subsequent NF treatment of the injected *Phaseolus vulgaris* root hairs, shows an increase of fluorescent glow at the root hair tip (Cárdenas *et al*., 1998). This glow, like the results of Allen and Bennet (1996) has been interpreted as NF-induced fragmentation of F-actin. However, when actin filaments are broken down, cytoplasmic strands disappear (Valster *et al*., 1997; Miller *et al*., 1999), which is completely in disagreement with the fact that NF induces more cytoplasmic strands in the tip of growth terminating root hairs (*M. truncatula*: Sieberer and Emons, 2000), and that no fragmented actin filaments have been found after NF treatment (*V. sativa*: Miller *et al*., 1997; 1999; de Ruijter *et al*., 1999). In addition, microinjected Alexa-488-phalloidin in growing *Arabidopsis* root hairs (Ketelaar *et al*., 2002), and untreated *M. truncatula* root hairs (Esseling, unpublished observations), labels the actin cytoskeleton for a short time, but rapidly leads to cell death, or degradation of the labelling into fluorescent aggregates, which are taken up in the cytoplasmic strands (Esseling *et al*., 2000).

Using the optimised ester-aldehyde-choline fixation method (Miller *et al*., 1999), de Ruijter *et al*. (1999) were able to show that NF application to *V. sativa* root hairs results in the increase of bundles of fine F-actin in the subapical area of the root hairs within 3 minutes (Figure 2). This rapid NF induced increase in fine F-actin has been observed in all stages of root hair development. After quantification of this increase, an interesting difference appeared (de Ruijter *et al*., 1999). Upon NF treatment, the number of bundles of fine F-actin in full-grown root hairs did not exceed the number of bundles in fine F-actin in untreated growth terminating hairs.

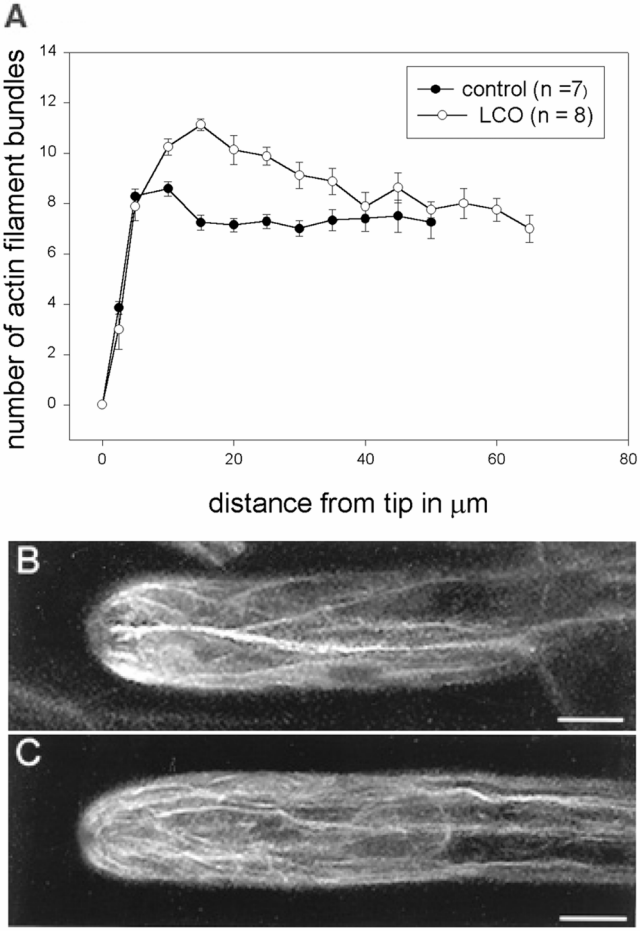

Figure 2. Increase in subapical fine F-actin in *Vicia sativa* root hairs within minutes after application.

A. Mean densities of actin filament bundles in growing root hairs (y-axis) *versus* distance from the root hair tip (x-axis). Error bars indicate SE. B. Actin cytoskeleton in a growing *Vicia sativa* root hair, 3 minutes after medium refreshment. C. Actin cytoskeleton in a growing *V. sativa* root hair, 15 minutes after global NF (LCO) application. Bar = 10 μm. Reprinted, with permission, from De Ruijter *et al*. (1999), Rhizobium Nod factors induce an increase in subapical fine bundles of actin filaments in *Vicia sativa* root hairs within minutes, Molecular Plant-Microbe Interactions 12:829-832.

According to these numbers, full grown root hairs just do not reach the threshold number of fine F-actin counted in growth terminating root hairs, the hairs that respond with root hair deformation (de Ruijter *et al*., 1999).

From the configuration of the actin cytoskeleton during root hair development, and especially the comparison with the configuration of the actin in the outgrowth after NF treatment (Miller *et al*., 1999; reviews: Esseling *et al*., 2000; de Ruijter *et al*., 2001), we could hypothesise that fine F-actin retains Golgi vesicles in the subapical area, and delivers them to the basis of the vesicle rich region, and thus mediate root hair tip growth. This was confirmed with the use of low concentrations of the actin depolymerising drug cytochalasin D (CD). Application of 1.0 μM CD did not inhibit bulge formation or NF-induced root hair swelling in growth terminating hairs, but inhibited initiation of polar growth from

bulges, root hair elongation, and NF-induced outgrowths from swellings (Miller *et al*., 1999). Moreover, the cell architecture and the configuration of the actin cytoskeleton of the growing hairs changed into that of growth terminating hairs, and those of growth terminating hairs into full grown hairs (Miller *et al*., 1999; review: Esseling *et al*., 2000). More about the role of F-actin in root hair tip growth became clear when even lower concentrations of CD were used. These experiments showed that the site of exocytosis coincides with sites where the actin cytoskeleton is most unstable (Ketelaar *et al*., 2003).

4) NF-induced Gene expression, the early nodulins (ENODs)

One of the latest root hair responses to NF is the expression of various symbiosis specific plant genes Most of these genes are known as the so-called early nodulin genes, or ENODs (Compaan *et al*., 2001; Journet *et al*., 2001; Pichon *et al*., 1992; Pingret *et al*., 1998; Scheres *et al*., 1990; Yang *et al*., 1993). Their expression ranges from approximately one to several hours after NF application, depending on the gene. The most detailed studied ENOD is ENOD12, which was initially isolated from a pea nodule cDNA library (Scheres *et al*., 1990). Like most of the ENODs, ENOD12 is a hydroxyproline rich glycoprotein, from which it is thought that it is a cell wall protein (Hong *et al*., 1990).

ENODs have proven to be valuable tools to study NF-induced signalling: promoters of the *M. sativa* ENOD12 (Journet *et al*., 1994; Pingret *et al*., 1998) and *M. truncatula* ENOD11 genes are coupled to β-glucuronidase (GUS; Vernoud *et al*., 1999; Journet *et al*., 2001). As such they serve as reporter genes to study NF induced responses.

V Dissection of NF induced signalling

1) Mutant analysis

From a molecule which induces such a wide variety of responses at the cellular level as the NF, it can easily be expected that it activates a complicated and fine-tuned signal transduction cascade. The biggest scientific challenge at the moment is to find out how this signal transduction cascade is activated and organised. One way to tackle this problem is to make use of plants which are disturbed in the early steps of nodulation. Natural nodulation mutants in the economically important legumes, such as pea, soybean and alfalfa (review: Caetano-Anollés and Gresshoff, 1991) were already used to gain insight into, and to understand the signalling processes (e.g. the natural pea SYM2 allele in the accession Afghanistan (reviewed in Geurts and Bisseling, 2002)). However, their large genome size (pea, soybean), polyploidy (soybean and alfalfa), and inefficient transformation and regeneration (pea, soybean) makes molecular and genetic analyses in these species difficult. Therefore, the ‘scientific legume community’ has chosen for the two relatively easy to transform and regenerate, diploid and autogamous *M. truncatula* (Barker *et al*., 1990; Cook, 1999) and *Lotus japonicus* (Schauser *et al*., 1998, 1999; Stougaard, 2001) as the two model legumes. EMS and γ-ray mutagenesis screens of these two legumes (*M.*

truncatula: Sagan *et al*., 1995; Penmetsa and Cook, 1997; 2000; *L. japonicus*: Szczygłowski *et al*., 1998) have resulted in numerous mutants which are either non-nodulating, low nodulating, nodulating but non-nitrogen-fixing, and supernodulating.

The *M. truncatula* Does not Make Infection (*DMI*) mutants are a group of mutants which are non-nodulating. There are eight described infection-defective mutants from the three complementation groups *DMI1*, *DMI2* and *DMI3* (Catoira *et al*., 2000). Studies in which NF-induced calcium responses were visualised (Wais *et al*., 2000; Shaw and Long, 2003), revealed that in *DMI1* and *DMI2* the rapid NF-induced Ca^{2+} influx (Felle *et al*., 1998) is sustained, while Ca^{2+}-spiking is absent in these mutants (Wais *et al*., 2000; Shaw and Long, 2003). In *DMI3* however, NF is able to induce the Ca^{2+}-spiking response, even at an approximately 10 times higher responsiveness to NF than wildtype (Wais *et al*., 2000). All *dmi* mutants have in common that they are defective for NF-induced p*MtENOD11::GUS* expression (Vernoud *et al*., 1999; Catoira *et al*., 2000), and that upon global application of purified NF, all the mutants respond with a swelling of the root hair tip, and occasional short, stumpy outgrowths (Catoira *et al*., 2000). All this shows that in these mutants, NF is still recognised, and that DMI1, DMI2 and DMI3 are somehow involved in reinitiation and maintenance of root hair tip growth. In addition, DMI3 seems to be involved in a negative feedback loop that regulates the Ca^{2+}-spiking response (Wais *et al*., 2000).

DMI2, and its orthologues from alfalfa (Nodulation Receptor-like Kinase – NORK), *L. japonicus* (Symbiosis receptor-like kinase – SYMRK), and pea (sym19), is the first gene involved in the early signaling events which has been cloned to date (Endre *et al*., 2002; Stracke *et al*., 2002). It encodes a receptor-like kinase that contains a characteristic extracellular domain with three leucine rich repeats, a transmembrane domain and an intracellular serine/threonine kinase domain. This structure indicates a role for DMI2 in the perception of a signal and in further signal transduction.

The recently described *M. truncatula* NFP (Nod Factor Perception) mutant (Ben Amor *et al*., 2003) is a mutant which upon application of NF or rhizobia, does not show any (early) response at all. It lacks the rapid NF-induced Ca^{2+} influx, Ca^{2+}-spiking is absent, the root hairs do not deform and there is no ENOD11 expression. Clearly, this *nfp* gene seems to be involved in initiation of the early signalling events, and might be very close just after - or at the point of NF perception, and might even code for the earlier proposed signalling receptor (Ardourel *et al*., 1994).

From other studies, in which wildtype legumes were inoculated with bacteria which produce mutant NF molecules, it appeared that from all NF induced plant responses, infection thread formation depends most on the structure of the NF molecules (Ardourel *et al*., 1994; Limpens *et al*., 2003). Inoculation with a *nodFnodL S. meliloti* mutant which produces NF which lack an acetate group at the nonreducing terminal glucosamine residue, and in which the C16:2 acyl chain is replaced by vaccenic acid (C18:1), results in most early NF induced plant responses, like entrapment of the bacteria in a curled root hair and

activation of cortical cell division (Ardourel *et al*., 1994; Limpens *et al*., 2003). However, the initiation and formation of an infection thread is highly inhibited and disturbed. Therefore, it was proposed that besides the above mentioned signalling receptor (Ardourel *et al*., 1994), a more specific receptor is required for the induction of infection thread formation, which was named the entry receptor (Ardourel *et al*., 1994; Catoira *et al*., 2001). Recently, Limpens *et al*. (2003) provided evidence for the presence and the identity of such a molecule. With a combination of RNAi to knock down the root specific genes of the *SYM2* orthologous region of *M. truncatula*, and inoculation with a *S. meliloti* 2011 *nodFE* mutant, which is able to infect wildtype roots, they discovered that one of the seven LysM domain containing receptor-like kinases in this region, LYK3, is specifically involved in infection thread initiation and growth (Limpens *et al*., 2003). Mainly, infection thread formation was blocked at the point of initiation, and the thread-like structures that did form, aborted in the root hair and had aberrant morphologies with sac- and tube-like structures (Limpens *et al*., 2003).

2) Biochemical and pharmacological approaches

Further insight into the route of NF induced signalling comes from biochemical and pharmacological approaches, from which it appeared that NF induces phosphoinositide signalling via putative heterotrimeric proteins (Pingret *et al*., 1998; den Hartog *et al*., 2001; Engstrom *et al*., 2002). Pertussis toxin, the antagonist of animal heterotrimeric G-proteins, inhibits NF-induced ENOD12-GUS expression in *M. sativa* root hairs (Pingret *et al*., 1998), while mastoparan, a G-protein agonist, mimics NF application. Mastoparan is a cationic peptide of 14 amino acids which was isolated from wasp venom (Ross and Higashijima, 1994). In animal systems, it mimics the activated intracellular domain of G protein-coupled receptors, thus activating heterotrimeric G proteins (Ross and Higashijima, 1994). In *V. sativa*, application of mastoparan induces root hair deformation and activates PLC and PLD, resulting in an increased concentration of phosphatidic acid (PA) and DGPP, in an identical way to NF (den Hartog *et al*., 2001). In *M. sativa* root hairs, mastoparan induces *ENOD12::GUS* expression (Pingret *et al*., 1998). PLC - and PLD antagonists inhibit NF induced root hair deformation (den Hartog *et al*., 2001), the increase in PA (den Hartog *et al*., 2001) and ENOD12 expression (Pingret *et al*., 1998). Furthermore, the PLC antagonist U-73122 also inhibits NF elicited Ca^{2+}-spiking (Engstrom *et al*., 2002), showing that phospholipid signalling is, at least partially, essential for NF induced responses.

More evidence for the involvement of phospholipids in NF signalling comes from the NF induced *MtAnn1* gene (de Carvalho-Niebel *et al*., 2002). This gene belongs to the annexin gene family, which all encode calcium and phospholipid binding proteins. In response to inoculation with *S. meliloti* or NF application, *MtAnn1* is, in addition, mainly expressed in the endodermis and in the outer cortex, indicating that NF induced phospholipid signalling also plays a role in these deeper cell layers of the root.

VI Regulation of nodule numbers: the shoot takes care of the root

That legumes have the ability to live in symbiosis with rhizobia, does not mean that they always have to live in symbiosis with each other. In the presence of a fixed nitrogen source such as ammonia and/or nitrate in the growth medium, nodulation will not take place (Malik *et al*., 1987), and NF induced root hair deformation is blocked (Heidstra *et al*., 1997). Also, to keep the balance between costs and profit (carbon resources versus nitrogen), the plants can not and will not be endlessly nodulated. Once the plant senses there are enough nodules, a systemic response is induced which prevents further nodulation in newly formed root regions (van Brussel *et al*., 2002). This autoregulation of nodulation is, surprisingly, regulated by the shoot, and requires long distance communication between the root and the shoot (Searle *et al*., 2003). This was found out via grafting studies. Grafting of the shoot of the *L. japonicus* hypernodulation mutant *HAR1* (hypernodulation aberrant root 1) onto a wildtype root system resulted in hypernodulation of the root (Krusell *et al*., 2002; Nishimura *et al*., 2002). From positional cloning studies, it appeared that the *HAR1* gene encodes a serine/threonine receptor like kinase which is, like DMI2, characterized by leucine rich repeats. Furthermore, *HAR1* is orthologous to *Sym29* of pea (Krusell *et al*., 2002; Nishimura *et al*., 2002) and to the 'nodule autoregulation receptor kinase' (NARK) from soybean (Searle *et al*., 2003). *HAR1/NARK* shows a high level of similarity with the *Arabidopsis CLAVATA1* (*CLV1*), from which it is known that it negatively regulates the formation of shoot and floral meristems via cell-cell communication via the CLV3 peptide (DeYoung and Clark, 2001). It might be that a legume specific CLV3-like gene is involved in the long distance organ to organ communication, thus regulating nodule numbers.

VII Future perspectives

It is clear that with the isolation and description of more mutants, the cloning of nodulation genes and development and use of recently developed techniques such as RNAi and NF spot application, we are getting our finger on the complexity of legume – microbe interactions. Multiple receptors and signalling pathways seem to be simultaneously involved in setting up a successful symbiosis. It seems as if the tools are there, and it is now up to the scientists to find the perfect symbiosis between each other and their different research disciplines, to get the different pathways together, to understand the system.

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Chapter 7

Arabidopsis SHAGGY-like Kinases are necessary for proper root hair tip growth and are targets for lithium ions

John J. Esseling[1], Martin Kreis[2] and Anne Mie C. Emons[1]

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

[2]Institut de Biotechnologie des Plantes ERS/CNRS 569, Université de Paris-Sud, Bâtiment 630, F-91405 Orsay Cedex, France

ABSTRACT

Root hairs are tip growing tubular extensions of root epidermal cells. Tip growing cells are characterized by a polar cytoarchitecture, in which subapical fine bundles of net-axial actin filaments are involved in targeting Golgi vesicles to the tip (Miller *et al*., 1999; Ketelaar et al., 2003), where they incorporate into the plasma membrane and deliver their content into the existing wall. Arabidopsis root hairs that overexpress an antisense construct against the shaggy-like kinases etha (η), iota (ι) and dzeta (ζ) are not able to set up and maintain this polarity properly. These root hairs are swollen, without a fixed diameter, branched and are wavy. Lithium treatment of wild-type plants mimics these phenotypes, and the effect of short-term lithium treatment, root hair deformation, is stronger in the ASK-antisense mutants than in wild-type plants. Pharmacological studies show that the lithium effects are not caused by inhibition of IMPase of the IP_3 pathway or accumulation of ACC synthase of the ethylene pathway, which are described effects of lithium on signalling pathways in plant cells. The data demonstrate that shaggy-like kinases are involved in root hair tip growth, and are lithium targets, like in *Xenopus* (Klein and Melton, 1996; Stambolic *et al*., 1996) and *Dictyostelium* (Harwood *et al*., 1995; Maeda, 1970; Peters *et al*., 1989; Stambolic *et al*., 1996)

Introduction

In animal cells, glycogen synthase kinase 3 (GSK3)/SHAGGY kinases are involved in insulin signaling, embryonic development, oncogenesis, modulation of transcription factor activity, bipolar mood disorder, and they play a role in Alzheimers disease.
The plant SHAGGY-like protein kinases belong to a multigene family (Bianchi *et al*., 1994; Dornelas *et al*., 1997) that codes for proteins which share a protein kinase catalytic domain about 70% identical to those of *Drosophila* zeste-white 3/shaggy and mammalian GSK3β (Glycogen Synthase Kinase 3β) (Bourois et al., 1990; Woodgett, 1990; Siegfried *et al*., 1994).
To date, ten different ASK genes have been identified in Arabidopsis (Dornelas *et al*., 1998). Based on the sequence analysis of the catalitic domains, they can be subdivided in four subgroups (Dornelas *et al*., 1998; Dornelas *et al*., 1999), with ASK α and γ in group I and ASK η, ι and ζ in group II (Dornelas *et al*., 1998; Dornelas *et al*., 1999). Besides Arabidopsis, other plants also contain Shaggy/GSK3β homologues. They have been cloned from *Medicago* (Pay *et al*., 1993), *Nicotiana* (Einzenberger *et al*., 1995), *Petunia* (Decroocq-Ferrant *et al*., 1995) and rice (Dornelas *et al*., 1998). It appears that Shaggy-like kinases are involved in cell fate and pattern formation during plant embryogenesis (Dornelas *et al*., 1999). During seed development, embryogenesis, and early seedling growth, they have a spatiotemporal expression pattern. ASKα and ASKγ are expressed in the hypocotyl, and ASKι, ASKη and ASKζ are expressed in the trichoblasts (Dornelas *et al* 1999). This is comparable to *Drosophila Shaggy* / vertebrate GSK3β during early development (reviewed by Welsh *et al*., 1996). In animals, Shaggy / GSK3β is part of the canonical wingless/Wnt signal transduction pathway. In this signal transduction pathway, Wnt uses Frizzled as receptor. Frizzled signals to Dishevelled. Dishevelled in its turn inhibits the activity of GSK3β, which is in a complex of the two scaffold proteins APC and axin (Lee et al., 2003). When active, GSK3β phosphorylates β-catenin/armadillo, which enhances the β-catenin degradation by ubiquitin (Aberle *et al*., 1997).
Shaggy/GSK3β was identified as a direct lithium target (Klein and Melton, 1996; Stambolic *et al*., 1996; Hedgepeth *et al*., 1997), since lithium treatment induces the accumulation of hypophosphorylated β-catenin/armadillo in cultured cells (Stambolic *et al*., 1996) thus mimicking Wnt signalling. Moreover, lithium treatment phenocopies ectopic expression of Wnt genes in *Xenopus* (McMahon and Moon, 1989) and zebrafish embryos (van de Water et al., 2001), and null mutation of the *Dictyostelium discoideum* GSK3β homolog gskA (Harwood *et al*., 1995) phenocopies lithium treatment (Maeda, 1970; Peters *et al*., 1989). This provided evidence for the mechanism involved in lithium-induced teratogenesis during animal development, since the inositol-depletion hypothesis stated by Berridge *et al*. (1989) could not explain the lithium induced developmental defects in *Xenopus* (Klein and Melton, 1996) and *Dictyostelium* (Maeda, 1970; Drayer *et al*., 1994; Peters *et al*., 1989).

Reported effects of lithium ions on plant cells are disruption of mitotic progression in *Tradescantia* stamen hair cells (Wolniak, 1987), effects on microtubule depolymerization (Bartolo and Carter, 1992), disruption of cell proliferation (Bagga *et al*., 1987), accumulation and activation of ACC-synthase in Arabidopsis (Liang *et al*., 1996) and tomato (Boller, 1984), and inhibition of IMPases in tomato (Gillaspy *et al*., 1995). In this manuscript, we show that Arabidopsis SHAGGY-like Kinases are necessary for proper root hair tip growth, that treatment of wild-type plants with lithium mimicks the antisense ASK phenotype, and reinitiates tip growth in growth terminating hairs. These effects appear not to be caused by inhibition of the inositol - and / or stimulation of the ethylene signal transduction pathways.

Results

Antisense ASKη, ASKζ and ASKι plants are disturbed in root hair tip growth and their morphology is phenocopied when wild-type seedlings are grown in lithium-containing media

Four days old wild-type Arabidopsis seedlings have a straight root with a ‘cone’ of root hairs (Fig 1a). The short hairs in the cone are growing and the longest are full grown hairs. When full grown, the root hairs can reach a length of 0.7 mm and they are straight. Antisense ASKη, ASKζ and ASKι plants show no difference in morphology of the root, but do not have normally shaped root hairs. The root hairs develop at the correct position at the base of the trichoblast, but they are bottle shaped, branched and wavy (Fig 1B ASKζ). Plants which over express an antisense construct against ASKα or ASKγ, two ASK-genes which are not expressed in trichoblasts (Dornelas *et al*., 1999), develop normal root hairs when air grown (see fig 1c for ASKγ).

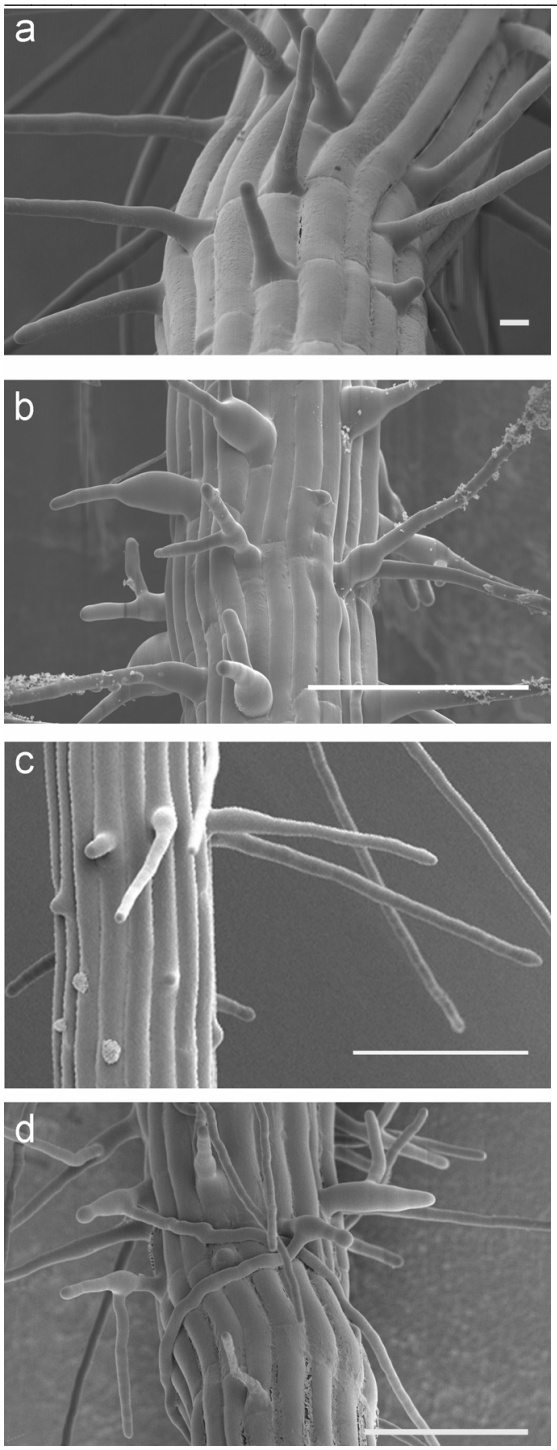

Figure 1. FESEM images of air grown Arabidopsis roots.
A Wild-type root grown on half strength MS medium in 1% agarose. Note the constant root hair diameter. **B** Antisense ASKζ root grown on half strength MS medium in agarose. The root hairs develop on their normal position on the trichoblast, but are bottle shaped and branched. **C** Antisense ASKγ root. ASKγ is not expressed in root hairs, and the root hairs have a normal morphology. **D** Wild-type grown on half strength MS medium in 1% agarose with 5mM LiCl. Like the antisense ASKζ root, these root hairs are bottle shaped, branched and can be wavy. Bar in a = 10µm, in b-d bar = 1 mm.

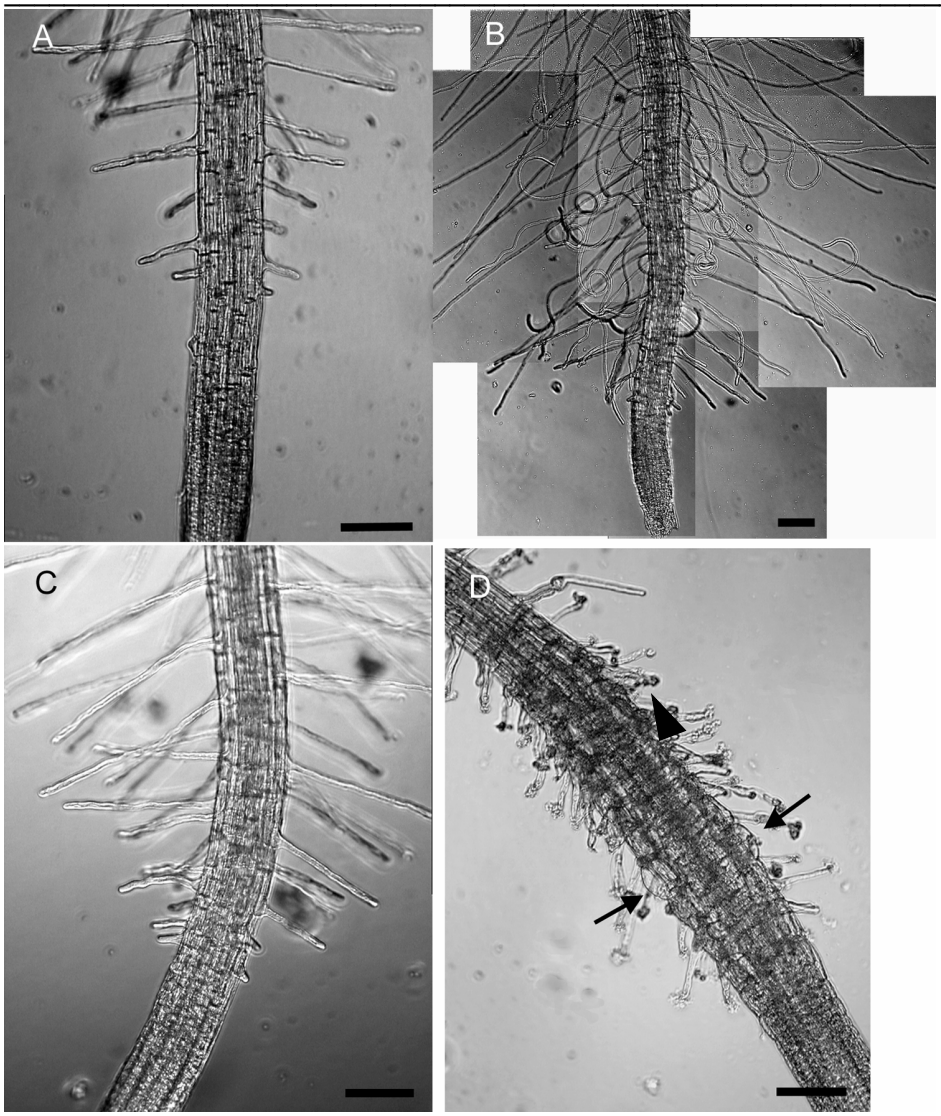

Figure 2. Roots grown in liquid medium in between glass slides
In **A**, an antisense ASDζ root is shown. Note that the root hairs have a wavy appearance. In **B**, a root with the enhanced curly root hair phenotype is shown. **C** shows a wild-type root that was growing 24h in 1mM LiCl. Note the wavy appearance of the root hairs. **D** Wild-type root grown for 24h in 50mM LiCl. Note that most root hairs bursted at their tip, and the swollen epidermal cells (arrows). The arrowhead points at a deformed hair with a random outgrowth.

Growing the wild-type seedlings in the air on agarose plates containing 5 mM LiCl phenocopies the antisense ASKη, ASKζ and ASKι plants, in that they have root hairs which are morphologically identical: bottle shaped, branched and wavy (Fig 1d). Wild-type seedlings grown on agarose plates containing 5 or 10 mM KCl / NaCl do not display such root hair morphologies (results not shown). When grown in liquid medium between glass slides (Ketelaar et al., 2002), four days old wild-type, ASKα - and ASKγ antisense seedlings develop a ‘cone’ of straight root hairs which contains a gradual range of stages of root hair development, from initiation as a bulge to full grown hairs. Of the antisense ASKζ and ASKη seedlings, 90 – 100% have wavy root hairs (Fig 2a). Of the antisense ASKι plants, 50% of the plants have wavy root hairs. In the other 50%

of the plants, 40% of the root hairs are curled and the other 60% of the root hairs are wavy (Fig 2b). When grown in 1 mM LiCl for 24 hrs, ASKαAS, ASKγAS and wild-type plants have wavy root hairs (fig 2c). For the ASKη, ζ and ι antisense seedlings, culturing them in 1 mM LiCl for 24 hrs does not change or enhance the wavy or curly root hair phenotype (not shown).
In higher concentrations of LiCl (10-50 mM), bulge development of wild-type plants is not affected, but at the onset of polar tip growth, the bulges burst at the tip. Also, epidermal cells are swollen (fig 2d).

Application of LiCl induces root hair deformation within one hour in a higher percentage in ASK antisense plants than in wild-type plants

When applied to four days old roots, 1 mM LiCl induces swelling of the root hair tip and new polar outgrowth from that swelling (fig 3)

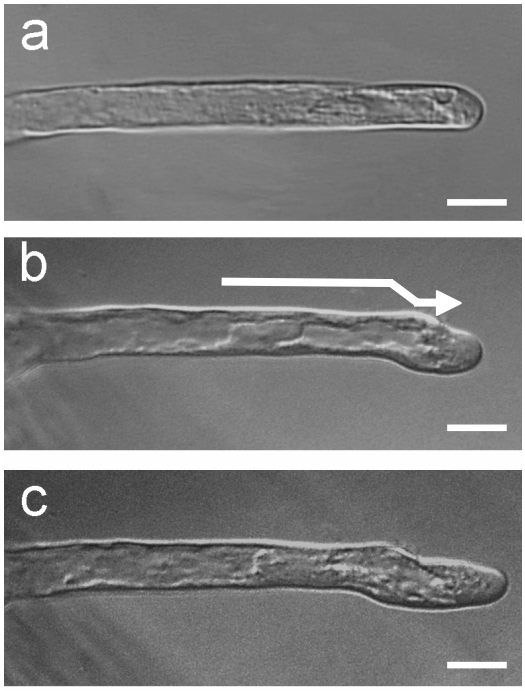

Figure 3 LiCl-induced root hair deformation.
A Growth terminating root hair. **B** Growth terminating root hair, 30 minutes after 1mM LiCl application. The tip of the root hair slightly swells, and an outgrowth starts to grow. This outgrowth may initially be a different direction, but recovers to the original growth direction of the root hair. This is visible in **C**, which is the same root hair as in **B**, 50 minutes after LiCl application. The white arrow represents the direction of root hair growth Bars = 10µm

in 20% (fig 4) of growth terminating wild-type root hairs. Such swelling and reinitiation of tip growth does also occur in the same developmental stage of vetch root hairs after application of Nod factor and has been called root hair deformation (Heidstra *et al*., 1994; De Ruijter *et al*., 1998) Also ASKα and ASKγ antisense seedlings show root hair deformation in 20% of the growth-terminating hairs. Since Li^+ ions inhibit animal GSK3 activity *in vitro* and *in vivo*,

LiCl treatment should give a more severe phenotype in ASKη, ζ and ι antisense root hair mutants, if it would inhibit plant ASK too.

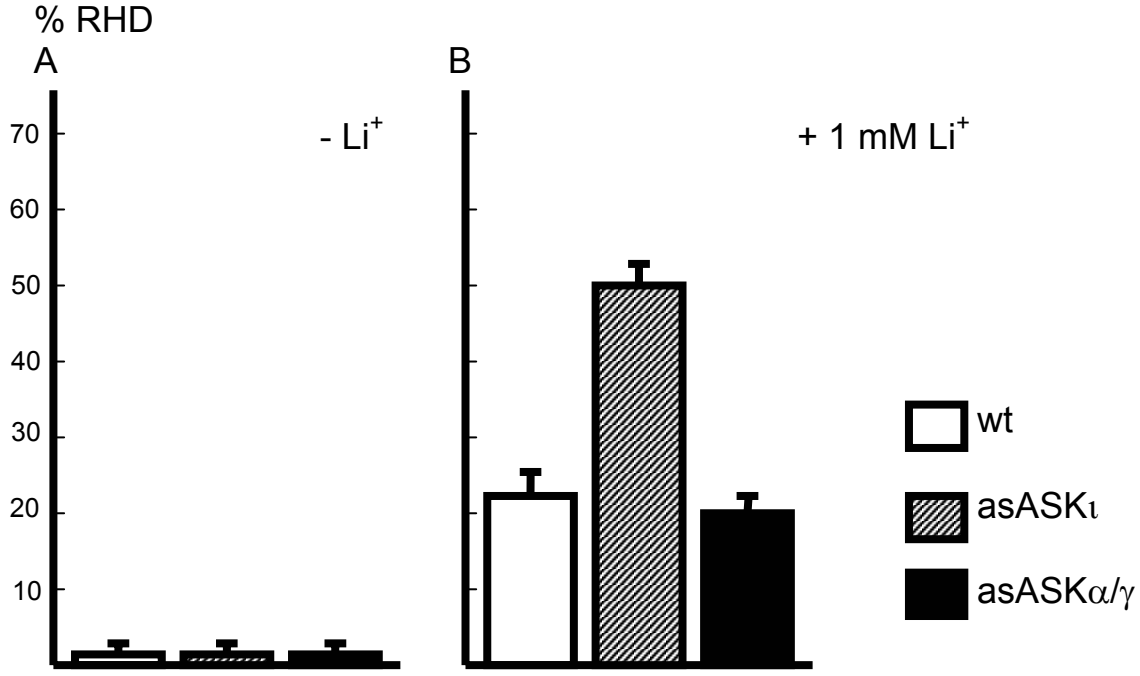

Figure 4. Percentages of RHD in wild-type and antisense root hairs after LiCl application. **A**. Control in which 1mM KCl or 1mM NaCl was applied, and root hair deformation was scored. Neither KCl nor NaCl application induced RHD in wild-type or antisense ASK plants. **B**. Application of 1mM LiCl to wild-type and antisense ASK plants induces root hair deformation. Wild-type and antisense ASKα or ASKγ (which are not expressed in the root hair) show a comparable percentage of RHD. LiCl treatment induces root hair deformation in a significant higher percentage root hairs in antisense ASKι. The graph represents 20 roots of each type for each treatment.

Indeed, 49% of the root hairs which are terminating growth of ASKι antisense seedlings show root hair deformation upon LiCl treatment. These experiments reveal that Li^+ ions may inhibit ASK in plant cells, like it inhibits mammalian GSK3β (Stambolic *et al*., 1996).

The phospholipase C inhibiting drug U-73122 does not mimic or inhibit lithium-induced root hair deformation

Since in tomato tissue, LiCl inhibits *myo*-Inositol monophosphatase (IMPase, Gillaspy *et al*., 1995), lithium-induced root hair deformation could be caused by inhibition of IMPase. To test the involvement of phospholipid signaling in lithium-induced root hair deformation, the phospholipase C (PLC) inhibiting drug U-73122, which has been shown to inhibit plant PLC in vitro and in vivo (Staxén *et al*., 1999), was used. In the inositol signal transduction pathway, PLC hydrolyzes phosphatidylinositolbisphosphate (PIP_2), which results in the formation of diacylglycerol (DAG) and inositol-trisphosphate (IP_3).

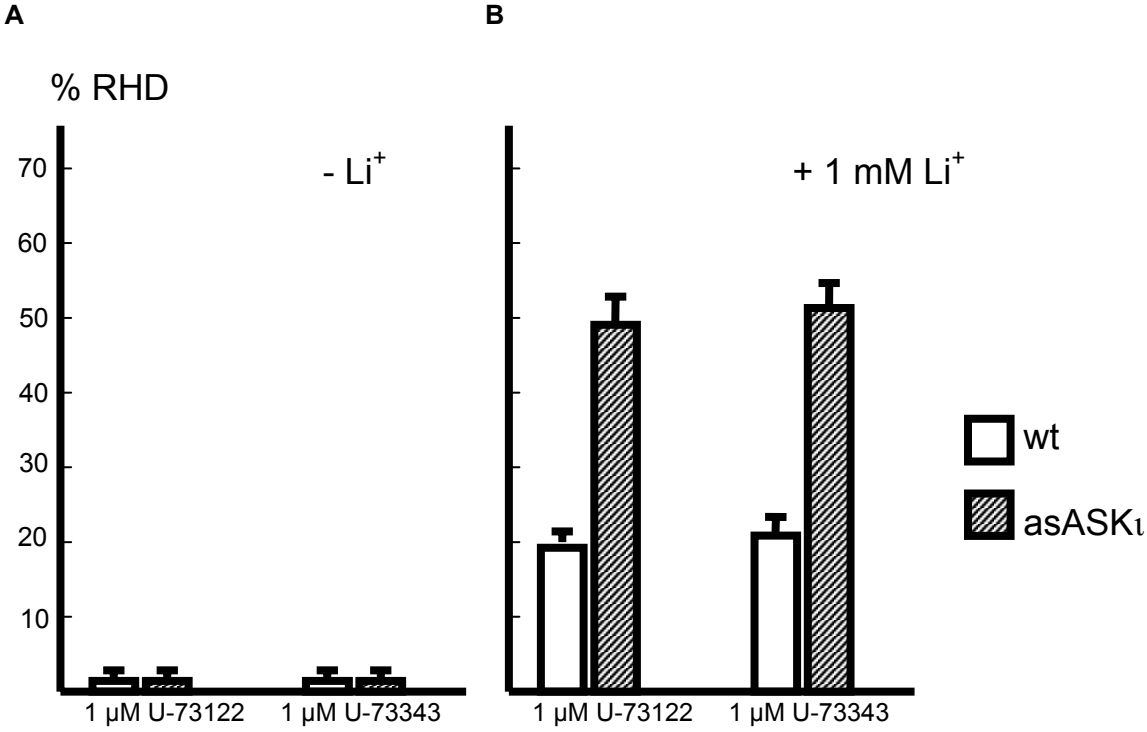

Figure 5. Percentage of RHD with or without Li+, in combination with U-73122, or U-73343.

Percentage of root hair deformation in wild-type and antisense ASKι Arabidopsis seedlings after application of 1µM of the phospholipase C inhibiting drug U-73122, or the non-active control drug U-73343. Virtually no root hair deformations were scored in the wild-type and the antisense after application of these drugs. **B**. Application of 1mM LiCl in the presence of 1µM U-73122 or U-73343 resulted in a percentage of RHDs that is comparable with LiCl treatment without U-73122 or U-73343, in other words, in these concentrations U-drugs have no effect on LiCl induced RHD. The graph represents 20 roots of each type for each treatment.

IP_3 is dephosphorylated into inositolbisphosphate (IP_2), which is dephosphorylated into inositolmonophosphate (IP). IMPase catalyzes the removal of the phosphate from IP, which results in the formation of inositol. Inositol is used for the formation of phosphatidylinositol (PI), which, after two phosphorylation steps, becomes PIP_2 (Berridge *et al*., 1989; Munnik *et al*., 1998).

Four days old liquid grown wild-type and antisense ASKι seedlings were treated with 0.1, 0.5, 1, 5 and 10 µM U-73122 or its almost inactive counterpart U-73433 in combination with or without 1 mM LiCl. Treatment with either concentration of U-73122 without lithium did not result in root hair deformation, in wild-type as well as in antisense ASKι seedlings (Fig 5A). At 10 µM U-73122, toxic effects were visible in the root hairs. These effects show that the drugs enter the root hairs. Some root hairs died, in others root hair growth stopped within 10 to 15 minutes after application, since the polar cytoarchitecture of growing hairs disappeared and the cytoplasmic streaming changed from reverse fountain to circulation (Miller *et al*., 1997; Sieberer and Emons, 2000). Also, cytoplasmic aggregates appeared in the cytoplasm. Treatment of 1mM LiCl combined with either 0.1, 0.5, 1 and 5 µM U-73122 or 0.1, 0.5, 1, 5 and 10 µM U-73433 resulted in root hair deformation in ~20% (for wild-type) and ~50% (for antisense ASKι) of the growth terminating hairs (Fig 5b). This indicates that inhibition of inositol signalling is not the working mechanism of lithium-induced root hair deformation, and that ASK enzymes are not downstream products of PLC.

Stimulation or inhibition of ethylene signalling did not result in an enhancement or inhibition of the ASKantisense – or lithium-induced root hair phenotype

Since in Arabidopsis, 10 mM and higher concentrations of LiCl induces accumulation of ACC synthase (Liang *et al*., 1996), we investigated whether application of ACC mimicks LiCl. Upon □ignalling□ti of wild-type or ASKι antisense Arabidopsis roots for 3 hours with either 10, 100µM or 1 mM ACC, neither concentration induced root hair deformation after reapplication (Fig 6). On the other hand, after reapplication of either concentration of ACC combined with 1mM LiCl, root hair deformation in wild-type was induced in 20% of the growth terminating hairs and in ASKι in 50% of the growth terminating hairs (Fig 6), showing that lithium-induced root hair deformation is ACC independent.

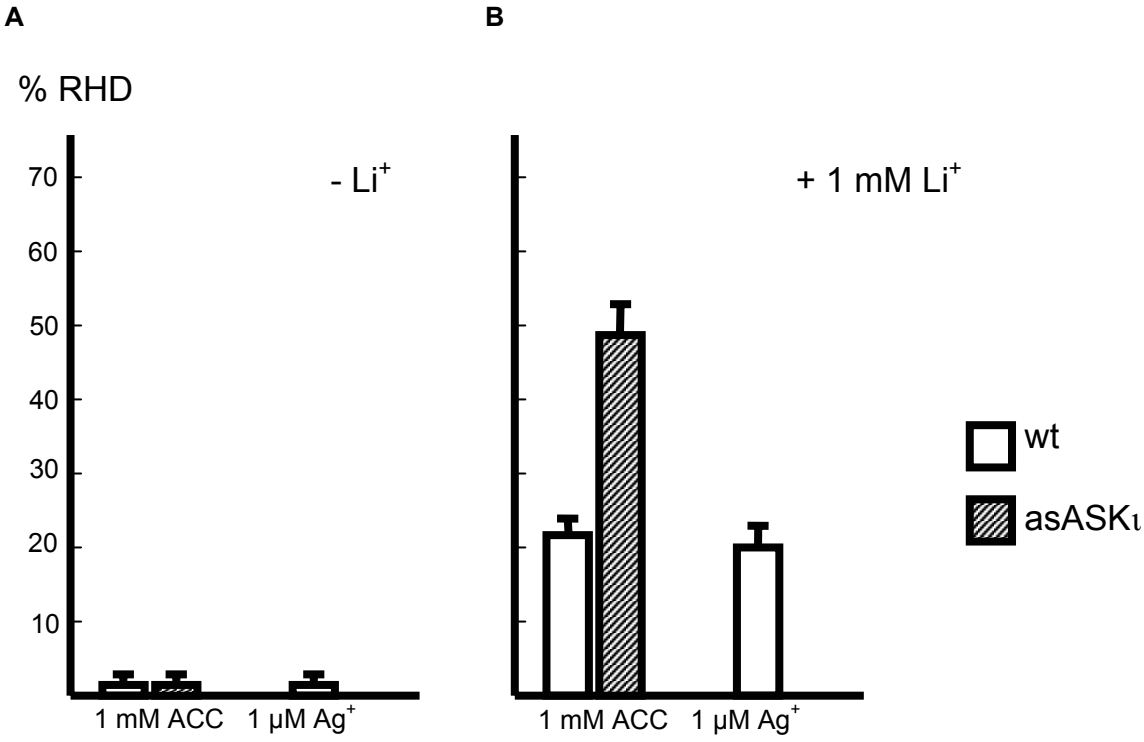

Figure 6. The effects of ACC or Ag^+ on LiCl-induced root hair deformation.
A. Application of ACC or Ag^+ does not induce root hair deformation in the wild-type and antisenseASKι root hairs. **B**. Application of LiCl to wild-type and antisense ASKι roots in the presence of ACC or Ag^+ induces RHD in a percentage of root hairs that is comparable with LiCl treatment without ACC or Ag^+.

Because Ag^+ blocks the ethylene receptor (Beyer, 1976), we investigated whether application of Ag^+ could inhibit lithium induced root hair deformation in wild-type root hairs. Application of 10 µM Ag^+/10 µM sodiumthiosulfate had an immediate effect on root hair growth. Within 10 minutes, root hair growth completely stopped. At 1 µM Ag^+/10 µM sodiumthiosulfate, root hair growth stopped between 1 and 1.5 hours. Application of 10 µM sodiumthiosulfate alone did not affect root hair growth. When 1 µM Ag^+/10 µM sodiumthiosulfate was applied in combination with 1 mM LiCl, root hair deformation was induced in 19% of the growth terminating root hairs, one hour after application (Fig 6).
Subsequently, we tested whether either the ASKι or lithium-induced air grown phenotype requires ethylene. Since AVG inhibits ACCsynthase, seedlings were grown on agarose plates containing 50, 20, 2, 0.2, 0.02 µM, 2 and 0.2 nM AVG in combination with 5mM LiCl for wild-type seedlings, and the same concentrations of AVG without lithium for the ASKι antisense and the wild type as a control.

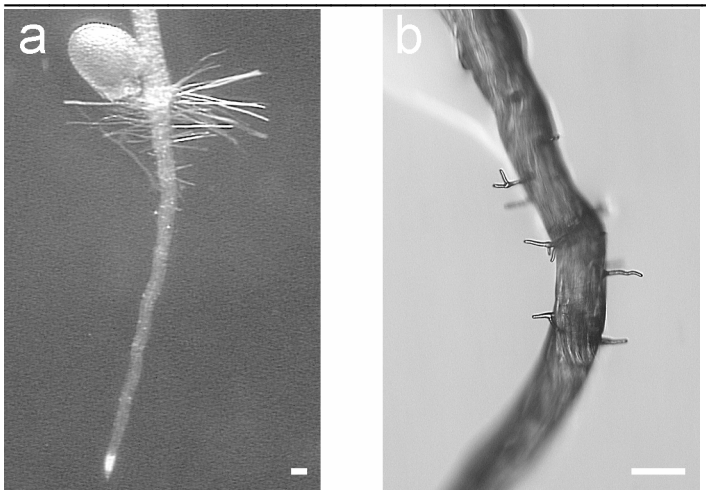

Figure 7 Wild-type and antisense ASKι develop root hairs on 20μM AVG.
A. Wild-type on agarose with 20μM AVG. The root hairs that develop have a normal appearance. **B**. Antisense ASKι on agarose with 20μM AVG. The root hairs that develop are branched and wavy. Inhibition of ethylene signalling does not rescue the ASKι antisense phenotype. Bars = 1 mm.

At 50 μM AVG, root hair growth was completely blocked, whereas at 20 μM some root hairs developed on the wild-type and ASKι antisense roots (fig 7). In wild-type plants, these root hairs were short and straight, whereas the ASK ι antisense roots develop short swollen, branched and wavy root hairs, showing that AVG does not rescue the ASK phenotype. Wild-type seedlings grown on 20 μM AVG with 5 mM LiCl, develop short swollen, branched and wavy root hairs, showing that AVG does not block the lithium induced phenotype. These results indicate that the lithium induced – and the ASKι root hair phenotype is ethylene independent.

Discussion

Arabidopsis shaggy-like kinases are regulators of proper tip growth

With the use of the antisense approach, we were able to study whether ASK genes function in root hair tip growth. In accordance with the expression patterns, antisense ASK α and γ root hairs appeared normal, whereas root hairs of the antisense ASK η, ζ and ι seedlings displayed various phenotypes. In air, the root hairs are bottle shaped, branched, wavy, or show a combination of these morphologies. In liquid, the root hairs are wavy and, for antisense ASK ι, curled. We discuss that these morphological disturbances indicate that ASK genes are needed for proper root hair tip growth.
Cell growth implies an increase in cell volume. Plant cells grow by insertion of new plasma membrane and exocytosis of cell wall material through Golgi vesicles (Roberts, 1994) into a flexible cell wall, while a vacuole under turgor pressure expands. The site of insertion of these vesicles into the plasma membrane determines the direction of cell growth and therefore mature cell shape. In tip growth, the cell expands at one side. Prior to such localized

exocytosis, vesicles are produced and budded from Golgi bodies, translocated to and released at the target plasma membrane, docked at, attached to and fused with this membrane. This means that Golgi vesicles should accumulate at the site of cell growth, and should insert into the plasmamembrane there. The first mentioned process requires a typical configuration of the actin cytoskeleton, namely subapical, net-axially oriented fine bundles of actin filaments (fine F-actin; Miller et al. 1999; Ketelaar et al., 2003). The insertion of the vesicles into the plasma membrane requires, surely among other things, a tip-focused calcium ion concentration gradient at the plasma membrane of the exocytosis site (De Ruijter et al. 1998), as well as an absence of bundles of actin filaments there (Miller et al. 1999, De Ruijter et al. 1999, Ketelaar et al., 2003).

The morphologies of the antisense mutants indicate problems with the targeting and docking of the exocytotic vesicles. An explanation for the bottle shaped root hairs, is that exocytosis occurs at a wider diameter than in normal hairs. In wavy root hairs, the direction of delivery and insertion of vesicles may be constantly changing its position. In curled root hairs, it seems that the position of delivery and insertion is re-directed towards the same side. In branched root hairs, the position of insertion and delivery alternates, which eventually leads to a splitting up of the tip, resulting in a branch. Combinations of the different morphologies can occur within the same root hair, so it is likely that a disruption of delivery and insertion can cause different phenotypes.

ASK deficiency in the antisense mutants and inactivation by lithium does not inhibit cell growth itself, but the proper direction of growth. Therefore, we conclude that the fine F-actin is intact, and that microtubules might be a target of ASKs. A role for microtubules in the direction of root hair tip growth has been shown before (Bibikova *et al*., 1999; Ketelaar et al., 2003). In the presence of oryzalin, root hairs grow wavy (Bibikova et al., 1999) and when tip growth recovers after a pulse treatment of cytochalasin D, in the constant presence of oryzalin, the tip grows in a random direction (Ketelaar et al., 2003). Furthermore, in high lithium concentrations, root epidermal cells swell, which is another effect of microtubule depolymerisation by oryzalin (Baskin *et al*., 1994). Also, Bartolo and Carter (1992) reported that Li^+ has effects on plant microtubule depolymerization. Therefore, disruption of microtubules could be involved in the ASK-antisense and the lithium induced phenotypes. From animal cells, it is known that GSK3β can phosphorylate the microtubule binding protein Tau, which results in microtubule depolymerisation (Lovestone *et al*., 1999). In addition, in a variety of animal cells, subpopulations of Tau can interact with microtubules and actin filaments (Henriquez *et al*., 1995), providing a link between GSK3β and the actin cytoskeleton. In plants, the Tau protein has not been found yet, but candidates could be the 83 kDa and 100 kDa maize proteins which cross-react with affinity purified rat brain Tau antibodies (Vantard *et al*., 1991; 1993; 1994). We hypothesize that ASKs are proteins that regulate one or more microtubule binding protein(s).

Lithium can be used as an inhibitor of Arabidopsis Shaggy-like Kinases

Now we show that culturing wild-type plants on lithium chloride containing media results in root hair phenotypes comparable to antisense ASK root hairs, and ASK ι antisense mutants are more sensitive to lithium than wild-type seedlings. Therefore, we conclude that lithium ions may inhibit ASK *in vivo*. Though it is known that lithium inhibits inositol monophosphatase (Gillaspy *et al*., 1995) and stimulates ethylene signalling (Boller, 1984), we have excluded that inositol- and ethylene signalling are involved in the response of root hairs to lithium.

Root hair deformation has also been described for growth terminating legume root hairs in response to lipochito-oligosaccharides, Nod factors (*Vicia sativa*: Heidstra et al., 1994; De Ruijter et al., 1998; *Medicago truncatula*: Sieberer and Emons, 2000), which are signal molecules excreted by rhizobia. Nod factor induced root hair deformation is also independent of ethylene (Heidstra *et al*., 1997), and Shaggy-like Kinases are present in *Medicago sativa* (MSKs; Pay *et al*., 1993). Together, these facts render the question whether MSKs are a step in the cellular signalling response of legume root hairs to Nod factor worth investigating.

Recent work has shown that brassinosteroids, plant steroid hormones that affect various processes in plant growth and development, activate a signal transduction pathway reminiscent of vertebrate Wnt signalling, and that ASKs are involved in this pathway (Yin et al., 2002). In response to brassinosteroids, ASKs are no longer able to promote degradation of BES1 (bri1-EMS-suppressor1) via phosphorylation, whereafter BES1 accumulates in the nucleus, where it activates transcription (Yin et al., 2002). Lithium treatment of BES1-GFP transgenic Arabidopsis seedlings, might give a direct answer if lithium has an effect on ASK *in vivo*.

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Materials and methods

Plant material

The antisense ASKα, ASKγ, ASKη, ASKι and ASKζ plants were obtained by transforming *Arabidopsis thaliana* (L.) Heynh, ecotype Columbia Co (Bechtold and Pelletier, 1998) with gene fragments cloned in the antisense orientation under the CaMV 35S promoter. By selfing the primary transformants, homozygous antisense transgenic inbred lines were obtained for all the constructs. In these experiments the lines ASKι 23i20-1 and ASKη 23y14-2 were used.

Growth conditions

Seeds were surface sterilized with 70% ethanol and 20% bleach / 0.01% Triton X100, and imbibed for 30 minutes in autoclaved 0.5 x strength Murashige-Skoog (half MS) medium (Duchefa).
To obtain air grown root hairs, seedlings were grown as previously described (Grierson *et al*., 1997) with minor changes. Briefly, after surface sterilisation and imbibition, seeds were plated on 1% agarose solidified half MS to which either nothing, LiCl (Merck), KCl (Merck), NaCl (Merck), AVG (Aminoethoxy-Vinyl-Glycine, Sigma) or a combination of the above compounds was added. Seeds were germinated at 25°C with the plates in a vertical orientation at a 16 hr light-8 hr dark cycle.
To obtain liquid grown root hairs, imbibed seeds were placed on modified Fåhraeus-slides (Heidstra *et al*., 1994), containing autoclaved 0.5 x strength Murashige-Skoog medium. For Arabidopsis, the space between the object slide and cover slip is decreased when compared with *Vicia*, so that the seeds can germinate on top of it and the root can grow in between the slides. Seeds were germinated at 25°C.

Drug treatments

LiCL, NaCl, KCl, U-73122 (Calbiochem) or U73433 (Calbiochem), ACC (1-AminoCyclopropane-1-Carboxylate, Sigma), $AgNO_3$ and sodium-thiosulfate were applied to four day old Arabidopsis roots and root hairs by dripping half MS medium containing one or a combination of the above compounds on top of the Fåhraeus-slides, thus replacing the medium in between the glass slides. For lithium treatment, several concentrations of LiCl were used: 0.1, 1, 10, 20 and 50 mM. In control experiments, the same concentrations of KCl and NaCl were added. To investigate the effects of Li+ on the inositol-signal transduction pathway, seedlings were double challenged with 1 mM lithium and 0.5, 1, 5 or 10 µM of the PLC-inhibitor U-73122 and the inactive control U-73433.
Short term effects including the ethylene pathway were carried out using silver-ions to block the ethylene receptor (Beyer, 1976) and ACC to stimulate the production of ethylene. ACC was applied in a concentration range of 0.1, 1 and 10 mM. In all experiments with ACC, seedlings were pretreated with ACC for 3 hours before either ACC or LiCl/ACC application, since for ACC it takes 1.5 to 2 hours before it becomes activated by the plant. Silver ions were applied in a concentration of 1 µM in combination with 10 µM sodiumthiosulfate to slow down the formation of non-soluble silver salts.

Microscopy

To monitor the short or long term effects of drug application of liquid grown root hairs, the phenotype of the root hairs was checked in the Fåhreus slides, either one hour or 24 hours after drug addition, with a Nikon 10x DLL 0.25 NA, 20x DIC 0.5 NA or a 40x DIC 0.7 NA objective on a Nikon Optiphot microscope. Images were recorded on a

Panasonic wv-E550 3-CCD camera with a Prysm framegrabber with AcQuis 2.0 software (Synoptics Ltd, Cambridge, UK).

Air grown root hairs were imaged either with cryo field emission scanning electron microscopy (cryo-FESEM) or a Nikon Diaphot 200 with Hoffmann modulation optics. For cryo-FESEM, air grown roots were stuck on stubs with carbon glue, plunged into liquid nitrogen, and coated with 2 nm platinum in an Oxford Instruments CT 1500 HF cryo-transfer unit mounted on a JEOL 6300F field emission cryo-scanning electron microscope.

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Samenvatting

In dit proefschrift wordt onderzoek beschreven dat bijdraagt aan een beter begrip van de effecten die nod factor als bacteriële signaalmoleculen hebben op wortelharen van vlinderbloemige planten. Als modelsysteem hebben we voornamelijk de wortelharen van de plant *Medicago truncatula* gebruikt. In de verschillende hoofdstukken komen de veranderingen in wortelhaarmorfologie en de signalen die daarbij betrokken zijn aan de orde, veranderingen die nodig zijn voor het opzetten van een succesvolle stikstofsymbiose.

Hoofdstuk 1 is een overzichtsartikel over het actine-cytoskelet in wortelharen. Het beschrijft de rol die filamenteus actine in wortelhaargroei heeft, dat actine filamenten de ruggegraat vormen van de cytoplasmadraden, dat het de snelweg voor transport binnen de cel is en dat het een “mikpunt” is van nod factor geinduceerde signalering.

In hoofdstuk 2 beschrijven we een nieuwe experimentele toets om effecten van nod factoren op wortelharen van vlinderbloemige planten te testen. Het voordeel van deze aanpak, in vergelijking met andere toetsen waarbij nod factor globaal aan een wortel met wortelharen wordt aangeboden, is dat het meer overeenkomt met de natuurlijke situatie waarbij de *Rhizobium* bacteriën lokaal aanwezig zijn op de wortelharen van hun gastheer. Met een micoinjectienaald wordt een klein druppeltje nod factor aan de zijkant van de top van een groeiende wortelhaar aangebracht. Deze ‘spot’ applicatie resulteert in een afbuiging van de groerichting van de wortelhaar naar de kant van de nod factor toe: de wortelhaar krult. In de wortelhaar komt het $Pro_{MtENOD11}$-*GUS* reporter-gen tot expressie: een teken dat het kleine druppeltje nod factor voldoende is om in de cellen symbiose-specifieke signalering aan te schakelen. De krullingsrespons na een lokale aanwezigheid van de nod factoren was al voorspeld met computersimulaties.

Mutanten maken en onderzoeken is tegenwoordig een veelgebruikte techniek om signaaltransductieroutes genetisch te ontrafelen. Hoofdstuk 3 beschrijft een niet symbiose-gerelateerd wortelhaarfenotype in de *Medicago truncatula dmi2/NORK* mutant, een mutant die gevonden is in zo’n screen. Het bleek dat deze mutant, net als dezelfde mutanten in de andere vlinderbloemigen *Lotus japonicus* en alfalfa, een verhoogde ‘touch response’ laat zien bij experimenteel handelen. Bij voorzichtig behandelen zodat de ‘touch response’ niet optreedt, reageren de mutante wortelharen op een normale manier op nod factor behandeling. Na globale nod factor toediening deformeren de wortelharen en na spot applicatie krult of vertakt de haar, afhankelijk van de plaats van nod factor toediening. Bovendien krullen de *dmi2/NORK* wortelharen in aanwezigheid van rhizobia, maar nooit verder dan 180˚. Waarschijnlijk stopt door de gevoeligheid voor aanraking de wortelhaar met

krullen (= groeien) zo gauw de wortelhaartop zijn eigen buis raakt en kan daarom nooit bacteriën opsluiten in een driedimensioneel kamertje. Omdat het *Pro$_{MtENOD11}$-GUS* reporter gen niet tot expressie komt na nod factor behandeling, stellen we een splitsing in nod factor geinduceerde signaaltransductie voor met een tak naar veranderingen in wortelhaarmorfologie, en de andere tak naar *Pro$_{MtENOD11}$-GUS* expressie.

Erwtenplanten, ook vlinderbloemigen, kunnen genoduleerd worden door bepaalde bacteriestammen die nauwelijks detecteerbare hoeveelheden nod factor produceren. Bovendien, in andere vlinderbloemigen zijn er maar heel lage concentraties nod factor nodig om toch veranderingen in wortelhaarmorphologie en expressie van nod factor specifieke genen te krijgen. Daarom hebben we getest wat de laagste concentratie nod factor is waarbij we nog wortelhaarreorientatie en *Pro$_{MtENOD11}$-GUS* expressie zien in *Medicago truncatula* na 'spot' applicatie. In hoofdstuk 4 beschrijven we het verrassende resultaat dat slechts één nod factor molecuul nodig is om wortelhaarreorientatie en *Pro$_{MtENOD11}$-GUS* expressie te krijgen.

In hoofdstuk 5 beschrijven we de resultaten die we behaald hebben door mengsels van nod factor en verschillende pharmacologische signaalremmers of stimulatoren op wortelharen te 'spotten'. Hiermee laten we zien dat de wortelhaarkrulling na nod factor geremd kan worden door gadolinium ionen, ionen die specifiek calcium influx blokkeren in plantencellen. Ook laten we zien dat we een aantal wortelharen kunnen laten reorienteren door aan één kant calcium aan te brengen, samen met de calcium ionofoor A23187. Verder laten we zien dat 'spot' applicatie van pertussis toxine, een remmer van zogenaamde heterotrimere G-eiwitten, wortelhaarreorientatie door nod factor niet afremt, maar dat *Pro$_{MtENOD11}$-GUS* niet tot expressie komt. 'Spot' applicatie van mastoparan, een eiwitje uit wespengif dat heterotrimere G-eiwitten activeert, laat wortelharen niet krullen, maar *Pro$_{MtENOD11}$-GUS* komt wèl tot expressie. Heterotrimere G-eiwitten activeren phospholipase C. Na 'spot' applicatie van een mengsel van nod factor en neomycine of U-73122, twee bekende remmers van phospholipase C, was *Pro$_{MtENOD11}$-GUS* expressie geblokkeerd, maar wortelhaarreorientatie niet. Phospholipase C is een enzym dat phospho-inositolbiphosphaat (PIP_2) 'knipt' in het secundair boodschapper molecuul inositoltriphosphaat (IP_3) en diacylglycerol (DAG). Door microinjectie van 'caged'- IP_3, niet actief IP_3 doordat er een ander molecuul aanhangt dat er met UV licht afgesplitst kan worden, en het vervolgens activeren van IP_3 met een UV laser kwam *Pro$_{MtENOD11}$-GUS* tot expressie in een aantal wortelharen. Dit laat allemaal zien dat heterotrimeer G-eiwit gekoppelde phosphaatsignalering betrokken is bij nod factor geinduceerde *Pro$_{MtENOD11}$-GUS* expressie in *M. truncatula* wortelharen.

Hoofdstuk 6 is een overzichtsartikel waarin de huidige stand van zaken beschreven wordt voor wat betreft het onderzoek naar de cellulaire signalering met betrekking tot stikstofsymbiose. In het hoofdstuk wordt niet

alleen de genetische dissectie via mutantenanalyse vergeleken met de pharmacologische aanpak, maar het behandelt ook de grondige celbiologische analyse die nodig is om tot een goed begrip van door nod factor geinduceerde signalering in vlinderbloemige wortelharen te komen.

Hoofdstuk 7 is een hoofdstuk waarin beschreven wordt dat de SHAGGY-kinase signalering betrokken is bij wortelhaardeformatie in de zandraket (Arabidopsis). Dit is een klein onkruidje dat als niet-vlinderbloemige plant als model gebruikt wordt. We laten zien dat toediening van lithium ionen wortelhaardeformatie veroorzaakt, en dat in wortels waarin SHAGGY kinases uitgeschakeld zijn, significant meer wortelharen deformeren na toediening van lithium ionen. In planten hebben lithium ionen ook invloed op ethyleen en phosphoinositol signalering, maar door het gebruik van specifieke remmers en stimulatoren, laten we zien dat lithium-geinduceerde wortelhaardeformatie niet veroorzaakt wordt door een verstoorde ethyleen of phosphoinositol signalering.

Summary

In this thesis, research is presented which contributes to a better understanding of nod factor (NF) induced signalling in legume root hairs, leading to a successful symbiosis. We mainly use root hairs of the model legume *Medicago truncatula* ('barrel medic') as an experimental system. In the different chapters, different aspects of the NF induced changes in root hair morphology that are required for establishing a successful symbiosis between rhizobia and legumes are covered.

Chapter 1 is a review article that describes the different roles of the actin cytoskeleton in legume root hairs: its different configurations in relation to root hair growth, its function as backbone of cytoplasmic strands and highway for cellular transport, and its target for NF-induced signalling.

Chapter 2 describes a new experimental assay to test the effects of NF on legume root hairs. The advantage of this assay, in comparison with the classical global application assays, is that it better mimics the natural situation in which rhizobia are locally present on the hosts' root hairs. It tests a theoretical computer model explaining root hair curling around bacteria. With a microinjection needle, a small droplet of purified NF was applied on the side of the tip of growing root hairs. The result of this is that the root hair under study reoriented its growth axis – it curls, toward the site of NF application, and it expresses the early nodulin gene $Pro_{MtENOD11}$-*GUS*.

Mutagenesis screens are nowadays widely performed to genetically dissect signal transduction pathways. In chapter 3, we studied the root hair phenotype in the non nodulating *M. truncatula dmi2/NORK* mutant which was found in such a screen. This mutant, and its two orthologues in alfalfa and *Lotus japonicus*, appeared to exhibit an enhanced touch response to experimental handling. When care was taken to not induce this touch response, the mutant root hairs responded morphologically like wild-type root hairs to NF application. A global application resulted in root hair deformation and NF spot application induced root hair reorientation or – branching, depending on the position of application on the root hair. In addition, *dmi2/NORK* root hairs make 180° curls in the presence of rhizobia, but as soon as the root hair tip touches its own shank, the root hair stops growing/curling, and as such is unable to entrap bacteria in a three-dimensional pocket. Because *dmi2* root hairs do not express the $Pro_{MtENOD11}$-*GUS* reporter gene after NF application, we propose a split in NF-induced signalling, with one branch to root hair curling, the other to $Pro_{MtENOD11}$-*GUS* expression.

Pea plants can be successfully nodulated by certain strains of rhizobia that produce hardly detectable amounts of NF. In addition, very low concentrations

of purified NF elicit changes in root hair morphology and gene expression in other legume species. Therefore, we tested what is the lowest NF concentration at which root hair reorientation and *Pro*$_{MtENOD11}$*-GUS* expression still occur. In chapter 4, we show the exciting result that one single NF molecule is sufficient to induce root hair reorientation and *Pro*$_{MtENOD11}$*-GUS* expression.

In chapter 5 we describe the results that we obtained after spot application of nod factor mixed with pharmacological agonists or antagonists of signal transduction pathways. As such, we show that NF-induced root hair reorientation can be blocked with gadolinium ions, ions that specifically block calcium influxes in plant cells. Moreover, we show that we can induce root hair reorientation in a number of hairs after spot application of a mixture of calcium ions and the ionophore A23187. Pertussis toxin specifically inhibits heterotrimeric G-proteins. Upon spot application of a mixture of NF and pertussis toxin, root hairs do reorient their growth axis, but do not express the *Pro*$_{MtENOD11}$*-GUS* reporter gene. Spot application of mastoparan, a small peptide from wasp venom that activates heterotrimeric G-proteins, does not result in root hair reorientation, but does induce *Pro*$_{MtENOD11}$*-GUS* expression. Heterotrimeric G-proteins activate phospholipase C. Upon spot application of a mixture of NF and neomycin or U-73122, two known antagonists of phospholipase C, *Pro*$_{MtENOD11}$*-GUS* expression was inhibited, but root hair reorientation not. Phospholipase C is an enzyme that cleaves phosphoinositolbisphosphate (PIP_2) into diacylglycerolphosphate (DAG) and inositoltriphosphate (IP_3). With microinjection of caged IP_3 into growing *M. truncatula* root hairs and subsequent uncaging with an UV laser, we got expression of *Pro*$_{MtENOD11}$*-GUS* in a number of root hairs. This all shows that heterotrimeric G-protein coupled phosphoinositide signalling is involved in NF-induced *Pro*$_{MtENOD11}$*-GUS* expression in *M. truncatula* root hairs.

Chapter 6 is a review which covers the current state of the art in the research of the *Rhizobium*-legume symbiosis, with a special focus on signal transduction. It not only compares genetic dissection with pharmacological approaches, but also covers the cell biological aspects that are necessary to fully understand NF induced signal transduction.

Chapter 7 is a chapter which describes that SHAGGY-kinase signalling is involved in root hair deformation in thale cress (Arabidopsis). This is a small weed that is used as a non-legume model plant. We show that application of lithium ions induces root hair deformation, and that in roots which lack a SHAGGY-kinase, a significant higher percentage of root hairs deform upon lithium application. In plant cells, lithium ions influence ethylene and phosphoinositide signalling, but with the use of specific agonists and antagonists of these pathways, we show that lithium induced root hair deformation in Arabidopsis is not caused by disrupted ethylene or phosphoinositide signalling.

Dankwoord

Na de vele woorden die ik in dit proefschrift al geschreven heb, wil ik het kort maar krachtig afsluiten door iedereen, die op welke wijze dan ook heeft bijgedragen aan de totstandkoming van dit boekje, heel heel erg te bedanken. Zonder jullie was het niet gelukt!

John, 18 mei 2004.

Curriculum vitae

Ik ben geboren op 30 januari 1974 in Oostburg. In datzelfde Oostburg heb ik in 1993 mijn Gymnasium-diploma gehaald aan Scholengemeenschap ’t Zwin. In het najaar van dat jaar ben ik met de studie Biologie begonnen aan de Universiteit Utrecht. Tijdens mijn studie heb ik mezelf volledig gericht op de ontwikkelingsbiologie van vertebraten. Tijdens mijn eerste afstudeervak bij de vakgroep Embryologie, Universiteit Utrecht, heb ik onder begeleiding van Dr. Wim Dictus en Dr. Erwin Houtzager microinjectie geleerd om cel-cel contacten in delende embryo’s van de klauwkikker, *Xenopus laevis*, te onderzoeken. Mijn tweede afstudeervak heb ik op het Hubrecht laboratorium in Utrecht gedaan onder begeleiding van Dr. Dana Jongejan-Zivkovic. Hier heb ik, onder andere, microinjectie en micromanipulatie-technieken toegepast om de ontwikkeling van de voorhersenen in zebravisembryo’s te bestuderen. Het was de combinatie van ontwikkelingsbiologie en micromanipulatie die me na mijn afstuderen eind januari 1998 naar Wageningen Universiteit bracht voor een door NWO gefinancierd promotieonderzoek bij het laboratorium voor Plantencelbiologie. Onder begeleiding van Prof. Dr. Anne Mie Emons heb ik het onderzoek uitgevoerd dat in dit proefschrift is beschreven.

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