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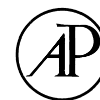

REVIEW

Time Course of Cell Biological Events Evoked in Legume Root Hairs by *Rhizobium* Nod Factors: State of the Art

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In many common legumes, when host-specific nodule bacteria meet their legume root they attach to it and enter through root hairs. The bacteria can intrude these cells because they instigate in the hairs the formation of an inward growing tube, the infection thread, which consists of wall material. Prior to infection thread formation, the bacteria exploit the cell machinery for wall deposition by inducing the hairs to form a curl, in which the dividing bacteria become entrapped. In most species, Nod factor alone (a lipochito-oligosaccharide excreted by bacteria) induces root hair deformation, though without curling, thus most aspects of the initial effects of Nod factor can be elucidated by studying root hair deformation. In this review we discuss the cellular events that host-specific Nod factors induce in their host legume root hairs. The first event, detectable only a few seconds after Nod factor application, is a Ca^{2+} influx at the root hair tip, followed by a transient depolarization of the plasma membrane potential, causing an increase in cytosolic [Ca^{2+}] at the root hair tip. Also within minutes, Nod factors change the cell organization by acting on the actin cytoskeleton, enhancing tip cell wall deposition so that root hairs become longer than normal for their species. Since the remodelling of the actin cytoskeleton precedes the second calcium event, Ca^{2+} spiking, which is observed in the perinuclear area, we propose that the initial cytoskeleton events taking place at the hair tip are related to Ca^{2+} influx in the hair tip and that Ca^{2+} spiking serves later events involving gene expression.
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Key words: Review, Nod factor, tip growth, root hair, *Rhizobium*, legume, cytoskeleton, calcium, symbiosis.

INTRODUCTION

The symbiotic relationship between legumes and rhizobia (legume root nodule bacteria, now classified in five genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*) is a complex process that results in the formation of nitrogen-fixing nodules within the host plant roots. In many common legumes, infection by rhizobia occurs through root hairs [for a description of other routes of infection see for example Sprent (1992)]. Successful infection of the roots by rhizobia is dependent upon a reciprocal molecular dialogue between the host plant and the rhizobia (Fisher and Long, 1992; Bladergroen and Spaink, 1998; Schultze and Kondorosi, 1998). Central molecules involved in the mechanisms leading to nodule formation are the so-called Nod factors, which are lipochito-oligosaccharides. Nod factor production is induced in the rhizobia by molecules that are produced by the host plant. These molecules are phenolic compounds, mainly flavonoids (Djordjevic *et al.*, 1985; for review, see Fisher and Long, 1992; Schultze and Kondorosi, 1998). Nod factors are excreted by the rhizobia and, even in the absence of the bacteria, trigger root hair deformation, cortical cell division and pre-infection thread formation (Fisher and Long, 1992; Heidstra and Bisseling, 1996; Schultze and Kondorosi, 1998). Preinfection threads are cytoplasmic bridges in the root cortical cells (Timmers *et al.*, 1998, 1999) which are reminiscent of phragmosomes, the first structures formed when cells initiate cell division (Venverloo and Libbenga, 1987). Infection threads, walled tubes formed by the plant cells in the root hairs and root cortical cells, and nodules, are formed in the root cortex only in the presence of bacteria. The appearance of all these cellular changes in the presence of bacteria is highly dependent upon the chemical structure of the Nod factor (Ardourel *et al.*, 1994; Demont-Caulet *et al.*, 1999). The first events triggered by Nod factors take place only a few seconds after their application (Ehrhardt *et al.*, 1992; Felle *et al.*, 1995), whereas some other events occur only after a few hours (Heidstra *et al.*, 1994; Heidstra and Bisseling, 1996; De Ruijter *et al.*, 1998; Schultze and Kondorosi, 1998; Downie and Walker, 1999; Jahraus and Bisseling, 2000; Sieberer and Emons, 2000). While studying Nod factor signalling, one has to bear in mind the spatio-temporal distribution of the induced responses. In this review, we describe the time course of the currently known events evoked by isolated Nod factors in root hairs, the first root cells to respond to Nod factors.

Calcium (Ca^{2+}) appears to be crucial in the Nod factor-induced changes. Therefore, the other Nod factor-induced changes, i.e. root hair deformation, actin cytoskeleton remodelling, endoplasmic reticulum re-orientation, vacuolation, changes in the pattern of cytoplasmic streaming,

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nuclear movement, cell wall deposition and cell wall loosening, will, if relevant, be related to Ca^{2+} dynamics.

NOD FACTOR PERCEPTION IN ROOT HAIRS

The fact that specific Nod factors trigger downstream events in their host plants at doses as low as 10^{-12}M (Lerouge *et al*., 1990; Heidstra *et al*., 1994), and that chito-oligosaccharides (the common inactive backbone of all Nod factors) are unable to trigger any, when externally applied, suggests the requirement of a receptor for the perception of Nod factors and the transduction of the signal. Though Nod factor receptors have not yet been cloned, two mechanisms have been proposed for the mediation of the Nod factor signal (Heidstra and Bisseling, 1996; Long, 1996; Bladergroen and Spaink, 1998). The first hypothesis involves the presence of a single receptor, the activity of which is dependent on the structure of the Nod factor (Hirsch, 1992). The Nod factor acyl chain could serve to anchor Nod factors into the plasma membrane close to a receptor (Bladergroen and Spaink, 1998). The fact that Nod factors integrate spontaneously in membranes but that transbilayer 'flip-flop' does not occur (Goedhart *et al*., 1999) is in good agreement with this hypothesis, but does not exclude the possibility of a multiple-receptor hypothesis. The second hypothesis suggests that two receptors are required to trigger all the downstream events (Ardourel *et al*., 1994; Heidstra and Bisseling, 1996; Long, 1996). The first receptor (signalling receptor) exhibits a low specificity for Nod factors (i.e. Nod factors lacking some substituted groups can bind to the signalling receptor) and induces root hair deformation, pre-infection thread formation, and cortical cell division in the presence or absence of the bacteria. The second receptor (uptake receptor) exhibits a high specificity for Nod factors (i.e. only host specific Nod factors can bind to the uptake receptor) and induces infection thread formation and nodule formation, but only in the presence of bacteria.

To date, two Nod factor-binding sites (NFBS1 and NFBS2) have been identified in microsomal preparations of *Medicago sativa* spp. *varia* (Niebel *et al*., 1997; Gressent *et al*., 1999). NFBS1 exhibits a low affinity for Nod factors, whereas NFBS2 exhibits a high affinity for Nod factors. Both binding sites can discriminate between host-specific and other forms of Nod factors. As such, they are potentially good candidates for Nod factor receptors. However, neither site is able to discriminate between sulfated (active) and non-sulfated (inactive) forms of Nod factors. The two-receptor model is attractive, since it can explain why structurally different Nod factors can elicit different sets of responses; nevertheless, no clear evidence has proven that downstream events triggered by Nod factors are mediated through one or more receptors. The acyl tail of Nod factors has been proposed to be involved in uptake and transport of Nod factors, whereas the substituted oligosaccharide moiety could activate an intracellular receptor (Philip-Hollingsworth *et al*., 1997; Schlaman *et al*., 1997). This hypothesis is very attractive as it could explain the fact that no Nod factor receptors have been clearly identified so far, but does not fit with the very early events that take place within a few seconds of Nod factor application (Ehrhardt *et al*., 1992; Felle *et al*., 1995).

Beside the possible involvement of receptors, several studies have shown that lectins might play a role in the recognition of Nod factors and/or bacteria (Diaz *et al*., 1986, 1995; Etzler *et al*., 1999; Hirsch, 1999). Indeed, Etzler *et al*. (1999) have shown that Nod factors from *Bradyrhizobium japonicum* bind to a *Dolichos biflorus* lectin and that this lectin possesses an apyrase (nucleotide phosphatase) activity that is stimulated by the addition of Nod factor. An enzymatic activity is quite unusual for a lectin but, if correct, might be a link between Nod factor recognition and signal transduction. However, no other lectins have been found so far with such an enzymatic activity. Thus, lectins are more likely to be involved in the interaction with the bacterial lipopolysaccharide in a host-specific manner, as proposed by Hirsch (1999).

CALCIUM CHANGES AT THE PLASMA MEMBRANE OF THE ROOT HAIR TIP: THE FIRST ROOT HAIR RESPONSE TO NOD FACTORS

The first event occurring a few seconds after Nod factor application, namely a transient influx of Ca^{2+} into the root hairs, was reported for *Medicago sativa* by Felle *et al*. (1998), who used non-invasive ion-selective microelectrodes. An inwardly directed Ca^{2+} influx was observed for at least 1 h in root hairs of *Phaseolus vulgaris* (Cárdenas *et al*., 1999). The increase in cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) within 3 min in root hair tips of *Medicago sativa* (Felle *et al*., 1999*a*) and the four-fold increase within 5 min in *Phaseolus vulgaris* root hair tips (Cárdenas *et al*., 1999), could activate anion channels in the plasma membrane. This could lead to an efflux of chloride ions (Cl^-) which is responsible for plasma membrane depolarization, as reported upon application of Nod factors by Ehrhardt *et al*. (1992) in *Medicago sativa*, Felle *et al*., (1995) and reviewed by White (1998) and Downie and Walker (1999).

The mechanism(s) involved in Nod factor-induced Ca^{2+} influx remain unclear, but it has been proposed that Ca^{2+} fluxes across the plasma membrane could be mediated directly via G-protein regulation of the plasma membrane Ca^{2+} channels (reviewed by Jan and Jan, 1997). Thuleau *et al*. (1998) reported the existence of two types of voltage-activated Ca^{2+} channels, hyperpolarization-activated Ca^{2+} channels and depolarization-activated Ca^{2+} channels, the latter are good candidates for the generation of the high $[Ca^{2+}]_c$ induced by Nod factors at the tips of root hairs. Nod factor-induced transient depolarization might thus activate depolarization-activated calcium channels leading to an increase in the $[Ca^{2+}]_c$ at the root hair tip. Nod factor-induced depolarization of the plasma membrane is a transient process that lasts between 15 and 30 min (Ehrhardt *et al*., 1992; Felle *et al*., 1995; Kurkdjian, 1995). Therefore, other mechanisms are likely to be involved to sustain the high $[Ca^{2+}]_c$ over the entire growth period—they are likely to be the same mechanisms as occur in normally-growing hairs prior to Nod factor application.

Microtubules have been proposed as potential regulatory elements of Ca^{2+} channel recruitment from experiments using the *Arabidopsis thaliana ton* mutant in which cortical microtubules are constitutively disorganized (Thion *et al*., 1998). In this mutant, Ca^{2+} channel activities were ten-times higher, and their half-life three times longer than recorded in the *Arabidopsis thaliana* wild type (Thion *et al*., 1998). So far, the molecular basis of this recruitment process remains largely obscure, but would imply that Nod factors may influence the organization of the root hair microtubule cytoskeleton.

In an assay in which root hair bearing roots were grown between glass slides, De Ruijter *et al*. (1998) showed that Nod factor-induced deformation of root hair tips was a re-initiation of tip growth in *Vicia sativa* growth-terminating hairs. Indeed, several studies have shown that, in tip-growing cells, there is a close correlation between growth and the presence of an elevated $[Ca^{2+}]_c$ at the tip (Schiefelbein *et al*., 1992; Bibikova *et al*., 1997; Wymer *et al*., 1997; De Ruijter *et al*., 1998; Cárdenas *et al*., 1999; Felle *et al*., 1999*a*, *b*, for review see Miller *et al*., 1997). Wymer *et al*. (1997) showed that tip growth in *Arabidopsis thaliana* root hairs is correlated with a high $[Ca^{2+}]_c$ at the tip and that growth stops when the Ca^{2+} channel-blocker verapamil is added to the external medium. This cessation of growth is accompanied by a decrease in the $[Ca^{2+}]_c$ at the root hair tip. Using $MnCl_2$ to quench the fluorescence signal from the fluorescent calcium reporter dye indo-1 in the cytoplasm, these authors determined that there was a higher density of open manganese ion (Mn^{2+})-permeable channels at the root hair tip than at its base. Moreover, the controlled asymmetric photo-release of the caged Ca^{2+}-ionophore, Br-A23187, generated asymmetric $[Ca^{2+}]_c$ gradients in the tip of *Arabidopsis* root hairs and also in *Tradescantia* pollen tubes (Bibikova *et al*., 1997). In both cases, this led to a re-orientation of growth towards the site of elevated $[Ca^{2+}]_c$. Together these results suggest that the presence of a high $[Ca^{2+}]_c$ in the tip of tip-growing cells is a requirement for tip growth. The Nod factor-induced increase of $[Ca^{2+}]_c$ in the root hair tips of legumes is consistent with this idea.

NOD FACTOR-INDUCED CALCIUM-MEDIATED SIGNALLING IN THE ROOT HAIR TIP

Using the generic activator of animal G-proteins, mastoparan, Pingret *et al*. (1998) showed that mastoparan could mimic Nod factor-induced transcription of MtENOD12-GUS. In addition, pertussis toxin, which interferes with the interaction between G-proteins and receptors, inhibited the Nod factor-induced MtENOD12-GUS expression. Furthermore, the widely used eukaryotic phospholipase C (PLC) antagonist, neomycin, and the aminosteroid PLC inhibitor, U73122, inhibited Nod factor-elicited MtENOD12 expression. From these results, the authors concluded that part of the cascade involved in Nod factor signalling is mediated by a G-protein and PLC. However, one has to keep in mind that G-proteins may also activate phospholipase D (PLD) (De Vrije and Munnik, 1997; Van Himbergen *et al*., 1999) and phospholipase A2 (PLA2) (Munnik *et al*., 1998). Thus, the inhibition of MtENOD12 expression by G-protein inhibitors does not exclude the possibility that PLD and/or PLA2 are involved in Nod factor-induced processes. However, the experiments indicate that the inositol 1,4,5-trisphosphate (IP_3) signal transduction cascade may well be employed by Nod factors, at least for gene expression.

Type II PLCs are predominantly bound to the plasma membrane, use polyphosphoinositides as preferred substrate, and are fully activated in the presence of micromolar Ca^{2+} concentrations. Hydrolysis of phosphatidylinositol bisphosphate (PIP_2) by PLC leads to the production of the water-soluble IP_3 and the lipid product diacylglycerol (DAG), which remains in the plasma membrane (Fig. 1). In mammalian cells, the increase in DAG is rapidly counteracted by its reversible conversion to phosphatidic acid (PA) by the enzyme diacylglycerol kinase (DGK). DGK has been identified in various plant systems (for review see Munnik *et al*., 1998). In addition, PA can be produced by stimulation of PLD. Several plant PLD genes have been cloned (Pappan *et al*., 1997; Qin *et al*., 1997; Munnik *et al*., 1998) and the enzyme can be found either in soluble form or associated with the plasma membrane (see Table 3 in Munnik *et al*., 1998). *In vitro*, both forms have a strict requirement for millimolar $[Ca^{2+}]$, too high for an activity *in vivo*. However, Dyer *et al*. (1995) and Qin *et al*. (1997) reported the presence of three novel PLDs, namely PLDα, β and γ. Whereas the α form is active at millimolar $[Ca^{2+}]$, the β and γ forms are active at submicromolar $[Ca^{2+}]$ (Pappan *et al*., 1997; Qin *et al*., 1997). Interestingly, the activity of the β and γ forms is dependent on PIP_2, whereas the activity of the α form is independent of PIP_2 (Pappan *et al*., 1997; Qin *et al*., 1997).

In animal cells, a group of PLDs is activated by Rho GTPases and PKC (Munnik *et al*., 1998). Interestingly, a Rho-like GTPase has been identified in pollen tubes, which is involved in tip growth (Lin *et al*., 1996; Lin and Yang, 1997; Yang, 1998). On the other hand, PA, the product of PLD activity, can be dephosphorylated to give DAG (Fig. 1) (McCormac *et al*., 1993; Voisine *et al*., 1993; Griebau and Frentzen, 1994; Malherbe *et al*., 1995) which can, in turn, activate PKC. Several roles have been described for PA in plant cells, but one of them is of great interest in the context of Nod factor-related signalling. PA is an endogenous Ca^{2+} ionophore (Munnik *et al*., 1998; Wang, 1999). Thus, PA could autoamplify PLD signalling by increasing Ca^{2+} influx. Furthermore, PA can amplify the PLC signalling cascade by activating the enzyme phosphatidylinositol 4-phosphate 5-kinase (PIP-kinase), leading to the production of PIP_2, and/or PLC, leading to an increased production of DAG and IP_3. This enhanced production of PA by either PLC alone or both PLC and PLD together might be part of the mechanism involved in sustaining the high $[Ca^{2+}]_c$ at the root hair tips during normal and Nod factor-induced tip growth.

Another mechanism that might be involved in sustaining the tip elevated $[Ca^{2+}]_c$ is the calcium-induced calcium release (CICR) from internal stores. This process has been extensively studied in animal cells and is potentially

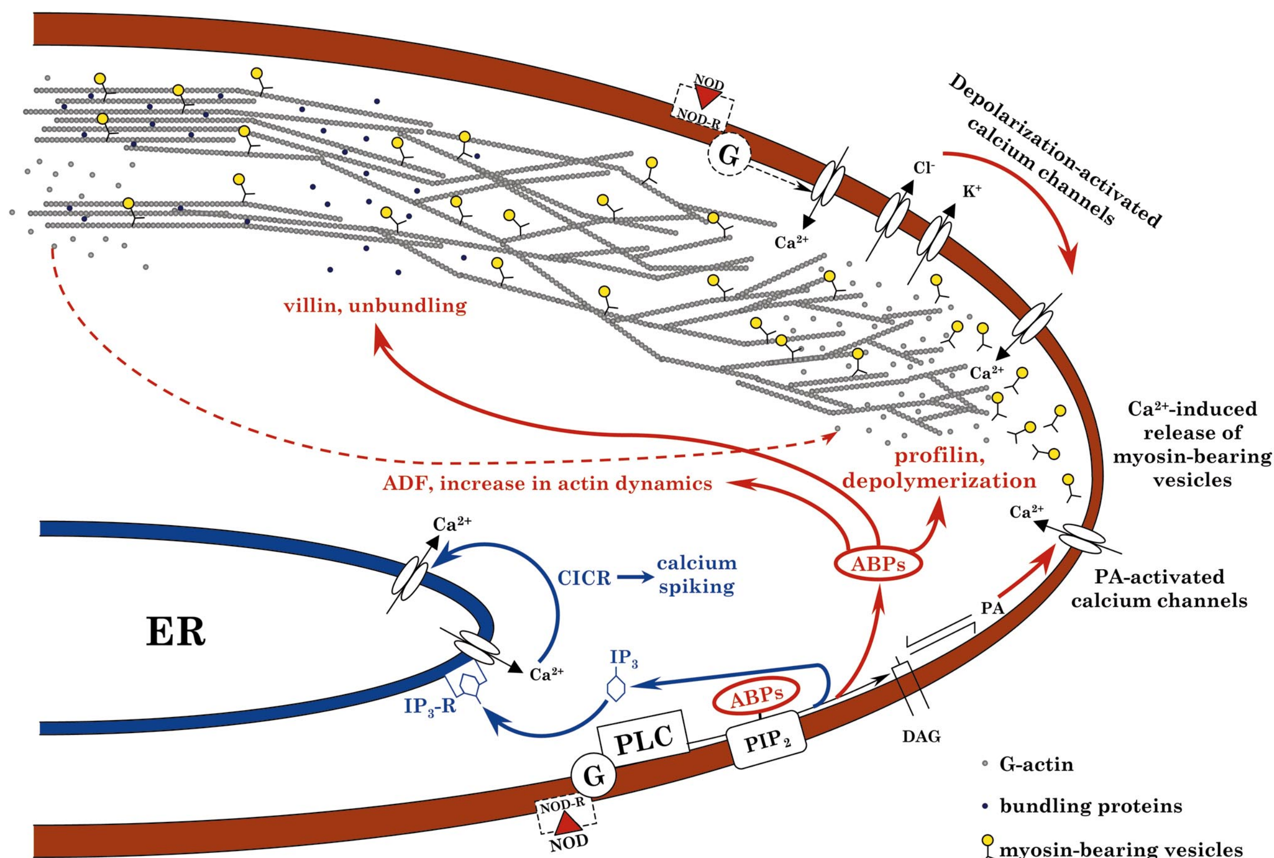

FIG. 1. Scheme showing the putative mechanisms involved in the generation and the maintenance of the high $[Ca^{2+}]_c$ at the tip of root hairs after Nod factor application and the consequences of a high $[Ca^{2+}]_c$ on the actin cytoskeleton. See text for details. NOD, Nod factor; NOD-R, putative Nod factor receptor; G, G-protein; PLC, phospholipase C; PIP_2, phosphatidylinositol bisphosphate; ABPs, actin binding proteins; IP_3, inositol 1,4,5-trisphosphate; IP_3-R, inositol 1,4,5-trisphopshate receptor; DAG, diacylglycerol; PA, phosphatidic acid.

important for amplifying changes in $[Ca^{2+}]_c$ in plants (Bush, 1995). IP_3 is known, mostly from animal studies [reviewed by Bush (1995) and Munnik *et al.* (1998)], to induce Ca^{2+} release from internal stores. Furthermore, it has been shown to exhibit a similar activity in plants (Alexandre *et al.*, 1990; Alexandre and Lassalles, 1990; Bush, 1995; Munnik *et al.*, 1998). Malhó (1998) reported that asymmetric photo-release of caged-IP_3 in pollen tube tips resulted in a transient $[Ca^{2+}]_c$ elevation and also induced slight or transient growth re-orientation. However, the photo-release of caged-IP_3, either in the sub-apical area or in the nuclear area, induced transient $[Ca^{2+}]_c$ elevation in the nuclear region followed by slow waves, and random sustained growth re-orientation as observed by both Malhó and Trewavas (1996) and Franklin-Tong *et al.* (1996). From these results the authors suggest that, in pollen tubes, IP_3 does not seem to be required for activation of Ca^{2+} entry at the tip, one of the primary events leading to re-orientation. Instead, IP_3-induced Ca^{2+} release seems to play a vital role in the transduction of the signal to the body of the tube.

SIGNALLING TOWARDS THE ACTIN CYTOSKELETON

The actin cytoskeleton reacts to Nod factors within 3 min of their application (*Phaseolus vulgaris*: Cárdenas *et al.*, 1998; *Vicia sativa*: De Ruijter *et al.*, 1999). In *Vicia sativa*, this reaction is manifested as an increase in the density of subapical fine bundles of actin filaments (De Ruijter *et al.*, 1999) called FB-actin (Miller *et al.*, 1999). Subapical FB-actin is always observed in growing root hairs. Also, there is an area, distal from it, at the extreme tip, which is devoid of bundles of actin filaments (Miller *et al.*, 1999). Neither of these features is ever seen in full-grown hairs (Miller *et al.*, 1999). Root hairs of all developmental stages (growing, growth-terminating and full-grown hairs), respond to Nod factors with an increase in length and density of FB-actin (Compare Fig. 2B, D, F and H with A, C, E and G, respectively). Interestingly, after Nod factor application, the FB-actin density in full-grown hairs never reaches the FB-actin density observed in control growing hairs (Compare Fig. 2B and A). This could be one reason why full-grown hairs do not usually exhibit deformation after Nod factor application. Alternatively, the presence of a secondary cell wall at the tip of full-grown root hairs—which has not yet been shown for legumes but is present in other species—may be another reason for the inability of these hairs to deform (Emons and Wolters-Arts, 1983).

How does the process of re-organization of the actin cytoskeleton, following Nod factor application, take place? Actin exists in plant cells as either filaments or monomers. The filaments (F-actin) consist of monomers (G-actin) and

possess a determined polarity, a fast growing 'plus end' (barbed end) and a slow growing 'minus end' (pointed end). Actin filaments are dynamic structures controlled by an unstable equilibrium between polymerization and depolymerization. Actin monomers are present in the cytoplasm either bound to ADP or ATP; ATP-actin polymerizes more easily than ADP-actin. The function and organization of actin filaments is largely determined by their regulatory proteins, the so-called actin binding proteins (ABPs). Actin-binding proteins that also have phospho-inositide binding sites are good candidates for regulators of changes in the actin cytoskeleton after Nod factor application. Profilin, actin-depolymerizing factor (ADF), spectrin, gelsolin, the arp2/3 complex and myosin will be discussed with regard to their possible role(s) in actin cytoskeleton remodelling.

Profilin

Profilin is a 12–15 kDa protein that binds to monomeric actin to form profilactin. Two distinct activities have been reported for profilin: firstly, by binding actin monomers, profilin may promote actin filament depolymerization in plant cells, as shown by Staiger *et al.* (1994) and Valster *et al.* (1997). On the other hand, by modifying the ratio between ATP-actin and ADP-actin in favour of ATP-actin, profilin promotes actin polymerization *in vitro* (Goldschmidt-Clermont *et al.*, 1992). Profilin can bind to poly-L-proline and to PIP_2 (Machesky and Pollard, 1993; Staiger *et al.*, 1997). Binding to PIP_2 results in a dissociation of the profilactin complexes (Machesky and Pollard, 1993). Since Nod factors induce Ca^{2+} influxes (Cárdenas *et al.*, 1999; Felle *et al.*, 1999*a*, *b*), possibly activated by PLC (Pingret *et al.*, 1998), Nod factors may induce the hydrolysis of PIP_2 by PLC. The hydrolysis of PIP_2 releases bound profilin which can form profilactin complexes, thus displacing the equilibrium between F-and G-actin in favour of G-actin formation. This leads to the depolymerization of F-actin. The depolymerization of F-actin could then (1) induce disintegration of bundles of actin filaments at the tip of root hairs to increase the length of the actin filament-free area and (2) provide actin monomers for actin cytoskeleton re-arrangement. The time course of the appearance of FB-actin at the tips of *Vicia sativa* root hairs (Fig. 2; De Ruijter *et al.*, 1999) and of the formation of the elevated tip focused $[Ca^{2+}]_c$ after Nod factor application (*Phaseolus vulgaris*: Cárdenas *et al.*, 1999; *Medicago sativa*: Felle *et al.*, 1999*b*) are consistent with the idea that the elevated $[Ca^{2+}]_c$ causes re-arrangement of the actin cytoskeleton. However, to date, there is no hard proof that functional profilin is indeed localized in the growing root hair tip. The report by Braun *et al.* (1999), showing the localization of profilin in maize root hairs, may indicate such a presence. However, accessible cell volume was not taken into account in this research, which may be relevant for the determination of the localization of a small, difficult-to-fix cytoplasmic protein like profilin (see discussion in Emons and De Ruijter, 2000). The presence of a weak tip-to-base gradient of PIP_2 in these hairs, which is lacking in full-grown hairs (Braun *et al.*, 1999), is interesting, but should be interpreted with care.

Gelsolin

A second Ca^{2+}-regulated ABP that requires attention is gelsolin and its superfamily, including villin and supervillin (Robinson *et al.*, 1999; Cooper and Schafer, 2000). These proteins possess severing and capping properties which model actin filaments. Activation of heterotrimeric and small GTP binding proteins dissociates gelsolin from the barbed ends of actin filaments (Cooper and Schafer, 2000). In addition to the severing and capping activities of the gelsolin-related portion of the protein, villin has a small headpiece that bundles actin filaments *in vitro* (Cooper and Schafer, 2000). In the presence of Ca^{2+}, villin can sever actin filaments into shorter filaments, whereas in the absence of Ca^{2+}, it bundles actin filaments (Yao and Forte, 1996). Recently, the presence of proteins from the villin-gelsolin family has been shown in *Lilium longiflorum* pollen tubes (Vidali *et al.*, 1999), maize pollen (Wu and Yan, 2000) and *Hydrocharis* root hairs (Tominaga *et al.*, 2000). For *Hydrocharis* root hairs, it was shown that a villin homologue is involved in the bundling of actin filaments which are present in transvacuolar strands (Tominaga *et al.*, 2000). This is an interesting observation for us, since the rearrangement of the actin cytoskeleton after Nod factor application may be described as bundling/unbundling of actin filaments at the base of the FB-actin area (arrow Fig. 2, see also De Ruijter *et al.* 1999), i.e. the interface between the subapical FB-actin and the thicker bundles of actin filaments in the cytoplasmic strands.

Actin-depolymerizing factor (ADF)

A third group of ABPs that may be relevant in Nod factor-induced actin cytoskeleton remodelling is the actin depolymerizing factor (ADF) family, which includes destrin, cofilin and actophorin (Staiger *et al.*, 1997). ADF/cofilin is a 15–22 kDa protein that possesses an actin- and a PIP_2-binding domain (Sun *et al.*, 1995; Staiger *et al.*, 1997; Welch *et al.*, 1997; for review on actin and ABPs in plant cells see De Ruijter and Emons, 1999; for review of ABPs see Cooper and Schafer, 2000). ADF binds to phosphoinositides and promotes actin dynamics *in vitro*. The ability of ADF to sever and depolymerize FB-actin is not directly dependent on the $[Ca^{2+}]_c$, but on pH (Staiger *et al.*, 1997; Maciver *et al.*, 1998). In maize growing root hairs, ADF can be localized in the apex and sub-apex (Jiang *et al.*, 1997), where FB-actin is also present (Miller *et al.*, 1999), whereas it is uniformly distributed in bulging trichoblasts in which no FB-actin has been detected (Miller *et al.*, 1999). It has been shown that upon application of Nod factors a sustained alkalinization of the root hair cytoplasm occurred (Ehrhardt *et al.*, 1992; Felle *et al.*, 1995, 1996). This increase in pH might activate ADF, thus leading to F-actin depolymerization and subsequent remodelling of the actin cytoskeleton.

Spectrin

Animal spectrins possess at least two actin filament-binding sites, as well as binding sites for calcium and

calmodulin (Tanaka *et al*., 1991; Puius *et al*., 1998). Spectrins are proteins involved in cross-linking actin filaments through Ca^{2+}/CaM-regulated coupling of plasma membrane proteins to actin (Tanaka *et al*., 1991) and also in mediating signal transduction through interactions with extracellular proteins via integrins (Burridge *et al*., 1988). Spectrin-like antigens are present in growing, but not in full-grown *Vicia sativa* root hair tips and reappear in hairs that are terminating growth when Nod factor is applied (De Ruijter *et al*., 1998). Unfortunately, a plant spectrin gene has not yet been cloned and its function is not known.

Arp2/3 complex

A protein complex that should be studied in root hairs before and after Nod factor application is the Arp2/3 complex. *In vitro*, purified Arp2/3 complex binds to the side of actin filaments and nucleates the formation of actin filaments with barbed ends (Mullins *et al*., 1998; Mullins, 2000). Mullins (2000) proposes that, in animal cells, prenylated GTP-bound CDC42 (a member of the Rho-GTPases family) localizes to the plasma membrane where it recruits and activates WASP (Wiskott-Aldrich Syndrome Protein). WASP then recruits the Arp2/3 complex and stimulates its nucleation activity. The Arp2/3 complex then nucleates formation of actin filaments and cross-links them into a branching network. Finally, the elongation of free barbed-ends causes protrusion of the plasma membrane. A similar process of actin filament nucleation from existing filaments may be involved in FB-actin density increase after Nod factor application. The hypothesis cannot be extrapolated as such to root hairs, however, since in the hairs with FB-actin protrusion, i.e. in the growing and Nod factor stimulated hairs, the actin filaments do not appear to be close enough to the plasma membrane (Miller *et al*., 1999). The presence of a putative Arp2 homologue has been demonstrated in *Arabidopsis* (Klahre and Chua, 1999; Lin *et al*., 1999) and the study of its involvement in relation to tip growth and the rearrangement of the actin cytoskeleton after Nod factor treatment would be an exciting challenge.

Myosin

Remodelling of the actin cytoskeleton is necessary for the Nod factor-induced root hair deformation to take place, but might not be sufficient in itself to explain the entire process. Yokota *et al*. (1999) reported that the absence of cytoplasmic streaming at the tip of lily pollen tubes is due to the inhibition of a 170 kDa-myosin. This 170 kDa-myosin from lily pollen tubes possesses calmodulin (CaM) as a light chain. Binding of Ca^{2+} to this CaM induced a partial dissociation of the light chain from the heavy chain (Yokota *et al*., 1999)—this was sufficient to inhibit myosin activity. These authors also reported that in *Characean* cells the inhibition of myosin is triggered by phosphorylation through a Ca^{2+}-dependent protein kinase (CDPK). Moutinho *et al*. (1998) studied the distribution of CDPK activity in growing pollen tubes of *Agapanthus umbellatus* and found a higher CDPK activity at their tip. In non-growing pollen tubes, CDPK activity was uniformly distributed throughout the cell. The photo-release of caged Ca^{2+} on one side of the apical dome resulted in a local increase of CDPK activity at the site of release. From these results, it can be hypothesized that Ca^{2+} is responsible for the release of vesicles from actin filaments through inhibition of a 170 kDa-myosin homologue; myosin isoforms responsible for the movement of bigger organelles might exhibit a lower or no sensitivity to Ca^{2+}. This hypothesis is in good agreement with the presence of a vesicle-rich region (clear zone), devoid of large organelles, at the tip of tip-growing cells.

CYTOPLASMIC STREAMING, ENDOPLASMIC RETICULUM RE-ORIENTATION, VACUOLATION AND NUCLEAR MOVEMENT

During root hair growth, large organelles are absent from the tip proper which contains only vesicles (Fig. 3A, see also Miller *et al*., 2000). Organelles in the rest of the root hair move upward towards the tip in the tube flanks. The organelles reverse the direction of movement in the cytoplasmic dense subapex, inside a cytoplasmic strand; this can occur either in the cell centre or near the flank of the tube. This pattern of organelle movement is called reverse fountain streaming (*Medicago truncatula*: Sieberer and Emons, 2000). The endoplasmic reticulum (ER) in the central part of the cytoplasmic dense region, the subapex of the cell, is aligned longitudinally (Fig. 3B; Miller *et al*., 2000). The large central vacuole is located below the nucleus at the base of the cytoplasmic dense region and has thin protrusions into this region. When root hairs terminate growth, the vacuole gradually overtakes the nucleus and moves towards the tip while the cytoplasmic dense region decreases in size (Sieberer and Emons, 2000). This process continues until the central vacuole fills the hair tip at hair maturity, except for a thin surrounding layer of cytoplasm. During growth termination, the ER becomes aligned transversely to the cell axis (Fig. 3C, see also Miller *et al*., 2000). When comparing actin data (Fig. 2, see also Miller *et al*., 1999) with TEM observations of ER (Fig. 3B and C, see also Miller *et al*., 2000), one can deduce that ER co-aligns with the actin filaments; however, this still needs to be proven in double-labelling experiments.

When Nod factors are applied to roots growing between glass slides, the central vacuole in hairs that are terminating growth expands towards the hair tip more rapidly than in normal growth-terminating hairs (Sieberer and Emons, 2000). At the same time, the vesicles that were present at the tip seem to be spread out over a larger plasma membrane area, as seen in TEM images (Miller *et al*., 2000). This could cause the swelling of the tip of these hairs, always seen in this assay. In the swelling the ER is aligned with the plasma membrane, meaning that at the cell tip the ER is transverse to the hair's long axis (Fig. 3C), as it was in these growth-terminating hairs before Nod factor application. Bundles of actin filaments in the swelling also have the same orientation. When the outgrowth emerges from the swelling, a new cytoplasmic dense region is rebuilt with reverse

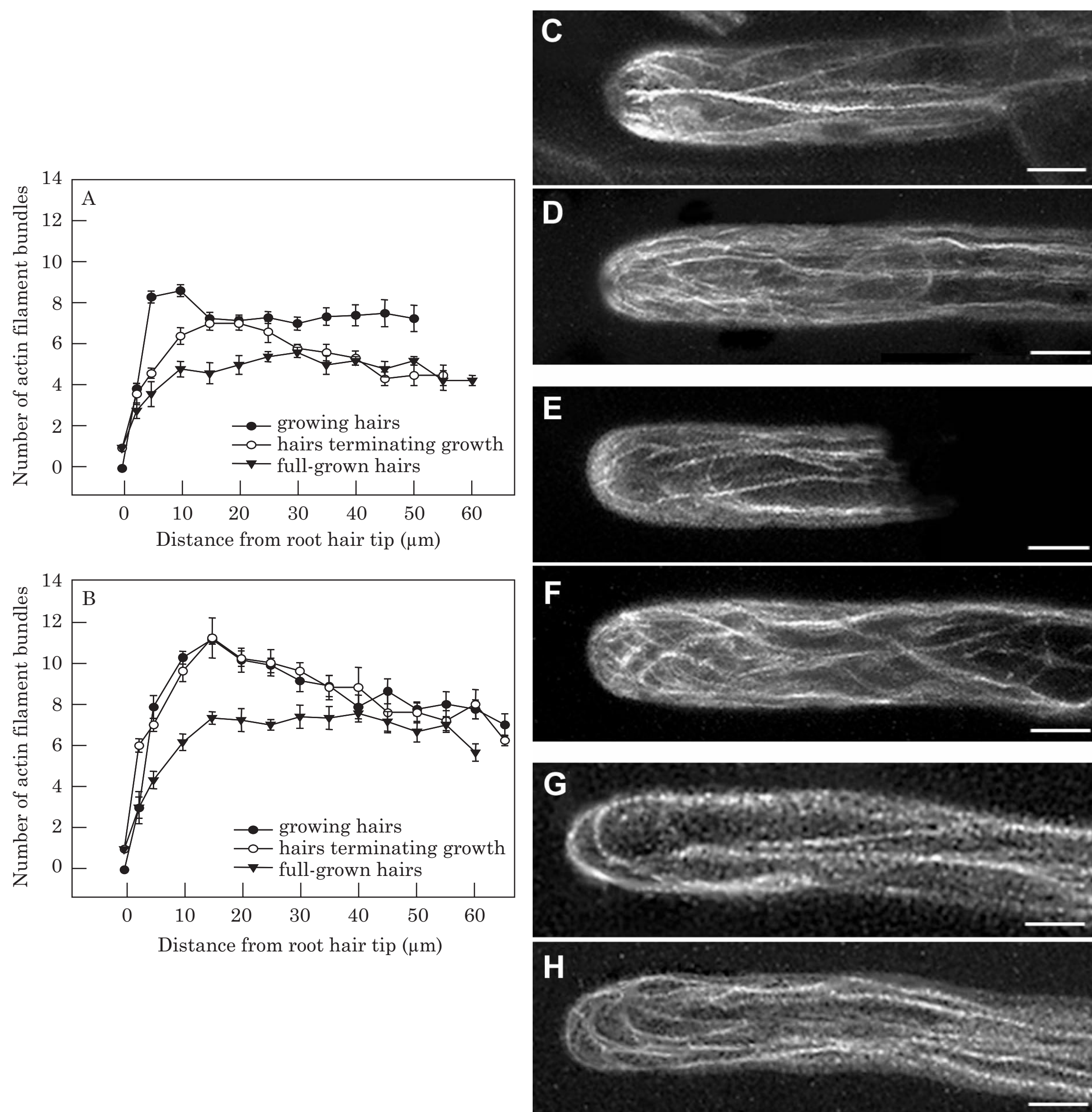

FIG. 2. Changes in the density of fine bundles of actin filaments in the subapical area of *Vicia sativa* root hairs after Nod factor application. Graphs showing the increase in the number of actin filament bundles before (A) and between 3 to 15 min after (B) Nod factor application. C–H, Visualization of actin filaments after staining with rhodamine-phalloidin before (C, E and G) and after (D, F and H) Nod factor application to growing (C and D), growth-terminating (E and F) and full grown (G and H) *Vicia sativa* root hairs. Bar = 10 µm. (Adapted from De Ruijter *et al.*, 1999).

fountain cytoplasmic streaming, longitudinal ER and longitudinal FB-actin. These results suggest that ER re-orientation upon application of Nod factors is likely to be related to the re-arrangement of the actin cytoskeleton. The new outgrowth can become as long as a normal root hair, meaning that the final length is twice that of normal. Studying the dynamics of the actin cytoskeleton in living root hairs upon Nod factor application would lead to a better understanding of these processes. In this context, the use of transformed plants, able to produce green fluorescent protein-tagged actin, is very promising.

In a growing root hair, the nucleus exhibits an almost fixed position at the base of the cytoplasmic dense region, approx. 30 µm below the tip in *Medicago truncatula* (Fig. 4A; Sieberer and Emons, 2000). The nucleus, therefore, moves with the pace of tip growth (Ketelaar and Emons, 2000). When root hairs terminate growth, the nucleus loses this position and moves backwards in the shank (Fig. 4B; Sieberer and Emons, 2000) of the root hair where it assumes a random position. During Nod factor induced tip swelling, the nucleus is positioned at the base of the swelling (Fig. 4C; Sieberer and Emons, 2000). When the

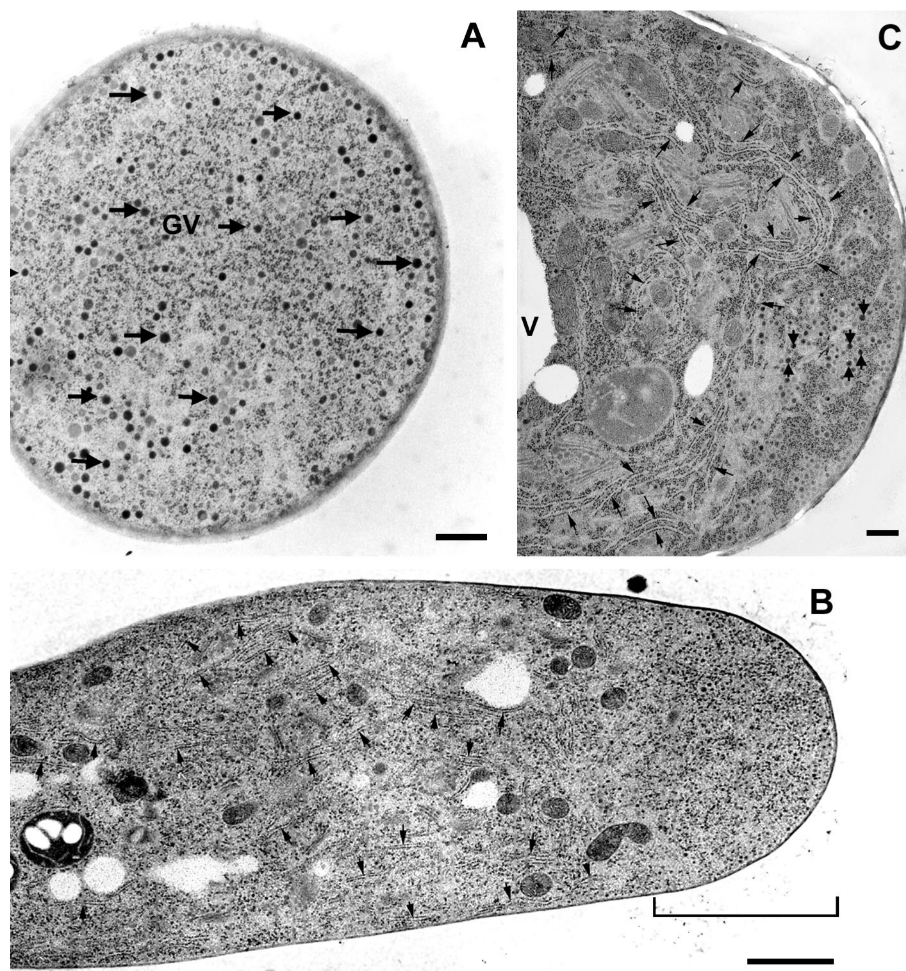

FIG. 3. Electron micrographs showing the ultrastructure of *Vicia sativa* root hairs. A, Transverse section through the apical dome of a growing root hair showing numerous vesicles (arrows) and the absence of any large organelles. GV, Golgi vesicles. Bar = 100 nm. B, Longitudinal median section through the apex and sub-apex of a growing root hair showing the presence of numerous longitudinally aligned ER cisternae in the sub-apex (arrows) and their absence in the apex (bracket). Bar = 2 µm. C, Longitudinal section through the tip of a growth-terminating root hair. Bar = 1µm. The vacuole is close to the tip and the cytoplasm is reduced to a thin layer along the plasma membrane. ER (small arrows) at the tip is transversely aligned to the root hair axis. The vesicle-rich region is considerably reduced compared to that in a growing hair (B). Large arrows, Golgi vesicles. For Materials and Methods, see Miller *et al.* (2000).

length of the new outgrowth has reached approx. 20 µm, the nucleus enters it. The new outgrowth continues to grow and the nucleus again assumes a fixed position approx. 30 µm below the tip (Fig. 4D; Sieberer and Emons, 2000). When the outgrowth stops growing, the nucleus again moves backward in the shank of the root hair where it again assumes a random position. Although these results are not yet fully understood, they indicate that the position of the nucleus in the root hairs is important in the process of tip growth (Derksen and Emons, 1990; Ketelaar and Emons, 2000).

CALCIUM SPIKING

The above-mentioned cellular changes in actin, ER orientation, cytoplasmic streaming and vacuolation are rapid; they occur within minutes. They are related to Ca^{2+} by means of the $[Ca^{2+}]_c$ gradient at the cell tip. However, a different Ca^{2+} phenomenon has been observed approx. 9 min after Nod factor application. Ehrhardt *et al.* (1996) reported the presence of Ca^{2+} spikes originating from the perinuclear region of *Medicago sativa* root hairs to which host specific Nod factors were applied. The spikes propagated bi-directionally from the perinuclear area. Measurements taken along the root hairs showed that the amplitude of the spikes decreased with distance from the perinuclear area and that no spikes were detected at the tip. A non-nodulating *Medicago sativa* mutant failed to show such Ca^{2+} spiking, whereas seedlings from the parental line from which the mutant was isolated showed a normal pattern of spiking. From these results, the authors suggested that the initiation of Ca^{2+} elevation in the region of the cell nucleus implies that either Ca^{2+} stores or channels, which mediate Ca^{2+} release, are localized in this region and are probably internal. Similar spiking has been observed in the root hairs of other species, e.g. *Vicia*, *Lotus*, *Medicago* and *Pisum* (Downie and Walker, 1999). IP_3 is known to induce Ca^{2+} release from internal stores such as vacuoles (Alexandre and

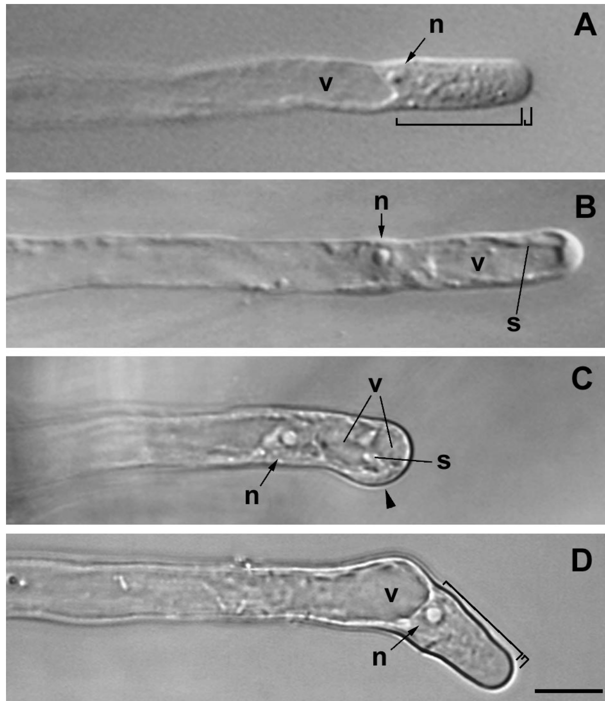

FIG. 4. DIC microscopy images of control and Nod factor-induced root hair deformation in *Medicago truncatula* root hairs. A, Untreated growing hair; B, Untreated growth-terminating root hair; C, Growth-terminating root hair approx. 105 min after Nod factor application with swollen tip; D, Nod factor-induced outgrowth from swelling approx. 210 min after Nod factor application. Note the similar cytoarchitecture of the growing hair (A) and of the outgrowth after Nod factor application (D). n, Nucleus; v, vacuole; s, strands of cytoplasm. Large bracket, cytoplasmic dense region; small bracket, vesicle-rich region; black arrowhead, site of new outgrowth emergence from the swollen tip. Bar = 20 µm. (Adapted from Sieberer and Emons, 2000).

Lassalles, 1990; Alexandre *et al*., 1990) and ER (in animal cells: De Young and Keizer, 1992; in plant cells: Bush, 1995; Muir and Sanders, 1997; Gong *et al*., 1998). Because ER is especially located in the perinuclear area of root hairs (not shown), the spikes observed by Ehrhardt *et al*. (1996) and Cárdenas *et al*. (1998) are possibly initiated by Ca^{2+} release from IP_3-sensitive ER stores. The lag phase between Nod factor application and the appearance of the spikes, and the fact that they are only clearly detectable in the vicinity of the nucleus, indicate they are unlikely to be related to the early Nod factor-induced cellular changes discussed above. The role of the spikes is currently unclear; however, they may carry information to other parts of the cell or be involved in gene expression (Schultze and Kondorosi, 1998; Felle *et al*., 1999*a*, *b*) and subsequent protein synthesis. The spikes originate from the perinuclear region and a high $[Ca^{2+}]$ can be observed in the nucleus 5 s after the initiation of spiking (Ehrhardt *et al*., 1996), which is consistent with mechanisms involving the nucleus. Whether the experiments of Pingret *et al*. (1998) showing the involvement of G-proteins and PLC are related to the high $[Ca^{2+}]_c$ at the cell tip or calcium spiking around the nucleus should be studied further by application of drugs and the scoring of cellular events over time.

Spikes were observed for at least 60 min and up to 3 h (Ehrhardt *et al*., 1996). It seems unlikely that the internal stores contain such high amounts of Ca^{2+} as to sustain spiking for hours. Thus a process for store refilling is required. Felle *et al*. (1999*a*) suggested that at least a part of the Nod factor response is triggered by Ca^{2+} from internal stores. This response was blocked using the endomembrane Ca^{2+}-ATPase inhibitor, 2,5-di(t-butyl)-1,4-benzohydroquinone, which presumably mobilizes Ca^{2+} from IP_3-sensitive stores. Their results suggest that Ca^{2+}-ATPase might be involved in refilling of internal stores as described for animal cells (Alberts *et al*., 1994). In addition, the mathematical model of De Young and Keizer (1992) shows that a single pulse of IP_3 is enough to generate spikes through a self-sustained mechanism involving Ca^{2+}-activation of PLC and Ca^{2+}-ATPase. In this model, a single pulse of IP_3 is sufficient to induce Ca^{2+} spiking according to the following process: binding of IP_3 to its receptor on the endoplasmic reticulum membrane induces the opening of Ca^{2+} channels and a subsequent rapid release of Ca^{2+} into the cytosol. The increasing $[Ca^{2+}]_c$ slowly inactivates the IP_3 receptor and activates Ca^{2+}-ATPase pumps on the ER membrane, thus initiating the refilling of the ER. When the $[Ca^{2+}]_c$ has been reduced sufficiently, the inactivation disappears and the channel again quickly activates. In addition, elevated $[Ca^{2+}]_c$ can directly activate PLC leading to an increased production of IP_3. Ca^{2+} spikes can thus be generated and maintained by a single pulse of IP_3 and a single Ca^{2+} store (ER) using a mechanism involving positive and negative feedback of Ca^{2+} on PLC and the IP_3-receptor. This model is highly consistent with the spikes observed by Ehrhardt *et al*. (1992) and Cárdenas *et al*. (1999), which were initiated approx. 9 min after Nod factor application and lasted for several hours. According to this model, IP_3 in root hairs might be responsible for Ca^{2+} spikes, but not for the elevated tip-focused $[Ca^{2+}]$.

EXOCYTOSIS AND CELL WALL FORMATION

In tip growing cells, growth takes place by the fusion of exocytotic Golgi vesicles at the tip. The membrane of the vesicles is inserted into the plasma membrane and the vesicle content is delivered into the extracellular matrix and assembles within the cell wall. Using differential interference contrast (DIC) microscopy, we have shown that just below the tip of a growing root hair, a thin area devoid of large organelles is present, the so-called clear zone (Miller *et al*., 1997; *Vicia sativa*: De Ruijter *et al*., 1998; *Medicago truncatula*: Sieberer and Emons, 2000). The area is in fact filled completely with vesicles (Fig. 3A; Miller *et al*., 2000; for more references see reviews by Miller *et al*., 1997; Emons and De Ruijter, 2000), and, thus, is also called the vesicle-rich region. We do not know how the vesicles move within the vesicle-rich region. If they are continually delivered at the base of this region by the FB-actin and consumed at the plasma membrane by exocytosis, they may not need a transport system.

As far as is known now, the docking and fusion of vesicles with the plasma membrane involves Ca^{2+}

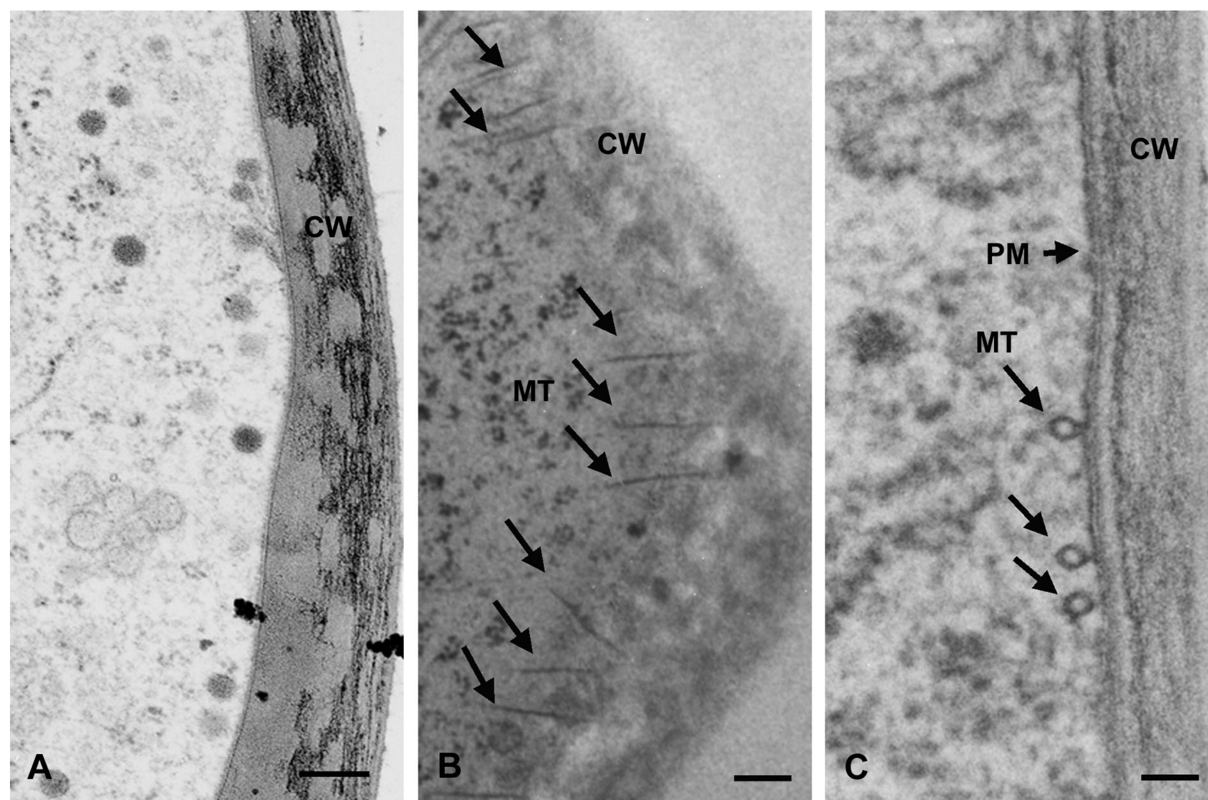

FIG. 5. Electron micrographs of longitudinal sections of *Vicia sativa* root hairs. A, Swollen tip of *Vicia sativa* root hair 52 min after Nod factor application. Bar = 200 nm. Note that the mottled aspect of the cell wall at the tip is similar to the aspect of cell wall during bulge formation (B, Bar = 100 nm) and completely different from the cell wall of an epidermal cell before bulge formation (C, Bar = 100 nm). CW, Cell wall; MT, microtubules; PM, plasma membrane; arrows, microtubules. For Materials and Methods, see Miller *et al.* (2000).

(Blackbourn *et al.*, 1991, 1992; Blackbourn and Battey, 1993; Clark and Roux, 1995; Lin and Yang, 1997; Carroll *et al.*, 1998; Yang, 1998; Battey *et al.*, 1999), annexins (Blackbourn *et al.*, 1991, 1992; Blackbourn and Battey, 1993; Clark and Roux, 1995; Carroll *et al.*, 1998; Yang, 1998; Battey *et al.*, 1999), Rho-GTPases (Lin *et al.*, 1996; Lin and Yang, 1997; Yang, 1998; Battey *et al.*, 1999; Sanderfoot and Raikhel, 1999) and proteins from the Soluble *N*-ethylmaleimide-sensitive factor Attachment Protein Receptor (SNARE) family (Battey and Blackbourn, 1993; Battey *et al.*, 1999; Sanderfoot and Raikhel, 1999). A general hypothesis for the mechanism of exocytosis has been proposed for animal cells (for review see Sanderfoot and Raikhel, 1999), which is in good agreement with what is known about the control of exocytosis in plant cells (for review see Battey and Blackbourn, 1993), and with other work that has shown the presence of Rho-GTPases in pollen tubes (Lin *et al.*, 1996; Lin and Yang, 1997; Yang, 1998) and t-SNARE-like proteins in plant cells (Battey *et al.*, 1999). The mechanism behind Rho-GTPases-Ca^{2+} interaction is still unknown; an explanation could be that Rho-GTPases regulate the accumulation of intracellular Ca^{2+} which itself is thought to be involved in the regulation of exocytosis through annexins (Blackbourn and Battey, 1993). The fact that in tip growing cells a high tip focused $[Ca^{2+}]_c$, Rho-GTPases (in *Pisum sativum* pollen tube: Lin *et al.*, 1996; Lin and Yang, 1997) and annexin-like proteins (in *Pisum sativum* pollen tube: Blackbourn *et al.*, 1991) can be detected at the tip only during growth supports the suggested mechanism.

Cell wall deposition and exocytosis are inter-related phenomena. Golgi vesicles carry newly synthesized cell wall materials which are delivered to the extra-cellular matrix by exocytosis. Meanwhile, the Golgi vesicle membranes carry the rosettes (Haigler and Brown Jr, 1986) which are the cellulose synthases (Kimura *et al.*, 1999). Assembly of the cell wall material within the existing wall contributes to its rigidification. In tip growing cells, there is a delicate balance between cell wall rigidification and cell expansion (pollen tube: Derksen, 1996): a rapid cell wall rigidification will prevent cell expansion, whereas a high degree of membrane insertion coupled to slow cell wall rigidification will lead to a swollen tip and even bursting (Schnepf, 1986; Battey and Blackbourn, 1993). To date, little research has been done on the molecular composition and subsequent alterations in the cell wall during root hair development.

After application of Nod factors to *Vicia sativa* or *Medicago truncatula* roots growing between glass slides, growth-terminating root hair tips swell (Fig. 4C) and the cell wall of the swelling exhibits a mottled aspect (Fig. 5A, see also Miller *et al.*, 2000). This is comparable to the appearance of the cell wall during bulge formation (Fig. 5B), but different from the wall before bulging or swelling (Fig. 5C, see also Miller *et al.*, 2000). Cell wall

acidification has also been demonstrated during bulge formation (Bibikova *et al.*, 1998); the initiation of root hair formation was arrested when acidification was prevented. A role for acidification in cell wall relaxation has been proposed (Pritchard, 1994) and expansins—proteins that trigger cell wall relaxation under acidic pH—are likely to be involved in this process (Cosgrove, 2000). Thus, it can be suggested that Nod factors induce cell wall relaxation during swelling of root hair tips and that this mechanism may be comparable to that of bulge formation. Immunogold electron microscopy studies are required to further identify the molecules, both structurally and enzymatically, involved in the synthesis and Nod factor-induced modification of the root hair cell wall.

Early nodulin genes (*ENOD*s) are induced shortly (a few hours) after Nod factor application (Nap and Bisseling, 1990; Hadri and Bisseling, 1998). Most of the *ENOD* genes are hydroxyproline-rich glycoproteins (HPRG-proteins) and are thought to be cell wall proteins, which are extensively post-translationally modified (Hadri and Bisseling, 1998; Schultze and Kondorosi, 1998; Jahraus and Bisseling, 2000). Due to their sequence, these genes are thought to be involved in the formation of the infection thread (extensins, glycine-rich proteins and a peroxidase) (for a review of Nod factors-induced gene expression see Hadri and Bisseling, 1998; Schultze and Kondorosi, 1998; Jahraus and Bisseling, 2000). Interestingly, the first gene expressed (Vb1) encodes for a leghaemoglobin; its expression is even faster than ENOD12. Vb1 is expressed in root hairs, but its expression is markedly higher in nodules (Hadri and Bisseling, 1998; Schultze and Kondorosi, 1998).

FUTURE PROSPECTS

When rhizobia colonize roots, only growing root hairs become infected and curl around the colony of dividing bacteria (Kijne, 1992). However, in assays in which the purified Nod factor is added to roots growing between glass slides, root hair deformation is induced in growth-terminating root hairs, as discussed above. From these experiments it is clear that Nod factor reinforces tip growth. This can be observed in hairs that are terminating growth, but we know that Nod factor gives the same membrane depolarization, calcium ion influx, increase in FB-actin density and gene expression in growing hairs. It seems that tip growth is also reinforced in the growing hairs. Therefore, we propose that curling is due to reinforcement of tip growth, though only at one side of the hair. This curling can be understood as follows. When a host-specific bacterium attaches close to the root hair tip hemisphere and excretes Nod factors, which are immobile inside the cell wall (Goedhart *et al.*, 1999), tip growth is stimulated but only at the attachment side (Emons and Mulder, 2000). The bacteria create a new centre of influence, thus redirecting tip growth towards them. Because the bacteria multiply, the surface contact area between the two organisms enlarges. Therefore, the new cell tip will touch the new bacteria, which excrete Nod factors too, and again redirect root hair tip growth towards the colony of bacteria. Since this happens continuously in time, the orientation of tip growth rotates in one direction and this gives rise to a tight curl in which the bacteria are entrapped (Fig. 6), a so-called shepherd's crook. It is essential that experiments to verify the redirection of the FB-actin and the tip-localized $[Ca^{2+}]_c$ in the direction of a colony of bacteria are now performed. Other fields for future research are the occurrence and mode of action of actin binding proteins, Rho-GTPases, and, directly related to this point, the role of second messengers, especially IP_3 and PA. In our laboratory, work is currently in progress to elucidate the involvement and role of these molecules in studies that combine the use of drugs and mutant plants.

FIG. 6. DIC microscopy image of a *Medicago truncatula* root hair 2 d after spot inoculation with *Rhizobium meliloti*. The root hair has curled forming a pocket in which the bacteria are entrapped, the so-called 'shepherd's crook'.

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