Symbiosis, 14 (1992) 359–373
Balaban, Philadelphia/Rehovot

A Model of Legume Root Hair Growth and *Rhizobium* Infection

ROBERT W. RIDGE
Plant Microbe Interactions Group, Research School of Biological Sciences, Australian National University, Canberra, ACT 2601, Australia
Tel. (61) 6 249 3087/4054, Fax (61) 6 249 0754/2603, Email: ridge@rsbs0.anu.edu.au

Received November 18, 1991; Accepted November 25, 1991

Abstract

The root hair is the main entry point for *Rhizobium* in forming a symbiosis with many important legume crops, via an infection thread that is built from plant cell wall material. Using rapid-freeze and freeze-substitution for electron microscopy, anti-cytoskeletal drug treatments, and immunofluorescence microscopy, a basis for the role of the cytoskeleton in root hair tip growth has been established in the literature. The nucleus migrates in-step with the growing tip, and anti-cytoskeletal drug studies have shown that the distance between nucleus and tip is controlled by microtubules and actin-based microfilaments. Endoplasmic microtubules delineate the internal route for precursors of tip growth, and together with cortical microtubules maintain the integrity of the hair, while cytoplasmic streaming (through microfilaments) is responsible for the flow of those precursors. After freeze-substitution, clathrin-coated vesicles and coated pits were found at the base of the tip dome, and may be involved in membrane recycling. Rapid-freeze treatment also revealed three different kinds of vesicle at the tip, including a pyriformis vesicle non-continuous with the Golgi apparatus. Cytochalasin-D studies showed that cessation of streaming causes random cell wall precursor dumping at sites of cytoplasmic accumulation. During infection by *Rhizobium*, the initiation of infection threads may occur when tip growth is sequestered by appropriation of the cytoskeleton, and microfilaments unload their vesicle passengers at the penetration site. *Rhizobium* may also switch off the cell wall 'plasticizing' effect of normal tip growth, but not the deposition of

0334-5114/92 /$03.50

cell wall precursors, allowing the inward growth of an infection thread. As the infection thread grows, there is a migration of the nucleus in front of the thread towards the base of the infected hair, and this is presumably maintained by the cytoskeleton. Evidence in the literature shows that cytoplasmic/cytoskeletal 'bridges' are formed in the dividing cortical tissue ahead of the infection threads, perhaps acting as guides for the direction of infection thread growth.

Keywords: clathrin, cytoskeleton, freeze-substitution, infection thread formation, *Rhizobium* infection, root hair

1. Introduction

One of the most important aspects of *Rhizobium*/legume research is in trying to understand the very earliest interactions that occur as *Rhizobium* takes over the cell machinery of root hairs and stimulates the cell to produce an infection thread, through which the bacteria are able to penetrate the dividing cortical cells of the root that will form a nitrogen fixing nodule.

The very earliest interactions are particularly important to study, because it is during these stages that the effects of the expression of the host-specific nodulation (hsn) genes can modulate the infection, and manipulation of host-specificity is the key to broadening *Rhizobium* host range.

Root hairs are tip-growing cells that have a finite length and do not divide or form transverse cell walls along the tube. Only pollen tubes have a similar type of structure. Other tip growing cells (such as moss protonemata and fungal mycelium) have very similar modes of growth, but usually have fewer vesicles at the tip (Staiger and Schliwa, 1987). Tip growing cells grow by the addition of cell wall material delivered by vesicles to the tip. It is known that a calcium gradient exists at the tip (Jaffe, 1982) and that vesicles within this gradient are shuttled to the growing wall. How this mechanism works is still an open question.

During the *Rhizobium* infection of many of the agriculturally important legumes, the hair is distorted, branched and curled by the presence of *Rhizobium*. Some rhizobia are entrapped by curled hairs and within this enclosed space the rhizobia are able to loosen and penetrate the hair cell wall (Bauer, 1981; Callaham and Torrey, 1981; Ridge and Rolfe, 1985, 1986). An infection thread is initiated and the rhizobia penetrate the plant by division within the confines of the thread, which grows to the base of the hair and penetrates the dividing cortical tissue that will become the plant's supply centre for fixed atmospheric nitrogen.

In this paper I put forward a hypothesis, based on recent published and unpublished evidence, that the cytoskeleton plays a pivotal role in the establishment and development of the infection thread. I suggest that one of the essential aspects of the *Rhizobium* infection process is the initial communication between *Rhizobium* and the cytoskeleton, perhaps through modulation of the calcium-based system that allows polarisation (and therefore movement) of vesicles to the tip. One of the most important roles of the cytoskeleton in the hair is in the maintenance of the nucleus/hair tip distance. This relationship may also be true for the infection thread/nucleus distance that is essential for successful infection of many legumes by rhizobia.

2. Materials and Methods

Vicia hirsuta was cultivated and prepared as described in Ridge (1988). Rapid-freeze, freeze substitution was performed and described in Ridge (1988, 1990a). Rhodamine phalloidin staining was performed as described in Ridge (1990b) except for Fig. 2 in which tissue was first prepared by the rapid-freeze method described in Roberts et al. (1988).

3. Basis of Root Hair Growth

Microtubule system

From immunofluorescence studies (Lloyd, 1983; Lloyd and Wells, 1985) and anticytoskeletal drug studies (Lloyd et al., 1987) the hair cell has been shown to have a complex but easily understood cytoskeletal system for controlling the position of the nucleus, which has a central role in the positioning of the infection thread as it grows down the hair. An important component of this is an endoplasmic system of microtubules that forms columns between the tip and nucleus (Fig. 14B). Cortical, helically-arranged microtubules (Fig. 14A) (Lloyd, 1983) appear to be continuous with the endoplasmic microtubules (Lloyd et al., 1987) the latter reflexing back toward the cortex within the apical dome of the cell. A possible implication of these results is that the cortical array is formed by microtubules that radiate from the nucleus before associating with the membrane.

Drug studies have shown that the endoplasmic microtubules actually function to regulate the nucleus-tip distance. Under conditions that specifically de-polymerise microtubules (leaving cytoplasmic streaming unaffected) the nucleus becomes uncoupled from the apex and migrates towards the base of the hair (Lloyd et al., 1987).

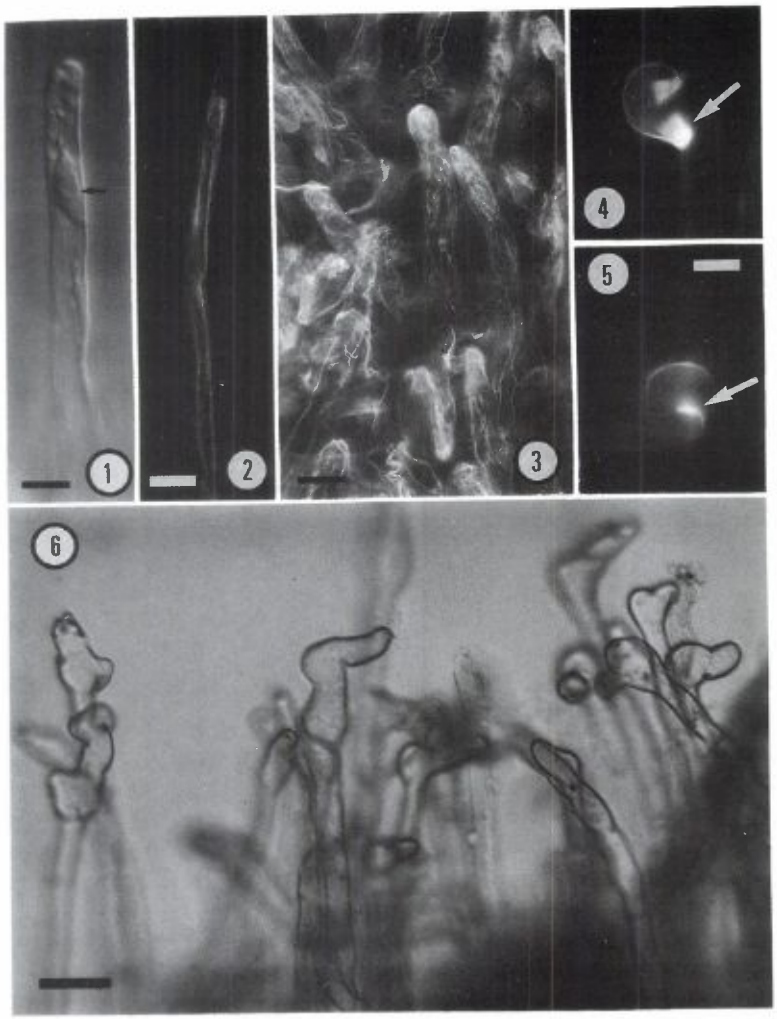

Figure 1. DIC image of a root hair of *Vicia hirsuta*. The cytoplasm was streaming well at the time it was photographed. The nucleus is arrowed. Bar = 15 μm.

Figure 2. Rhodamine-phalloidin staining of microfilaments after freeze-substitution treatment. Long cables of filaments are apparent but do not enter the apical dome. Bar = 20 μm.

Figure 3. Rhodamine-phalloidin staining of root hairs after conventional fixation. There is fluorescence within the apical dome. Bar = 15 μm.

Figure 4&5. Curled root hairs after exposure to *Rhizobium* have been stained for microfilaments (no fixation). There is an apparent loss of filaments in the hairs and a concentration of stained material (arrows) (G-actin?) at the pivot of the curl. Bar = 12 μm.

Figure 6. Distorted and branched hairs after *Rhizobium* treatment. Bar = 20 μm.

Electron microscope studies (conventional and freeze-substitution fixation procedures) have shown that the endoplasmic microtubules associate in bundles, specifically between the nucleus and tip (Lloyd et al., 1987). Microtubules are closely associated with the nucleus, and at the plasma membrane (cortical microtubules) often have closely associated microfilaments (Ridge, 1988, 1990a). Microtubules, together with microfilaments, are also associated with clathrin (see below).

Microfilament system

Using immunofluorescence techniques, Lloyd et al. (1987) showed that the f-actin system enters the apical dome; but using rapid-freeze, freeze-substitution techniques, Ridge (1988, 1990a) was unable to find either microfilaments or microtubules in the apical dome (cf. Figs. 2&3). The rapid-freeze technique was, however, able to confirm that long cables of f-actin fibres exist in the hair (Figs. 2&7) and that they associate with vesicles and organelles (Figs. 7, 10–12) presumably in the transport of these cell components around the cell (Ridge, 1988, 1990a). Association of cell components with the plasma membrane were also found (Fig. 13) and I suggest the possibility of transport of cell components along the inner surface of the plasma membrane.

After freeze-substitution, long cables of f-actin were found between the tip and nucleus (Fig. 7) and in cross section these individual fibres were found to maintain separation in a hexagonal arrangement (Ridge, 1988).

When cytochalasin-D was used to fragment f-actin, aggregations of organelles and vesicles occurred, mostly away from the tip region (Ridge, 1990a). At these sites, abnormal cell wall ingrowths occurred (Fig. 9) and it is possible that, with the lack of cytoplasmic streaming, cell wall precursors are deposited at the closest available site, guided by microtubules. This probably occurs as the cytoplasmic streaming gradually ceases on exposure to the drug. No cell wall ingrowths occurred after microtubules were depolymerised at the same time as microfilaments were fragmented, nor when only microtubules were depolymerised and streaming allowed to continue. Thus, the occurrence of ingrowths requires function microtubules after microfilament fragmentation.

Vesicles and clathrin

Conventionally-fixed hairs for electron microscopy have a single vesicle type with undulating membranes. Freeze-substitution for electron microscopy has revealed a number of vesicle types in the root hair (Ridge, 1988). Specifically, a pyriform vesicle (Fig. 8) exists at a lower frequency that other vesicle types in

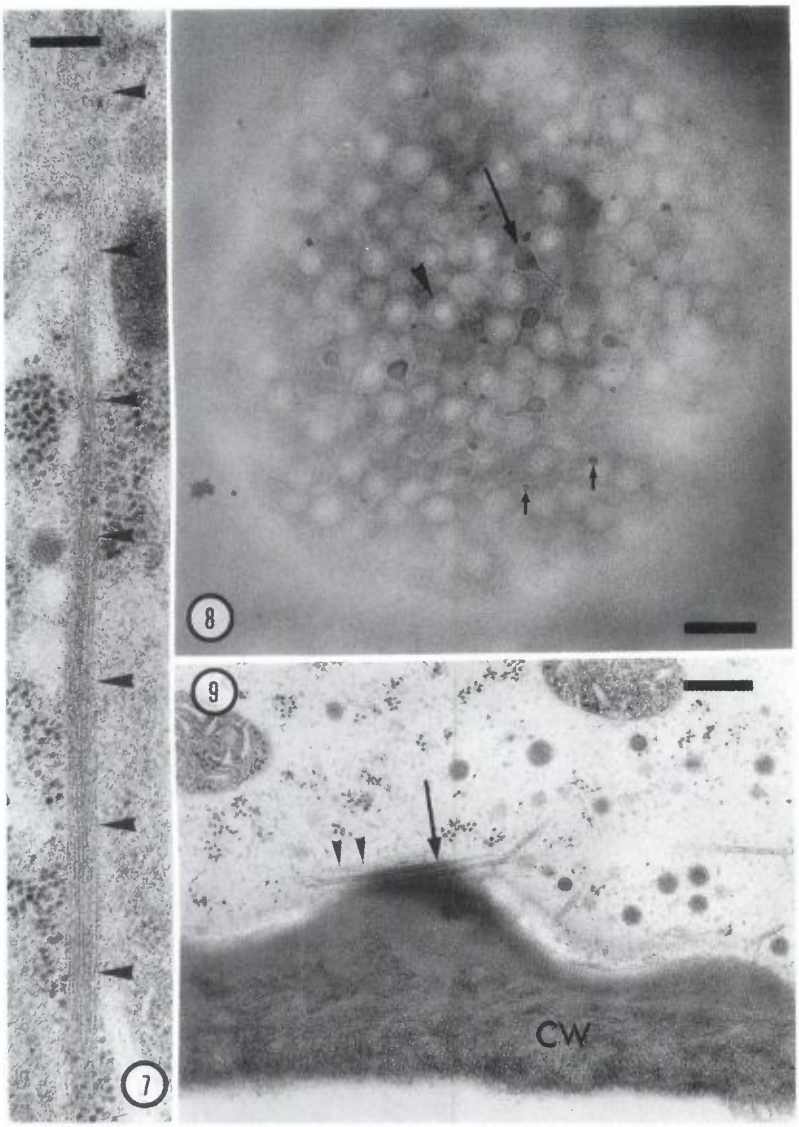

Figure 7. Cable of microfilaments (arrowheads) after freeze-substitution. These cables could not be found after conventional fixation procedures. Note the proximity of several vesicles next to the cable. Bar = 70 nm.

Figure 8. Section close to the very tip of the apical dome. Note the pyriform vesicles (arrow) with a distribution that implies a specialised role. The smaller arrows indicate transections of the narrow neck of the pyriform vesicle. The other, electron transparent vesicles may be the main contributors of cell wall precursors at the tip. Bar = 120 nm.

Figure 9. After cytochalasin-D treatment, the microfilaments (arrowheads) are fragmented, and abnormal wall ingrowths are deposited randomly in areas where organelles and vesicles accumulate. Note the microtubules (arrow) and the well-preserved mitochondrial ribosomes. CW = normal cell wall. Bar = 250 nm.

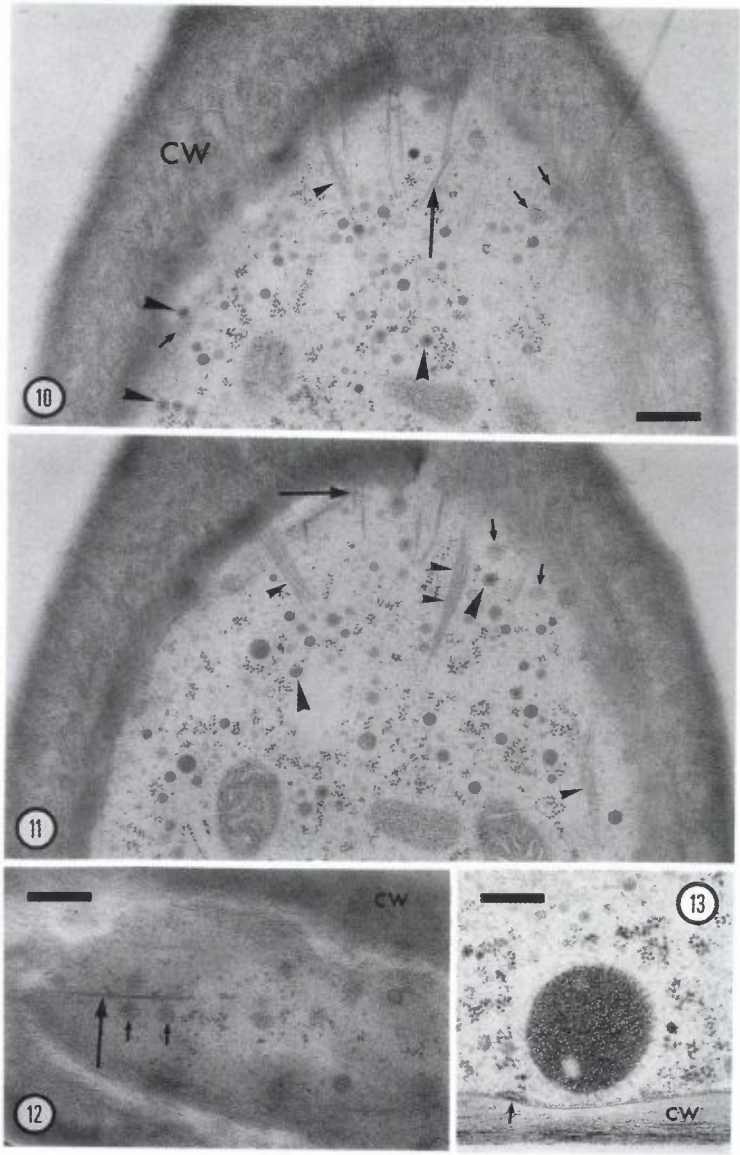

Figure 10–12. Figs. 10&11 are two consecutive grazing sections of the base of the apical dome: Fig. 12 is another example. This region is particularly rich in clathrin and cytoskeleton close to the plasma membrane. Large arrowheads = coated vesicles; small arrows = coated pits; small arrowheads = microfilaments; large arrows = microtubules; CW = cell wall. For Figs. 10&11, bar = 320 nm; For Fig. 12, bar = 350 nm.

Figure 13. Organelles were found to be associated with the plasma membrane after freeze-substitution. This example shows an amyloplast in contact distance of the membrane. Arrow indicates section of coated pit. CW = cell wall. Bar = 250 nm.

the apical dome. The pyriform vesicle appears to be distributed non-randomly in some parts of the cell, but there is no known role for this kind of vesicle. Other vesicle types have spherical profiles, possibly with coatings, and different types of content and distribution. The more 'common' spherical vesicles are most likely the delivery system for cell wall precursors.

Coated pits were found predominantly at the base of the apical dome, closely associated with microfilaments, microtubules and coated vesicles (Figs. 10–12). The protein clathrin is well characterised as having a role in the recirculation of membrane (e.g., in the secretory cell) (Alberts et al., 1989). It has been shown by others (Picton and Steer, 1982) that there is an excess of membrane delivered to the tip of tip-growing cells. If a clathrin-based system for recycling membrane exists in the hair, it is clear that the cytoskeleton is intimately involved with it, and may act as the transport and guidance system for the recycled membrane to return to the endomembrane system.

Summary of root hair growth (Figs. 14 and 15)

1. There would appear to be a synergy between the two cytoskeletal systems. The simplest explanation is that the endoplasmic microtubules delineate the internal route for precursors of tip growth and, together with the cortical microtubules, help maintain the integrity of the tip area.
2. Cytoplasmic streaming is responsible for the flow of tip growth precursors.
3. Cortical microtubules probably maintain the integrity of the hair's cylindrical shape.
4. The presence of clathrin at the base of the dome implies recycling activity of membrane, and it is likely that a clathrin-based membrane recycling system exists to return excess membrane flowing from the tip into the endomembrane system, guided by the cytoskeleton.

4. *Rhizobium* infection

The root hair curling, cell wall degradation, and formation of infection threads at the beginning of the *Rhizobium*/legume symbiosis is well characterised (e.g. Callaham and Torrey, 1981; Ridge and Rolfe, 1985, 1986). It is also well known that the proximity of the nucleus is essential for both root hair growth and infection thread growth, and that the maintenance of a specific distance between the nucleus and these growing points is essential for growth to continue.

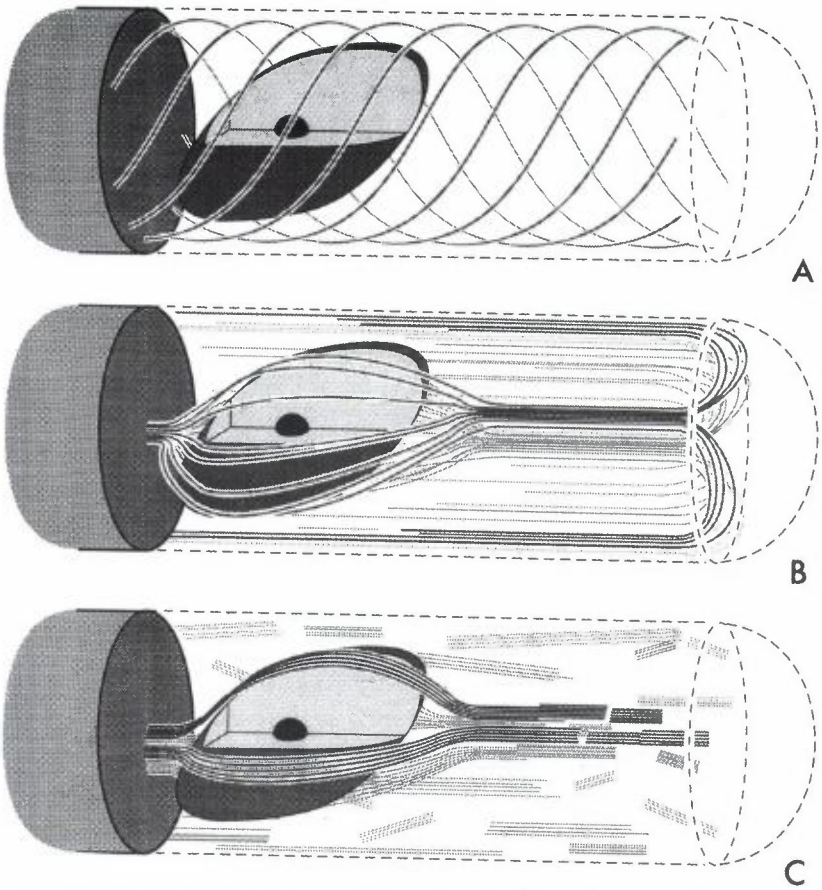

Figure 14. Model of the cytoskeleton on the root hair. Cortical microtubules (A) lie close to the plasma membrane and have been shown to be helically-arranged (Lloyd, 1983). Endoplasmic microtubules (B) form columns between the nucleus and tip and reflex back close to the base of the apical dome. Microfilaments (C) form long cables along the length of the hair and, like endoplasmic microtubules, are closely affiliated with the nucleus. None of these cytoskeletal elements have been seen within the apical dome at the ultrastructural level after freeze-substitution.

From evidence of the role of the cytoskeleton in maintaining the distance between nucleus and hair tip, it can be deduced that the cytoskeleton may play a major role in the initiation and development of the infection thread.

Earlier studies of infection using conventional fixation for electron microscopy showed that at the site of penetration by *Rhizobium*, microtubules are present (Ridge and Rolfe, 1985, 1986). From the drug studies described above, where the vesicle system dumps cell wall material at the closest site after fragmentation of microfilaments, and where the presence of microtubules

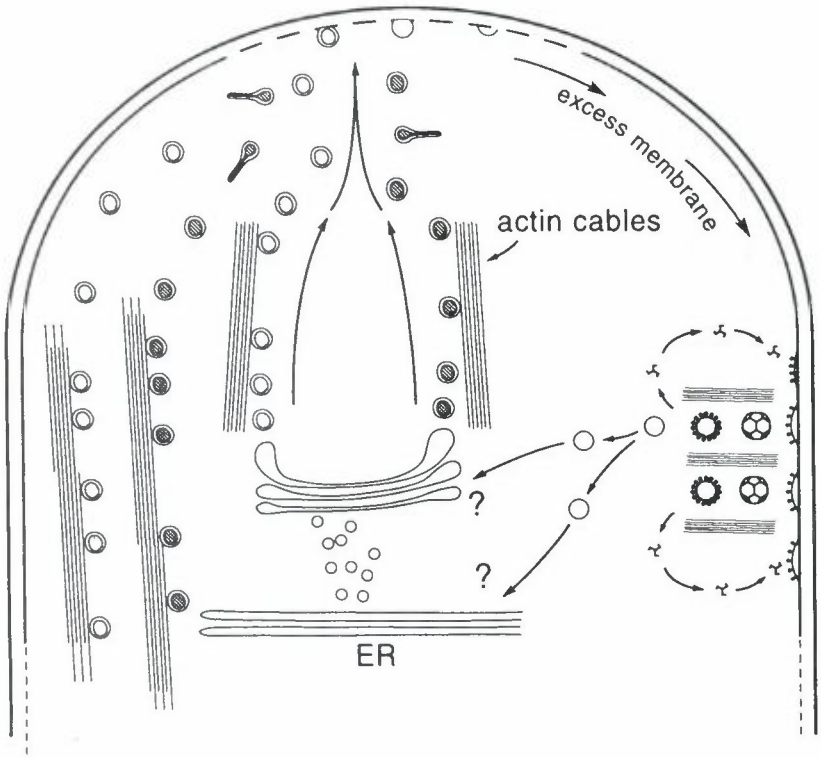

Figure 15. Model of how clathrin may function in the root hair tip. Freeze-substitution studies have shown that coated pits and coated vesicles can be found in abundance at the base of the apical dome (Ridge, 1988). Other studies have shown that there is an excess of membrane in tip-growing cells (Picton and Steer, 1982). It is therefore feasible that clathrin is picking up the excess membrane that is flowing along the plasma membrane from the tip and forming vesicles that return membrane to the endomembrane system. The cytoskeleton provides the direction (via microtubules) and transport (via microfilaments) and the clathrin detaches from the vesicles once formed to produce more coated pits and coated vesicles to continue the cycle.

is needed for such dumping, it would seem possible that *Rhizobium* causes both a localised fragmentation of microfilaments (Figs. 4&5) and appropriation of cell wall material by sequestering the microtubule system that usually guides vesicles to the growing hair tip (Fig. 15). Thus, effects caused by the disruption of the cytoskeleton by anti-cytoskeletal drugs may be similar to early interactions of *Rhizobium* and the root hair.

If the cortical microtubules in the normal hair are essential for the maintenance of the hair's cylindrical shape, then it is quite possible that a similar set of microtubules maintain infection thread integrity, following the growth of the thread much as their formation would follow the growth of the normal hair.

There is evidence that microtubules exist around infection threads of *Pisum sativum* (Bakhuizen, 1988) and white clover (Margreet De Boer, unpublished data) but as yet their structure is not established. I suggest that the curling, bending and branching of root hairs (Fig. 6) caused by interaction with rhizobia is a direct result of interference with the normal cytoskeletal structure. That is, compounds (signals) released by rhizobia are able to affect the biology of the hair cell before physical contact.

Summary of how Rhizobium *may infect hairs (Fig. 16)*

1. A perturbation of normal tip growth may be the consequence of both a modulation of the calcium balance at the tip, as well as interference in the usual way the cytoskeleton delivers vesicles to the tip. *Rhizobium* may also interfere with the way cortical microtubules extend with hair growth, allowing the normal polarity of growth to deviate, causing curling, branching and distortion.
2. Entrapped rhizobia are able to dissolve components of the cell wall, allowing the microfibrils to loosen and stretch under pressure of rhizobial growth (Fig. 16A).
3. At the same time the rhizobia are able to sequester the vesicle delivery system, possibly by (at least initially) causing a localised fragmentation of microfilaments. This causes deposition of cell wall material at the site of penetration (Fig. 16A).
4. The rhizobia are able to re-polarise the microtubules to the same point, uncoupling normal tip growth and re-coupling at the invasion site. As the nucleus can be uncoupled by microtubule-depolymerising drugs in uninfected hairs, it is possible that the rhizobia also temporarily depolymerise microtubules at the local penetration site (Fig. 16A).
5. Inward growth of the infection thread is an inside-out version of normal tip growth. Thus, hairs grow from the inside and infection threads grow from the 'outside' (Fig. 16B,C).
6. The essential presence of the nucleus at a fixed distance from the tip of the thread is presumably maintained by the cytoskeleton, as the nucleus/hair tip distance is in uninfected hairs (Fig. 16C).
7. The integrity of the infection thread may be maintained by a similar cortical microtubule system (a helix?) as in normal hairs (Fig. 16C).

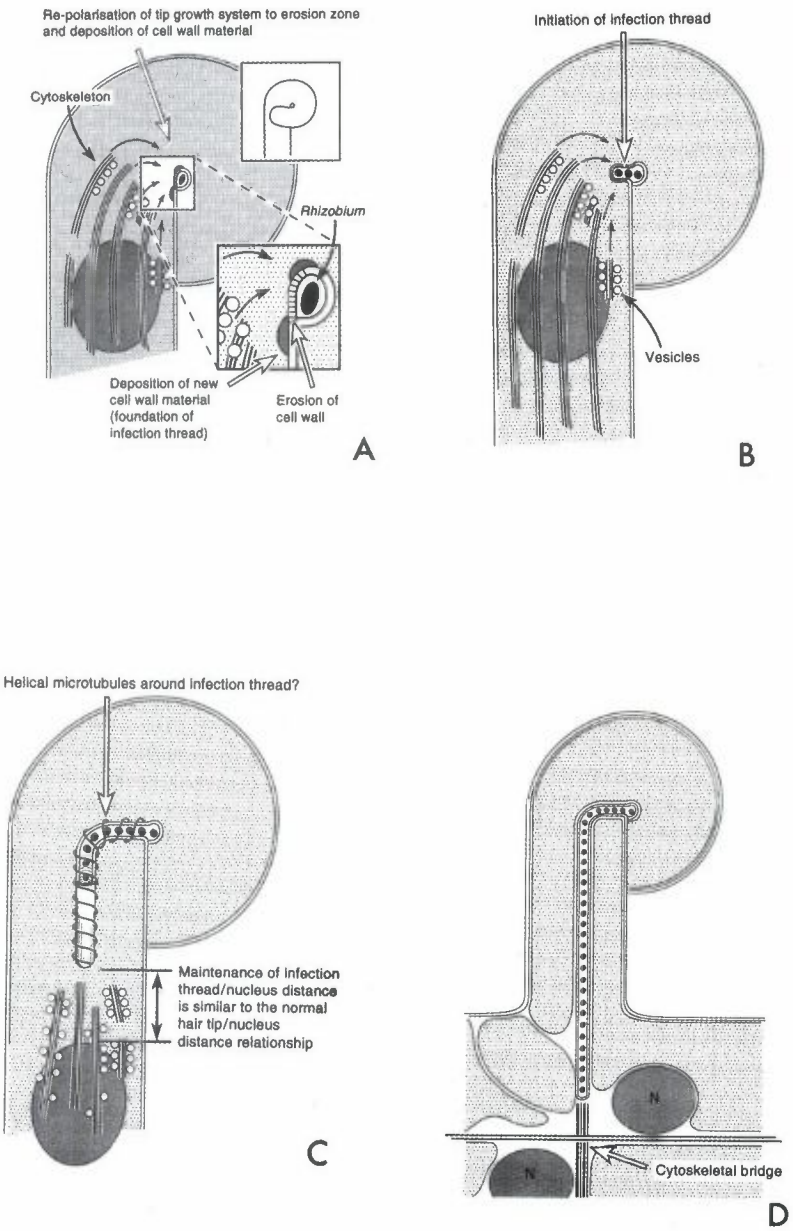

Figure 16. Model of how *Rhizobium* may infect legume root hairs. Please refer to the text for details.

8. In some failed infections, where the thread/nucleus distance changes to either too far or too close, the rhizobia presumably have lost control of directionality, although they have not necessarily lost control of the cytoskeletal system. The latter because infection threads that lose their nucleus can continue to grow, though usually in a twisted and 'lost' fashion; they can sometimes grow back up the hair.

   Thus the nucleus and microtubules are essential for directionality of growth of both root hairs and infection threads.

9. As for the role of clathrin in infection thread growth, there would be no reason why a clathrin-based recycling of membrane shouldn't work as well with an infection thread as with a normal tip. However, if any excess membrane occurred during infection thread growth, it would have to travel the complete length of the infection thread and around the hair wall to reach the region where the infection thread tip is being formed, unless there are cross-bridging cytoplasmic strands that follow the thread as it grows. If not, then the distance of excess membrane would be continuously increasing as the infection thread grows. It seems unlikely that such a recycle of excess membrane would work as well as in normal tip growth.

10. Recent evidence (Bakhuizen, 1988) shows that the cytoskeleton may play a role in aligning the cytoplasm for future infection thread growth, because the position of these bridges predicts the future site of infection thread growth (Fig. 16D).

5. Concluding Comments

A model of root hair growth and *Rhizobium* infection can provide ideas on how to further examine the system. In early interactions, signals from rhizobia affect normal root hair growth, and we should look for abilities of these compounds to modulate the cytoskeleton. In the context of trying to expand the range of *Rhizobium* interactions, it may be possible to use such knowledge to construct a *Rhizobium* strain to interact with non-legumes. The development of models and hypotheses can thus give aid to effective strategies in research.

The rapid-freeze and freeze-substitution technique has been an invaluable tool for improving knowledge of root hair biology. The technique eliminates plasmolysis and greatly improves the preservation of the cytoskeleton, notably microfilaments and clathrin-based structures. The method gives increased confidence in the interpretation of organelle positioning and interactions, because

disruption and possible re-positioning of cell components by slowly penetrating fixative is eliminated. Freeze-substitution has proven to be a very successful approach to understanding the biology of root hairs and will help in demonstrating the role of the cytoskeleton in the development of the symbiosis.

REFERENCES

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. 1989. *Molecular Biology of the Cell.* 2d ed. Garland Publishing, Inc., New York.

Bakhuizen, R. 1988. The plant cytoskeleton in the *Rhizobium*-legume symbiosis. Ph.D. thesis, University of Leiden.

Bauer, W.D. 1981. Infection of legumes by rhizobia. *Ann. Rev. Plant Physiol.* **32:** 407–449.

Callaham, D.A. and Torrey, J.G. 1981. The structural basis for infection of root hairs of *Trifolium repens* by *Rhizobium. Can. J. Bot.* **59:** 1647–1664.

Jaffe, L.F. 1982. Developmental currents, voltages and gradients. In: *Developmental Order: its Origin and Regulation.* S. Subtelny and P.B. Green, eds. Alan R. Liss, New York, pp. 183–215.

Lloyd, C.W. 1983. Helical microtubular arrays in onion root hairs. *Nature* **305:** 311–313.

Lloyd, C.W. and Wells, B. 1985. Microtubules are at the tips of root hairs and form helical patterns corresponding to inner wall fibrils. *J. Cell Sci.* **75:** 225–238.

Lloyd, C.W., Pearce, K.J., Rawlins, D.J., Ridge, R.W., and Shaw, P.J. 1987. Endoplasmic microtubules connect the advancing nucleus to the tip of legume root hairs, but F-actin is involved in basipetal migration. *Cell Motility and Cytoskeleton* **8:** 27–36.

Picton, J.M. and Steer, M.W. 1982. A model for the mechanism of tip extension in pollen tubes. *J. Theor. Biol.* **98:** 15–20.

Ridge, R.W. 1988. Freeze-substitution improves the ultrastructural preservation of legume root hairs. *Bot. Mag. Tokyo* **101:** 427–441.

Ridge, R.W. 1990a. Cytochalasin-D causes organelle-crowding and abnormal ingrowths in legume root hairs. *Bot. Mag. Tokyo* **103:** 93–102.

Ridge, R.W. 1990b. A simple apparatus and technique for the rapid freezing and freeze-substitution of single-cell algae. *Japanese J. Electron Microscopy* **39:** 121–125.

Ridge, R.W. and Rolfe, B.G. 1985. *Rhizobium* sp. degradation of legume root hair cell wall at the site of infection thread origin. *Appl. Envtl. Micro.* **50:** 717–720.

Ridge, R.W. and Rolfe, B.G. 1986. Sequence of events during the infection of the tropical legume *Macroptilium atropurpureum* Urb. by the broad-host-range, fast-growing *Rhizobium* ANU240. *J. Plant Physiol.* **122:** 121–137.

Roberts, K.R., Farmer, M.A., Schneider, R.M., and Lemoine, J.E. 1988. The microtubular cytoskeleton of *Amphidinium rhynchocephalum* (Dinophyceae). *J. Phycol.* **24:** 544–553.

Staiger, C.J. and Schliwa, M. 1987. Actin localisation and function in higher plants. *Protoplasma* **141:** 1–12.