

Refined analysis of early symbiotic steps of the *Rhizobium-Medicago* interaction in relationship with microtubular cytoskeleton rearrangements

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Accepted 8 June; published on WWW 19 July 1999

SUMMARY

In situ immunolocalization of tubulin revealed that important rearrangements occur during all the early symbiotic steps in the *Medicago/R. meliloti* symbiotic interaction. Microtubular cytoskeleton (MtC) reorganizations were observed in inner tissues, first in the pericycle and then in the inner cortex where the nodule primordium forms. Subsequently, major MtC changes occurred in outer tissues, associated with root hair activation and curling, the formation of preinfection threads (PITs) and the initiation and the growth of an infection network. From the observed sequence of MtC changes, we propose a model which aims to better define, at the histological level, the timing of the early symbiotic stages. This model suggests the existence of two opposite gradients of cell differentiation controlling respectively the

formation of division centers in the inner cortex and plant preparation for infection. It implies that (i) MtC rearrangements occur in pericycle and inner cortex earlier than in the root hair, (ii) the infection process proceeds prior to the formation of the nodule meristem, (iii) the initial primordium prefigures the future zone II of the mature nodule and (iv) the nodule meristem derives from the nodule primordium. Finally, our data also strongly suggest that in alfalfa PIT differentiation, a stage essential for successful infection, requires complementary signaling additional to Nod factors.

Key words: Symbiotic association, *Rhizobium*, *Medicago*, Microtubular cytoskeleton

INTRODUCTION

The symbiotic association between plants of the Leguminosae family and soil bacteria belonging to the genera *Rhizobium*, *Azorhizobium*, *Bradyrhizobium* and *Sinorhizobium* (here referred to as rhizobia) results in the development of nodules, root organs in which rhizobia convert nitrogen into ammonia, which is used by the plant as a nitrogen source.

Nodulation is initiated by a molecular dialogue involving plant-secreted flavonoids and rhizobia-synthesized lipochitooligosaccharidic Nod factors (reviewed by Dénarié et al., 1996; Long et al., 1996; Mylona et al., 1995; Schultze and Kondorosi, 1998; Spaink, 1996) and takes place through a series of developmental stages elicited in different plant tissues (reviewed by Brewin, 1991; Hirsch, 1992; Kijne, 1992; Nap and Bisseling, 1990). In temperate legumes such as alfalfa, vetch and pea, symbiotic responses are generally observed, including root hair deformations, the formation of a nodule primordium by the division of inner cortical plant cells and plant infection by infection threads, which are initiated in a curled root hair and then grow centripetally towards the cortex of the plant. Early steps are completed with the formation of the nodule meristem, which remains active for several weeks thus leading to the formation of an elongated indeterminate nodule.

The fact that some of the early events mentioned above are

elicited almost simultaneously in the same root area, makes the study of the temporal timing of early nodulation difficult. Despite this, previous descriptive studies in temperate legumes have clearly shown that (i) the nodule primordium forms in the inner cortex prior to infection (Dudley et al., 1987; Libbenga and Harkes, 1973), (ii) infection threads grow into the activated cortex through cytoplasmic bridges (van Brussel et al., 1992), and (iii) successful infection is not required for the nodule meristem to form, as shown by the fact that nodules can be formed in the absence of rhizobia (Truchet et al., 1989), by infection-deficient rhizobia (Leigh et al., 1985, 1994; Yang et al., 1992) or by purified Nod factors (Truchet et al., 1991). In contrast, it still remains unclear (i) which root tissues are reactivated, (ii) what is the fate of Nod factor-activated cortical cells during early symbiosis, (iii) what discriminates the nodule primordium and the nodule meristem, (iv) how, where and when the latter differentiates, and (v) what the spatio-temporal relationship is between infection and meristem formation.

Cytoskeletal reorganizations have been described during the various stages of the symbiotic interaction or in legumes treated with Nod factors. A transient actin fragmentation is observed in *Medicago sativa* root hairs responding to a treatment with Nod factors from *Rhizobium meliloti* (Allen et al., 1994; Cardenas et al., 1998), changes in actin filament configuration occur in root hairs of *Vicia sativa* treated

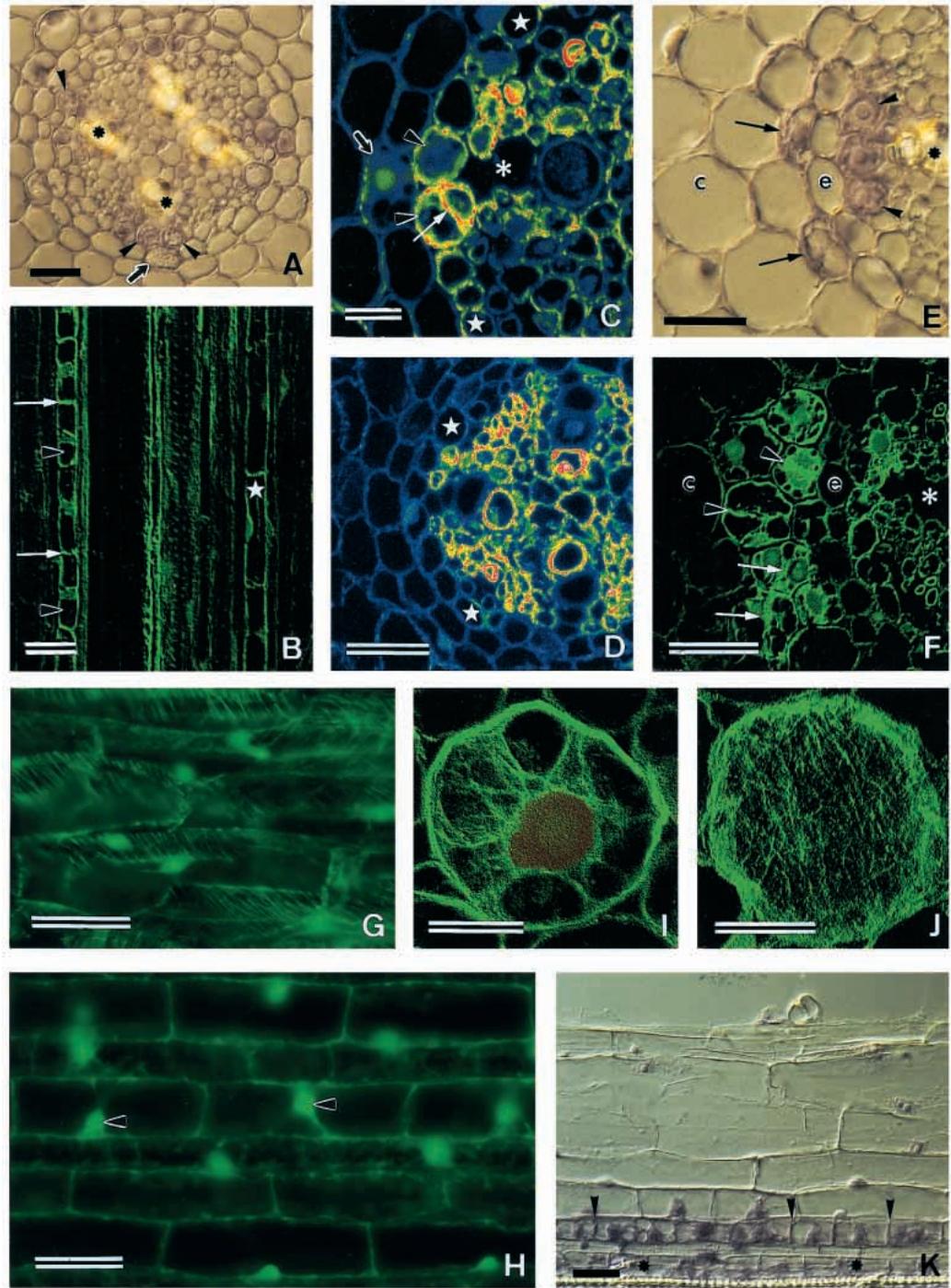
Fig. 1. Pericycle and cortex activation. (A,E,K) Cellular changes, Nomarski optics; (G,H) MtC immunolocalization, fluorescence microscopy; (B,C,D,F,I,J) MtC immunolocalization, laser confocal microscopy.

(A-D) Pericycle activation in the *M. truncatula skl* mutant.

(A) Transverse section of a root showing the activation of pericycle cells (arrowheads) facing protoxylem poles (asterisk). (B) Longitudinal section. Anticlinal divisions (arrows) are seen in pericycle activated cells (arrowheads). (C) Detail of an activated site on a transverse section. A strong MtC labeling is seen in pericycle cells (arrowheads) facing a protoxylem pole (asterisk). The arrow points to a periclinal division. Fluorescence intensity is indicated by colours from low (blue) to high (red).

(D) Transverse section of a non-inoculated control root. Note the very low level of fluorescence in the pericycle (stars). (A-D) stars: non-activated pericycle cells; black arrows: activated endodermis cells. Bars, 25 μ m, except C, 10 μ m. (E,F) Inner cortex activation. Transverse sections of a *M. truncatula skl* mutant (E) and alfalfa (F) roots showing the activation of inner cortical cells (arrows) facing activated pericycle cells (arrowheads). In F, anticlinal (black arrowheads) and periclinal (arrows) cell divisions are seen. (E,F) Asterisks, protoxylem pole; e: endodermis; c: root cortex. Bars, 25 μ m.

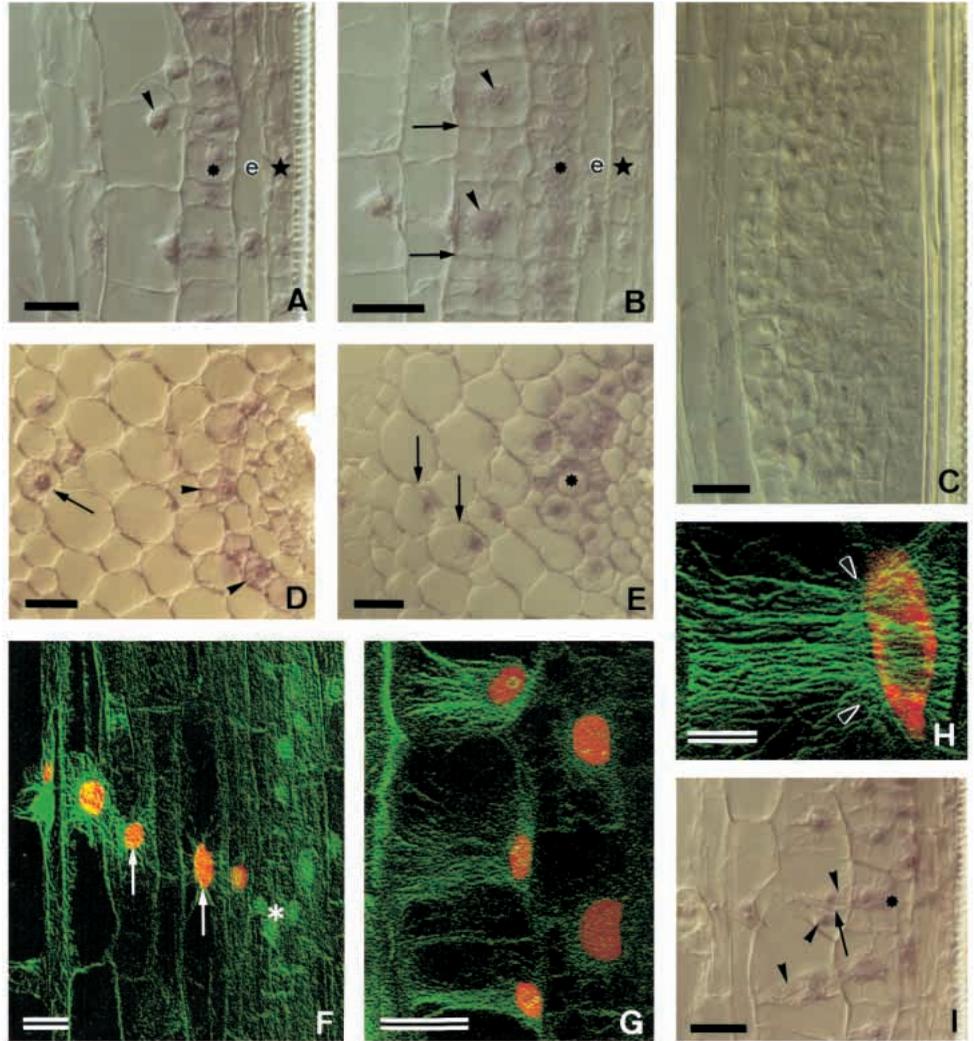
(G-J) MtC changes during cortical cell activation. (G,H) Longitudinal root sections (*M. truncatula skl* mutant) showing MtC changes during cortical cell activation. The slightly oblique organization of the cortical MtC in the non-inoculated control root (G) is no longer seen in activated cortex (H). A fluorescence signal (arrowheads) is seen surrounding the nuclei in activated cells (H). Bars, 50 μ m. (I,J) Extended focus images of transverse sections of isodiametric cortical cells in the *M. truncatula skl* mutant, showing (I) the endoplasmic MtC with microtubule arrays (green) radiating from the central nucleus (red) and (J) the randomly organized cortical MtC network. Bars, 10 μ m. (K) An initial primordium in a *M. truncatula* sickle mutant (longitudinal section). Anticlinal divisions (arrowheads) are shown. Asterisks, activated pericycle. Bar, 25 μ m.



with lipo-chito oligosaccharides (Miller et al., 1999), and microtubular cytoskeleton (MtC) rearrangements take place in the cortex of *Vicia hirsuta* either inoculated with rhizobia (Bakhuizen, 1988, van Spronsen et al., 1995) or treated with purified Nod factors (van Spronsen et al., 1995). The microtubular cytoskeleton (MtC) also changes during nodule

differentiation. Thus, MtC disorganization concomitant with Nod factor internalization occurs in the invaded cells of zone II of alfalfa, vetch and clover indeterminate nodules, while a newly patterned MtC reforms in the nitrogen-fixing zone III (Timmers et al., 1998). Moreover, the fact that the cytoskeleton is involved in the regulation of cellular features such as cell

Fig. 2. Primordium formation, inward gradient of differentiation and PIT formation. (A-E,I) Cellular changes, Nomarski optics; (F,G,H) MtC immunolocalization, laser confocal microscopy. In this figure, nuclei are in red. (A-C) Primordium formation. Longitudinal sections. (A,B) Sequential organization of the nodule primordium in *M. truncatula skl* mutant by progressive activation (arrowheads) and division (arrows, B) of the cortical cells adjacent to the initial primordium (asterisks). In (B), note the cytoplasmic strands (isodiametric organization) radiating from the nuclei of activated cortical cells (arrowheads). Stars, pericycle; e, endodermis. (C) A nodule primordium in alfalfa. (D-F) Inward gradient. (D) *M. truncatula skl* mutant. Transverse section in a root showing an early activation in the outer cortex (arrow) facing a reactive site in the inner cortex (arrowheads). (E,F) Transverse (E, *M. truncatula skl* mutant) and longitudinal (F, alfalfa) sections. Inward activation of cortical cells (arrows) facing the initial primordium (asterisks). (G) Extended focus images of differentiated PIT cells in the outer cortex (longitudinal sections, alfalfa). Note the absence of the cortical MtC. (H,I) PIT cell division. (H) Formation of a cortical preprophase band in a PIT cell, prior to cell division. Arrowheads point to endoplasmic microtubules radiating from the nucleus (red). (I) PIT cells (arrowheads) at the outer border of a nascent primordium (asterisk). The arrow points to a newly formed cell plate. Bars, 25 μm , except (H), 10 μm .



division, cell shape and polarity and cell spatial organization (reviewed by Cyr and Palevitz, 1995; Seagull, 1989), means that it is likely to be involved in the following early symbiotic steps, activation of cortical cells, which re-enter the cell cycle to form nodulation-related divisions centers (Dudley et al., 1987; Libbenga and Harkes, 1973; Yang et al., 1994), root hair deformation and infection (reviewed by Kijne, 1992) and oriented growth of an infection network in plant tissues (van Brussel et al., 1992).

In this study, we have looked at MtC changes in order to investigate the events occurring during early nodulation and, subsequently, provide a better insight into their histological timing. We report that in *Medicago* sp., MtC rearrangements can be observed during all the early symbiotic steps related to pericycle, cortex and root hair activation, the formation of cortical division centers and plant infection. A model for the histological timing of early symbiotic steps is proposed.

MATERIALS AND METHODS

Bacterial strains and plant assays

Rhizobial strains used in this study were as follows: wild-type

R. meliloti RCR2011(pXLGD4) GMI6526 (Ardourel et al., 1994); *R. meliloti* RCR2011 δ (*nodF*)13*nodL*::Tn5(pXLGD4) GMI6630 (Ardourel et al., 1994); *R. meliloti* 1021*exoA32*::Tn5(pXLGD4) (provided by J. Leigh, Seattle, Washington University). All strains were grown on TY medium supplemented with 6 mM calcium chloride and appropriate selective antibiotics.

Surface-sterilized seeds of *Medicago sativa* cv Europe (Florimont Desprez, F59242 Cappelle en Pévèle, France) and *M. truncatula* (cv. Jemalong genotype j5) sickle mutant (kindly provided by D. Cook; Penmetza and Cook, 1997) were germinated and grown in test tubes or Petri dishes as described by Ardourel et al. (1994). Plants were either flood inoculated with exponentially growing bacteria resuspended in water (approximately 10^7 bacteria per ml, 100 μl per plant), or spot inoculated in the zone of root hair development (0.3 μl per spot). Alfalfa plants were flood treated with purified *R. meliloti* Nod factors (10^{-7} M) and grown as described by Truchet et al. (1991).

Immunolocalization of microtubules

In situ visualization of the microtubular cytoskeleton was performed as follows. Root segments were fixed in 2% glutaraldehyde, 5% DMSO, 0.1% Triton X-100 in phosphate buffer (pH 7.2) 30 minutes at room temperature, followed by a subsequent 60-minute fixation in fresh fixing medium. After rinsing, the specimens were embedded in

low melting point wax (Vitha et al., 1997), and cut into 20 µm thick sections which were deposited on poly-L-lysine-coated slides and dewaxed prior to the immunolocalization procedure. Tubulin immunolocalization was performed on sections by treating with a monoclonal anti- α -tubulin (Sigma T-5168) (1/500 in PBS buffer with 0.1% Triton X-100 and 0.2% gelatin, pH 7.4, 2 hours at 37°C), rinsed in PBS (3 times 15 minutes, pH 7.4) and incubated with an anti-mouse IgG-FITC antibody (Boehringer Mannheim, 821462) (1/100 in PBS with 0.1% Triton X-100, pH 7.4, 2 hours at 37°C). After staining, autofluorescence was quenched using Evans blue (Sigma E-2129; 0.5% in PBS, 5 minutes at room temperature), and the sections were mounted in mowiol 4-88 (Calbiochem 475904) containing 1,4 diazabicyclo-(2.2.2) octane (Sigma D-2522) as anti-fading agent and 4,6-diamino-2-phenylindole (Sigma D-9542) as nuclear stain.

Microscopic methods

Sections were viewed by light microscopy using Nomarski optics. Tubulin-stained sections were viewed either by fluorescence microscopy (Zeiss, Axiophot 2) or confocal laser scanning microscopy (Zeiss, LSM 410 Invert) as already described (Timmers et al., 1998). As the treatment with Evans blue leads to an intense fluorescence in the red part of the spectrum, it was not possible to use red fluorescent DNA stains compatible with the excitation wavelength of the laser of the confocal microscope to observe plant cell nuclei or bacteria inside infection threads. Therefore, root sections previously treated for MtC immunolocalization were post-treated with DAPI and images obtained by normal fluorescent microscopy were digitized using a Polaroid SprintScan 35/LE and merged in the corresponding confocal images by using Adobe PhotoDeluxe 1.0 software. In the final image, MtC appears green, while nuclei are arbitrarily represented in red, and infection threads in blue.

RESULTS

Cytoskeletal changes related to cell differentiation during early symbiotic events

The objective of this work was to describe the microtubular cytoskeleton (MtC) changes and the correlative cytoplasmic spatial reorganization occurring during early symbiosis in *Medicago* sp. The data presented below were obtained from repeated microscopic observations of longitudinal or transverse sections of the zone of the root most susceptible to infection (i.e. the region of root hair development; Bhuvanewari et al., 1981) collected at various times, from 6 hours to 14 days after inoculation. We used both *M. sativa* (alfalfa) and *M. truncatula*, two *Medicago* species which are widely studied in the field of rhizobia/legume interactions and which are both efficiently nodulated by *R. meliloti*. All experiments were performed on wild-type *M. sativa* and the majority of them repeated on the *M. truncatula* sickle (*skl*) mutant, which is phenotypically characterized by the formation of a high number of root infections and hyper nodulation limited to a restricted zone of the root (Penmetsa and Cook, 1997). Use of this mutant facilitated the observation of the early symbiotic stages, and particularly of the infection process. Results described below were similar for both species, unless otherwise stated.

Cell activation and cell division in the pericycle

In non-inoculated control plants of both *Medicago* species, pericycle cells of the susceptible zone are small and contain one large vacuole and an ellipsoid nucleus generally positioned

against the inner periclinal cell wall. In this cell type, the MtC is organized as in cortical cells (Baluska et al., 1992), i.e. with short cytoplasmic microtubules forming a loose perinuclear network interconnected with cortical microtubules generally orientated in parallel arrays slightly oblique to the elongation axis of the cell (data not shown).

The first MtC changes were observed at 16-18 hours post-inoculation in pericycle cells positioned opposite protoxylem poles (Fig. 1A). A significant increase in MtC immunolabeling identified these activated pericycle cells characterized by an endoplasmic MtC around a round-shaped and centrally positioned nucleus (Fig. 1B,C). These features were not observed in the pericycle of non-inoculated control roots (compare Fig. 1C and 1D). In alfalfa, pericyclic activation was often observed simultaneously with inner cortex activation (see below). However, histological studies of the hypernodulating *M. truncatula skl* mutant, clearly showed that pericyclic activation preceded cortical activation (compare Fig. 1A,C with 1E). This pericyclic activation resulted first in anticlinal (Fig. 1B) and then in periclinal cell divisions, thus generating a bilayered pericycle at specific sites where activation has occurred (Fig. 1C; see also Fig. 1K). The loci generated from this cell division extended over the entire pericycle cell layer facing the protoxylem pole in the *M. truncatula skl* mutant (Fig. 1B; see also Fig. 1K), while they appeared as discrete sites in alfalfa (data not shown). Although of a smaller size, pericycle cells that had divided displayed typical features of cell differentiation such as the presence of a large vacuolar system occupying most of the cell volume (Fig. 1B). Occasionally, at this stage cellular changes were observed in the endodermis adjacent to activated pericycle cells (Fig. 1A,C).

The formation of the initial primordium

Following pericycle activation, MtC rearrangements were next observed in the inner cortex. Cortical cell activation started around 18-24 hours post-inoculation and was observed in inner cortex cells facing the activated pericycle cells on the other side of the endodermis (Fig. 1E,F).

In control plants, cortical cells of the susceptible zone contain one large vacuole and an ellipsoid nucleus positioned against one of the longer cell walls (data not shown). In these cells, the MtC appears essentially as a cortical MtC made of parallel microtubules perpendicular or slightly oblique to the elongation axis of the cell (Fig. 1G). In activated cortical cells, confocal microscopy showed first an increase in fluorescence around the nucleus (Fig. 1H) and then perinuclear radiating endoplasmic arrays located in cytoplasmic strands (Fig. 1I). At this stage, cortical microtubules progressively lose the parallel organization of non activated cells (compare Fig. 1G and H), leading to a random organization (Fig. 1J). Such MtC changes correlated with a pronounced rearrangement of the cytoplasm of the activated cells leading to an isodiametric appearance (Fig. 1I). Fully activated cortical cells contained a round-shaped central nucleus with a large nucleolus, and a vacuole traversed by cytoplasmic strands radiating from the nucleus to the cell wall (Fig. 1I; see also Fig. 2A).

In general, activated inner cortical cells first divided anticlinally and then periclinally, to generate square-shaped cells remaining aligned in the same cell layer with adjacent non-activated inner cortical cells and forming what we propose

to call the initial nodule primordium (Fig. 1K; see also 2A). Cytologically, initial primordium cells resembled their activated mother cells, with a similar isodiametric organization. In conditions of flood inoculation, the initial nodule primordium appeared in alfalfa as discrete loci involving only a limited number of inner cortical cells (data not shown), whereas in the *M. truncatula skl* mutant, all cells of the inner cortex of the susceptible zone reacted simultaneously to form one continuous initial primordium (Fig. 1K).

The completion of the nodule primordium: an outwardly directed differentiation process

Activation in the middle and outer cortex started immediately after the formation of the initial primordium (24-48 hours post-inoculation; Fig. 2A). The cells that had initially differentiated into isodiametric cells, underwent subsequent differentiation according to their location in the root. An outward gradient of cell differentiation was observed in those cortical cell layers adjacent to the initial primordium, where cells divided once or twice anticlinally (Fig. 2B). This resulted in the primordium increasing in size and appearing as a larger division center, extending to the middle cortex (Fig. 2C). The outer cortical cells did not become involved in primordium formation (Fig. 2C). In flood inoculated *M. truncatula skl* mutant roots, cortical cell activation occurred over the whole cortex, but nodule primordia formed only at distinct sites (data not shown).

PIT formation: an inwardly directed differentiation process

Simultaneously to the formation of the initial primordium, we observed an inward gradient of differentiation, from the subepidermal cortical cell layer towards the primordium, which indicated that the outer cortex was preparing for infection (Fig. 2D,E). Thus, the two opposing gradients of differentiation partially overlapped at their respective extremities (Fig. 2E,F). Inward cell differentiation resulted first in an isodiametric organization (see above) which was then progressively lost as the cells reorganized to form a pre-infection thread (PIT; van Brussel et al, 1992), i.e. a cytoplasmic bridge joining the outer and the inner sides of the cell (Fig. 2G-I). During PIT formation, cortical MtC disappeared (Fig. 2G). The complete disappearance of the MtC was deduced from the observation of extended focus images of several PIT cells viewed by laser confocal microscopy. In PIT-containing cells, the endoplasmic microtubules are oriented anticlinally and parallel to each other in the centrally positioned cytoplasmic bridge (Fig. 2G) and run either from one side of the cell to the other (Fig. 2G), or between the nucleus and the plasma membrane (Fig. 2H). In both *Medicago* species studied, PITs formed essentially above the center of the developing primordium although, for the *M. truncatula skl* mutant, isodiametric cell activation occurred in all outer cortical cells (data not shown). Finally, we found that (i) PIT formation was an irreversible differentiation trait, (ii) PITs were never observed in the inner cortex-derived initial primordium, which always retained an isodiametric organization (Fig. 2I), and (iii) most outer cortical PIT cells eventually divided to generate two daughter cells keeping the same PIT organization (see Fig. 2I; see also Fig. 4D). PIT cell division was preceded by the formation, in the plane where the

endoplasmic PIT had previously formed, of a cortical preprophase band made of transverse microtubules (Fig. 2H).

Root hair activation and root hair curling

MtC rearrangements in root hairs were observed at the time of initial primordium formation (approximately 18-24 hours post-inoculation; Fig. 3A). In root hairs of uninoculated control plants, the MtC is organized as described for a number of other plants (Lloyd and Wells, 1985; Traas et al., 1985). Briefly, in emerging root hairs cortical microtubules are parallel to the elongation axis of the cell and the spherical nucleus is close to the growing tip of the developing root hair (data not shown). In elongating and mature root hairs, the MtC becomes progressively helical and the nucleus, which becomes ellipsoid, finally moves to the proximal half of the root hair where it is positioned against the cell wall (Fig. 3B).

Remarkably, the first changes were seen exclusively in root hairs lying above an activated area (Fig. 3A). In activated root hairs which were presumably preparing to curl, but which had not yet curled, the helical organization of the MtC (Fig. 3B) was progressively replaced by (i) endoplasmic microtubules forming a network around the spherical nucleus and (ii) a network of cortical microtubules parallel to the elongation axis of the root hair (Fig. 3C). Then, the nucleus migrated to the tip of the root hair, and during this time the MtC gradually concentrated in the region between the nucleus and the root hair tip (Fig. 3D). In root hairs which were mature at the time of inoculation, activation was characterized by the formation of a MtC network surrounding the nucleus and extending to a point of the root hair where growth was initiated adjacent to the position of the nucleus. This resulted in the development of a branch containing a dense network of microtubules at its tip, similar to that described above (data not shown). Finally, during root hair curling, endoplasmic microtubules which had accumulated between the nucleus and the tip of the root hair (see above), organized into arrays (Fig. 3E), which progressively converged to the center of the curl, although microtubules were also seen still attached to the hair tip during this step (Fig. 3F).

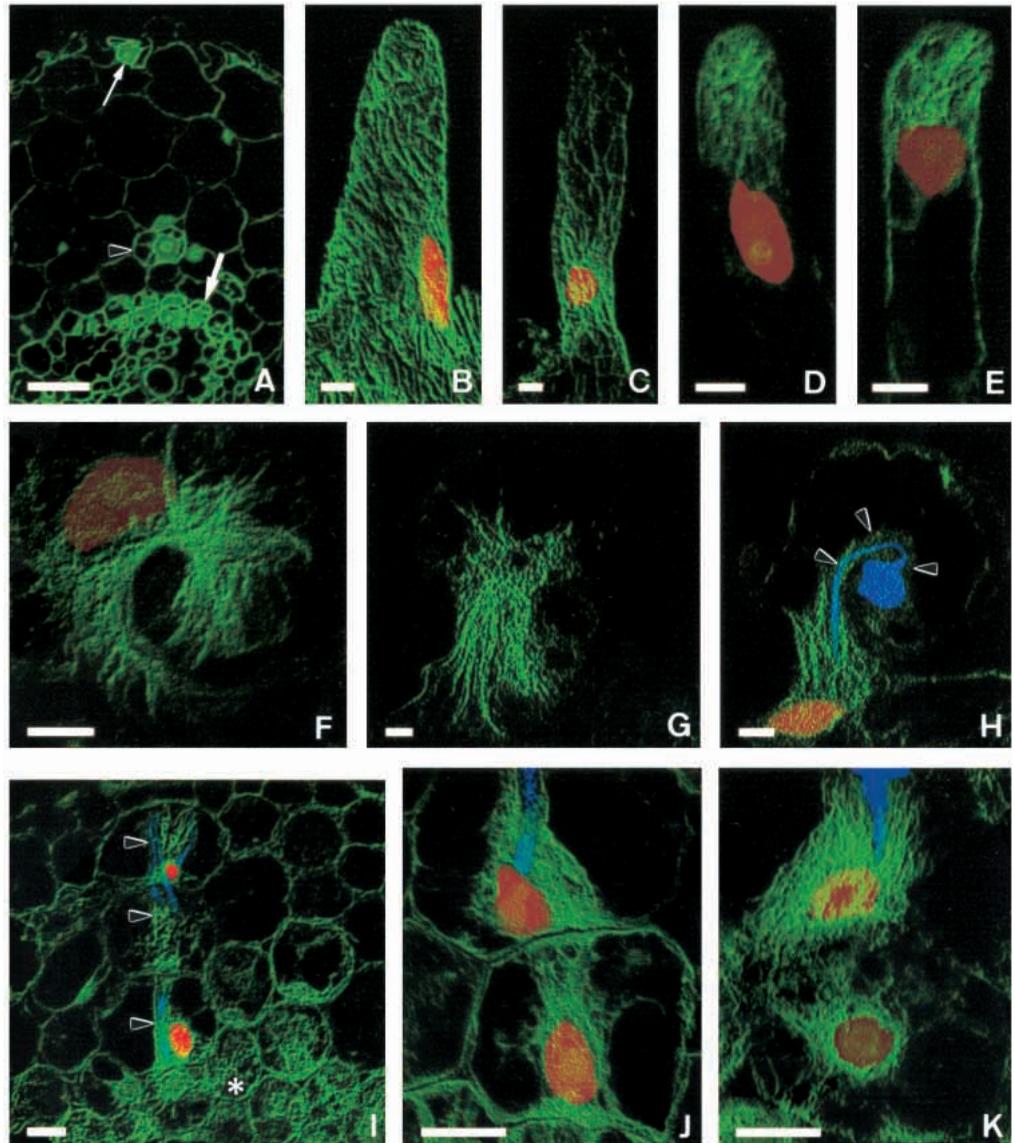
Infection-related MtC reorganization

In *Medicago* species, the infection process *sensu stricto* (reviewed by Kijne, 1992) starts shortly after PIT formation (approximately 48 hours post-inoculation) and infection threads are initiated at the center of curled root hairs. Here we observed infection-related MtC reorganization both in activated root hairs and in cortical cells.

In root hairs, microtubules were recruited for the formation of an infection thread and accumulated to form dense parallel arrays extending from the curl, indicating the beginning of the infection process (Fig. 3G). As infection proceeded, the MtC reorganized to form (i) a dense network surrounding the growing infection thread and connecting the nucleus to the infection thread tip (Fig. 3H), and (ii) longitudinal microtubules parallel to and in close contact with the infection thread (Fig. 3H). Similar MtC organization was seen during the growth of infection threads through activated cortical PIT cells (Fig. 3I,J). In addition, we noted that microtubules accumulated at both sides of the crossing point between adjacent recipient cells (Fig. 3I,J). The inward growth of the infection network terminated once infection threads had

Fig. 3. Infection-related MtC reorganization in alfalfa (A,G,H) and in the *M. truncatula skl* mutant (B-F,I-K). Extended focus images by laser confocal microscopy. In this figure, MtC appears green, nuclei, red, and infection threads, blue.

(A) Activation in a root hair (small arrow) facing a reacting site. The activated pericycle (large arrow) and the differentiating initial primordium (arrowhead) are seen. Bar, 25 μm . (B-F) Micrographs showing the intermediate stages in MtC organization in activated root hairs, from a control root hair (B) to a root hair at the end of the curling process (F). (G) MtC in a curled root hair, just prior to the formation of an infection thread. (H) Infected root hair. Note the MtC network at the tip of the infection thread. The arrowheads show microtubules applied against the thread body. Bars, 5 μm . (I-K) Transverse sections of roots showing the infection thread growth in the root cortex. (I) Infection thread is growing centripetally through PITs (arrowheads) to the initial primordium (asterisk). (J) Details showing MtC organization at a crossing point between two activated cortical cells. (K) Variations in MtC organization at the interface between a cortical activated cell (above) and an initial primordial cell (below). Bars, 15 μm .



penetrated into initial primordium cells in which microtubules concentrated in the region of the cell facing the advancing thread (Fig. 3K). Finally, with the exception of the infection-related MtC changes described above, both the cortical and the endoplasmic MtC could barely be observed in cortical cells through which the infection thread had passed (Fig. 3I).

The formation of the nodule meristem

The next symbiotic step results in the formation of the nodule meristem, approximately 60 to 72 hours post-inoculation. The meristem originates from a number of middle cortex-derived primordial cells which have not been traversed by an infection thread (Fig. 4A,B). At the very beginning of meristem formation each cell divided many times in various directions to generate numerous islets of meristematic cells which then fused together into a unique division center making up the nodule meristem (Fig. 4A,B). There was no indication that activated pericycle-derived cells contribute to the formation of the meristem. Cytologically, nodule meristematic cells (i) clearly differed from adjacent cells of the initial primordium,

on the proximal side, and activated isodiametric and PIT cells, on the distal side (Fig. 4B-D) and (ii) displayed the typical features of root meristematic cells, i.e., small-size, actively dividing and possessing a dense cytoplasm and a large central nucleus (Fig. 4A,B). In nodule meristematic cells, as in other plant meristematic centers (Baluska et al., 1996), the MtC appeared as a randomly localized fluorescent signal due to extensive radiating endoplasmic MtC and randomly oriented cortical microtubules (data not shown). Generally one meristem per activated area was formed in both flood-inoculated *Medicago* sp. studied. In the *M. truncatula skl* mutant, nodule meristems differentiated later than in wild-type alfalfa (4-5 days vs. 2.5-3 days, as previously reported by Penmetsa and Cook, 1997), and formed several discrete loci located side by side in the susceptible zone of the root (data not shown). Finally, during meristem formation, the outermost primordial cell layer divided anticlinally once or twice, to form a cap layer of smaller cells at the distal border of the meristem (Fig. 4B-D). This layer may play a role in protecting the meristem during outgrowth from the root. PIT cells located

Fig. 4. Meristem formation. Cellular changes in the *M. truncatula skl* mutant (A, transverse section) and in alfalfa (B-D, longitudinal sections). Nomarski optics. (A) Division plane (arrowhead) inside an individual primordial cell (arrows). (B) Fusion between meristematic cells to form the meristem (star). (C) A nodule meristem (star) delimited by the initial primordium (asterisk) at its proximal part and a cap layer (arrowheads) at its distal part. (D) Histological organization of outer tissues at the distal part of a nodule meristem: PIT cells (black arrows), cap layer (arrowheads) and distal meristematic cell layer (star). The white arrow points to a new cell plate. Bars, 25 μ m.

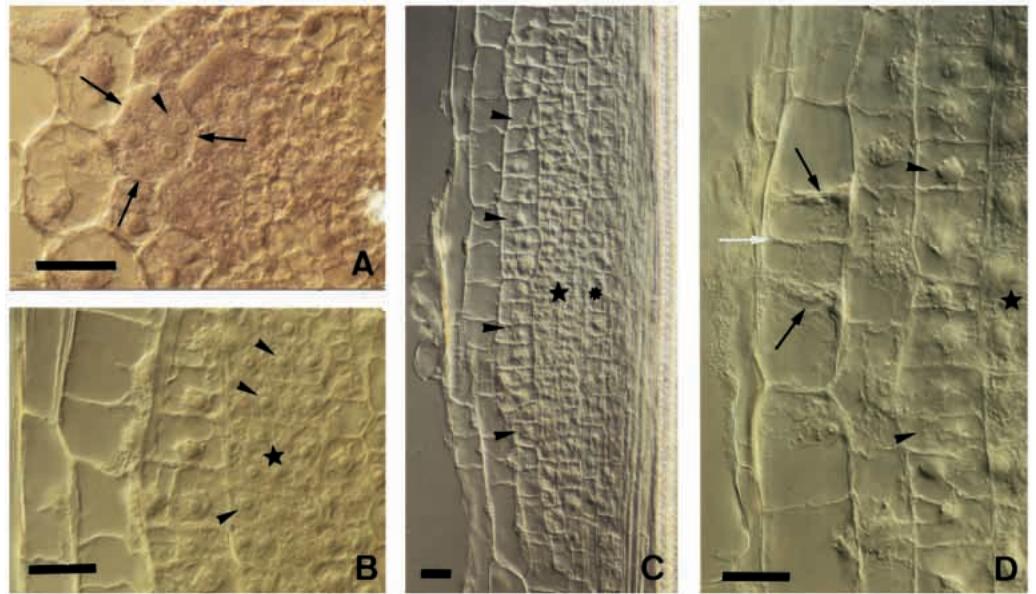
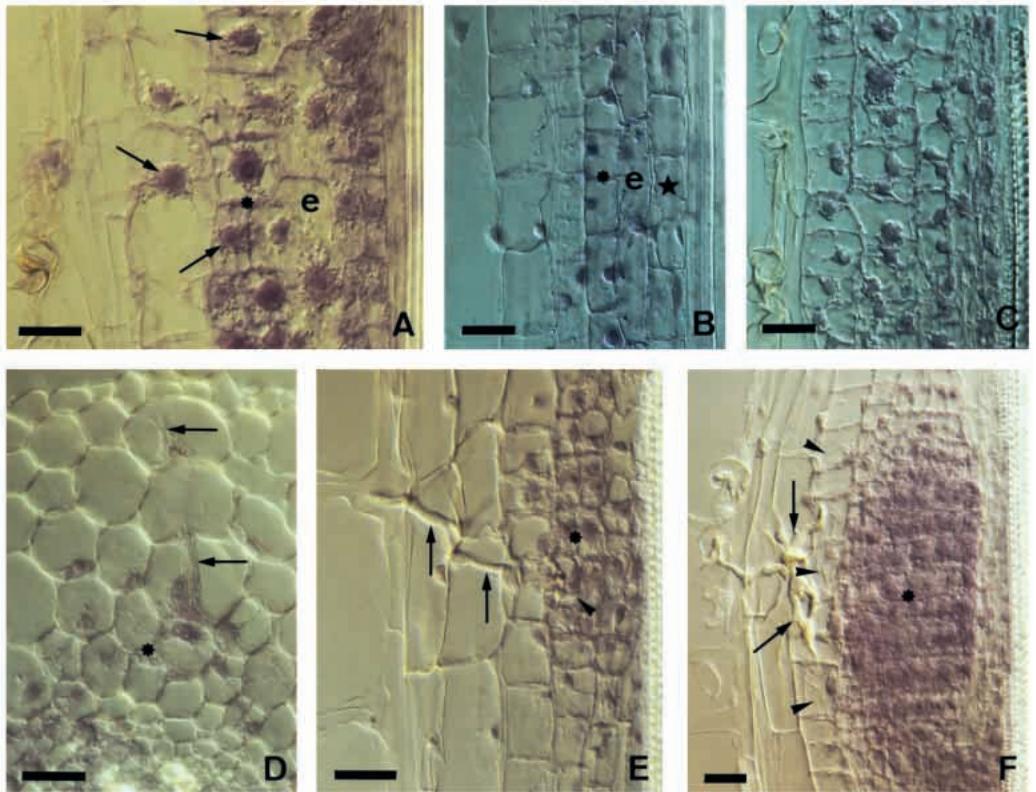


Fig. 5. (A-C) Cell activation in alfalfa roots inoculated with a *R. meliloti* double mutant *nodF/nodL* (A) or treated with Rm Nod factors (B,C). Longitudinal sections. Nomarski optics. Note the isodiametric cell differentiation (arrows) and the lack of PIT formation. In B, anticlinal and periclinal divisions are seen in the pericycle (star); e: endodermis; asterisk: initial primordium. (D-F) Correlation between infection and meristem formation in the *M. truncatula skl* mutant inoculated with the wild-type *R. meliloti* 2011 strain. Transverse (D) and longitudinal (E,F) sections. Nomarski optics. (D) Infection thread (arrows) in the activated inner cortex (asterisk) prior to meristem formation. (E) Successful infection (arrows) passing through a nodule primordium (asterisk). The arrowhead points to the growing tip of the thread. (F) Abortive infection (arrows) which does not traverse the cap layer (arrowheads) at the distal part of a meristem (asterisk). Bars, 25 μ m.



distally to the meristem in the outer cortex and the meristem cap layer were lost during the course of nodule growth.

Correlation between early symbiotic steps

To study further the interrelationship between early symbiotic steps, we examined MtC and cellular changes in plants treated with wild-type *R. meliloti* Nod factors, which elicit the development of bacteria-free nodules in alfalfa (Truchet et al., 1991), or inoculated with the following *R. meliloti*

strains: (i) a double *nodF/nodL* mutant which synthesizes structurally modified Nod factors that are non O-acetylated and N-acylated by vaccenic acid instead of unsaturated C16 fatty acid. This mutant does not infect or nodulate, but elicits a strong activation of the epidermal and cortical cells in alfalfa (Ardourel et al., 1994), and (ii) an *exoA* mutant which is defective in exopolysaccharide synthesis (Leigh et al., 1985), but produces wild-type Nod factors (Lerouge et al., 1990). The *exoA* mutant is able to initiate infection, but

infection threads abort in root hairs (Leigh et al., 1994), and it elicits small, bacteria-free nodules (Leigh et al., 1985; Niehaus et al., 1994).

In alfalfa, PIT formation is elicited by infecting rhizobia but not by purified Nod factors

Our first objective was to determine the relationship between PIT differentiation and infection. In plants inoculated with the *R. meliloti* *exoA* mutant PIT and meristem formation, along with MtC and cellular changes, were similar to those described above for the wild-type interaction. The non-infective double mutant *R. meliloti* *nodF/nodL* was also able to elicit the activation and division of the pericycle (Fig. 5A) and the cortex (as already described by Ardourel et al., 1994; Fig. 5A) but, surprisingly, did not elicit PIT, primordium or meristem formation, at least during the 8 days following inoculation (Fig. 5A). Since this double mutant synthesizes modified Nod factors (Ardourel et al., 1994), we tested whether purified wild-type *R. meliloti* Nod factors could induce PIT formation in alfalfa. We found that Nod factors induced pericycle activation and division (Fig. 5B), cortical cell activation (Fig. 5B,C), primordium and meristem formation (as already described in Truchet et al., 1991; data not shown), but not PIT formation (Fig. 5C). Thus, Nod factors are able to elicit most of the early symbiotic events in alfalfa, with the exception of PIT formation.

Correlation between the infection process and meristem formation

Our second goal was to examine the temporal relationship between efficient infection and meristem formation. In plants inoculated with wild-type *R. meliloti* 2011, infection starts and proceeds through the activated cortex and nodule primordium before the meristem forms (Fig. 5D,E). In contrast, a delay in infection progression following inoculation by either a wild-type strain (Fig. 5F), or by a deficient bacterial strain such as an *exoA* mutant (data not shown), results in the differentiation of the meristem before the infection network has reached the initial primordium, and subsequently, in the abortion of infection threads in the upper cortical cell layers (Fig. 5F). These data suggest that to be successful, infection must reach the initial primordium before the meristem forms.

DISCUSSION

In this study, we have made use of the MtC architectural rearrangements and correlated cellular changes occurring during the early stages of nodulation in two *Medicago* species to provide a detailed description and timing of the events during early ontogeny of *Medicago* indeterminate nodules.

Nodulation-related developmental changes

We have made the novel observation that the pericycle is the first cell layer to display MtC changes, and to divide. This occurs prior to activation of the inner cortex, which was previously considered to be the first tissue to proliferate during nodule organogenesis (Dudley et al., 1987; Libbenga and Harkes, 1973). In alfalfa, cell division in the pericycle has been reported to occur simultaneously with division in the inner cortex (Thornton, 1930), while pericycle division has not been described in pea (Bond, 1948; Newcomb et al., 1979). Our results correlate with

recent data showing that bacterial inoculation (or treatment with Nod factors) elicits a strong and rapid pericyclic expression of the early nodulin gene *ENOD40* (Asad et al., 1994; Kouchi and Hata, 1993; Minami et al., 1996; Yang et al., 1993). The majority of nodules (Libbenga and Harkes, 1973) and lateral roots (Sussex et al., 1995) are initiated in front of protoxylem poles, i.e. in the part of the pericycle which is the most sensitive to the action of auxin (Sussex et al., 1995). This co-localization and similar division pattern suggest a common mechanism for initiation of both organs. Using an auxin-responsive reporter construct, Mathesius et al. (1998) have shown that, in the case of nodulation, Nod factors elicit an auxin transport inhibition resulting in an accumulation of auxin. This might result in initiation of cell division in the pericycle opposite protoxylem poles described in this study. In contrast, a transient decrease in auxin-responsive reporter gene expression is observed in the inner cortex, which precedes the activation and the formation of division centers (Mathesius et al., 1998). Such a decrease may render differentiated cortical cells more sensitive to the following increase in auxin, stimulating them to re-enter the cell cycle and divide to form the nodule primordium.

Major cytoskeletal changes and dramatic reorganization of the cell cytoplasm are seen in the inner cortex shortly after those in the pericycle. This leads to an isodiametric organization with cytoplasmic strands and microtubules radiating from the central nucleus (Bakhuizen, 1988; this paper). Inner cortical activation (i) is associated with the expression of several nodulin genes such as *PsENOD12* (Scheres et al., 1990), two lectin genes *Mtlec1* and *3* (Bauchowitz et al., 1996), *MsENOD40* (Asad et al., 1994; Yang et al., 1993) and *MtENOD20* (Vernoud et al., 1999) and (ii) results in the formation of a division center which is commonly referred to as the nodule primordium (Dudley et al., 1987; Libbenga and Harkes, 1973; Yang et al., 1994; see reviews by Brewin, 1991; Nap and Bisseling, 1990). We propose to distinguish between the initial primordium, i.e., the very first primordial cells that arise from the division of the inner cortex, and the nodule primordium itself, the division center extending to the middle cortex once properly formed. The nodule primordium is a structural entity which forms according to a multi-step process and comprises cells with different fates generating the first recipient cells for infection threads and intracellular rhizobia (see below), PIT cells for infection, meristematic cells and cap cells.

Activated outer cortical cells probably correspond to those cells described by Yang et al., (1994) as re-entering the cell cycle and staying blocked in G₂. Further differentiation in such cells results in the formation of pre-infection threads (PITs), as previously described in pea and vetch (van Brussel et al., 1992). In this study, we show that PITs form in plants inoculated with infective *R. meliloti* strains (wild-type and *exoA* mutant) but do not form in plants inoculated with the non-infective *R. meliloti* *nodF/nodL* double mutant or after treatment with purified wild-type Nod factors. This last observation, which does not agree with a previous report showing that PITs form in pea and vetch treated with *R. leguminosarum* bv. *viciae* Nod factors (van Brussel et al., 1992), may indicate that requirements for PIT induction vary even in closely related legumes. In *Medicago* sp., our results lead to the conclusions that (i) purified *R. meliloti* Nod factors which elicit the nodule primordium (Truchet et al., 1991; this study) are not sufficient to induce PIT formation and (ii) PIT

formation and infection are tightly coupled early events. Moreover, they prompt us to speculate that a second rhizobial signal, possibly different from and acting together with Nod factors, is necessary for PIT formation. This putative additional signal could also be under the control of *nod* genes. Finally, the fact that Nod factors are able to elicit primordium-meristem formation in alfalfa (Truchet et al., 1991) but are not sufficient for PIT formation (this study), confirms our previous results showing that Nod factor structural requirements to elicit nodulation-related events vary according to the symbiotic step which is activated (Ardourel et al., 1994).

One aim of this study was to discriminate between the two division centers which are referred to as the nodule primordium and the nodule meristem respectively (Libbenga and Harkes, 1973; for reviews see Brewin, 1991; Hirsch, 1992; Kijne, 1992). Our results show that the primordium and the meristem are closely related in *Medicago* sp. Thus, the primordial cells located in a medial position within the nodule primordium act as founder cells for nodule meristem formation. The relationship between the two cortical division centers is also strengthened by data showing that (i) a nodule meristem always forms on plants where a primordium has been elicited, such as plants either inoculated with a *R. meliloti exoA* mutant (Leigh et al., 1985) or treated with Nod factors (Truchet et al., 1991), and (ii) no meristem develops on plants where formation of the primordium is impaired (*R. meliloti nodF/nodL* mutant; Ardourel et al., 1994 and this study).

The hypothesis of molecular gradients controlling the development of nodules (Thimann, 1936), was substantiated first by Libbenga et al. (1973), who predicted that the pattern of cortical cell division during early nodulation required both bacterial signaling and plant vascular substances. This prediction has received strong support with the discovery of rhizobial Nod factors which elicit cortical cell division (reviewed by Dénarié et al., 1996; Schultze and Kondorosi, 1998; Spaik, 1996), the characterization of uridine as a stele factor able to elicit cell growth and division in root explants (Smit et al., 1995), and the involvement of plant hormones in the formation of cortical division centers (Libbenga et al. 1973; Mathesius et al., 1998). We suggest that molecular gradients and the two opposite gradients of cell differentiation described in this study, are tightly coupled. Nod factors and presumably plant hormones are involved in the accomplishment of the outward gradient of differentiation which results in the formation of the primordium and the meristem. As mentioned above, it seems that Nod factors alone are not sufficient for the completion of the inward gradient of differentiation leading to preparation for infection and PIT formation, but that additional signals are necessary.

Infection as an integrated process during cortical activation

We have shown that, in *Medicago* sp., infection occurs shortly after the pericycle and the inner cortex are activated and is limited to root hairs which are located directly above an activated area. This is in line with previous results showing that (i) the infection-related nodulin genes *ENOD12* (Pichon et al., 1992; Scheres et al., 1990) and *MtENOD20* (Vernoud et al., 1999) and the lectin genes *Mtlec1* and *3* (Bauchrowitz et al., 1996), are strongly expressed in root hairs located directly above root sites where the inner cortex has divided, and (ii) in

pea, bacteria bind preferentially to the tip of root hairs which are located in front of protoxylem poles (Diaz et al., 1986).

To understand early nodule ontogeny, it is important to understand the interrelationship between infection and meristem formation. It is obvious that (i) the infection network reaches the initial primordium (this study); (ii) the meristem forms independently of the completion of the infection process (this study) and (iii) the meristem is located distal to infected tissues in indeterminate nitrogen-fixing nodules (Vasse et al., 1990). Taken together, these results suggest that there is an open window during which infection must occur prior to the formation of the meristem. In the case where infection occurs normally, the infection network reaches the initial primordium before the meristem forms distally to the growing part of the infection threads. In the case where the infection process is absent, delayed or arrested (as for example with a *R. meliloti exoA* mutant), the meristem forms proximally to the infection network and abortive nodules are consequently formed (Arcondéguy et al., 1997; Leigh et al., 1985, 1994; Niehaus et al., 1994; Yang et al., 1992). Finally, the above interpretation prompts us to hypothesize that the role of the initial primordium is to provide the first recipient cells for bacterial release, prefiguring the prefixing (infection) zone II, which therefore differentiates prior to the apical meristem. Interestingly, this relationship is reversed in the mature nodule where zone II derives from the apical meristem (Vasse et al., 1990).

Specific symbiosis-related MtC changes

This study clearly shows that the MtC rearranges throughout all early steps preceding nodule development, particularly during PIT formation and infection thread growth.

MtC changes which occur during PIT formation include the gradual breakdown of the cortical MtC (this study), and a dramatic extension and reorganization of the endoplasmic MtC (Kijne, 1992; this study). Since a very intense anti-tubulin staining is seen initially around nuclei of activated cells, it is likely that the MtC forms from newly synthesized microtubules radiating from microtubular organizing centers located at the nuclear envelope. Furthermore, the fact that MtC reorganization is similar to changes accompanying the preparation of a cell to divide and the formation of a phragmosome during the cell cycle (Staiger and Lloyd, 1991), strongly suggests that a PIT is analogous to a phragmosome (Kijne, 1992), but without a cortical MtC.

We found that the presence of PITs does not prevent cells from dividing and forming two daughter cells displaying the same PIT organization. This shows that PIT cells are not necessarily blocked in the G₂ phase of the cell cycle (Yang et al., 1994). During preparation for cell division, a circular band of cortical microtubules, the preprophase band, (PPB) forms just before nuclear envelope breakdown (Goddard et al., 1994). This is also the case for the division of PIT cells. Our results indicate that PPBs are not present at the time of PIT formation, but form as the PIT cells prepare to divide. The fact that PITs and PPBs occur simultaneously only at the onset of cell division, strongly suggests that they are different structures.

Our results confirm and extend previous electron microscopy studies showing that microtubules are present at the site of penetration by infection threads (Ridge and Rolfe, 1985) and in the vicinity of infection (Bakhuizen, 1988) and are in agreement with the model of Ridge (1992) proposing a

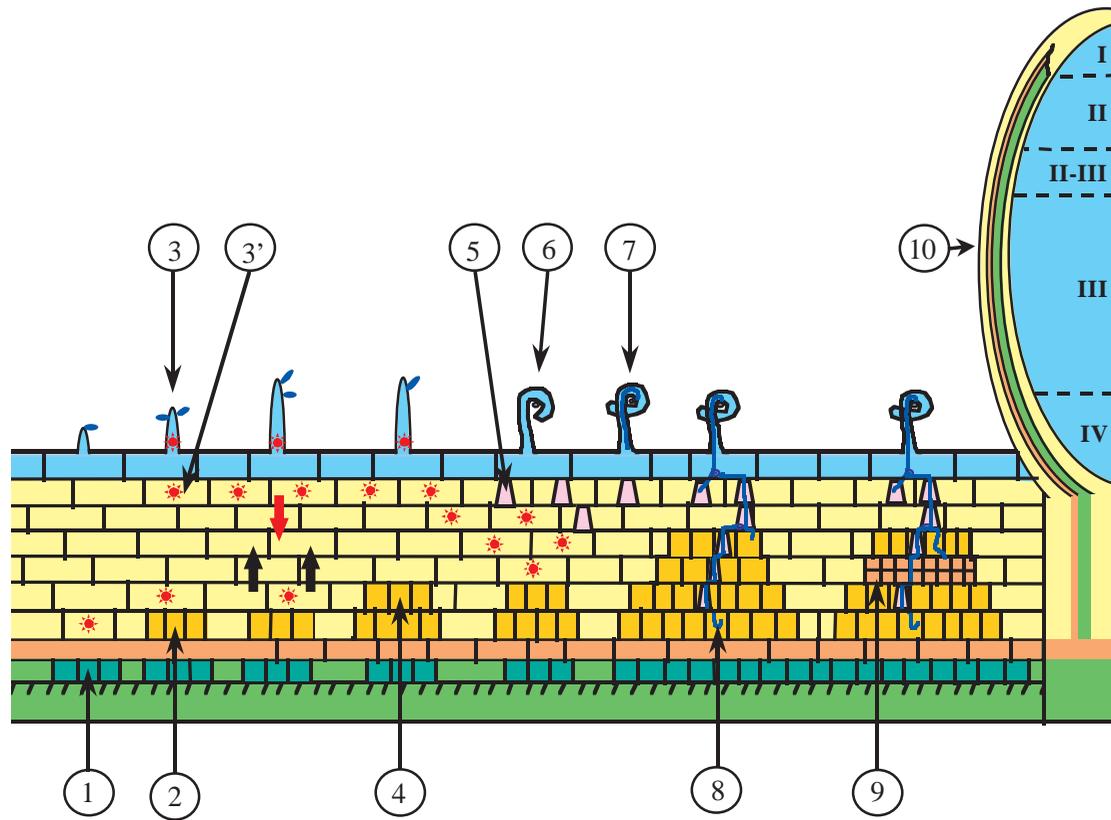


Fig. 6. Model for successful infection of *Medicago* species by *Rhizobium meliloti*. The sequence of the developmental stages as deduced from changes in the MtC are indicated with numbers. For explanation see text.

role for the cytoskeleton during root hair infection. We hypothesize that (i) the dramatic MtC reorganization at the crossing point between two adjacent cells is correlated with the partial digestion of the cell wall that precedes the arrival of the infection thread (IT) as shown by Van Spronsen et al. (1994) and (ii) the interconnection between the microtubules located at the growing tip of an IT and the MtC network surrounding the nucleus, is essential for maintaining the nucleus at the appropriate distance to sustain infection thread growth (Nutman, 1959). In conclusion, the MtC seems to play a key role in the infection process, including infection thread growth, as it has been shown to be involved in the development of other tip growing structures such as root hairs and pollen tubes (reviewed by Miller et al., 1997).

Can IT development be considered as a kind of inverted root hair growth? Although the function of the MtC may be the same in both situations, i.e. determining the site and the width of the growing structures (Miller et al., 1997), fundamental differences exist: (i) in root hairs the cell machinery responsible for growth acts inside the elongating cell itself, while for IT growth it operates outside the growing structure; (ii) the MtC organization is different at the tip of both structures; (iii) in contrast to root hair growth, the infected plant cell has to change its internal organization, including its cytoskeleton, to allow the IT to grow against the turgor pressure of the cell. Interestingly, a similar MtC organization is described in barley coleoptile cells in response to infection attempts by the fungus *Erysiphe pisi* (Kobayashi et al., 1997). In this non-pathogenic association, the MtC plays the role of a counter force to the entry attempts of the

fungus and successfully prevents the penetration of the hyphae, probably by forming a rigid structure located at the site of appressorium formation. It is possible that in legume-rhizobia interactions, IT growth results from a similar, although modified, mechanism. In symbiosis, the cytoskeleton would not oppose the penetration of the microsymbiont but would direct the growth of the IT in the opposite direction, i.e. into the plant cell.

A model for successful infection of *Medicago* sp. by *Rhizobium meliloti*

Using the novel observations of MtC rearrangements made in this study, we propose a model aimed at improving our understanding of the early symbiotic interaction in *Medicago* sp. (Fig. 6). Following inoculation, Nod factors secreted by *Rhizobium* induce a series of rapid changes at the root level, such as variations in cytosolic Ca^{2+} and pH, a depolarization of the plasma membrane, a depolymerization of microfilaments and the induction of various nodulin genes. However, the pericycle is the first tissue in which microtubular cytoskeleton rearrangements and corresponding cellular changes take place (Fig. 6, no. 1). Cell activation then occurs in the inner cortex, in which cells divide to form the initial primordium (Fig. 6, no. 2) and shortly after, in root hairs (Fig. 6, no. 3), the target cells for infection, and in the outer cortical cells (Fig. 6, no. 3') which are always located distally to the activated area of the root. Cell activation then extends progressively to the middle and the outer cortex according to two opposite gradients of cell differentiation: an outward gradient (Fig. 6, black arrows) resulting in the formation of the nodule primordium extending to the middle

cortex (Fig. 6, no. 4), and an inward gradient (Fig. 6, red arrow) which starts in the outer cortex and results in the infection-related PIT cell differentiation (Fig. 6, no. 5). Simultaneously to PIT differentiation, root hairs curl (Fig. 6, no. 6). Infection threads, which are initiated in curled root hairs (Fig. 6, no. 7), grow centripetally through cortical PIT cells down to the initial primordium (Fig. 6, no. 8). At this stage, middle primordial cells which have not been traversed by infection threads dedifferentiate to form the nodule meristem which therefore develops in a distal position to the growing part of the infection network (Fig. 6, no. 9), i.e. in the same spatial organization as in nitrogen-fixing indeterminate nodules. After meristem formation, cell activation is progressively lost as the nodule grows and emerges from the root surface (Fig. 6, no. 10).

We are very grateful to Doug Cook for providing seeds of the *M. truncatula* sickle mutant, and to Fabienne Mailet and Jean Dénarié for purified Nod factors. We thank Philippe Cochard for advice in laser confocal microscopy and Jérôme Juille for help in digitizing and treating micrographs. We appreciate the helpful comments and the valuable criticism on the manuscript made by numerous colleagues from our laboratory. Our particular thanks go to Clare Gough and Dave Barker for English reviewing. A. C. J. T. was supported by a TMR fellowship and this work was funded by a grant from the European Community TMR Programme (FMRW-CT96-0039).

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