A Non-nodulating Alfalfa Mutant Displays neither Root Hair Curling nor Early Cell Division in Response to *Rhizobium meliloti*

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The early events in the alfalfa-*Rhizobium meliloti* symbiosis include deformation of epidermal root hairs and the approximately concurrent stimulation of cell dedifferentiation and cell division in the root inner cortex. These early steps have been studied previously by analysis of *R. meliloti* mutants. Bacterial strains mutated in *nodABC*, for example, fail to stimulate either root hair curling or cell division events in the plant host, whereas exopolysaccharide (exo) mutants of *R. meliloti* stimulate host cell division but the resulting nodules are uninfected. As a further approach to understanding early symbiotic interactions, we have investigated the phenotype of a non-nodulating alfalfa mutant, MnNC-1008 (NN) (referred to as MN-1008). Nodulating and non-nodulating plants were inoculated with wild-type *R. meliloti* and scored for root hair curling and cell divisions. MN-1008 was found to be defective in both responses. Mutant plants inoculated with Exo- bacteria also showed no cell division response. Therefore, the genetic function mutated in MN-1008 is required for both root hair curling and cell division, as is true for the *R. meliloti nodABC* genes. These observations support the model that the distinct cellular processes of root hair curling and cell division are triggered by related mechanisms or components, or are causally linked.

INTRODUCTION

Members of the plant family Leguminosae can establish a symbiosis with compatible species of the soil-dwelling bacteria, *Rhizobium*. During the development of the symbiotic organ, the root nodule, the prokaryotic partner differentiates and gains the ability to fix atmospheric dinitrogen into ammonia. This symbiotic association provides usable nitrogen to the plant and provides a carbon and energy source for the bacterium.

Although the morphology and morphogenesis of nodules can vary considerably, the early stages of nodulation always involve two events (reviewed by Newcomb, 1981): (1) The bacterium invades the plant root (in many cases this occurs by means of an infection thread, formed by plant cell wall deposition around invading and proliferating bacteria); and (2) The plant host initiates organogenesis by cortical cell dedifferentiation and division.

Successful infection of the *Medicago sativa* (alfalfa) root during *Rhizobium meliloti*-alfalfa noduleation is characterized by an early nodulation-specific host response at the plant surface, root hair curling (Yao and Vincent, 1969). Growing epidermal root hair cells that are competent to respond to *R. meliloti* may develop a characteristic 360° curled deformation of the root hair (known as the "shepherd's crook"; also called marked root hair curling [Yao and Vincent, 1969; Truchet et al., 1985] and Hac [as in Vincent, 1980]). A microscopically defined bright or refractile spot in the crook of the curled hair is believed to correlate with the point of infection thread initiation (Callahan and Torrey, 1981; Truchet et al., 1985). Less striking reactions are also characteristic of early symbiosis (Yao and Vincent, 1969). Such events include branching and partial curling and will be referred to here as root hair deformation. There is an excellent correlation between the ability of a bacterium to infect a host and its ability to induce marked root hair curling. For that reason, and the relative ease of scoring the root hair phenotype compared with observing infection events, root hair curling is monitored routinely as an indication of an early nodulation-specific event.

Studies of several systems have shown cell divisions also to be a diagnostic early event. In the *Bradyrhizobium japonicum*-soybean symbiosis, plant cortical cell divisions occurred by 12 to 24 hr, as early as the epidermal root hair cell reactions (Turgeon and Bauer, 1982; Calvert et al., 1984). Early cell divisions occur in the alfalfa-*R. meliloti* symbiosis within 21 to 24 hr after inoculation of the plant with bacteria (Dudley et al., 1987). This also is concurrent with, or precedes, root hair curling at the surface. Thus,
during nodulation of both soybean and alfalfa, the initial cell divisions within the root occur characteristically as early as epidermal events.

The cellular mechanisms that underlie either root hair deformation or plant cell division are not known. Whether the two events are coupled mechanistically is also of interest. Dissection of the nodulation process using bacterial genetic mutants has demonstrated that some mutants, such as Exo− strains, cause root hair deformation without full curling, yet provoke complete root nodule morphogenesis. However, other genes such as nodABC are required for cell division and for even minimal root hair deformation to occur.

A complementary approach to analyzing the relationship of early events in the plant epidermis and cortex is to dissect the plant’s genetic contribution to each. Non-nodulating plant mutants have been recovered after mutagenesis in soybean (Gresshoff et al., 1987; Mathews et al., 1987) and pea (Kneen et al., 1987). Genetic analysis of these mutants has shown that several plant loci are involved in control of nodulation. Although the precise block to nodulation of the Hac+, non-nodulating soybean has not yet been identified, grafting experiments that replace one plant shoot system with the shoot system from a genotypically different plant have demonstrated that control of the non-nodulating trait in soybean resides in the plant root, not the shoot (Mathews et al., 1987).

We have studied the phenotype of a mutant alfalfa line relative to the early events infection. We have investigated the response to inoculation by both wild-type and Exo− mutants of R. meliloti on a non-nodulating alfalfa mutant, MnNC-1008 (NN). Our results demonstrate that non-nodulating plants are deficient in expression of the earliest observable phenotypes, both the initiation of cell division and the root hair curling responses. This implies that common plant components or mechanisms, possibly those responsive to the bacterial nodABC-produced signal, may be involved in or precursors to the epidermal and cortical events.

**RESULTS**

**Root Hair Curling**

The ability of homologous bacteria (R. meliloti 1021) to cause root hair curling on wild-type or non-nodulating alfalfa was assayed by scoring for the presence of root hair deformation and shepherd’s crook formation (marked root hair curling) at 3 days after bacterial inoculation.

Within each genotype, and to a lesser extent along any one root, extensive variation in density and length of hairs was observed. Despite this variability in root hair morphology, a clear difference in response to inoculation was observed between the two genotypes. For eight out of nine wild-type plants scored, the shepherd’s crook morphology was observed in at least one and usually several root hairs (Figure 1A). In all nine wild-type alfalfa plants, an extensive zone of deformed root hairs was observed.

In contrast, no shepherd’s crooks at all were observed on any plant in the non-nodulating sample group. The root hairs in almost all microscopic fields along all 10 of the non-nodulating roots displayed no deformation. (A representative sample is shown in Figure 1B.) Root hair deformation was observed at levels comparable to uninoculated wild-type controls (Vincent, 1974). Nonrepresentative fields showing the maximum observed deformation are also displayed in Figure 1: two out of 10 non-nodulating plants displayed root hair twining (Figure 1C). Branching (Figure 1D) was observed on two other non-nodulating plants. Short root hairs with bulging tips, a phenotype not associated specifically with nodulation, was observed on seven out of 10 non-nodulating plants.

**Cell Division**

The ability of wild-type and non-nodulating plants to initiate nodule-specific cortical cell divisions was also examined. Aseptically grown plant roots were inoculated using the spot inoculation technique, with homologous bacteria (R. meliloti 1021 or 1021 [pRmSL26]) grown under inducing conditions. At 2 and 3 days after inoculation, a 0.5-cm segment of root containing each spot was harvested, fixed, and embedded in plastic. Serial sections were cut and stained. Each section was scored for any indication of cortical cell activation, such as mitotic figures, brightly staining cytoplasm, or small cell size indicative of prior division. Three repetitions, each comprising six non-nodulating and three wild-type alfalfa plants, were performed.

Eight of nine wild-type plants displayed extensive regions of cell activation (Figure 2A); one wild-type plant showed no signs of any activation or cell division. In contrast, no signs of cortical cell activation were observed in any of 18 non-nodulating root segments scored (Figure 1B). Cell divisions of preemergent lateral root meristems were observed occasionally in roots of both wild-type and non-nodulating plants across the root axis from the inoculum spot (Figure 1C).

Exo− bacteria cause dedifferentiation and division of wild-type host cells but do not establish normal infections. To determine whether the formation of uninfected nodules nonetheless involves the same morphogenetic process as wild-type nodulation, we examined whether Exo− Rhizobium mutants were subject to the same block to cell division exhibited by MnNC-1008 (NN) alfalfa. Wild-type alfalfa plants were inoculated with Exo− mutants and, after 12 days, a characteristic “empty nodule” (an amorphous, uninfected outgrowth of the plant root) was evident at the spot of inoculation in 26 out of 28 inoculated plants (Figure 3A). In contrast to the response of wild-type plants, all
MnNC-1008 (NN) plants inoculated (nine with Rm7031 and seven with Rm7055) displayed no response. At the level of resolution permitted by the optics of the dissecting microscope, there were no indications of any swelling of the root or any morphological aberration (Figure 3B).

We also examined the reaction of cortical cells of the non-nodulating alfalfa to inoculation by Exo⁻ R. meliloti. Six plants, three inoculated with an exoA mutant (Rm7031) and three with an exoF mutant (Rm7055), were embedded 12 days after inoculation and sectioned for high resolution scoring. No evidence of cell division or cell activation of the inner cortex was observed (Figure 3C).

**DISCUSSION**

Root hair curling and cortical cell activation are the earliest known plant responses in a successful R. meliloti-alfalfa symbiosis. The specific mechanisms leading to these events are not known; in part this reflects our limited understanding of how plant cell growth and cell division are controlled at the molecular level. The relationship between root hair deformation and cell division is also not understood. It is possible to hypothesize either (1) separate causes for each or (2) common requirements for both processes (for instance, sequential causation of one by the other or common cellular mechanisms underlying both phenotypes).

**Phenotypes of Rhizobium nod Gene Mutants**

In the R. meliloti-alfalfa symbiosis, several observations indicate that root hair deformation and cell division appear to be tightly coupled processes. Marked root hair curling is induced on alfalfa only by those Rhizobium species that are able to induce nodule organogenesis as well (Vincent, 1974). The hypothesis that cell division and root hair deformation are coupled is supported further by phenotypic analysis of non-nodulating R. meliloti mutants. Specifically, phenotypic analysis of Rhizobium mutants has shown that the genes nodABC are needed for nodule formation and for root hair curling in many plant-Rhizobium

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**Figure 1.** The Root Hairs of Nod⁺ and MN-1008 Alfalfa Exhibit Different Responses to Inoculation with R. meliloti 1021 When Viewed Under Nomarski Differential Interference Contrast Optics (Magnification, ×125). Scale Bar = 100 μm.

(A) Wild-type alfalfa root hairs displayed marked curling (shepherd's crook, at least 360° curling) at the root hair tip (arrows).
(B) to (D) Nod⁻ alfalfa shows no marked curling.
(B) Typical field of undistorted Nod⁻ alfalfa root hairs after inoculation.
(C) Root hair twining (arrow) was observed rarely.
(D) Root hair branching (arrow) was also rare.
The Plant Cell

Figure 2. Nod⁺ Mn-1008 Alfalfa Failed to Initiate Cell Divisions in Response to Wild-Type R. meliloti 1021.

Three-micrometer longitudinal sections through alfalfa roots after inoculation, viewed with fluorescence optics. Inoculum spots stain as a bright patch on the root surface (arrows). Magnification (all), X270.

(A) Control plants (Nod⁺) harvested 70 h after inoculation display an extensive activated area including mitotic cells and brightly staining cytoplasm. Scale bar = 50 μm.

(B) No characteristics of cell division or cytoplasmic activation were evident in roots of the Nod⁻ alfalfa harvested 70 h after inoculation.

(C) Presence of a lateral root primordium in MN-1008 (open arrow) across the root axis from the spot of inoculum contrasts with the lack of nodule-specific activation of cortical cells.

Additional Rhizobium nod genes are needed for normal efficiency and kinetics of nodulation; these appear also to affect host range: examples are nodFE, nodH, nodU, and nodLMN (Djordjevic et al., 1985; Downie et al., 1985; Truchet et al., 1985; Debelle et al., 1986; Swanson et al., 1987; Surin and Downie, 1988). Strains with mutations in some of these cause abnormal, in some cases exaggerated, root hair deformation on their host plant and/or on nonhost plants. Often, these strains form nodules at lower efficiency.

On the other hand, at least some uncoupling between full root hair curling and nodule morphogenesis has been demonstrated. The genes encoding functions for the synthesis of the acidic exopolysaccharide (exo genes; Leigh et al., 1985; Finan et al., 1985) are also required for root hair curling and infection in R. meliloti. Exo⁻ mutants provoke the formation of an "empty nodule," a nodule-like organ that is not infected by bacteria. Alfalfa inoculated with exoB mutants show an incomplete root hair curling phenotype, or hair deformation (Had*), but no marked curling or infection threads (Finan et al., 1985). Other exo mutants display some marked root hair curling (Leigh et al., 1987). In summary, some Rhizobium mutants (Exo⁻) uncouple organogenesis from marked root hair curling; however, the Exo⁻ mutants reported to date do stimulate at least some root hair deformation.

Thus, there are many bacterial genes required for full host-specific root hair curling and infection, whereas only a subset of these (nodABC) are required for early cell division. This might indicate that cell division is the primary event, independent of root hair curling. However, mutants that cause cell division cause some root hair deformation. Also, the requirement for bacterial "common" nod genes (e.g., nodABC) in the initiation of both the root hair deformation and curling response and the cortical cell division response suggests that bacterial nod genes act via a mechanism that is required for both cellular phenotypes. The products of these genes must be required for both root responses either through sequential activation or directly.

Phenotype of the Alfalfa Non-nodulating Mutant

To investigate the possibility that there are common mechanistic antecedents to both events, it is useful to examine plant genetics. We have analyzed a non-nodulating genetic variant of alfalfa (M. sativa). Alfalfa is a tetraploid (4N) species that exhibits inbreeding depression. These factors and the lack of established genetic markers make the genetic analysis of alfalfa a technically challenging under-
The non-nodulating line, MnNC-1008 (NN) (or MN-1008), was isolated during a breeding program designed to produce alfalfa with increased nitrogen-fixing potential (Peterson and Barnes, 1981). The non-nodulating clone was isolated from a population containing equal amounts of germplasm from six alfalfa cultivars. Peterson and Barnes (1981) conclude that the non-nodulating trait is conditioned by two unlinked, tetrasomically inherited, recessive genes termed $nn_1$ and $nn_2$. The nulliplex condition of both genes is required for the non-nodulating trait to be expressed. It is possible that $nn_1$ and $nn_2$ are redundant genes encoding the same function. The results of $F_2$ crosses between MN-1008 and four strains that produce ineffective nodules (in mutants) suggest that MN-1008 is also homozygous for one recessive, tetrasomally inherited ineffective locus, $in_1$, and heterozygous at the $in_2$ locus. Data from the $F_2$ and backcross generations of these crosses and of $F_2$ crosses between MN-1008 and normal lines indicate that the four identified $in$ loci and the $nn_1$ and $nn_2$ loci are unlinked. In addition, the $nn$ recessive alleles occur frequently in the normal alfalfa germplasm from several sources. Peterson and Barnes suggest, however, that the requirement for both genes to be nulliplex reduces greatly the chance for the non-nodulating trait to be expressed.

Our results support the proposal that root hair deformation and cell division during nodule initiation are coupled mechanistically. The MN-1008 alfalfa line, in which root hair growth and root morphogenesis appear normal, responds to inoculation with neither root hair curling nor cell division. None of the 10 plants examined displayed curled root hairs in response to inoculation with wild-type \textit{R. meliloti} 1021. None of the 18 non-nodulating alfalfa plants spot-inoculated with \textit{R. meliloti} 1021 displayed cell divisions. To assure that bacterial nod genes were active, the bacteria that were spot-inoculated were pre-induced; in addition, the root exudate of the MN-1008 seedlings is capable of inducing expression of the nod genes of \textit{R. meliloti} 1021 (Peters and Long, 1988). Therefore, it is unlikely that the non-nodulating phenotype can be explained as a lack of induction of the \textit{Rhizobium nod} genes by the MN-1008 plants.

The non-nodulating alfalfa also showed no response to Exo$^-$ bacteria (which provoke empty nodule formation on wild-type alfalfa hosts). This result suggests that the same plant component is involved in the morphogenesis stimulated by Exo$^-$ (empty nodule) and wild-type bacteria (infected nodule). The simplest interpretation of the MN-1008 non-nodulating phenotype is that MN-1008 is blocked in the “perception,” or transduction and transmission, of the signal from the bacterial nodABC genes.

**Figure 3.** Difference between Response of Nod$^+$ and Mn-1008 Alfalfa after Inoculation with Exo$^-$ \textit{R. meliloti}.

(A) Nod$^+$ alfalfa displays large, nodule-like outgrowth at the site of the spot. Magnification, $\times 12$. Scale bar = 1.0 $\mu$m.
(B) MN-1008 alfalfa display no signs of nodulation. Dark spot of marker dye is visible on the roots at the point of inoculation (arrows) ($\times 12$).
(C) Inoculation of MN-1008 alfalfa with Exo$^-$ \textit{R. meliloti} (arrow) failed similarly to provoke any sign of cell activation (root harvested 12 days after inoculation). Magnification, $\times 270$.

**Relationship between Root Hair Events and Cortical Cell Division**

The ability of Exo$^-$ bacterial mutants to induce cell division and root hair deformation on wild-type plants, but not to
infect plant roots, has demonstrated clearly that morphogenesis can be uncoupled from infection. However, the partial root hair deformation induced by Exo^- mutants (Finan et al., 1985) may reflect events that are insufficient for full root hair curling and infection but that are nonetheless sufficient to cause nodule initiation (Finan et al., 1985). This is consistent with the proposal that cell divisions are caused by events occurring in the Rhizobium-root hair interaction.

Two general models potentially could explain a relationship between root hair deformation and cell division. One model predicts that a primary event occurs only in the developing root epidermal cell. The root hair reaction is an appealing candidate for the primary event. The specificity of root hair curling, the large number of bacterial genetic factors influencing it, and the fact that root hair curling occurs at the surface of the root and thus is a candidate for local bacterial action, all would support this order of causation. This primary event would result in the commitment of the root hair cell to deform (and in the right circumstances [other nod and exo genes] to curl) and would also cause cell division within the root cortex, perhaps by production of a secondary signal such as a cytokinin or some novel growth regulator. The second model predicts that root hair curling and cell division are cell-autonomous events, but each involves the same host factor—such as a receptor—missing in MN-1008, and each responds to a “signal” from the nodABC genes. Further research into the nature of the bacterial signal supplied by the nodABC genes and of the cellular response of the host should help resolve this issue.

METHODS

Bacterial Strains

*R. meliloti* 1021 is a streptomycin-resistant derivative of wild-type strain SU47 (Meade et al., 1982). Rm7031 and Rm7055 are deficient in the synthesis of acidic exoploysaccharide (exoA::Tn5, exoF::Tn5; Leigh et al., 1985). GM255 contains a large deletion of the megaplasmid including common nod genes (Truchet et al., 1985). Plasmid pRmSL26 (Long et al., 1982) is a broad host range plasmid containing *R. meliloti* common nod genes sequences. Bacterial inocula were grown in 3-ml cultures of M9 minimal medium (Meade et al., 1982) with 0.5 µg/ml streptomycin (and with 10 µg/ml tetracycline for 1021 (pRmSL26)) and 0.2% alfalfa seed exudate (Mulligan and Long, 1985).

Plant Material

The wild-type alfalfa used in this study was *M. sativa* L., cv AS-13R (Ferry Morse Co., Mountain View, CA). All seeds of the non-nodulating alfalfa line MnNC-1008 (NN) (Peterson and Barnes, 1981) were a generous gift of Dr. Donald Barnes. Plants were grown as described previously (Dudley et al., 1987). Briefly, seeds were surface sterilized in 70% ethanol for 30 min and then in 6% NaClO, for 20 min. Seeds were washed several times in sterile, distilled water and germinated on a vertical nodulation plate for several days. Individual seedlings were then transplanted, without their seed coats, to a fresh nodulation plate in such a manner that their shoot system extended from the top of the plate while their roots remained enclosed in the aseptic nodulation plate. Seedlings of either genotype whose overall growth was abnormal were discarded and not sectioned or scored.

Root Hair Curling

Seedlings were inoculated by applying 1 ml of inoculum to each plant root. Inocula were prepared by dilution (1:50) of a late-log phase culture of *R. meliloti* 1021 into 10 mM MgSO₄ (final bacterial concentration approximately 10⁷ ml⁻¹). The position of the meristem at the time of inoculation was marked on the bottom of the plate. Seedlings were allowed to grow for 3 days after inoculation; roots were then harvested by excising the root about 2 cm above the previously marked spot on the plate where the meristem had been at the time of inoculation. Each root, from the point of excision to the growing meristem, was moved carefully from the nodulation plate to sterile water. Seedlings were washed gently in sterile water once, and then mounted for microscopic observation. For each genotype of alfalfa, two groups of four or five plants were observed. Each root was scored along its entire length for the presence of any “shepherd’s crook” deformations of the root hairs, using bright-field, phase contrast, or Nomarski differential interference contrast optics on a Nikon Optiphot microscope. Scoring of root hair curling was performed “blind” to ensure objective evaluation. (The investigator did not know which plant genotype was being observed in each sample.) Photomicrographs were taken using a Nikon AFX camera in automatic exposure mode, and Kodak Ektachrome ISO 150 (tungsten) or Tri-X-Pan ISO 400 film (Eastman-Kodak, Rochester, NY).

Cell Division

Root segments were inoculated and prepared for scoring as described previously (Dudley et al., 1987). Briefly, a spot inoculation technique (Turgeon and Bauer, 1986) was used to apply approximately 1 to 10 nl of a 10-fold concentrated solution of bacteria (estimated final concentration at 10¹⁵ ml⁻¹) to a small, developmentally competent area of alfalfa root, and the spot of application was marked with waterproof ink (Dudley et al., 1987). High resolution scoring for individual mitotic events was performed by harvesting a 2- to 3-mm segment of root at the point of inoculation (50 or 70 hr after application of the bacteria for wild-type *R. meliloti* 1021 inocula; 12 days after inoculation for the Exo^- bacteria). Root segments were fixed overnight in 4% (w/v) paraformaldehyde in 20 mM PBS (20 mM sodium phosphate, pH 7.2, 150 mM sodium chloride). Segments were dehydrated in an ethanol series to 95% ethanol, and then infiltrated for at least 24 hr in catalyzed JB-4 resin (Polysciences, Inc., Warrington, PA). Segments were embedded in JB-4 resin according to the manufacturer’s instructions and stored at room temperature for sectioning. Serial sections of 6 µm were cut using a glass knife, and the sections were affixed to gelatin-coated slides. The sections...
were stained with a combination of the fluorescent dyes acridine orange and 4′, 6-diamidino-2-phenylindole hydrochloride (Sigma). Sections were viewed under epifluorescent illumination on a Nikon Optiphot microscope with a UV\textsuperscript{10} excitation filter as described previously (Dudley et al., 1987). Low-resolution scoring of nodule initiation was performed by allowing the spot-inoculated plants to grow for 12 days after inoculation. Approximately 1 cm of root surrounding the spot was harvested into distilled water, gently washed once, and viewed in this paper. We also thank Robert Fisher, Ann Hirsch, and Paul Green for general discussions and specific comments on this manuscript, and Alexandra Bloom for her patience and careful work during its preparation. This research was supported by National Science Foundation Presidential Young Investigator award (to S.R.L.) with matching funds supplied generously by the Agrigenetics Corporation and the DuPont Corporation. M.E.D. was supported in part by a training grant in Cell and Molecular Biology from the National Institutes of Health to Stanford University.

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