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Linda J. Utrup
University of Rhode Island

Andrew J. Cary
University of Rhode Island

Joanna Hanks Norris

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Five Nodulation Mutants of White Sweetclover (*Melilotus alba* Desr.) Exhibit Distinct Phenotypes Blocked at Root Hair Curling, Infection Thread Development, and Nodule Organogenesis

Linda J. Utrup[1], Andrew J. Cary[2], and Joanna Hanks Norris*

Botany Department, University of Rhode Island, Kingston, Rhode Island 02881–0812

In an effort to obtain a developmental sequence of mutations in the *Rhizobium*-legume interaction within a single legume species, we have characterized the early events of nodule development in 10 nodulation mutants of sweetclover, *Melilotus alba* Desr. cv U389, representing five genetic loci. Both seed and root exudates from all of the sweetclover mutants induced expression of the *nod* genes of *Rhizobium meliloti*. Mutants in three loci were blocked in the early stages of root hair curling. Of these, a mutant in the *sym*-3 locus exhibited root hair deformations in response to inoculation with *R. meliloti* but produced no nodules or emerging nodule primordia, suggesting a blockage in the signal transduction events leading to nodule organogenesis. In contrast, mutants in both the *sym*-1 and *sym*-5 loci formed ineffective nodules in response to inoculation but differed slightly in the type of root hair response observed. None of these three early mutants formed infection threads. Infection threads were observed in mutant *sym*-2 as well as in ineffective nodules. Mutant *sym*-4 also formed infection threads but lacked nodules. The phenotypes observed for mutants from these five loci suggest that a secondary receptor or signal produced by the plant is required for nodule development.

The development of nitrogen-fixing root nodules during the *Rhizobium*-legume symbiosis requires a complex sequence of interactions between the host plant and bacterium (reviewed in Brewin, 1991; Caetano-Anollés and Gresshoff, 1991a; Sanchez et al., 1991; Hirsch, 1992). Mutants in the *nod* genes of *Rhizobium* are unable to induce nodulation or root hair curling on appropriate host legumes (Long et al., 1982). Flavonoid compounds secreted by the host plant induce expression of the *nod* genes before infection (Peters et al., 1986; Kosslak et al., 1987; Maxwell et al., 1989). The product of the *nod* genes in *Rhizobium meliloti*, a sulfated and acylated glucosamine oligosaccharide signal called NodRm-IV(S), elicits root hair deformation (Lerouge et al., 1990) as well as root nodule organogenesis in alfalfa (Truchet et al., 1991). The response of the root hair cell to inoculation with *Rhizobium* includes characteristic deformations, some of which result in tightly curled root hairs. The bacteria enter the plant through a host-derived infection thread that grows through the curled root hair cell into the cortex of the root. Simultaneously, a meristematic region forms in advance of the penetrating infection thread, either within the inner cortex, in the case of legumes that form indeterminate nodules, or within the hypodermal layer in legumes that form determinate nodules. The initial cell divisions within the cortex occur at the same time as root hair cell responses (Turgeon and Bauer, 1982; Calvert et al., 1984; Dudley et al., 1987).

A developmental sequence of mutants in one species of legume blocked early in infection and nodule initiation would be useful in understanding the role and order of expression of the host genes required for nodule development. Nonnodulating and other symbiotic mutants have been reported for several legumes (reviewed by Vance et al., 1988; Caetano-Anollés and Gresshoff, 1991a; Sanchez et al., 1991), including pea (Weeden et al., 1990), soybean (Gresshoff et al., 1988), and alfalfa (Peterson and Barnes, 1981), indicating that a number of plant loci are involved in nodule development.

Analysis of the phenotypes of several of these plant mutants has shown that nodule development can be blocked at root hair curling, infection thread formation, or induction of cortical cell divisions. Both root hair curling and cortical cell division responses are lacking in the nonnodulating alfalfa mutant MN-1008 (Dudley and Long, 1989). Several nonnodulating soybean mutants representing two complementation groups that epistatically suppress the supernodulation phenotype (Carroll et al., 1986; Mathews et al., 1990) are unable to respond to *Bradyrhizobium* inoculation by root hair curling (Mathews et al., 1987). Inoculation of plants mutated in the *nod49* locus results in pseudoinfections with no infection threads, whereas inoculation of mutant *nod139* elicits neither pseudoinfections nor actual infections (Mathews et al., 1989a, 1989b, 1989c). All of the soybean mutants and the alfalfa mutant were normal in their ability to induce the *nod* genes of their respective bacterial microsymbiont, implying that the defect was specifically in the host response (Peters and Long, 1988; Mathews et al., 1989a; Sutherland et al., 1990).

Root hair curling is observed in additional mutants including the nonnodulating pea mutant K24, which exhibits root hair curling but lacks infection threads, whereas mutant K5 forms infection threads and demonstrates delayed nodulation

[1] Present address: Microbiology Research, SmithKline Beecham, King of Prussia, PA 19406–1539.

[2] Present address: Field of Botany, Section of Plant Biology, Cornell University, Ithaca, NY 14853.

* Corresponding author; fax 1–401–792–5974.

in soil upon inoculation with *Rhizobium* (Postma et al., 1988). In the strain-specific pea cv Afghanistan, noninfective rhizobia elicit deformed root hairs and induce abortive nodule meristems, but infection threads are not present (Le Gal and Hobbs, 1989). A nonnodulating mutant of chickpea, PM233B, is blocked in development before infection thread formation (Matthews and Davis, 1990). Normal infection threads are found in mutant E2 (*sym*-5) of pea, but cortical cell divisions rarely occur, resulting in few or no nodules (Guinel and LaRue, 1991). Although analysis of all of the above mutants has contributed greatly to our understanding of the infection process, a group of mutants from a single species with phenotypes representing the sequence of events occurring early in nodule development has not been characterized.

In an effort to identify such a group of mutants, we have characterized 10 nodulation mutants of sweetclover (*Melilotus alba* Desr. cv U389), a forage legume infected by *R. meliloti*. Originally isolated as 10 nonnodulating mutants after ethyl methanesulfonate and neutron radiation treatments of seed (Kneen and LaRue, 1988), these mutants have recently been shown by complementation analysis to represent five different genes (Miller et al., 1991a).

Like *Medicago truncatula* (Barker et al., 1990), sweetclover is a useful model system for analysis of the host genes involved in nodule development. Because of its diploid and self-fertilizing nature, sweetclover is more amenable to genetic analysis than the autotetraploid alfalfa (Miller et al., 1991b). Sweetclover is an annual of small size, suitable for test tube growth, setting ripe seed in less than 3 months. A number of nonsymbiotic mutants have been isolated (Ronnenkamp et al., 1975). Sweetclover is nodulated by *R. meliloti* and exhibits a phenotype similar to alfalfa when infected with *exo* and other mutants of *R. meliloti*. Sweetclover nodulins identified by two-dimensional gel analysis of in vitro translation products (L. Utrup and J. Norris, unpublished data) are similar in pattern and size to those previously identified in alfalfa (Dickstein et al., 1988; Norris et al., 1988).

In this study, we have analyzed seed and root exudate from the sweetclover nodulation mutants for induction of *nod* gene expression. One mutant from each of the five loci has been examined for root hair deformation, branching or curling, infection thread formation, and nodule formation. Each of the mutants exhibited a discrete phenotype characteristic of a blockage early in nodule development.

MATERIALS AND METHODS

Plant Material

Seeds of white sweetclover (*Melilotus alba* Desr. cv U389, PI 165554) and 10 symbiotic mutants (Kneen and LaRue, 1988) were generously provided by Dr. Tom LaRue (Boyce Thompson Institute for Plant Research, Ithaca, NY). Seeds were scarified between sandpaper, surface sterilized in ethanol followed by bleach for 30 min, rinsed, and transferred aseptically either to sterile 1-quart glass jars containing vermiculite and 160 mL of modified Jensen's nutrient media (Vincent, 1970) or to nodulation plates (Dudley et al., 1987). All plants were grown in a chamber with an approximate light intensity of 80,000 lux in a 16-h light (20°C)/8-h dark (18°C) daily cycle. Actual temperature around the plant roots did not exceed 24°C. Acetylene reduction to ethylene was measured on a Varian model 3700 gas chromatograph after plants were incubated overnight with 1 mL of acetylene generated from calcium carbide (Postgate, 1972).

For exudates, 30 surface-sterilized seeds were rinsed and allowed to imbibe in 1.5 mL of sterile distilled water and placed in the growth chamber. Seed exudate was collected after 24 h and replaced with an equal volume of sterile water for collection of root exudate 4 d after imbibition.

Bacterial Inocula and Root Hair Assays

Seedlings were inoculated 48 h after planting with late log phase cultures of *Rhizobium meliloti* SU47 grown in yeast mannitol media (Zurkowski and Lorkiewicz, 1978), to enhance attachment and infectivity (Kijne et al., 1988). More than 300 seeds of a single mutant representing each locus were grown in vermiculite (above) to test for nodulation.

For root hair assays, seedlings on nodulation plates (Dudley et al., 1987) were inoculated with 0.1 mL of a culture containing approximately 10^8 bacteria mL^{-1}. The entire length of the root (less than 1 inch) was inoculated. The excess inoculum drained away when the plates were returned to a vertical position in the growth chamber after 2 h. Root hairs along the entire length of cleared roots were observed (below) daily, beginning 12 h after inoculation and continuing until d 12 after germination. Additional time points up to 4 weeks were observed. From 10 to 20 mutant plants were examined in a typical experiment, which also included an equivalent number of inoculated wild-type plants as well as uninoculated controls for all mutants and wild-type plants. More than 100 plants of at least one mutant from each locus were assayed for root hair curling.

nod Induction

R. meliloti strain Rm1021/pRmM57, containing a *nodC-lacZ* fusion (Mulligan and Long, 1985), was obtained from Dr. Sharon Long (Stanford University) and was induced in early log phase for 3 h by addition of seed or root exudate. The β-galactosidase assay (Miller, 1972) was performed with permeabilized cells (Mulligan and Long, 1985). Assays were performed in duplicate.

Microscopic Examination

Whole roots were harvested and cleared in 85% lactic acid (Dudley et al., 1987) or in 5% sodium hypochlorite under vacuum (Truchet et al., 1989) and briefly stained with methylene blue. Nodules were fixed in formalin-acetic acid-alcohol solution (Berlyn and Miksche, 1976), dehydrated through a graded alcohol series to 100% *tert*-butyl alcohol, embedded in Paraplast, sectioned 10 μm thick, and stained with Safranin O and Fast Green according to the procedure of Jensen (1962). Whole preparations of entire cleared root systems and stained sections were observed for root hair curling and emerging nodule primordia using a Zeiss standard light microscope (Rainin Instrument Co., Woburn, MA) under bright-field

illumination and photographed with a Nikon Microflex UFX photomicrographic attachment using Plus-X pan film (Kodak).

RESULTS

Induction of *nod* Genes

Seed exudate collected from wild-type sweetclover line U389 induced the *nod* genes of strain Rm1021/pRmM57 at 60 to 70% of the level of induction found for an exudate sample collected from the same number of alfalfa seeds. Wild-type sweetclover seed exudate collected during the first 24 h of imbibition induced *nod* expression at twice the level found with root exudate collected at 3, 5, and 7 d after imbibition, as measured by β-galactosidase activity (data not shown). Both seed and root exudate from all 10 sweetclover nodulation mutants representing five loci induced bacterial *nod* gene expression above background level, as shown in Table I.

Morphological Analysis

One mutant from each of the five genetic loci was analyzed for the response to inoculation with *R. meliloti* SU47 by comparison with wild-type sweetclover U389. Root hair responses were observed daily, beginning 12 h after inoculation and continuing for up to 12 d after germination, with additional weekly time points up to 4 weeks. The number of infection threads observed along the entire root system of wild-type plants ranged from 3 to 10 threads per plant, most easily seen 3 d after inoculation, as shown in Figure 1A. The entire root system of more than 100 plants of a single mutant line representing each locus was examined.

Table I. *Induction of nod genes by seed and root exudates from sweetclover mutants*

Mutant	Seed Exudate	Root Exudate
	% of wild-type activity[a]	
sym-1		
BT35	84 ± 25	109 ± 13
BT58	56 ± 25	73 ± 27
BT62	63 ± 6.3	89 ± 44
BT64	59 ± 1.3	105 ± 44
sym-2	92 ± 17	81 ± 17
sym-3		
BT61	92 ± 32	110 ± 10
BT69	93 ± 1.3	94 ± 1.3
BT70	85 ± 0.56	106 ± 42
sym-4	65 ± 15[b]	98 ± 42
sym-5	116 ± 2.1	88 ± 0.71

[a] Measured as β-galactosidase activity on pRmM57 in *R. meliloti* 1021 in Miller units (Miller, 1972). Values represent the means ± SD of three experiments for seed exudate and two experiments for root exudate, in which samples were assayed in duplicate. [b] Mean ± SD of five experiments.

sym-1

Mutant BT62 from the *sym*-1 locus exhibited root hair deformations in response to inoculation with *R. meliloti* SU47 but did not produce infection threads (Fig. 1, B and C). The deformations observed in this mutant often consisted of a proximal swollen region followed distally by one to three deformed protrusions (Fig. 1B). Alternately (Fig. 1C), the root hairs were slightly swollen in comparison with uninoculated root hairs (Fig. 1D) and bent at angles of 90° or greater. Uninoculated control plants for mutants from all five loci as well as wild-type sweetclover exhibited only straight root hair cells as shown for BT62 only (Fig. 1D).

White, ineffective nodules were also observed on inoculated BT62 plants (Fig. 1E). Nitrogen-fixing nodules on wild-type sweetclover plants had a structure similar to alfalfa nodules and other indeterminate nodules (not shown; Thornton, 1930; Truchet et al., 1989). The ineffective *sym*-1 nodules possessed typical indeterminate nodule features including distal apical meristem, peripheral vascular bundles, nodule parenchyma, nodule endodermis, and outer nodule cortex (Fig. 1E). The parenchyma cells of these nodules were often filled with starch grains (not shown).

sym-2

As shown in Figure 2A, the single mutant isolated at the *sym*-2 locus displayed root hair curling and infection thread formation similar to that of wild-type sweetclover (Fig. 1A). This mutant also formed white, ineffective nodules (Fig. 2B) similar to wild-type nodules (Fig. 2C). Nitrogen-fixing nodules were also found on 5% of 300 inoculated *sym*-2 plants grown in jars of vermiculite (acetylene reduction data not shown).

sym-3

Mutant BT70 at the *sym*-3 locus responded to inoculation with root hair deformations of a more limited nature than those observed in other mutants and wild-type sweetclover. As shown in Figure 3, two typical responses were observed; in the first, termed "rippley," the root hairs had a serpentine pattern, successively bent at angles between 45 and 90° in one direction and then another (Fig. 3A). The second response was termed "arcing" because the root hairs formed long, slightly swollen arcs and were often observed in tightly clumped groups (Fig. 3B). A combination of rippling and arcing was often observed. Neither infection threads, nodules, nor round-shaped, emerging nodule primordia were observed on more than 100 entire root systems of this mutant using established clearing and staining methods for identification of nodule primordia (Dudley et al., 1987; Truchet et al., 1989; Caetano-Anollés and Gresshoff, 1991b). Emerging nodule primordia were easily observed in cleared wild-type roots 3 d after inoculation (not shown, see preceding refs.).

sym-4

Root hair curling and infection thread formation appeared normal in the single *sym*-4 mutant as shown in Figure 4. The total number of infection threads per mutant plant was

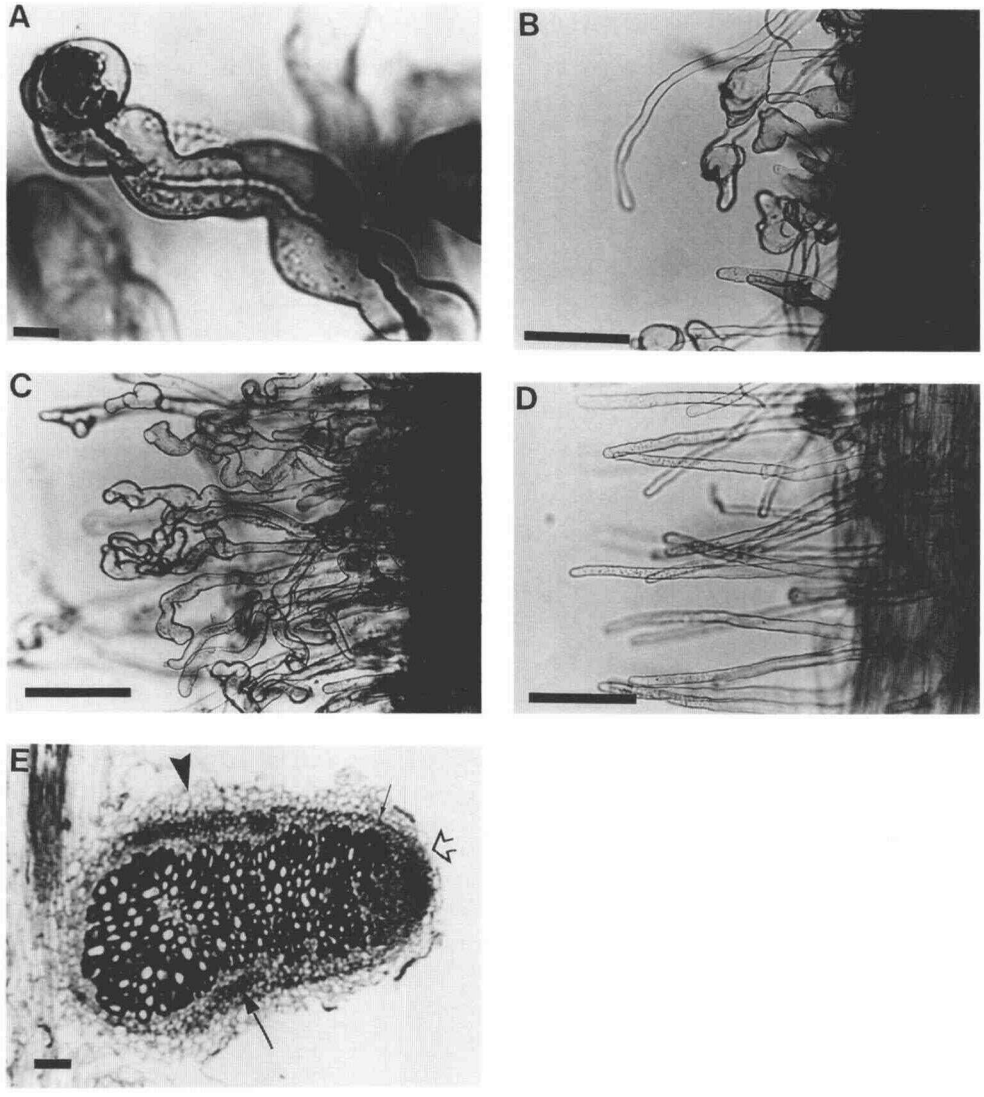

Figure 1. Phenotype of wild-type sweetclover and a *sym*-1 mutant. A, Light micrograph of curled root hair and infection thread on wild-type sweetclover U389. Bar = 10 μm. B, Root hair deformations on mutant BT62 in the *sym*-1 locus 3 d after inoculation showing swollen regions with protrusions. Bar = 100 μm. C, Inoculated mutant BT62 showing markedly bent root hair deformations. Bar = 100 μm. D, Uninoculated mutant BT62 root hairs at 3 d. Bar = 100 μm. E, Longitudinal section of a mutant BT62 nodule. Open arrow, Distal apical meristem; large arrow, peripheral vascular bundles; small arrow, nodule endodermis; arrowhead, nodule cortex. Bar = 100 μm.

comparable to that observed on wild-type plants in the same experiment. Nodule formation, however, was almost completely blocked. Ineffective nodules were found on only 3% of 300 plants grown in vermiculite in jars. Only 3 plants of 300 developed nitrogen-fixing nodules (acetylene reduction data not shown).

sym-5

The most common response of the single *sym*-5 mutant to inoculation was root hair branching as shown in Figure 5A. Often, the majority of the root hairs were branched in areas of the root that were just above the root tip at the time of inoculation. Occasionally, root hairs also formed loose hooks and rippling responses. No infection threads were observed on more than 100 mutant plants assayed for root hair curling. Numerous white, ineffective nodules were also induced on this mutant by inoculation with *R. meliloti* SU47 (Fig. 5B).

DISCUSSION

The phenotypic characteristics of the sweetclover nodulation mutants representing five genetic loci (Miller et al., 1991a) are summarized in Table II. All of the mutants induced expression of the *nod* genes of *R. meliloti*. Both seed and root exudates from all 10 mutants induced *nod* expression in strain Rm1021/pRmM57 at levels comparable to wild type. We found higher levels of *nod* induction for seed exudates than

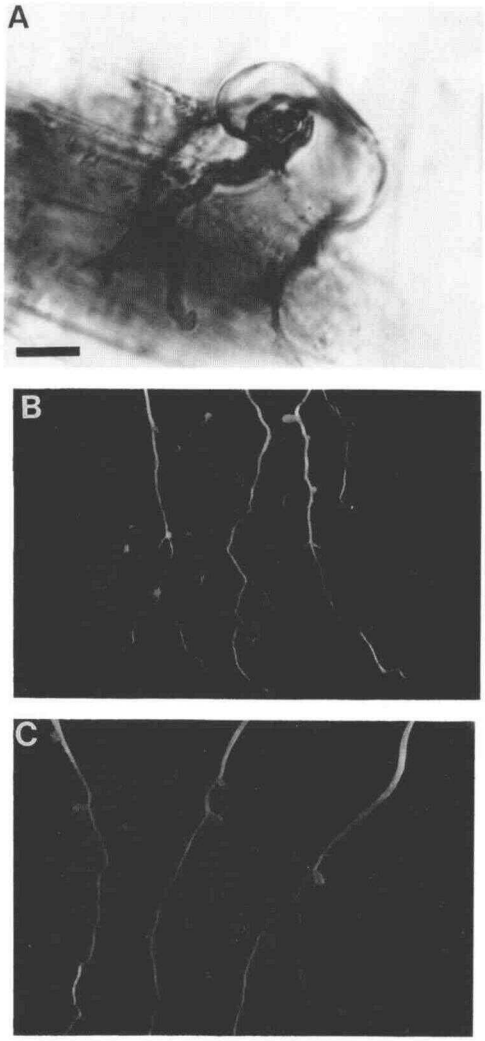

Figure 2. Phenotype of the *sym*-2 mutant. A, Light micrograph of curled root hair and infection thread on sym-2 mutant 3 d after inoculation. Bar = 10 μm. B, Photograph of entire root system of three *sym*-2 mutant plants containing numerous ineffective nodules 3 weeks after inoculation. C, Entire root system of three wild-type U389 sweetclover plants exhibiting nitrogen-fixing nodules.

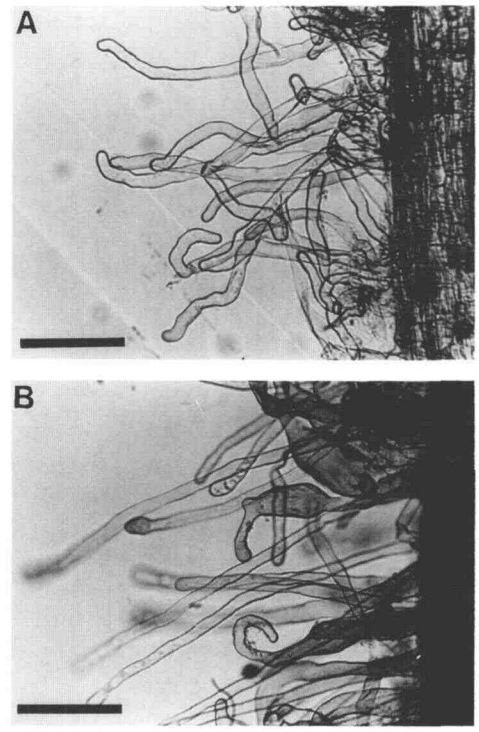

Figure 3. Phenotype of a *sym*-3 mutant. Light micrographs of mutant BT70 3 d after inoculation. Bar = 100 μm. A, Deformed root hairs exhibiting the rippley response. B, Swollen root hairs demonstrating the arcing response.

for root exudates, as was previously reported for alfalfa (Hartwig et al., 1990). Root exudates were analyzed in addition to seed exudates because the inducers identified in alfalfa root exudate differ from those found in the seed (Maxwell et al., 1989). Alfalfa root exudate collected 3 d after imbibition produces maximal *nod* induction, followed closely by exudate collected at 4 d (Maxwell et al., 1989), the age at which we collected the sweetclover root exudate. We also found somewhat lower levels of *nod* induction for sweetclover exudates than for those of alfalfa, as was previously reported (Gyorgypal et al., 1988).

Although some variation in the level of *nod* induction among mutants was observed (Table I), the variation present in the values for all of the mutants at a single locus, such as *sym*-1, for example, suggests that these differences fall within the biological range. Moreover, the mutant with the lowest *nod* induction value, *sym*-4, had normal root hair curling and infection threads, suggesting that the level of *nod* gene induction was sufficient for infection.

Models have been proposed in which root hair curling and cortical cell divisions are triggered by a single signal synthesized by *Rhizobium* or, in an alternative model, root hair-curling responses result in a signal transduction event (second signal) leading to cortical cell divisions (Long and Cooper, 1988; Dudley and Long, 1989). In the Nod factor-receptor model (Hirsch, 1992), the strength of the interaction between the factor produced by the *nod* genes of *Rhizobium* and its

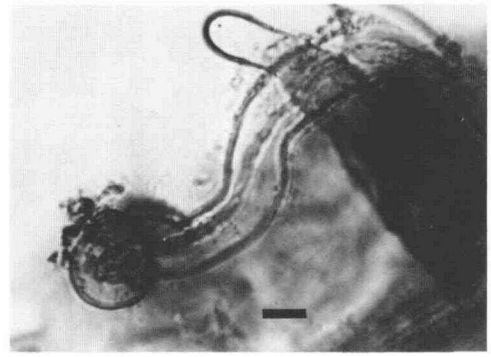

Figure 4. Phenotype of the *sym*-4 mutant. Light micrograph showing root hair curling and infection thread 3 d after inoculation. Bar = 10 μm.

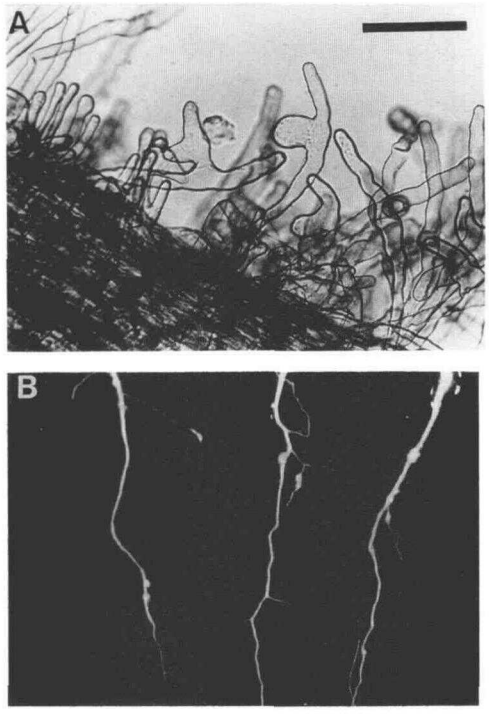

Figure 5. Phenotype of the *sym*-5 mutant. A, Light micrograph of branched and deformed root hairs 3 d after inoculation. Bar = 100 μm. B, Photograph of entire root system of three *sym*-5 mutant plants exhibiting numerous ineffective nodules 3 weeks after inoculation.

receptor on the root hair cell surface regulates infection thread formation and root hair curling and may generate a second signal that induces cortical cell divisions. This model predicts that induction of cortical cell divisions may depend on an alternative receptor or, more simply, the degree of receptor cross-linking. Plant hormones or flavonoids might also play a role in propagation of the second signal (Hirsch, 1992). The Nod factor-receptor model is partially based on recent studies that have shown that NodRm-IV(S), the major alfalfa-specific signal produced by *R. meliloti*, elicits root hair deformation (Lerouge et al., 1990), as well as root nodule organogenesis in alfalfa (Truchet et al., 1991). That nodules can be generated in the absence of *Rhizobium* is additionally demonstrated by uninfected nodules elicited by *Agrobacterium* transconjugants carrying *R. meliloti nod* genes (Hirsch et al., 1985) and nodules elicited by auxin transport inhibitors (Hirsch et al., 1989). Uninfected nodules are also elicited on alfalfa by complementation of *nod* mutants of *R. meliloti* with a strain carrying the *tzs* (*trans*-zeatin secretion) gene from *Agrobacterium* (Long and Cooper, 1988).

Comparison of the five phenotypes observed in the sweetclover mutants (Table II) appears to support models requiring a second signal for induction of nodule organogenesis. The mutant *sym*-3, in which no nodules or nodule primordia were observed, appears to be the earliest phenotype represented among the sweetclover mutants. Although more than 100 entire cleared root systems of the *sym*-3 mutant were examined for nodule primordia 3 d or more after inoculation by methods previously used to observe cortical cell divisions 2 d after inoculation in alfalfa (Caetano-Anollés and Gresshoff, 1991b), we cannot exclude the possibility that some early cortical cell division foci may have been missed by this method. Further analysis of inoculated *sym*-3 roots by spot-inoculation assays (Dudley et al., 1987) for cortical cell divisions at earlier times will determine whether an aborted cortical cell response occurs in this mutant.

Unlike the nonnodulating alfalfa mutant MN-1008 (Dudley and Long, 1989) and the soybean mutant *nod139* (Carroll et al., 1986; Mathews et al., 1989a, 1989b, 1989c), in which no root hair curling or cell divisions are observed, *sym*-3 exhibited root hair deformations in response to inoculation. Thus, we speculate that mutant *sym*-3 responded to NodRm-IV(S) with root hair deformations but lacked the secondary receptor or signal transduction event necessary to trigger nodule organogenesis.

Mutants in the *sym*-1 and *sym*-5 loci have phenotypes similar to that of wild-type alfalfa treated with purified NodRm-IV(S) (Truchet et al., 1991). Both mutants responded to inoculation with *R. meliloti* by root hair deformations in *sym*-1 and predominantly by root hair branching in *sym*-5. No infection threads were observed in either of these mutants. Both *sym*-1 and *sym*-5 also formed ineffective nodules in response to inoculation. In comparison, the soybean mutant *nod49* forms subepidermal cell divisions resulting in pseudoinfections and also forms nodules at higher levels of inoculum but lacks root hair curling (Carroll et al., 1986; Mathews et al., 1989a, 1989b, 1989c). The phenotypes of the *sym*-1 and *sym*-5 mutants, as well as that caused by *sym*-3, indicate that at least three host plant genes are involved in the early stages of root hair curling and bacterial infection in sweetclover. Whereas *sym*-3 appears to be important at an earlier stage (above), *sym*-1 and *sym*-5 cannot be developmentally ordered by our current data.

Mutant *sym*-2 had a more advanced phenotype in which infection threads as well as ineffective nodules were formed. Further analysis of these nodules will determine whether *sym*-2 is similar to any of the alfalfa mutants that form ineffective nodules (Peterson and Barnes, 1981; Vance and Johnson, 1983; Egli et al., 1991). A few (5%) *sym*-2 plants form nitrogen-fixing nodules. We speculate that the *sym*-2 mutant may be defective in some aspect of bacterial release or late nodule development. Further morphological analysis

Table II. *Summary of sweetclover mutant phenotypes*

+, Trait observed in the majority of 100 plants tested; −, trait not observed in any of 100 plants tested.

Trait	*sym*-1	*sym*-2	*sym*-3	*sym*-4	*sym*-5
Induction of bacterial *nod* genes	+	+	+	+	+
Branched root hairs	−	−	−	−	+
Root hair deformations	+	+	+	+	+
Infection threads	−	+	−	+	−
White non-nitrogen-fixing nodules	+	+	−	3%[a]	+
Nitrogen-fixing nodules	−	5%[a]	−	1%[a]	−

[a] Percentage of 300 plants tested.

of the ineffective nodules found on the *sym*-2 mutant, as well as those found on *sym*-1 and *sym*-5 mutants, for infection threads, bacteroids, and other structural features of nitrogen-fixing nodules will better distinguish the phenotypes of these mutants.

Mutant *sym*-4 is interesting in that abundant infection threads were observed, but nodules (ineffective) were rarely found. The phenotype of mutant *sym*-4, like that of nodules elicited by *R. meliloti exo* mutants (Finan et al., 1985), *Agrobacterium* transconjugants carrying *R. meliloti nod* sequences (Hirsch et al., 1985), auxin transport inhibitors (Hirsch et al., 1989), and sweetclover mutant *sym*-3, among others, demonstrates that root hair curling and infection thread formation can be uncoupled from nodule formation. Mutant *sym*-4, however, exhibits an apparently opposite phenotype in which infection threads are present, but nodules are not.

A pea mutant (*sym*-5) similar in phenotype to sweetclover *sym*-4 forms nodules in response to treatment with Ag^+, suggesting a role for ethylene in the inhibition of nodule formation (Guinel and LaRue, 1991). The ethylene inhibitor aminoethoxyvinylglycine has also been reported to stimulate nodule formation (Peters and Crist-Estes, 1989) and overcome nitrate inhibition of nodulation in alfalfa (Ligero et al., 1991), although findings from a recent study do not support a role for ethylene in nitrate inhibition (Lee and LaRue, 1992b). Application of exogenous ethylene results in inhibition of nodulation in both pea and sweetclover (Lee and LaRue, 1992c), although the pleiotropic phenotype of the pea *sym*-17 mutant suggests that other factors may be involved as well (Lee and LaRue, 1992a). It will be of interest to determine whether ethylene is also implicated in the sweetclover *sym*-4 phenotype.

In summary, we have reported here the phenotypes of sweetclover mutants representing five loci affecting early nodule development. Three of the mutants responded to inoculation with *R. meliloti* with root hair deformations lacking infection threads; two of these mutants formed ineffective nodules (Table II). Of two additional mutants that formed infection threads, only one formed numerous ineffective nodules. The range of phenotypes present in these mutants demonstrates that sweetclover is a valuable model system for analysis of the role of the plant in nodule development.

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