Theor Appl Genet (1992) 84:624–632

THEORETICAL AND APPLIED GENETICS

Plant genetic suppression of the non-nodulation phenotype of *Rhizobium meliloti* host-range *nodH* mutants: gene-for-gene interaction in the alfalfa-*Rhizobium* symbiosis?

G. Caetano-Anollés and P. M. Gresshoff

Plant Molecular Genetics, Institute of Agriculture and Center for Legume Research, The University of Tennessee, Knoxville, P.O. Box 1071, TN 37901-1071, USA

Received October 28, 1991; Accepted December 19, 1991
Communicated by I. Potrykus

Summary. *Rhizobium* nodulation genes can produce active extracellular signals for legume nodulation. The *R. meliloti* host-range *nodH* gene has been postulated to mediate the transfer of a sulfate to a modified lipo-oligosaccharide, which in its sulfated form is a specific nodulation factor for alfalfa (*Medicago sativa* L.). We found that alfalfa was capable of effective nodulation with signal-defective and non-nodulating *nodH* mutants (Nnr) defining a novel gene-for-gene interaction that conditions nodulation. Bacteria-free nodules that formed spontaneously at about a 3–5% rate in unselected seed populations of alfalfa cv 'Vernal' in the total absence of *Rhizobium* (Nar) exhibited all the histological, regulatory and ontogenetic characteristics of alfalfa nodules. Inoculation of such populations with *nodH* mutants, but not with *nodA* or *nodC* mutants, produced a four- to five-fold increase in the percentage of nodulated plants. Some 10–25% of these nodulated plants formed normal pink nitrogen-fixing nodules instead of white empty nodules. About 70% of the S_1 progeny of such Nnr^+ plants retained the parental phenotype; these plants were also able to form nodules in the absence of *Rhizobium*. If selected Nar^+ plants were self-pollinated almost the entire progeny exhibited the parental Nar^+ phenotype. Segregation analysis of S_1 and S_2 progeny from selected Nar^+ plants suggests that the Nar character is monogenic dominant and that the nodulation phenotype is controlled by a gene dose effect. The inoculation of different S_1 Nar^+ progeny with *nodH* mutant bacteria gave only empty non-fixing nodules. Our results indicate that certain alfalfa genotypes can be selected for suppression of the non-nodulation phenotype of *nodH* mutants. The fact that the Nnr plant phenotype behaved as a dominant genetic trait and that it directly correlated with the ability of the selected plants to form nodules in the absence of *Rhizobium* suggests that the interaction of plant and bacterial alleles occurs early during signal transduction through the alteration of a signal reception component of the plant so that it responds to putative signal precursors.

Key words: *Medicago sativa* – Nodulation (spontaneous) – Nitrogen fixation – Symbiosis

Abbreviations: *Nar*, Nodulation in the absence of *Rhizobium*; *Nar*, nodulation with non-nodulating Rhizobium
Correspondence to: G. Caetano-Anollés

Introduction

Soil bacteria of the genus *Rhizobium* or *Bradyrhizobium* infect legume roots and induce the formation of nitrogen-fixing nodules (Caetano-Anollés and Gresshoff 1991 a). The symbiosis has been viewed as a developmental model system in which genes in both partners specify the exchange and interpretation of chemical signals. This complex process is carefully regulated at different stages during infection, as judged by the number of bacterial and plant mutants that have been isolated which differentially arrest nodule development. Such mutants can be used to dissect nodulation into its different regulatory components and to provide insight into the role of bacterial and plant signals that are crucial for the establishment of a functional nodule.

Rhizobium meliloti induces cellular redifferentiation in the root cortex of alfalfa roots leading to cell division and nodule organogenesis (Truchet et al. 1980, 1984; Dudley et al. 1987; Caetano-Anollés and Gresshoff 1991 b). Anticlinal and periclinal cell divisions initiate a nodule primordium that forms a persistent nodule meristem in its distal end and gives rise to a central tissue with

both infected and uninfected cells. Nodules without infection are elicited by a number of agents. Ineffective bacteria-free nodules are produced in response to *Agrobacterium tumefaciens* transconjugants carrying *R. meliloti* nodulation (*nod*) genes (Truchet et al. 1984; Wong et al. 1983; Hirsch et al. 1984, 1985) and *R. meliloti* mutants defective in exopolysaccharide synthesis (Finan et al. 1985; Leigh et al. 1987). *Rhizobium* and *Bradyrhizobium* can also secrete cytokinins (Phillips and Torrey 1971; Sturtevant and Taller 1989) and extracellular nodulation signals (Lerouge et al. 1990; Philip-Hollingsworth et al. 1991; Truchet et al. 1991; Spaink et al. 1991) that are able to induce cortical cell division and in some cases empty nodules. Root-derived structures that fulfill most of the histological and molecular criteria defining an indeterminate nodule also appear in alfalfa upon treatment with auxin-transport inhibitors (Hirsch et al. 1989).

The recent discovery that *Rhizobium* is not obligate for nodule initiation (Truchet et al. 1989) has given a new perspective to the contribution of the plant to the symbiotic process (reviewed in Caetano-Anollés et al. 1991 b). Nodulation in the absence of *Rhizobium* (Nar) can occur spontaneously in alfalfa (Truchet et al. 1989; Caetano-Anollés et al. 1991a). Cytological studies have shown that spontaneous nodules have all the normal histological and regulatory features of normal indeterminate alfalfa nodules (Truchet et al. 1989; Caetano-Anollés et al. 1991 a; Joshi et al. 1991) and share the same ontogeny (Joshi et al. 1991).

Although little is known about the mechanisms that determine and control nodule initiation, there is evidence that *nod* gene-related extracellular signaling is required to elicit root-hair deformation and curling in alfalfa (Faucher et al. 1988, 1989; Banfalvi and Kondososi 1989). One such signal, a sulfated and acylated glucosamine oligosaccharide (Lerouge et al. 1990) can also induce cortical cell division and nodule organogenesis (Truchet et al. 1991). This alfalfa-specific nodulation signal, NodRm-IV(S), requires the *nodABC* genes for its synthesis and the host-range *nodH* and *nodQ* genes for its activity. The *nodP* and *nodQ* gene products in conjunction with *nodH* activate inorganic sulfate (Schwedock and Long 1990) and could introduce it into a precursor of the nodulation signal, turning it from vetch-specific into alfalfa-specific. Thus, genes like *nodH* and *nodQ* can change or extend the host-range by altering the nature of an extracellular symbiotic effector.

It can be postulated that a certain genetic alteration in one partner should be compensated for (or suppressed) by an appropriate genetic alteration in the other partner, in a lock-and-key relationship akin to the gene-for-gene relationships specifying pathogenic plant-microbe interactions. In the present article we describe the isolation of dominant alfalfa genotypes having the ability to form normal nitrogen-fixing nodules with the same *R. meliloti nodH* mutants used to elucidate the structure-function relationship of NodRm-IV(S). These genotypes possessed the ability to nodulate spontaneously in the absence of *Rhizobium*. We propose that the selected alfalfa genotypes are altered in a gene product intimately related with the plant transduction mechanisms triggered by the NodRm family of bacterial nodulation signals.

Materials and methods

Microorganisms

Rhizobium meliloti RCR2011, the non-nodulating *nodH*::Tn5 mutant derivatives GMI5375 and GMI5429, the *nodA*::Tn5 mutant GMI5382 and the *nodC*::Tn5 mutant GMI5387 were obtained from J. Dénarié, CNRS-INRA, Castanet-Tolosan, France. Stock cultures were maintained and grown as described previously (Caetano-Anollés and Bauer 1988).

Plant material and inoculation procedures

Alfalfa (*Medicago sativa* L.) cv 'Vernal' seeds were obtained from R. Van Keuren, Agronomy Department, The Ohio State University, Wooster, Ohio. Seeds were surface sterilized with ethanol and mercuric chloride and germinated in water agar plates (Caetano-Anollés et al. 1990). Seeds from progeny were scarified before surface sterilization. Seedlings were transferred in groups of five to ethylene oxide-sterilized plastic growth pouches (Vaughan's Seed Co, Downers Grove, Ill.) containing 10 ml of Jensen nitrogen-free medium (Jensen 1942) 2 days after seed imbibition. Plants were grown at 25 °C day/22 °C night temperatures, 60–70% relative humidity, a 16-h photoperiod and a photosynthetically active radiation of 500 $\mu mol \cdot s^{-1} \cdot m^{-2}$. Plants were watered when required and low levels of nitrogen were provided by adding 2 m*M* KNO_3 at the beginning of the 3rd, 4th and 5th weeks post-germination. The final levels of fixed nitrogen achieved in the pouches did not affect spontaneous nodulation. When demanded by the protocol, plants were inoculated 5 days after seed imbibition with 100 µl of a bacterial suspension containing 10^6 viable cells diluted from a late-exponential phase bacterial culture (Caetano-Anollés and Bauer 1988). The location of the root tip (RT) was marked on the surface of the plastic pouch at that time and 24 h later. Uninoculated and inoculated plants were examined at regular intervals for the appearance of spontaneous or normal nodules.

Plants were also grown in a modified Leonard jar assembly. Two plastic Magenta jars (Magenta Corp, Chicago, Ill.) were stacked together. The upper jar was filled with 200 cm^3 horticulture grade vermiculite and was connected through a wick to the lower reservoir containing Herridge solution (in $mg \cdot l^{-1}$: KH_2PO_4, 17; K_2HPO_4, 21.8; KCl, 18.7; $MgSO_4 \cdot 7H_2O$, 123.3; $CaCl_2$, 27.7; ferric monosodium EDTA, 8.7; H_3BO_3, 0.715; $MnCl_2 \cdot 4H_2O$, 0.453; $ZnCl_2$, 0.028; $CuCl_2 \cdot 2H_2O$, 0.013; $NaMoO_4 \cdot 2H_2O$, 0.006; Herridge 1977). Once assembled the jars were sterilized by autoclaving. Five 2-day old seedlings were planted in each jar and were inoculated 3 days later with 10 ml bacterial suspension containing 10^8 cells.

Plant screening

Individual plants grown in uninoculated plastic growth pouches were selected for their ability to nodulate in the absence of *Rhizobium*. Similarly, plants inoculated with non-nodulating mutants that exhibited bacteria-free nodules or nitrogen-fixing red nodules were also selected. Plants were transferred to 25-cm diameter pots filled with 3S soil mixture (Conrad Fafard,

Springfield, Mass.); grown in the greenhouse (temperature range between 25 °C and 30 °C and supplemented lighting for a 16-h photoperiod) and watered weekly with Herridge solution. Nitrogen was provided with the controlled release fertilizer Osmocote 14-14-14 (Sierra Chemical Co, Milpitas, Calif.).

Crosses and progeny screening

Selected plants exhibiting the same phenotype were either crossed or selfed. Cross-pollinations were made following emasculations with fine forceps. S_1 seeds were produced by tripping the flowers with an applicator or in some cases by gently rolling racemes between the fingers. F_1 and S_1 progeny were analyzed by growing the plants in plastic growth pouches as described. Individuals expressing the phenotype were selected, transferred again to pots and self-pollinated, and S_2 progeny were analyzed in pouches.

Isolation of nodule bacteria, acetylene-reduction assays and microscopy

Nodules were excised, removed from the roots, and crushed individually (Caetano-Anollés et al. 1991 a). The suspensions were used to inoculate groups of five 5-day-old plants in growth pouches. The suspensions were also plated in YEMG medium (Caetano-Anollés and Bauer 1988), isolated colonies resuspended and the bacterial suspensions used to inoculate alfalfa plants. Nodules from 4-week or 6-week-old seedlings were excised and tested for acetylene reduction (Caetano-Anollés et al. 1991 a) or were prepared for microscopy. Nodules were fixed with Karnovsky fixative, post-fixed with 2% osmium tetroxide, dehydrated and embedded in Spurr's plastic as previously described (Caetano-Anollés et al. 1991 a). Thick sections (1–3 μm) were stained with toluidine blue for light microscopy, and thin sections (50–60 nm) were post-stained with uranyl acetate for electron microscopy (Caetano-Anollés et al. 1991 a).

Results

Nodulation in the absence of Rhizobium

A small subpopulation of alfalfa cv 'Vernal' plants (between 3 and 5%) grown aseptically in plastic growth pouches formed white nodule-like structures during the first 30–45 days of growth in the absence of *R. meliloti* and combined nitrogen (Caetano-Anollés et al. 1991 a; Table 1). The histology, ultrastructure and ontogeny of these spontaneous nodules has been examined in detail (Joshi et al. 1991). Spontaneous nodules first appeared on the primary root usually between 10 and 15 days after seed inbibition. After the first month of growth new plants continue to be nodulated but with nodulation confined to lateral roots. Usually, 20% of nodulated plants had nodules on the primary roots 45 days after seed imbibition.

Based on the recent observation that the requirement of the plant for a functional *R. meliloti nodH* varied with the conditions of plant growth (Ogawa et al. 1991), we examined the expression of the Nar phenotype in vermiculite-grown plants. When 306 plants were grown in modified Leonard jar assemblies, 21.5% were spontaneously nodulated with 39% of them harboring nodules on the primary roots 45 days after seed imbibition. Nodulated plants had 6.6±1.0 (SE) nodules per plant, a number similar to that found in plastic growth pouches (Table 1). Thus, the expression of Nar is also influenced by the plant growth substrate.

Table 1. Formation of bacteria-free structures in the presence and absence of *R. meliloti nod* mutants. Groups of 200–400 alfalfa plants grown in plastic growth pouches were inoculated 5 days after seed imbibition with a sham inoculum (as defined in Caetano-Anollés and Bauer 1988) or with 10^6 bacteria · plant^{-1} of the nodulation mutants GMI5387 and/or GMI5375. Nodules were scored 30 and 45 days after seed imbibition

Inoculum	Number of nodules per nodulated plant		% Nodulated plants	
	30 days	45 days	30 days	45 days
Sham	3.5±0.4 *a*	4.6±0.06 *a*	3.3±0.6 *a*	5.7±0.2 *a*
nodC	2.8±0.7 *a*	3.7±0.7 *a*	2.7±0.7 *a*	3.8±0.9 *a*
nodH	4.1±0.2 *a*	4.8±0.4 *a*	15.2±2.7 *b*	17.2±4.2 *b*
nodC and *nodH*	4.3±0.7 *a*	5.3±1.0 *a*	14.3±2.2 *b*	16.3±2.0 *b*

Values are means±SE for three independent experiments. Means in a column followed by different letters were significantly different ($P<0.05$) after ANOVA and Fisher PLSD

Bacteria-free white nodules formed in the presence of non-nodulating Rhizobium mutants

White, usually multilobed nodule-like structures also appeared upon inoculation with *nodA, nodC* and *nodH* transposon Tn5 insertion mutants of *R. meliloti* (Fig. 1). Detailed microscopical studies have defined the ontogeny, anatomy and histology of these root structures on the basis of defined criteria (Dudley et al. 1987; Joshi et al. 1991). These nodules had no inter- or intracellular bacteria, but most of the histological characteristics of normal indeterminate alfalfa nodules [see Joshi et al. (1991) for comparable histological sections] and the nodular meristems originated from foci of dividing cells in the inner root cortex. They can be considered morphologically and histologically indistinguishable from spontaneous nodules formed in the absence of *Rhizobium* (Nar). The plating of suspensions from surface-sterilized and crushed nodule structures on YEMG medium showed that at least 99% of them did not produce colony-forming bacteria. Several of these nodule structures appeared as genuine nodules when examined by light and electron microscopy.

Inoculation of a *R. meliloti nodC* mutant allowed the formation of empty nodules in numbers similar to those of spontaneous nodules formed in the absence of *Rhizobium* in only a small fraction of the plants examined (Table 1). A similar result was found with a *nodA*::Tn5 mutant (data not shown). However, alfalfa plants inoculated with *nodH*::Tn5 mutants formed empty nodules at

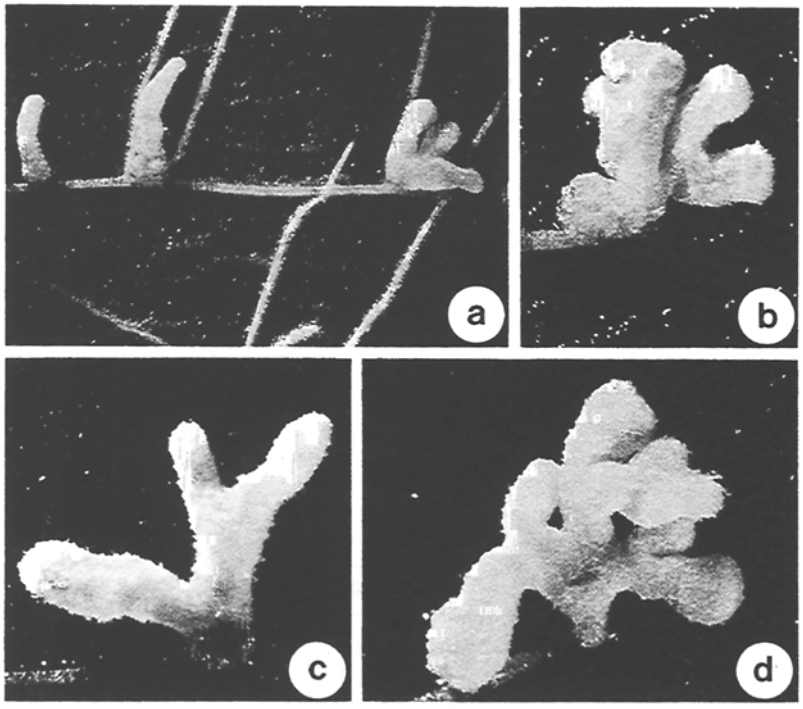

Fig. 1a–d. Morphology of bacteria-free nodules formed in the presence of *R. meliloti* GMI5375 on lateral roots of 30-day-old alfalfa plants. **a** Single and multilobed nodules on a lateral root; **b** typical multilobed structure; **c** trilobed alfalfa nodule; **d** nodule exhibiting 8 lobes probably resulting from the dicotomous activity of individual meristems of a trilobed nodule

a four- to five-fold higher frequency (Table 1). Generally one out of five plants from an unselected alfalfa population formed nodules late in root development upon inoculation with the mutant, almost exclusively on secondary roots. When plants were inoculated with the non-nodulating mutants, empty nodules appeared 5 days earlier than when plants were kept uninoculated. Co-inoculation of *nodC* and *nodH* mutants induced empty nodules to appear at a similar number and frequency as in *nodH*-inoculated plants (Table 1). However, 20% of such nodulated plants consistently formed nodules on the primary root, showing a synergistic action of *nodH* and *nodC* mutants in speeding spontaneous nodule development.

Inheritance of the Nar phenotype

Selected Nar^+ individuals were selfed or crossed. S_1 and F_1 progeny plants were germinated and analyzed for the Nar phenotype in plastic growth pouches. An average of 80% of plants from eight different S_1 seed populations formed nodules in the absence of *Rhizobium*, and 82% of the F_1 progeny was spontaneously nodulated. Root structures in S_1 and F_1 plants were of cortical origin and were anatomically and histologically indistinguishable from spontaneous nodules formed by the progenitors. The percentage of plants nodulated from individual S_1 populations ranged from 46% to 100%. The kinetics of nodule formation in representative S_1 populations showed that nodule emergence varied between populations (Fig. 2A), but that generally spontaneous nodulation of S_1 plants occurred earlier and to a greater extent than that observed in unselected alfalfa. Figure 2B shows that nodules emerged earlier in pooled S_1 and F_1 plants than in progenitor plants. However, both the number and the rate of nodule emergence were similar (data not shown). While the number of nodules per nodulated plant formed by the S_1 Nar population in plastic growth pouches was similar to the number obtained in the unselected alfalfa population grown in pouches or vermiculite, growth in vermiculite produced a four-fold increase in the number of spontaneous nodules (Table 2).

The dominant nature of the Nar trait was demonstrated by analyzing S_1 progeny from different plants that developed spontaneous nodules on the primary root in an unselected alfalfa population. In Table 3 we show that progeny from a single plant segregated 79 Nar^+:4 Nar^-, which approximates a 35:1 ratio. Progeny from several other plants showed similar ratios. Three phenotypic classes were observed, where plants developed spontaneous nodules on the primary root, on lateral roots, or were non-nodulated; these phenotypic classes distributed at a ratio of 27:8:1. Progeny from self-fertilized plants from this S_1 population again formed spontaneous nodules, indicating the stability of the Nar phenotype. Analysis of S_2 progeny showed that if progenitor plants nodulated on the primary root, the segregation of phenotypic classes followed the expected 27:8:1; but if progenitor plants nodulated only on lateral roots segregation followed a 1:2:1 ratio. In these experiments, despite the low number of individuals analyzed, the chi-square analysis indicated that the observed values fitted the expected ratios at a relatively high level of signifi-

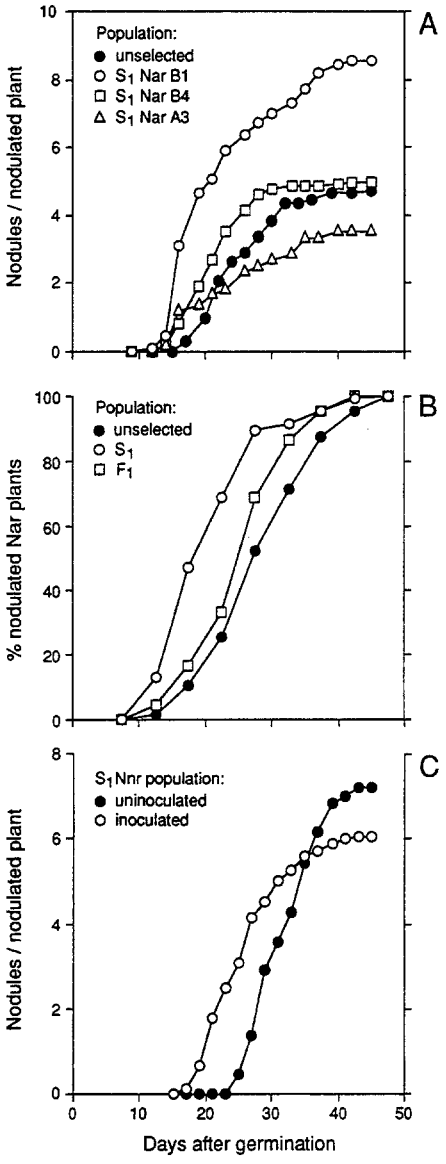

Fig. 2A–C. Time course of nodulation of Nar and Nnr alfalfa populations. **A** Spontaneous nodulation in unselected alfalfa plants (●) and the S_1 Nar populations B1 (○), B4 (□) and A3 (△). Groups of 11–16 plants were scored at regular intervals for the appearance of nodules. Nodulation is expressed as the number of nodules per nodulated plant found at the end of the experiment, 45 days after seed imbibition. In this experiment 5.5% of unselected alfalfa, 85% of B1 plants, 84% of B4 plants and 46% of A3 plants were nodulated. **B** Spontaneous nodulation in pooled S_1 Nar (○), F_2 Nar (□) and unselected Nar (●) alfalfa populations. Nar plants were examined at regular intervals for the appearance of nodules, and nodulation was expressed as the percentage of nodulated Nar plants at a particular time after seed imbibition. The number of plants examined for S_1, F_1 and unselected alfalfa was 123, 22 and 74, respectively. **C** Nodulation in the presence of *R. meliloti* GMI5375. Plants from the S_1 Nnr A7 population were inoculated in groups of 23–24 as described in the Materials and methods (○) or received a sham inoculum (●)

cance ($P<0.291-0.847$). The results demonstrate that the Nar character is monogenic dominant and that the nodulation phenotype is stable and controlled by a gene dose effect. The mean number of nodules per nodulated plant for the S_2NarC1, C3 and C2 progeny studied in these experiments was 5.5 ± 1.9, 5.6 ± 1.7 and 4.7 ± 0.7, respectively. There were no significant differences in nodulation between these three populations ($p>0.828$).

Plants with empty nodules formed and stimulated by the presence of the *nodH* mutants were also self-pollinated. All of the S_1 plants examined ($n=15$) developed empty nodules in the presence of the bacterial mutant, reflecting the parental phenotype. This indicates that the formation of empty nodules with non-nodulating bacterial mutants also segregates in progeny plants.

Nodulation with non-nodulating R. meliloti nodH mutants

About 10–25% of the nodulated plants that were inoculated with *nodH* mutants had pink, usually single-lobed nodules that generally appeared on lateral roots between 2 and 4 weeks after inoculation. These nodules fixed atmospheric nitrogen (measured by acetylene reduction) and supported the growth of their respective plants. The nodules were histologically indistinguishable from normal indeterminate alfalfa nodules (not shown). They usually had a single nodular meristem, cortex and endodermis and a central zone with vascular strands. There were infection threads and bacteroids enclosed singly in peribacteroid membranes. However, both infected and uninfected cells in the central zone had abundant starch grains (Joshi and Caetano-Anollés, in preparation). The ability to form nodules with non-nodulating *Rhizobium nodH* mutants (Nnr) was completely inhibited by 15 m*M* potassium nitrate. No nodules were formed when the contents of nonsterilized nodules were added directly to 5-day-old alfalfa plants or after inoculation with isolated nodule bacteria grown in YEMG medium (50 plants were examined in each experiment). Thus, pink nodules of Nnr^+ plants were not the result of bacterial reversion or contamination as the postulates of Koch were fulfilled.

Inheritance of the Nnr phenotype

Selected Nnr^+ plants that formed pink, nitrogen-fixing nodules upon inoculation with the *nodH* mutant GMI5375 produced S_1 progeny that were about 70% Nnr^+ when inoculated with *nodH* mutant bacteria (Table 2). Surprisingly, the same S_1 population yielded plants at about 70% frequency that were Nar^+ when left uninoculated. The kinetics of nodule formation of Nnr^+ plants in the presence or absence of *nodH* bacteria is shown in Fig. 2C. Nodules emerged earlier when the plants were inoculated, but nodule emergence rates were comparable.

Table 2. Nodulation of S_1 progeny from Nar^+ and Nnr^+ plants in the presence or absence of *R. meliloti nodH* bacteria

Plants[a]	Inoculation with strain GMI5375	% Plants nodulated	Number of nodules per nodulated plant[c]	Plants tested	Nodules:[d] morphology and histology	
Plastic growth pouches[b]						
S_1 Nar^+	–	93	6.6±0.9 *a*	27	White	Bacteria-free
S_1 Nar^+	+	92	6.0±0.7 *a*	40	White	Bacteria-free
S_1 Nnr^+	–	76	6.1±1.3 *a*	28	White	Bacteria-free
S_1 Nnr^+	+	67	6.1±0.8 *a*	37	Pink	Normal
Vermiculite jars						
S_1 Nar^+	–	94	23.6±3.9 *b*	17	White	–
S_1 Nar^+	+	100	12.6±2.2 *b*	16	White	–
S_1 Nnr^+	–	70	3.1±0.7 *a*	10	White	–
S_1 Nnr^+	+	73	3.2±0.8 *a*	11	Pink	–

[a] Progeny from self-fertilized plants were grown in plastic growth pouches or in vermiculite Magenta jars as described in Materials and methods and scored for nodulation 45 days after seed imbibition

[b] Results are from two independent experiments. A two-way analysis of variance indicated no significant differences between experiments ($P>0.485$). Groups of 12–17 control plants from S_1 Nnr^- and S_1 Nar^- progeny were not nodulated in the absence or presence of bacteria

[c] Values are means with standard errors. Means followed by the same letter are not significantly different ($P=0.05$)

[d] Nodules exhibited peripheral differentiation of a vascular system and an endodermis, and one or more apical nodule meristems. White nodules were usually multilobed structures. Pink nodules appeared as normal indeterminate nodules developed when plants were inoculated with wild-type bacteria. Bacteria-free nodules were defined as unable to (1) produce colony-forming units when suspensions from crushed surface-sterilized nodules were plated on YEMG medium and (2) show any bacteria by light and electron microscopy

Table 3. Segregation of Nar and Nnr traits in S_1 and S_2 populations. Progeny from self-fertilized S_0 and S_1 progenitor plants were grown in plastic growth pouches as described in Materials and methods and scored for nodulation 45 days after seed imbibition. Phenotypes were as follows: Nar^+, spontaneous nodulation on primary roots; Nar^{+d}, delayed spontaneous nodulation confined to lateral roots; Nar^-, no spontaneous nodules; Nnr^+, nitrogen-fixing nodules formed with *R. meliloti nodH* mutants. Assuming that the mutant allele *B* controls the response, the expected segregation ratios were 35 nodulated: 1 non-nodulated (27 Nar^+: 8 Nar^{+d}: 1 Nar^-) for the duplex condition or 3 nodulated: 1 non-nodulated (1 Nar^+: 2 Nar^{+d}: 1 Nar^-) for the simplex condition. The triplex and quadraplex conditions are expected to give all Nar^+ plants. The observed values were tested for goodness-of-fit to the expected by χ^2-analyses using the Yates correction term

Nodulation of selected progenitor	Population[a]	Number of plants			Expected ratio	Calculated[d] χ^2	*P*	Genotype of progenitor
		Nodulated on primary root	Nodulated on lateral roots	Non-nodulated				
Nar^+	S_1 Nar B4	57	22	4	35:1 27:8:1	0.644 1.496	0.422 0.473	*BBbb*
Nar^+	S_2 Nar C2[a]	21	4	2	35:1 27:8:1	0.833 2.266	0.361 0.322	*BBbb*
Nar^{+d}	S_2 Nar C1[a]	3	3	3	3:1 1:2:1	0.037 2.333	0.847 0.311	*Bbbb*
Nar^{+d}	S_2 Nar C3[a]	7	5	6	3:1 1:2:1	0.296 2.472	0.586 0.291	*Bbbb*
Nnr^+	S_1 Nnr A7	–	26	9	3:1	0.010	0.922	*Bbbb*
Nnr^+	S_1 Nnr A7[b]	–	26[c]	10	3:1	0.037	0.847	*Bbbb*

[a] S_2 progeny was from selected S_1 Nar B4 plants

[b] Plants were inoculated with *R. meliloti* GMI5375

[c] All plants harbored Nnr nodules

[d] Calculated χ^2 values indicated the segregation ratios were not significantly different from the expected ratios at 5% level of significance; tabulated χ^2 for 1 *df* is 3.84 and 2 *df* is 5.99

The inoculation of S_1 progeny from Nar^+ plants with *nodH* mutant bacteria did not result in the formation of pink Nnr nodules. To confirm this result, ten different S_1 populations from selected Nar^+ individuals were inoculated with GMI5375 bacteria. None of the screened plants from the different S_1 populations developed the Nnr trait.

Table 3 shows that the S_1 progeny from Nnr plants followed a 3:1 ratio of spontaneously nodulated to non-nodulated plants when uninoculated, and also a 3:1 ratio of Nnr^+ plants to non-nodulating plants when inoculated with *nodH* mutant bacteria. In both cases all nodules developed on lateral roots so only two phenotypic classes were visualized. This apparently results from the effect of bacterial inoculation during the initial selection of Nnr plants.

Our results suggest that both nodulation in the absence of *Rhizobium* and in the presence of host-range *nodH* mutants are heritable characters that appear to be stable, dominant and linked. We propose that all Nnr^+ are Nar^+, but that initially selected Nar^+ plants are not necessarily Nnr^+.

Discussion

The development of an effective nodule is under the inductive control of *Rhizobium* but represents an internal developmental program controlled by a more or less complicated set of plant genes. The study of spontaneous nodulation indicates that the presence of *Rhizobium* is not required for nodule organogenesis (Truchet et al. 1989; Caetano-Anollés et al. 1991 a) and the elicitation of autoregulatory control of nodulation (Caetano-Anollés et al. 1991 a). However, *Rhizobium* signals can be crucial for the efficient initiation of nodules and instrumental for overcoming plant barriers to infection.

To determine if nodule initiation was limited by the nature or concentration of certain bacterial nodulation signals, we initially co-inoculated *R. meliloti nod* gene mutants with suboptimal concentrations of wild-type bacteria (Caetano-Anollés and Bauer 1988, 1990). Pairs of host-specificity mutants helped wild-type *R. meliloti* initiate nodule formation, apparently by providing a sustained supply of different but complementary extracellular signals required for the induction of appropriate host responses during nodule initiation. These signals act synergistically to favor nodule initiation (Caetano-Anollés and Bauer 1990). In the study presented here we further investigated the plurality of *nod* gene-related signals. Inoculation of alfalfa plants with host-range *R. meliloti nodH* non-nodulating mutants resulted in a five-fold increase in the number of plants with empty nodules when compared to sham-inoculated treatments. Inoculation with *nodA* and *nodC* mutants gave no such increase. These results suggest that other bacterial signals, different from but perhaps precursors of the lipo-oligosaccharide bacterial signal NodRm-IV(S), are able to induce host responses in alfalfa. The *nodH*-dependent NodRm-IV(S) signal (Lerouge et al. 1990; Truchet et al. 1991) can exert its effects either directly or indirectly through more or less complicated transducing mechanisms. The plant should regulate the activity of this and related signal molecules at different levels, perhaps by controlling the availability of the putative signal receptors, by altering the release or production of secondary signals, or through plant factors that modulate the overall response. Nodulation in the absence of *Rhizobium* may be the endogenous expression of those control mechanisms in selected plants and may thus result from the alteration of functional genes in the plant. Our results suggest that some genotypes are able to express the "nodulation cascade" spontaneously, whereas other genotypes depend on extracellular nodulation signals. While bacteria and their *nod* genes fail to condition the ontogeny and morphogenesis of spontaneous nodulation, extracellular *Rhizobium* signals can determine the efficiency with which spontaneous nodules are formed. This suggests further co-evolution of plant and bacteria to optimize the development of the symbiosis.

We found that alfalfa not only formed bacteria-free nodules when inoculated with non-nodulating *R. meliloti nodH* mutants. Between 1% and 4% of the inoculated alfalfa plants grown in plastic growth pouches and in the absence of fixed nitrogen produced normal pink, nitrogen-fixing nodules. Our results indicate that spontaneous nodulation (the Nar trait) and the formation of nitrogen-fixing nodules in the presence of a *nodH* mutant (the Nnr trait) segregated in progeny plants of 'Vernal' alfalfa. While the Nar and Nnr traits can be the product of a yet undefined genetic element present in certain alfalfa genotypes, like a non-culturable endophytic bacterium, a mycoplasm, a plant virus or a viroid, it appears most likely that they result from a plant gene involved in the signal transduction chain that triggers nodule morphogenesis.

The mode of inheritance indicated that dominant genetic elements control Nar and Nnr in the tetraploid alfalfa. We will consider *a priori* that these genetic elements behave as genes and we will name them *nar*. While one or more of these *nar* genes could condition spontaneous nodulation, our observations suggest that several different allele doses of a single *nar* gene could be responsible for the Nar and Nnr phenotypes. On the basis of this hypothesis the selfing of Nar^+ plants should increase *nar* gene dose, thus enriching the initial and largely heterozygous alfalfa population with homozygous Nar^+ and Nar^- individuals. While the morphology, anatomy and ontogeny of nodules remained the same in progeny plants, possibly due to strong genetic control over the

ability of an alfalfa plant to induce the nodular structures, the number and location of nodules within the root system was skewed towards plants producing spontaneous nodules very early in root development (Fig. 2B). That allele dose conditions the expression of the Nar phenotype is also suggested by segregation studies (Table 3). Plants with a low allele dose (the simplex configuration) exhibited a delayed expression of spontaneous nodulation that confined nodules to lateral roots. Plants with higher *nar* allele dose (the duplex, and perhaps the triplex and quadraplex configurations) produced early expression of the Nar phenotype and early nodulation in the primary roots. In our experiments, S_1 and S_2 progeny from single plants fell in the different categories specified above and followed the expected segregation ratios (Table 3).

Progeny from alfalfa plants exhibiting the Nnr phenotype produced spontaneous nodules in the absence of *Rhizobium* late in root development (Fig. 2C). Nodules appeared in both Nar and Nnr plants exclusively on lateral roots. Our results indicate that the Nar and Nnr traits are linked. While the inoculation of Nar^+ plants with *nodH* mutant bacteria was unable to induce nitrogen-fixing nodules we found the Nnr phenotype only in plants with a low *nar* allele dose. Since the dose of the dominant allele at the *nar* gene locus appears to determine how rapidly spontaneous nodulation occurs in plant development and since spontaneous nodules are still able to suppress further nodulation in ontogenetically young tissue (Caetano-Anollés et al. 1991a), only plants exhibiting low allele doses will allow the induction of cell division in the root cortex late enough to permit infection of NodRm-IV(S) defective mutant bacteria but early enough to avoid autoregulation of nodule formation. Similarly, an early expression of Nar (conditioned by a high dose of the *nar* gene allele) should suppress nodulation in regions of the root that allow infection of spontaneous cell division foci by mutant bacteria. This implies a hypothesis where the Nnr trait is functionally conditioned by the number of dominant alleles at the *nar* loci. Further segregation studies of the Nnr trait will determine if the trait is determined by a putative plant gene that interacts with *nar*.

Our results suggest that the plant is able to overcome the absence of active NodRm-IV(S) signals secreted by *Rhizobium*. Following the gene-for-gene concept formulated by Flor (1955), genetic analyses of plant-bacterial interactions have demonstrated that race-specific disease resistance is specified by the presence of single avirulence genes in the bacterium that correspond to single resistance genes in the host (Lamb et al. 1989; Bent et al. 1991). In the *Rhizobium*-legume symbiosis, single dominant host genes in Afghanistan pea can also determine resistance to nodulation by strains of *R. leguminosarum bv. viciae* (Lie 1984). In contrast to the dominant nature of *nar*, recessive host resistance genes that limit nodulation by specific *Rhizobium* strains have been reported in pea and soybeans (reviewed in Caetano-Anollés and Gresshoff 1991a).

The *nar-nodH* interaction appears as another gene-for-gene relationship specifying infection in plant-microbe interactions. A major implication of the gene-for-gene hypothesis is that resistance will be functionally dominant and result from either direct or indirect molecular recognition between the products of the complementary genes in host and microsymbiont. Our results suggest that a delicate balance of functions, provided by bacterial host-range *nod* genes and plant genes, exists that can account for the success of the symbiosis. These functions involve the alfalfa-specific lipo-oligosaccharide signal and organogenesis in spontaneous nodulation. How plant growth conditions superimpose another variable on this interaction requires further investigation.

Acknowledgements. We thank J. Dénarié for bacterial strains, Marcia Young and Gloria Caetano-Anollés for help in the greenhouse, Priyavadan A. Joshi for histological studies, Noel Gerahty for acetylene-reduction assays, and Effin T. Graham for allowing the use of microscope facilities. This work was supported by an endowment to the Racheff Chair of Excellence of the University of Tennessee, and the Tennessee Soybean Promotion Board.

References

Banfalvi Z, Kondorosi A (1989) Production of root-hair deformation factors by *Rhizobium meliloti* nodulation genes in *Escherichia coli*: *hsnD* (*nodH*) is involved in the plant host-specific modification of the nodABC factor. Plant Mol Biol 13:1–12

Bent A, Carland F, Dahlbeck R, Innes R, Kearney B, Ronald P, Roy M, Salmeron J, Whalen M, Staskawicz B (1991) In: Hennecke H, Verma DPS (eds) Advances in molecular genetics of plant-microbe interactions, vol 1. Kluwer Academic Publ, Dordrecht, pp 32–36

Caetano-Anollés G, Bauer WD (1988) Enhanced nodule initiation on alfalfa by wild-type *Rhizobium meliloti* co-inoculated with *nod* gene mutants and other bacteria. Planta 174:385–395

Caetano-Anollés G, Bauer WD (1990) Host-specificity mutants of *Rhizobium meliloti* have additive effects in situ on initiation of alfalfa nodules. Planta 181:109–116

Caetano-Anollés G, Gresshoff PM (1991a) Plant genetic control of nodulation. Annu Rev Microbiol 45:345–382

Caetano-Anollés G, Gresshoff PM (1991b) Alfalfa controls nodulation during the onset of *Rhizobium*-induced cortical cell division. Plant Physiol 95:366–373

Caetano-Anollés G, Favelukes G, Bauer WD (1990) Optimization of surface sterilization for legume seed. Crop Sci 30:708–712

Caetano-Anollés G, Joshi PA, Gresshoff PM (1991a) Spontaneous nodules induce feedback suppression of nodulation in alfalfa. Planta 183:77–82

Caetano-Anollés G, Joshi PA, Gresshoff PM (1991b) Nodulation in the absence of *Rhizobium*. In: Gresshoff PM (ed) Current topics in plant molecular biology, vol 1. CRC Press, Boca Raton, pp 67–76

Dudley ME, Jacobs TW, Long SR (1987) Microscopic studies of cell divisions induced in alfalfa roots by *Rhizobium meliloti*. Planta 171:289–301

Faucher C, Maillet F, Vasse J, Rosenberg G, van Brussel AN, Truchet G, Dénarié J (1988) *Rhizobium meliloti* host range *nodH* gene determines production of an alfalfa-specific extracellular signal. J Bacteriol 170:5489–5499

Faucher C, Camut S, Dénarié J, Truchet G (1989) The *nodH* and *nodQ* host range genes of *Rhizobium meliloti* behave as avirulence genes in *R. leguminosarum* bv. *viciae* and determine changes in the production of plant-specific extracellular signals. Mol Plant-Microbe Interact 2:291–300

Finan TM, Hirsch AM, Leigh JA, Johansen E, Kuldau GA, Deegan S, Walker GC, Signer ER (1985) Symbiotic mutants of *Rhizobium meliloti* that uncouple plant from bacterial differentiation. Cell 40:869–877

Flor HH (1955) Host-parasite interaction in flax rust – its genetics and other implications. Phytopathology 45:680–685

Herridge DF (1977) Carbon and nitrogen nutrition of two annual legumes. Ph. D. thesis, University of Western Australia, Perth, Australia

Hirsch AM, Wilson KJ, Jones JDG, Bang M, Walker VV, Ausubel FM (1984) *Rhizobium meliloti* nodulation genes allow *Agrobacterium tumefaciens* and *Escherichia coli* to form pseudonodules on alfalfa. J Bacteriol 158:1133–1143

Hirsch AM, Drake D, Jacobs TW, Long SR (1985) Nodules are induced on alfalfa roots by *Agrobacterium tumefaciens* and *Rhizobium trifolii* containing small segments of the *Rhizobium meliloti* nodulation region. J Bacteriol 161:223–230

Hirsch AM, Bhuvaneswari TV, Torrey JG, Bisseling T (1989) Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors. Proc Natl Acad Sci USA 86:1244–1248

Jensen HL (1942) Nitrogen fixation in leguminous plants. I. General characters of root nodule bacteria isolated from species of *Medicago* and *Trifolium* in Australia. Proc Linn Soc NSW 66:98–108

Joshi PA, Caetano-Anollés G, Graham ET, Gresshoff PM (1991) Ontogeny and ultrastructure of spontaneous nodules in alfalfa (*Medicago sativa*). Protoplasma 162:1–11

Lamb CJ, Lawton MA, Dron M, Dixon RA (1989) Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell 56:215–224

Leigh JA, Reed JW, Hanks JF, Hirsch AM, Walker GC (1987) *Rhizobium meliloti* mutants that fail to succinylate their Calcofluor-binding exopolysaccharide are defective in nodule invasion. Cell 51:579–587

Lerouge P, Roche P, Faucher C, Maillet F, Truchet G, Promé J, Dénarié J (1990) Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature 344:781–784

Lie TA (1984) Host genes in *Pisum sativum* conferring resistance to European *Rhizobium leguminosarum*. Plant Soil 82:415–425

Ogawa J, Grierley HL, Long SR (1991) Analysis of *Rhizobium meliloti* nodulation mutant WL131: novel insertion sequence IS*Rm3* in *nodG* and altered *nodH* protein product. J Bacteriol 173:3060–3065

Philip-Hollingsworth S, Hollingsworth RI, Dazzo FB (1991) N-Acetylglutamic acid: an extracellular *nod* signal of *Rhizobium trifolii* ANU843 that induces root hair branching and nodule-like primordia in white clover roots. J. Biol Chem 266:16854–16858

Phillips DA, Torrey JG (1971) Studies on cytokinin production by *Rhizobium*. Plant Physiol 49:11–15

Schwedock J, Long SR (1990) ATP sulphurylase activity of the *nodP* and *nodQ* gene products of *Rhizobium meliloti*. Nature 348:644–647

Spaink HP, Sheeley DM, van Brussel AAN, Glushka J, York WS, Tak T, Geiger O, Kennedy EP, Reinhold VN, Lugtenberg BJJ (1991) A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature 354:125–130

Sturtevant DB, Taller BJ (1989) Cytokinin production by *Bradyrhizobium japonicum*. Plant Physiol 89:1247–1252

Truchet G, Michel M, Dénarié J (1980) Sequential analysis of the organogenesis of lucerne (*Medicago sativa*) root nodules using symbiotic-defective mutants of *Rhizobium meliloti*. Differentiation 16:163–172

Truchet G, Rosenberg C, Vasse J, Julliot JS, Camut S, Dénarié J (1984) Transfer of *Rhizobium meliloti* pSym genes into *Agrobacterium tumefaciens*: host specificity nodulation by atypical infection. J Bacteriol 157:134–142

Truchet G, Barker DG, Camut S, de Billy F, Vasse J, Huguet T (1989) Alfalfa nodulation in the absence of *Rhizobium*. Mol Gen Genet 219:65–68

Truchet G, Roche P, Lerouge P, Vasse J, Camut S, de Billy F, Promé J, Dénarié J (1991) Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature 351:670–673

Wong CH, Pankhurst CE, Kondorosi A, Broughton WJ (1983) Morphology of root nodules and nodule-like structures formed by *Rhizobium* and *Agrobacterium* strains containing a *Rhizobium meliloti* megaplasmid. J Cell Biol 97:787–794