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Expression of *MsLEC1* Transgenes in Alfalfa Plants Causes Symbiotic Abnormalities

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Legume lectins have been proposed to have important symbiotic roles during *Rhizobium*-legume symbioses. To test this hypothesis, the symbiotic responses of transgenic alfalfa plants that express a portion of the putative alfalfa lectin gene *MsLEC1* or *MsLEC2* in either the antisense or sense orientation were analyzed following inoculation with wild-type *Sinorhizobium meliloti* 1021. *MsLEC1*-antisense (LEC1AS) plants were stunted, exhibited hypernodulation, and developed not only abnormally large nodules but also numerous small nodules, both of which senesced prematurely. *MsLEC2*-antisense plants were intermediate in growth and nodule number compared with LEC1AS and vector control plants. The symbiotic abnormalities of *MsLEC1*-sense transgene plants were similar to but milder than the responses shown by the LEC1AS plants, whereas *MsLEC2*-sense transgene plants exhibited symbiotic responses that were identical to those of vector and nontransgenic control plants. *MsLEC1* mRNA accumulation was not detected in nodule RNA by Northern blot analysis but was localized to alfalfa nodule meristems and the adjacent cells of the invasion zone by in situ hybridization; transcripts were also detected in root meristems. A similar spatial pattern of *MsLEC2* expression was found by using a whole-mount in situ hybridization procedure. Moreover, mRNAs for an orthologous lectin gene (*MaLEC*) were detected in white sweetclover (*Melilotus alba)* nodules and root tips.

Additional keywords: nodule development, transgenic plants.

Rhizobium-legume symbioses are highly specific interactions between leguminous plants and *Sinorhizobium*, *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, and *Azorhizobium* bacteria (collectively known as rhizobia). These symbioses result in the development of root nodules in which the rhizobia convert atmospheric nitrogen into ammonia, a reaction catalyzed by nitrogenase. Detailed descriptions of the *Rhizobium*-legume symbioses have been presented in a number of recent reviews (Downie and Walker 1999; Hirsch et al. 2001; Perret et al. 2000).

Lectins are carbohydrate-binding proteins that are encoded by genes of plant, animal, microbial, and viral origin (Brewin and Kardalisky 1997; Pusztai 1991; Sharon and Lis 1990). The vast majority of those studied lack a transmembrane domain, and hence, these lectins are known as soluble lectins. One of the proposed functions of legume lectins is as a specificity determinant of the *Rhizobium*/legume symbiosis (Bauchrowitz et al. 1996; Bohlool and Schmidt 1974; Dazzo and Hubbell 1975; Díaz et al. 1989, 1996, 2000; Hirsch 1999; Hirsch et al. 1995; Kijne et al. 1997; van Eijsden et al. 1995; van Rhijn et al. 1998, 2001). The lectin recognition hypothesis originally proposed that lectins served in attachment and recognition of compatible rhizobia, leading to initiation of symbiosis (Bohlool and Schmidt 1974; Dazzo and Hubbell 1975). However, a lack of lectin specificity to ligands on homologous rhizobia has also been observed (Chen and Phillips 1976; Pueppke et al. 1980), challenging the lectin recognition hypothesis.

Subsequent lectin gain-of-function experiments, exemplified by the expression of the pea (*Pisum sativum*) seed lectin (*PSL*) gene in white clover (*Trifolium repens*), provided a different approach that gave strong support to the lectin recognition hypothesis, because the *Agrobacterium rhizogenes*-transformed "hairy" roots were nodulated by the pea symbiont *Rhizobium leguminosarum* bv. *viciae* (Díaz et al. 1989). Similarly, soybean seed lectin (*SBL*) expression in transgenic *Lotus* roots, a plant that is normally nodulated by *Mesorhizobium loti*, enabled the heterologous soybean symbiont *Bradyrhizobium japonicum* to elicit nodules, albeit uninfected ones, on these transgenic roots (Hirsch et al. 1995; van Rhijn et al. 1998). In addition, expression of either the *SBL* or *PSL* gene in *A. tumefaciens*-transformed alfalfa enabled *B. japonicum* or *R. leguminosarum* bv. *viciae*, respectively, to form nodules on the transgenic plants but only if the heterologous rhizobial strain harbored a plasmid that allowed the expression of the *S. meliloti nod* genes (van Rhijn et al. 2001). Thus, for both indeterminate and determinate nodule-forming legumes, introduction of a lectin transgene facilitated nodulation by the "wrong" rhizobia. However, lectins may also lower the threshold of responsiveness of legume root cells to rhizobial-derived lipochitin oligosaccharides (LCO), because transgenic red clover roots expressing PSL developed structures resembling nodule primordia, when treated with heterologous LCO (Díaz et al. 2000). These gain-of-function experiments demonstrate that the involvement of lectins in nodulation might be more complex than the attachment or recognition role that was originally envisioned for lectins in the lectin recognition hypothesis.

A differing but complementary approach involves lectin loss-of-function experiments as a way to test the roles of lectins in the *Rhizobium*-legume symbiosis. Few lectin-minus (Le1$^-$; *Le1* codes for SBL) mutants have been described, however. Boyd and Reguera (1949) reported that some lima bean (*Phaseolus lunatus*) cultivars lacked agglutinating lectins, and

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Brücher and associates (1969) noted a similar situation for *P. aborigineus*. In soybean (*Glycine max* L.), which, like *Phaseolus lunatus*, forms determinate nodules, at least five Le1$^-$ mutant cultivars have been identified; the most commonly studied one is 'Sooty' (Pull et al. 1978). The mutation in the Sooty cultivar is the result of a large insertion in the *SBL* gene (Goldberg et al. 1983; Vodkin et al. 1983). Yet, 'Sooty' nodulates in response to *B. japonicum* (Pull et al. 1978), suggesting that SBL is not essential for the initiation or persistence of the soybean-*Bradyrhizobium* symbiosis. Other lectins that facilitate the interaction or that could compensate for the loss of SBL might be present in the Le1$^-$ genome, but their identity has not been uncovered in spite of considerable effort (Wycoff et al. 1997).

To our knowledge, no loss-of-lectin-function mutants have been described for an indeterminate nodule-forming plant, although large-scale screening studies have identified pea cultivars with variable lectin levels (Boyd et al. 1961). We therefore constructed multiple transgenic alfalfa (*Medicago sativa*) lines that expressed either an *MsLEC1*- or *MsLEC2*-antisense transgene (LEC1AS and LEC2AS, respectively) to generate loss-of-function plants as well as the cognate sense transgene (LEC1ST and LEC2ST plants, respectively) (Brill and Hirsch 1999; Brill et al. 2001; Hirsch et al. 1995). Although there is, as yet, no direct proof that *MsLEC1* and *MsLEC2* gene products are lectins, we predicted that silencing the *MsLEC1* or *MsLEC2* genes might decrease nodulation of alfalfa, because gain-of-function studies with legume lectins resulted in nodules and nodule-like structures where no nodulation would otherwise occur (Díaz et al. 1989, 1996, 2000; van Rhijn et al. 1998, 2001). The predicted protein products of *MsLEC1* and *MsLEC2* are very likely to be lectins, because they are 77 and 73% similar, respectively, to the amino acid sequence of the α and β chains of PSL and demonstrate high homology to many other legume lectin genes (Brill et al. 2001). Furthermore, a candidate MsLEC1 protein was down-regulated specifically in LEC1AS plants. These plants displayed severe developmental and reproductive abnormalities (Brill et al. 2001). Because it is especially difficult to isolate soluble lectin proteins from alfalfa (L. M. Brill, *unpublished data*; J. Kijne, *personal communication*), we are currently expressing the *MsLEC1* and *MsLEC2* genes in a heterologous system to demonstrate unambiguously that they encode lectins (P. de Hoff and A. M. Hirsch, *unpublished data*).

In this report, we show that the involvement of the *MsLEC1* and *MsLEC2* genes in symbioses established on the roots of small-seeded legumes such as alfalfa is complex and, in the case of the *MsLEC1* gene, significant. Surprisingly, our results suggest that the *MsLEC1* gene product participates in negative regulation of nodule development, number, and size rather than facilitation of initial nodule development and could somehow assist in the maintenance of mature nodule function in alfalfa.

RESULTS

Development of nodules.

To assess the involvement of the *MsLEC1* and *MsLEC2* genes in nodulation of alfalfa, we examined the responses of rooted cuttings of transgenic vector control plants, plants expressing the antisense transgene for *MsLEC2* (LEC2AS), and plants expressing the antisense transgene for *MsLEC1* (LEC1AS) to inoculation with β-glucuronidase (GUS)- or green fluorescent protein (GFP)-marked strains of *S. meliloti* Rm1021. Because legume lectins have been associated with facilitation of nodulation, reduced nodulation of lectin loss-of-function plants was predicted. However, contrary to our expectations, all the transgenic plants, including the controls, were nodulated 7 days postinoculation (dpi). By this time, the LEC1AS plant lines had already developed abnormally large numbers of nodules (Fig. 1A). The colonized nodules, as evidenced by the presence of GUS- or GFP-marked rhizobia, were frequently adjacent to each other or directly opposite one another on the root. Infection thread development in root hairs, as viewed by fluorescent microscopy of GFP-marked Rm1021, was not impaired in the LEC1AS roots, although some of the nodules appeared to be uninfected (Fig. 1B). Occasionally, uninfected nodules also developed on the roots of LEC2AS plants (data not shown), but generally, the LEC2AS root nodules contained the marked strains (Fig. 1C, 1D). With one exception, line β49b, the LEC2AS roots developed markedly separate rather than clustered nodules.

By 12 to 14 dpi, many of the LEC1AS nodules were already beginning to show signs of senescence, as indicated by the reduction in overall staining in a nodule 13 dpi with a GUS-marked strain (Fig. 1E) and by the decrease in Rm1021 GFP fluorescence in the center part of a 2-week-old nodule (Fig. 1F). In the Rm1021 GFP nodules, there was a concomitant accumulation of autofluorescent compounds, presumably flavonoids, in the central and proximal parts of the nodule (Fig. 1F and G). Sections of senescent nodules demonstrated that the rhizobia had senesced from the inside outward (Fig. 1H). In contrast, the vector control and LEC2AS nodules as well as the plant lines containing the cognate sense transgenes did not show any symptoms of senescence until many weeks after inoculation. All nodules were colonized by rhizobia (data not shown).

Nodulation phenotypes in response to different growth conditions.

Potting soil and Turface. Nodules formed on vector control plants grown in potting soil (Fig. 2A) appeared identical, i.e. pink and elongated, to nodules formed by nontransgenic, wild-type alfalfa (data not shown). Similarly, nodules formed on the LEC2AS plants were elongated and pink in color (data not shown). In contrast, although some LEC1AS root nodules appeared morphologically normal, many LEC1AS nodules were large, multilobed structures that showed signs of senescence, including loss of pink color due to breakdown of leghemoglobin, whereas others showed arrested development at early stages of nodule formation (Fig. 2C and D). Nodulation was also examined in an inert root medium (Turface). The Turface-grown plants exhibited an identical nodulation phenotype (data not shown). The LEC1AS lines displaying the most severe developmental and reproductive abnormalities (Brill et al. 2001) had the highest proportion of abnormal nodules.

We also examined plants that expressed the sense transgene for *MsLEC1* (LEC1ST) and found that these roots also developed some large, albeit pink, multilobed nodules (Fig. 2B), suggesting that cosuppression might be the cause. We did not pursue this analysis further. In contrast, the LEC2ST plants produced nodules that were identical to those of the vector control (data not shown).

Hydroponic conditions. When grown hydroponically, the differences between inoculated versus uninoculated plants became very obvious. The shoots of the uninoculated plants were paler than the nodulated plants and, of the three types of transgenic plants, the LEC1AS plants were the most chlorotic (Fig. 3A and B). The differences between the vector control, LEC2AS, and LEC1AS plants 35 dpi, were striking (Fig. 3B). The LEC1AS plants were much less robust than either the vector control or the LEC2AS plants; the plants were consistently small and chlorotic, with a poorly developed root system. Nevertheless, a number of large, prominent nodules were observed on the roots of the LEC1AS plants (Figs. 2D and 3B), as well as small, senescent nodules (Fig. 2C). The abnormal nodulation

phenotype in LEC1AS plants was evident 15 to 20 dpi under hydroponic conditions. In contrast, nodulated vector control and LEC2AS plants appeared normal, and many fewer nodules formed on the root system (Fig. 3B).

To express these findings in a quantitative way, the nodules were removed from the roots of the hydroponically grown plants and were separated into pink and senescent categories. Table 1 shows that the mean number of pink nodules was significantly lower for the LEC1AS plants than for the vector control and the LEC2AS plants. In contrast, the mean number of senescent nodules (Fig. 2C) was significantly higher for the LEC1AS plants compared with the vector control and

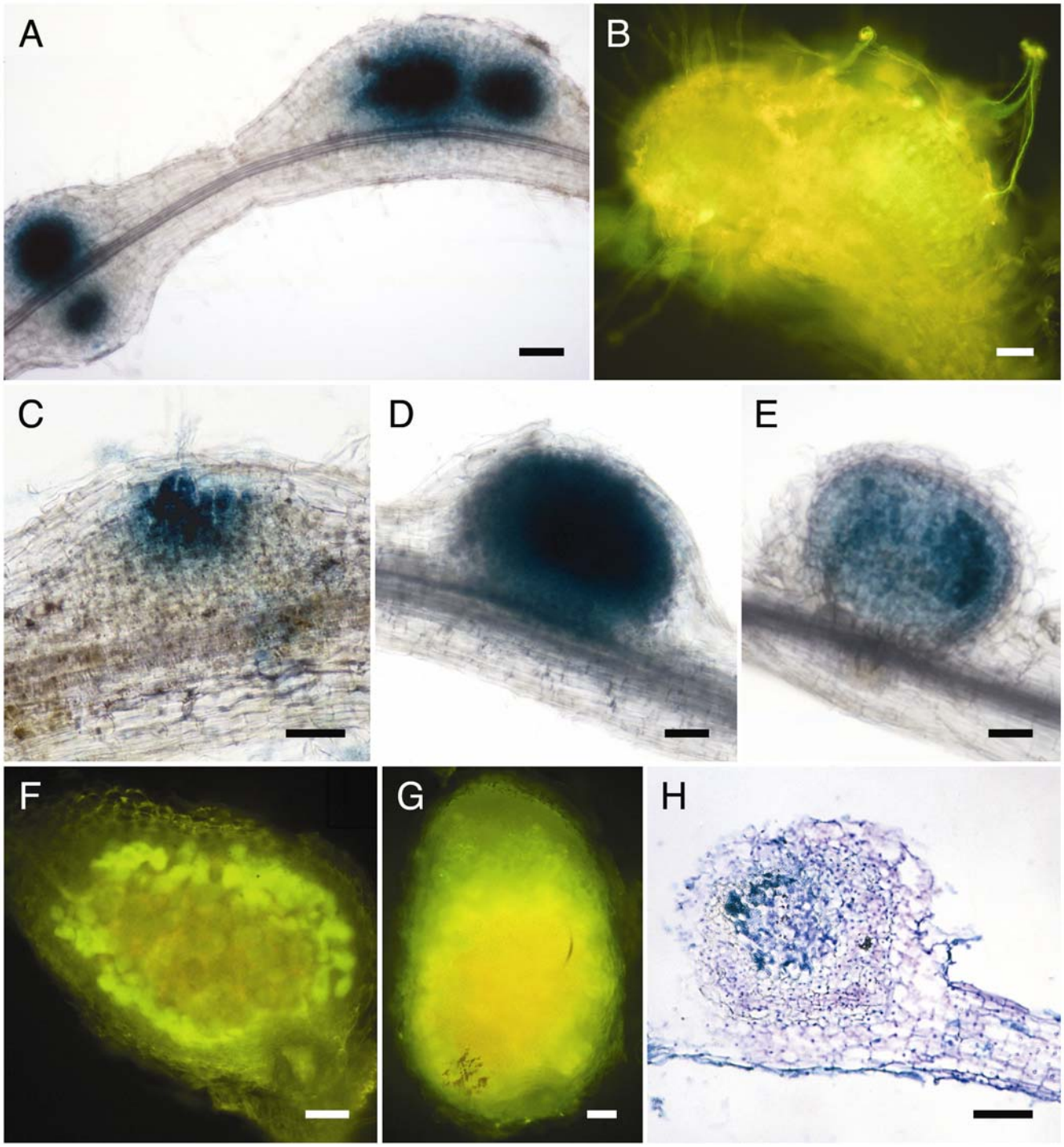

Fig. 1. Nodule development with either β-glucuronidase (GUS)- or green fluorescent protein (GFP)-marked Rm1021 on roots of LEC1AS and LEC2AS transgenic plants. **A,** A cluster of nodules inhabited by GUS-marked Rm1021 is present on a LEC1AS root 7 days postinoculation (dpi). Bar = 40 μm. **B,** Infection thread development 13 dpi, as evidenced by the GFP-marked Rm1021 in the curled root hairs appears normal, although the nodule is uninfected and the cells are accumulating flavonoids (yellow autofluorescence). Bar = 40 μm. **C,** A nodule formed on a LEC2AS root 7 dpi, colonized by GUS-marked Rm1021. Bar = 40 μm. **D,** Another LEC2S nodule 7 dpi. This nodule is more developed than the one in C. Bar = 40 μm. **E,** Reduction in GUS-staining in a LEC1AS nodule 13 dpi. Bar = 40 μm. **F,** Section through a LEC1AS nodule 14 dpi, showing green fluorescence of GFP-labeled Rm1021 cells in the peripheral nodule cells and yellow autofluorescence of flavonoid compounds in the center. Bar = 40 μm. **G,** LEC1AS nodule 14 dpi. The major fluorescence is due to the autofluorescence of the accumulating compounds in the nodule, presumably flavonoids. Bar = 40 μm. **H,** Section through a senescent nodule from a LEC1AS plant. Bar = 40 μm.

LEC2AS plants (Table 1). The mean for the total number of nodules for the three plant groups, which included pink and senescent nodules, did not differ significantly (Table 1). However, when the mean total nodule number was normalized to grams of root dry weight, the value was significantly higher for LEC1AS plants compared with vector control and LEC2AS plants; the vector control and LEC2AS plants did not differ significantly from each other (Table 1). These results demonstrate that the LEC1AS plants produced more nodules, even though their overall root mass was less than the vector control and LEC2AS plants.

Nodules from plants containing *MsLEC1* transgenes result in more CFU of *S. meliloti.*

We reasoned that if the nodules produced on LEC1AS plants senesced faster than vector control and LEC2AS plants, then more rhizobial colony-forming units (CFU) would appear on agar medium after squashing the LEC1AS nodules. Moreover, we anticipated that the large pink nodules would also have an increased number of CFU.

Nodules were collected, weighed, surface-sterilized, and crushed, and the resulting extracts were diluted and plated on RDM culture plates. LEC2AS, LEC2ST, and vector control plants all had similar numbers of culturable *S. meliloti* cells per mg of nodule tissue (Table 2). In contrast, there were large (albeit highly variable) mean levels of culturable *S. meliloti* cells in LEC1ST and especially in LEC1AS nodules. These results suggest that the excessive proliferation of the nodule tissue on LEC1ST and especially on the LEC1AS plants was accompanied by an extensive increase in the numbers of culturable bacteria. Both pink and senescent nodules contained large numbers of viable bacteria.

RNA analysis of lectin genes.

Northern blot analysis. To examine the activity of endogenous *MsLEC1* and *MsLEC2* gene expression, Northern blot analyses were performed under high-stringency conditions, using gene-specific probes (Hirsch et al. 1995). When probed with the *MsLEC2* probe, the roots from all three transgenic plant types (vector control, LEC1AS, and LEC2AS) that had nodules were found to contain abundant transcripts of approximately 1.3 kb, the correct size for the *MsLEC2* mRNA (Fig. 4A, top panel). Hybridization with *Msc27* (Kapros et al. 1992) showed that RNA loading was relatively even among the lanes (Fig. 4A, bottom panel).

We previously reported that distinct signals were detectable for endogenous and transgenic RNAs within the stably transformed plants (Brill and Hirsch 1999; Brill et al. 2001; Hirsch et al. 1995). This is also shown in the Northern blot of RNA isolated from LEC2AS roots that developed nodules. As seen in the blot, the majority of the LEC2AS lines accumulated both the endogenous and the antisense *MsLEC2* mRNAs (Hirsch et al. 1995) (Fig. 4A, top right panel).

MsLEC2 RNA accumulated to very low levels in the nodules of vector control plants, which is consistent with what we previously reported for wild-type, nontransgenic alfalfa (Fig. 4B, top panel). In contrast, RNA isolated from nodules formed on roots of the LEC1AS plants showed a highly variable level of *MsLEC2* mRNA that depended on the plant line (Fig. 4B, top panel). Although the signal was detectable in all of the lanes, it was very intense in some (Fig. 4B, lanes 12, 18, and 19), suggesting that introduction of the antisense-*MsLEC1* transgene affected *MsLEC2* mRNA accumulation in a plant line-dependent way (Fig. 4B). The most abundant *MsLEC2* mRNA in the LEC1AS nodule samples (Fig. 4B, lane 18) was found in RNA isolated from one very large nodule. Nodule RNA from

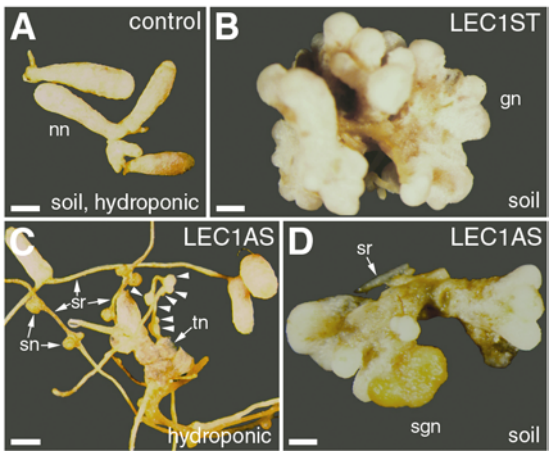

Fig. 2. Overviews of nodules formed on the different types of transgenic plants. **A,** Vector control nodules collected 48 days postinoculation (dpi). Nodule morphology did not differ in hydroponic or solid-rooting media. nn = normal nodule. Bar = 1 mm. **B,** LEC1ST nodules collected from potting soil 48 dpi. Giant nodule (gn), consisting of many lobes. Bar = 1 mm. **C,** LEC1AS nodules obtained from cuttings grown hydroponically 48 dpi. Arrowheads point to small, senescent nodules. sn = senescent nodules, tn = tumorous nodules, sr = senescent roots. Some potentially normal, pink nodules were also observed. Bar = 1 mm. **D,** LEC1AS nodule removed 34 dpi from roots growing in potting soil. sgn = senescent giant nodule. Bar = 1 mm.

Fig. 3. Hydroponic plants. **A,** Plants are shown growing in tanks. Fluorescent light grates were covered in aluminum foil, plant crowns were anchored in the openings of fluorescent light grates, and an aquarium pump connected to air-hoses provided constant aeration of the medium in the tanks. Vector control, LEC1AS, and LEC2AS plants were alternated in the rows. An uninoculated control tank (right) and a tank of nodulated plants (left) are shown. Bar = 15 cm. **B,** Nodulated plants 35 days postinoculation. Representative vector control (left), LEC2AS (center), and LEC1AS (right) plants are shown. Arrows point to large nodules on the LEC1AS plant. Bar = 10 cm.

LEC2AS plants contained highly variable levels of both endogenous and antisense (Fig. 4, marked with an asterisk) *MsLEC2* mRNAs (Fig. 4B, upper right panel).

RNA from LEC1AS roots with nodules contained detectable levels of the antisense-*MsLEC1* transgene (expected size of 0.7 kb) (Fig. 5A); no endogenous sense mRNA (1.3 kb) was observed. The nodulated roots from the different LEC1AS plants showed considerable variability in the amount of accumulation of this transgenic RNA. We had found earlier that there was a correlation between plants that demonstrated moderate to severe developmental and reproductive abnormalities (Brill and Hirsch 1999; Brill et al. 2001) and those with low accumulation of *MsLEC1*-antisense RNA in nodulated roots (Fig. 5A, lanes 4, 6, and 7). Similarly, low levels of *MsLEC1*-antisense RNA (Fig. 5A, indicated by an asterisk) were detected in nodules using Northern analysis. The transgenic plants were stably transformed, and different lines contained varying numbers and positions of transgene insertions (Brill et al. 2001), which may have contributed to variability in transgenic and endogenous lectin mRNA expression (Figs. 4 and 5). Transcripts hybridizing to *MsLEC1*-sense mRNA (1.3 kb) were not detected in RNA isolated from nodules of LEC1AS plants (Fig. 5B).

In situ hybridization analysis. Because of the difficulty in detecting *MsLEC1* transcripts in nodules using Northern blot analysis, we performed in situ hybridization experiments on nontransgenic alfalfa nodules to get a better idea of the spatial expression pattern of this gene. Transcripts hybridizing to *MsLEC1* were detected in alfalfa nodule meristems (zone I) and adjacent cells of the invasion zone (zone II) (Fig. 6B and C), whereas no transcripts were observed in the comparable cells of the sense controls (Fig. 6A). We also examined *MsLEC1* expression in alfalfa roots and found that this lectin gene was expressed in the root apical meristem and also in cells of the elongation zone (Fig. 6E and G); no transcripts were observed in the sense controls (Fig. 6D and F). It was difficult to evaluate the difference in the extent of *MsLEC1* expression in individual uninoculated (data not shown) versus inoculated roots. The two sets of roots looked almost identical.

For *MsLEC2*, essentially the same pattern of transcript localization was observed. Figure 6J illustrates an entire alfalfa nodule primordium 7 dpi; *MsLEC2* mRNA was detected throughout the developing nodule using the WISH (whole-mount in situ hybridization) method. As the nodule matured, the signal became more concentrated in the cells of zones I and II, the nodule meristem and the invasion zone, respectively (Fig. 6K). More mature nodules showed the same pattern of *MsLEC2* mRNA localization (data not shown). *MsLEC2* transcripts were also detected in the root meristems and adjacent regions (Fig. 6H) and lateral root tips (Fig. 6I). Similar to the *MsLEC1* results, there was no obvious difference in the amount of transcript observed in inoculated versus uninoculated roots. There was no signal detected in the nodule hybridized with the sense probe or in the comparable control for the root (data not shown).

To help confirm the spatial expression pattern of the soluble lectin genes in indeterminate nodules, we examined white sweetclover (*Melilotus alba*) roots and nodules. This legume appears to have only a single copy of a putative seed lectin gene, termed *MaLEC* (Brill 1997; W. M. Karlowski and A. M. Hirsch, *unpublished data*). This contrasts with alfalfa and *Medicago truncatula,* each of which has three different *LEC* genes (Bauchrowitz et al. 1992; Brill 1997). In white sweetclover nodules, *MaLEC* mRNA was detected in a 21-day-old nodule, in the cells of zones I and II, the nodule meristem and the invasion zone, respectively (Fig. 6M), in a pattern that was identical to that observed for alfalfa nodules (Fig. 6C and K). The nodule hybridized with the sense probe is depicted in Figure 6L; no signal was detected. Transcripts hybridizing to *MaLEC* were also detected in the main root tip (Fig. 6N) and in lateral root meristems (Fig. 6O) of both inoculated and uninoculated roots (data not shown); no transcripts were observed in the roots hybridized with the sense control (data not shown).

Taken together, these data indicate that *MsLEC1* and *MsLEC2* genes, as well as the orthologous gene *MaLEC*, are expressed in the youngest cells of indeterminate nodules of both alfalfa and white sweetclover. Moreover, the genes that encode these soluble lectins are also expressed in root tips.

MsENOD40 gene expression. We hypothesized that expressing the lectin transgenes would not only have an effect on overall nodule phenotype, but also on downstream nodulin gene expression. We analyzed the expression of *MsENOD40*, which is expressed within a few hours after rhizobial inoculation (Fang and Hirsch 1998). Similar to *MsLEC1*, *MsENOD40* is expressed in the youngest cells of indeterminate nodules and also in root meristems (Asad et al. 1994; Crespi et al. 1994; Fang and Hirsch 1998). Accumulation of *MsENOD40* RNA was significantly higher in RNA isolated from LEC1AS and LEC2AS nodules than in RNA derived from vector controls, in spite of high variability (Table 3). The highest mean *MsENOD40* RNA accumulation was found for LEC1AS plants, which exhibited the severest symbiotic abnormalities.

DISCUSSION

MsLEC2 symbiotic functions.

Seed lectin genes of both pea (Buffard et al. 1988) and soybean (Goldberg et al. 1989; Okamuro et al. 1986) are expressed

Table 1. Nodule numbers from hydroponically grown transgenic plants

Parameter	Control plants	LEC1AS plants	LEC2AS plants
Pink nodules	1,024a[y] ± 419[z]	650b ± 309	1,027a ± 388
Senescent nodules	25.3a ± 57.8	152.6b ± 148.2	17.8a ± 25.2
Total nodules	1,050a ± 459	803a ± 395	1,045a ± 399
Total nodules/g dry roots	5,232a ± 1,070	8,929b ± 2,804	6,886a ± 1,899

[y] Different letters follow means that differed significantly in analysis of variance (ANOVA) at $P < 0.05$.
[z] Standard deviation.

Table 2. *Rhizobium meliloti* CFU per mg of nodule tissue

Vector Control Nodules	LEC1AS Nodules (Pink)	LEC1AS Nodules (Senescent)	LEC1ST Nodules	LEC2AS Nodules	LEC2ST Nodules
8.2a [x,y]± 6.2[z]	545.0ab ± 722.0	320.3b ± 316.3	219.4b ± 104.8	8.3a ± 2.0	5.9a ± 1.1

[x] $\times 10^{-3}$.
[y] Different letters follow means differing significantly in ANOVA, $P < 0.05$.
[z] Standard deviation.

at low levels in roots. Lectin concentration is very low in the roots of *Phaesolus vulgaris* in the absence of *Rhizobium phaseoli*, but infection causes an increase in the quantity of lectin in the roots (Pusztai 1991). However, lectin levels remain relatively constant in pea roots upon inoculation with *R. leguminosarum* bv. *viciae* (Díaz et al. 1986). Thus, the significance of changes or lack thereof in the regulation of lectin expression levels during nodulation of legume roots is unclear.

Earlier, we found that *MsLEC2* mRNA accumulated in uninoculated alfalfa roots (Hirsch et al. 1995), and here, we report that its accumulation increased in the roots of nodulated plants as well, possibly due to the initiation of nodule primordia (Fig. 6J) or, in the case of sweetclover, more lateral roots (Fig. 6O) (Giordano et al. 2002). Nevertheless, it is difficult to assess the importance of *MsLEC2* function in the alfalfa-*S. meliloti* symbiosis. Most LEC2AS plants did not differ from vector controls in the symbiotic parameters examined, nor did they show any obvious alterations in nodule development or morphology. However, symbiotic parameters measured in some of the LEC2AS plants were at a level intermediate between that of the vector controls and LEC1AS plants. These data collectively suggest that *MsLEC2* may have a subtle role in the alfalfa-*S. meliloti* symbiosis.

In contrast, all of the symbiotic parameters that were examined in LEC1AS plants were clearly abnormal. In addition, *MsLEC2* mRNA accumulation was up-regulated in many of the LEC1AS nodules and roots of nodulated plants. Moreover, the most abnormal LEC1AS nodule used for Northern blot analysis had the highest level of *MsLEC2* mRNA accumulation. These results suggest that the *MsLEC1* gene product may repress *MsLEC2* expression during symbiosis and that there may be a relationship between the level of *MsLEC2* expression and normal symbiotic development.

The *Mtlec2* gene, which is 93% homologous to *MsLEC2*, may be inessential for nodulation in *M. truncatula* because it apparently is a pseudogene (Bauchrowitz et al. 1992). Although the *Mtlec2* promoter was active in mature *Medicago varia* nodules, it was not active in uninoculated or nodulated *M. varia* roots (Bauchrowitz et al. 1996). In alfalfa, *MsLEC3* is a pseudogene (W. M. Karlowski and A. M. Hirsch, *unpublished data*) whereas *MsLEC2* is not, a finding that is consistent with our results showing *MsLEC2* mRNA accumulation in nodules, as well as in uninoculated and nodulated alfalfa roots.

***MsLEC1* is expressed at a very low level in nodules.**

The expression of lectins within nodules has been detected in some cases but not in others (Kishinevsky et al. 1988). A lectinlike protein or vegetative lectin (nonseed lectin), encoded by *PsNlec1*, is expressed at very high levels in pea nodules

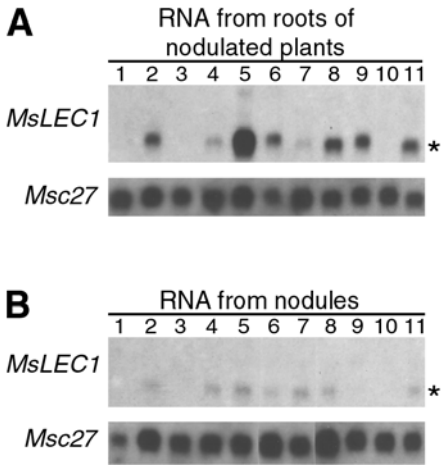

Fig. 5. Northern blot analysis of *MsLEC1*-antisense RNA accumulation in nodules and roots from nodulated LEC1AS plants. **A,** RNA isolated from roots of nodulated LEC1AS plants, and **B,** RNA isolated from nodules of LEC1AS plants. Top panels in A and B show *MsLEC1*-antisense mRNA signal (*) at approximately 0.7 kb, detected with the gamma-probe from *MsLEC1* (Hirsch et al. 1995). Bottom panels in A and B show Msc27 loading control signal. Tissues from independent LEC1AS plants were used for RNA isolation for each lane. High stringency washing conditions were used.

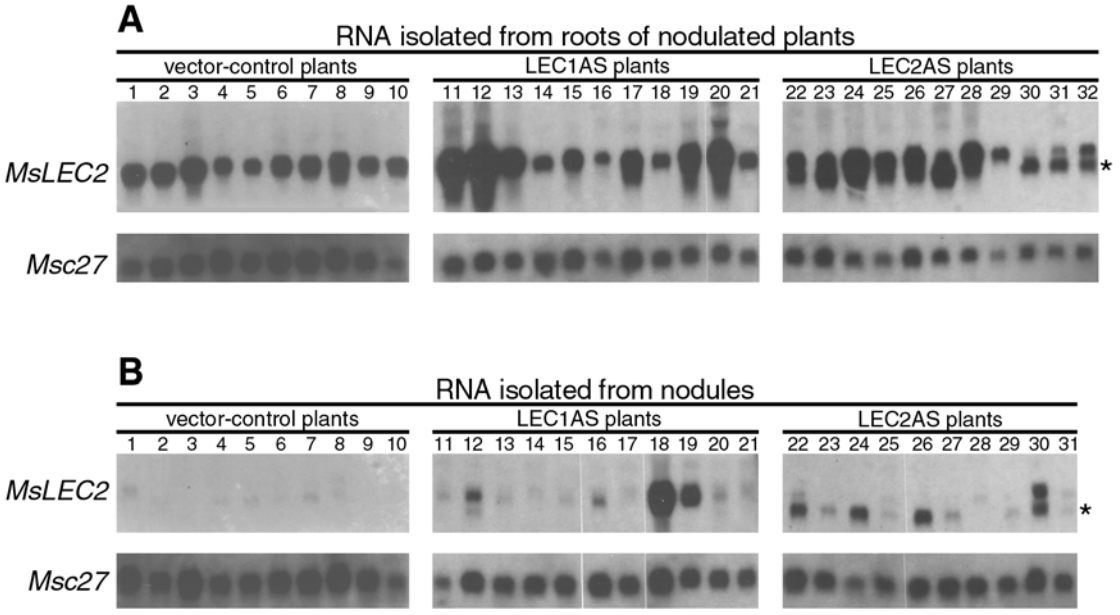

Fig. 4. Northern blot analysis of *MsLEC2* mRNA accumulation in nodules and roots from nodulated plants. **A,** RNA isolated from roots of nodulated plants, and **B,** RNA isolated from nodules. Top panels in A and B show *MsLEC2* mRNA signal, approximately 1.3 kb, detected with the β-probe from *MsLEC2* (Hirsch et al. 1995). Bottom panels in A and B show *Msc27* (Kapros et al. 1992) loading control signal. Lanes are numbered for clarity, and tissues used for RNA isolation for each lane were from independent plant lines. Each independent plant line was found to be stably transformed by one or more independent transgene insertions, verified by Southern blot analysis, as described previously (Brill and Hirsch 1999). High stringency washing conditions (Hirsch et al. 1995) were used. Transgene-encoded *MsLEC2* RNA (*, LEC2AS plants only).

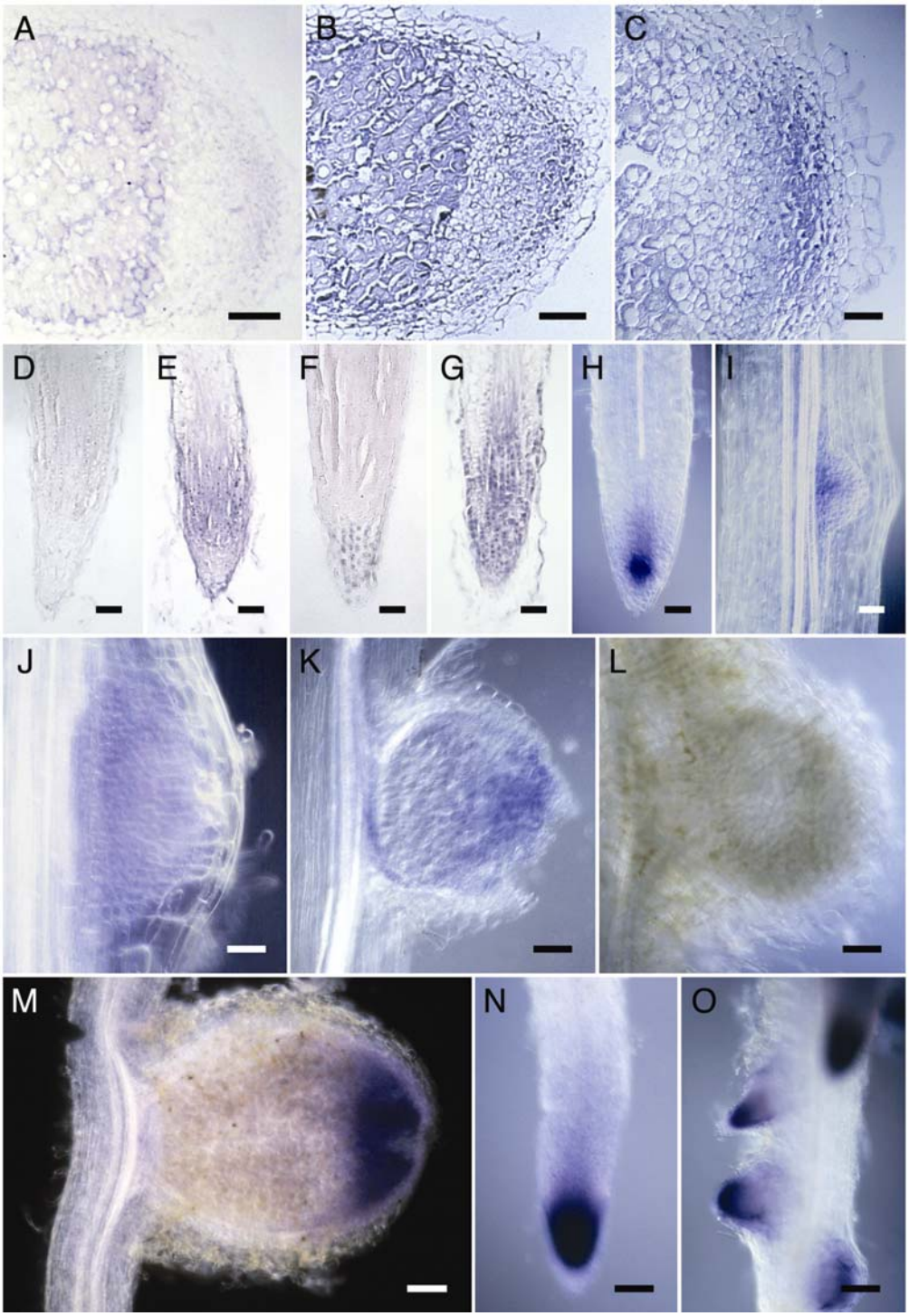

Fig. 6. A through **K,** In situ hybridizations of alfalfa and **L** through **O,** white sweetclover. **A,** In situ hybridization using a sense *MsLEC1* probe on a median longitudinal section of a control (nontransgenic) alfalfa nodule. No color is evident in zones I or II of the nodule. There is some nonspecific staining of starch grains in Interzone II-III. Bar = 20 µm. **B** and **C,** In situ hybridization using an antisense *MsLEC1* probe on longitudinal sections of control (nontransgenic) alfalfa nodules. Bar = 20 µm. B uses bright-field microscopy with signal evident in zones I and II of the nodule. C uses Nomarski optics with an older nodule. Signal is observed in zones I and II of the nodule. **D** through **G,** In situ hybridizations using either an antisense or sense *MsLEC1* probe on longitudinal sections of control (nontransgenic) alfalfa roots for different time periods after inoculation, using bright-field microscopy. Bar = 20 µm. Sense 6 days postinoculation (dpi), no color is evident in the root. Antisense 6 dpi, blue-purple color is present in the root meristem and adjacent cells. Sense 10 dpi, nonspecific staining of the starch grains in the root cap. Antisense 10 dpi, slightly off-median section. The root meristem and adjacent cells of the elongation zone are intensely blue-purple. **H** and **I,** WISH (whole-mount in situ hybridization) of alfalfa roots using the *MsLEC2* probe. With Nomarski optics, intense blue-purple color is detected in the root tip meristem and the cells above it (elongation zone). Bar = 20 µm. Blue-purple color indicating the presence of *MsLEC2* transcripts is seen in the developing lateral root. Bar = 10 µm. **J** and **K,** Whole nodules of alfalfa probed with antisense *MsLEC2*. Using Nomarski optics, J shows blue-purple color evident throughout the nodule primordium 7 dpi. Bar = 10 µm. K shows a young nodule (7 dpi)with *MsLEC2* transcripts accumulating in the cells of the nodule meristem and zone II, as indicated by the blue-purple color. Bar = 40 µm. **L** through **O,** WISH of sweetclover probed with either the sense (L) or antisense (M through O) *MaLEC* probe. Nomarski optics. Bar = 40 µm. L, shows a sweetclover nodule 21 dpi. The sense *MaLEC* probe was used. No blue-purple color is observed. M shows a sweetclover nodule 21 dpi. Antisense probe. Transcripts hybridizing to the *MaLEC* probe are found in the nodule meristem as shown by the blue-purple color. N shows a sweetclover root. Antisense probe. *MaLEC* transcripts accumulate in the root tip. O shows a sweetclover root. Antisense probe. Lateral root meristems accumulate transcripts of *MaLEC*, as indicated by the blue-purple color.

(Dahiya et al. 1997). This lectin has been proposed to function as a storage protein.

We did not detect *MsLEC1* mRNA accumulation in nodules using Northern blots containing total RNA, but we were able to detect it in both root and nodule meristems using in situ hybridization methods. These data correlate with those whereby a promoter-GUS fusion of the *Mtlec1* gene was found to be active in mature nodules of transgenic *M. varia* plants but not with its localization in the nodule peripheral tissue instead of the nodule meristem (Bauchrowitz et al. 1996). However, blue color indicating GUS expression was observed in developing nodule primordia of the *M. varia Mtlec1-gusA* transgenic plants (Bauchrowitz et al. 1996). Our in situ data also demonstrated *MsLEC1* mRNA accumulation in root tips, but the *Mtlec1-gusA* fusion was not expressed in the root meristems of transgenic *M. varia*. To help resolve these differences, we examined the expression of *MaLEC*, which codes for a putative soluble lectin, in white sweetclover. We detected *MaLEC* mRNA in white sweetclover nodule and root meristematic regions, suggesting that these are the main sites of expression for soluble lectin genes in these organs.

The symbiotic abnormalities of LEC1AS plants are consistent with the in situ hybridization data, demonstrating that *MsLEC1* is expressed in alfalfa nodules. It appears that *MsLEC1* expression at the correct level at nodule initiation and in cells in zones I and II of the nodule may be important for regulation of nodule number (Table 1), as well as for the regulation of nodule size and persistence (Figs. 1, 2B through D, and 6B through C). Interestingly, the antisense-*MsLEC1* mRNA also accumulated in nodules at almost undetectable levels (Fig. 5), in spite of its transcription being driven by the strong cauliflower mosaic virus (CaMV) 35S promoter. These results suggest that the accumulation of the antisense-*MsLEC1* mRNA is regulated in some unknown manner.

What is the function of the *MsLEC1* gene product?

The abnormally large number and size of nodules seen on LEC1AS plants were unexpected. Based on studies in which lectins promoted nodulation and nodulation-related responses (Díaz et al. 1989, 1996; Halverson and Stacey 1986; Hirsch et al. 1995; van Rhijn et al. 1998), we predicted that smaller, uninfected nodules would have developed. Indeed, the majority of nodules produced by LEC1AS plants were small, undeveloped, and senesced prematurely (Figs. 1H, 2C). However, although infection thread formation appeared normal, at least based on the organized arrangement of rhizobia in the curled root hairs (Fig. 1B), it is not known whether or not the premature senescence exhibited by the LEC1AS nodules is due to a lack of *MsLEC1* transcript accumulation in nodule meristematic tissues or to a defect in persistence of the infection threads. The latter seems less likely because the rhizobia were not affected by their course through the nodules; large numbers of bacteria were recovered from both LEC1AS and LEC1ST nodules (Table 2).

Based on our previous studies, when the *MsLEC1* gene is disturbed, a disorganized proliferation of embryonic and vegetative tissues results (Brill and Hirsch 1999; Brill et al. 2001). In this report, we have shown that following inoculation with Rm1021, the LEC1AS transgenic root nodules that result are also highly aberrant. In contrast, no abnormal vegetative or reproductive development was detected in the LEC1ST plants (Brill et al. 2001), although some abnormal nodulation was observed. This result is compatible with the finding that sense-suppression-induced symbiotic abnormalities are usually milder than those from antisense suppression and further suggests that symbiotic processes may be more sensitive to alterations in *MsLEC1* expression.

In addition to finding that *MsLEC2* gene expression was up-regulated in the LEC1AS transgene-containing tissues, we also found that *MsENOD40*, an early nodulin gene, was expressed at relatively high levels in LEC1AS nodules. The *MsENOD40* gene has been shown to be up-regulated in response to cytokinin application, and it has been proposed that nodule development may be influenced by changes in the endogenous cytokinin to auxin ratio (Hirsch and Fang 1994; Fang and Hirsch 1998). LEC1AS plants exhibit excessive nodule formation, a result that is consistent with an increase in the level or responsiveness to cytokinin. Moreover, LEC1AS plantlets frequently formed severe teratomas with minimal root development (Brill and Hirsch 1999; Brill et al. 2001), and mature LEC1AS roots were poorly developed (Fig. 2B), further suggesting an excessive cytokinin response. A mechanism whereby lectin could mediate phytohormone levels and interactions is hypothetical at this time, but hydrophobic ligands, including auxins and cytokinins, are known to bind to some soluble legume lectins, albeit to sites independent of the sugar-binding site (Edelman and Wang 1978; Gegg et al. 1992; Roberts and Goldstein 1983; Sharon and Lis 1990).

Taken together, our findings indicate that the expression of the *MsLEC1* and *MsLEC2* genes, especially the *MsLEC1* gene, is important in the compatible symbiotic interaction between alfalfa and *S. meliloti.* This hypothesis is consistent with both lectin gain-of-function (Díaz et al. 1989, 1996, Hirsch et al. 1995; van Eijsden et al. 1995; van Rhijn et al. 1998, 2001) and loss-of-function (this work) experiments. How lectins promote compatible symbiotic interactions is unclear, particularly because lectins with similarity to legume lectins have been found in plant families in addition to the Fabaceae (Wang et al. 2003). In *Arabidopsis* moreover, numerous genes encoding receptor kinases with legume lectin domains have been uncovered (Barre et al. 2002), and similar proteins have now been identified in *Medicago truncatula* (J. Cullimore, *personal communication*). The finding that other plant families have genes that encode proteins with legume lectin domains implies that legume lectins are derived from a lectin gene that was already present in an ancestral flowering plant (Wang et al. 2003). Indeed, many proteins that appear to be specific to the legume-*Rhizobium* interaction seem to be recruited from proteins that are common to both legumes and nonlegumes, e.g., *NORK* (nodulation receptor kinase) (Endre et al. 2002) and *HAR1/NARK* (nodule autoregulation receptor kinase) (Krusell et al. 2002; Nishimura et al. 2002; Searle et al. 2003). *NORK* extracellular sequencelike (*NSL*) genes are found not only in nonlegumes, including several grasses and *Arabidopsis*, but also in a gymnosperm (Endre et al. 2002). Similarly, *HAR1/NARK* genes are very similar to *CLAVATA1*, a serine-threonine kinase that is important for restricting the floral meristem in *Arabidopsis* (Clarke et al. 1997). It is clear that duplication of *NSL*, *CLAVATA1*, and other genes found in nonlegumes has taken place, along with a specialization of their respective proteins for the *Rhizobium*-legume symbiosis. Gene duplication events often result in the development of new functions for the new proteins. Thus, what makes the rhizobia-legume interaction specific may rely more on the details of the interactions between various legume proteins including lectins

Table 3. *MsENOD40* RNA accumulation in nodules[x]

Vector-control plants	LEC1AS plants	LEC2AS plants
2.91a[y] ± 1.48[z]	5.33b ± 5.35	4.62b ± 3.21

[x] *MsENOD40* RNA/*Msc27* mRNA (loading control).
[y] Different letters follow means that differed significantly in ANOVA at $P < 0.05$.
[z] Standard deviation.

and their ligands. Finding a lectinless mutant in an indeterminate nodule-forming legume such as white sweetclover, which appears to have only one gene coding for a soluble lectin, might be one strategy for testing this hypothesis. Alternatively, introducing a gene for a legume lectin, e.g., *SBL* or *PSL* into a nonlegume such as *Arabidopsis*, may also help elucidate whether or not legume lectins can promote colonization of a nonlegume root by rhizobia.

MATERIALS AND METHODS

Plant transformation, bacterial strains, and studies of early nodule development.

Construction of transgenic plants was described previously (Brill et al. 2001; Hirsch et al. 1995). Briefly, the *MsLEC1* transgene contained 420 bp of DNA encompassing the 3′ portion of the open reading frame plus 76 bp of predicted 3′ untranslated region. The *MsLEC2* transgene contained 400 bp from the 5′ portion of the open reading frame beginning 74 bp downstream of the predicted initiator codon (Hirsch et al. 1995). Sense or antisense orientations of transgenes were confirmed using DNA sequence analysis. The CaMV 35S promoter drove transcription of all the transgenes. One plant line of alfalfa cv. Regen SY (also used as the untransformed control line) (Bingham 1991) was used for transformation and regeneration of dozens of independent transformant lines of LEC1AS, LEC1ST, LEC2AS and LEC2ST plants, some of which were grown to maturity for use in nodulation assays. Further control lines containing only the vector used for lectin-transgene plants but lacking inserted genes following the promoter were also constructed (Hirsch et al. 1995) and were used in nodulation assays. Stable transgene integration and activity, as well as transgene-specific phenotypic effects, have been clearly demonstrated (Brill and Hirsch 1999; Brill et al. 2001; Hirsch et al. 1995).

For nodulation tests, stem cuttings of the transgenic alfalfa plants (Brill and Hirsch 1999; Brill et al. 2001; Hirsch et al. 1995) were placed in sterile 11-liter pans that contained 6 liters of a 1:1 mix of perlite/vermiculite saturated with 2.5 liters of complete Hoagland's $^1/_4$-strength nutrient solution (Machlis and Torrey 1956) and were allowed to root. Stem cuttings were from independent, primary transformant lines because they demonstrated developmental abnormalities that were very similar to progeny resulting from selfing (Brill et al. 2001; Brill and Hirsch 1999). However, because alfalfa is an outcrossing tetraploid and shows inbreeding depression, it was difficult to obtain progeny plants that survived to maturity.

The cuttings were transferred to sterilized Magenta jars (Magenta Corp., Chicago) containing a similar mix of perlite and vermiculite watered with Hoagland's $^1/_4$-strength nutrient solution minus nitrogen. A 5-ml suspension of *Sinorhizobium meliloti* wild-type strain 1021 (Rm1021, streptomycin resistant) cells at an optical density of 600 nm (OD_{600}) equal to approximately 0.1 to 0.2, labeled either with GUS (van Rhijn et al. 2001) or with GFP (Cheng and Walker 1998), was added to the Magenta jars after the bacteria were rinsed and diluted in sterile water. One and two weeks after inoculation, the roots were carefully removed from the Magenta jars, were rinsed, and were prepared either for GUS-staining (Jefferson 1987) or for viewing under a Zeiss Axiophot fluorescent microscope.

Nodulation studies.

Nodulation in potting soil. Stem cuttings of the transgenic alfalfa plants were made as described above and allowed to root. Cuttings were placed in pots with approximately 400 cm^3 of potting soil in a greenhouse. One week before inoculation, nitrogen nutrition was withdrawn from the plants, but other macronutrients were supplied. The potting soil was leached with large quantities of tap water four and one days before inoculation.

Rm1021 cells were grown in RDM medium (Vincent 1970), containing 100 mg of streptomycin per liter to an OD_{600} of 0.11 or 0.13, depending on the experiment. Rhizobia were pelleted in a clinical centrifuge and were suspended in sterile milli-Q water to an OD_{600} of 0.1 (approximately 5×10^7 cells per ml). Rm1021 suspension (5 ml) was placed on the surface of the potting soil of each plant.

The plants were grown for 21 dpi. Stems were cut off at the crown, and the potting soil was gently removed from the nodulated roots in standing tap water. The nodules were separated from the roots, were divided into pink and senescent types, and were counted. The external morphology of the nodules was also examined.

Nodulation in Turface rooting medium. Rooted cuttings were placed in pots with approximately 400 cm^3 of inert Turface rooting medium (Amcor, Inc., Buffalo Grove, IL, U.S.A.) and were allowed to grow in the presence of a complete, dilute nutrient solution. One day before inoculation, nitrogen was withdrawn from the plants. Rm1021, grown to early stationary phase, was prepared as described above. Rm1021 suspension (5 ml) was inoculated onto each plant. The plants grew for 34 more days, and then, the stems were cut off at the crown. The Turface was removed from the nodulated roots in standing tap water. The external nodule morphology was examined.

Nodulation under hydroponic conditions. Stem cuttings were allowed to root as described above. Fluorescent light grates were covered with aluminum foil, and individual square openings (approximately 2 cm) of the grate, five to six squares apart, were cut out for placement of plants. The rooting medium was gently removed from the roots of the cuttings by placing them in standing tap water. The crown of each rooted cutting was wrapped in cotton and firmly wedged into an opening of a fluorescent light grate. Rooted cuttings were spaced evenly, with 30 cuttings per grate. Each grate was placed on top of a tank containing 30 liters of complete $^1/_4$-strength Hoagland's medium. Tanks were continuously aerated with aquarium pumps. Six independent vector control, 12 independent LEC1AS, and 12 independent LEC2AS plant lines were used in each of six hydroponic tanks. The entire assembly of 30 plants could be removed and replaced relatively undisturbed from the medium. The complete nutrient solution was replaced with 30 liters of ¼-strength Hoagland's medium lacking nitrogen. Five days after medium replacement, a suspension of Rm1021, prepared as described above, was uniformly mixed into the medium, and the roots were returned to the solution. Rm1021 inocula from mid-lag, early-exponential, late-exponential, early-stationary, or stationary phase were used. The liquid level of the hydroponic tanks was maintained by adding deionized water.

The plants grew for an additional 28 to 37 dpi. Stems of nodulated plants were cut at the crown, were dried under vacuum at 45°C for 2 days, and were weighed. The nodulated roots were pooled for each plant type and were stored at –20°C; the nodules were later separated from the roots. Nodules and roots were dried under vacuum for 2 days at 45°C and were weighed. Some nodulated roots were left intact, were allowed to dry at room temperature (23 to 27°C) under ambient conditions for 14 days, and then were weighed. Uninoculated plants were removed from the hydroponic conditions, were dried in the greenhouse for 1 week, and then, the roots and vegetative tissues were separated at the crowns and weighed.

Recovery of bacteria from nodules.

Nodules were separated from roots of hydroponic plants at 21 dpi, were blotted dry, and were weighed. Nodules were sur-

face-sterilized for 1 min in 10% bleach, were rinsed three times in sterile, deionized water, and were crushed in 1 ml of sterile, deionized water with a pestle in a sterile microfuge tube. Suspensions containing crushed nodules were diluted 10-, 100-, and 1000-fold in sterile, deionized water. Undiluted and diluted nodule suspensions (100 µl each) were plated on a culture plate containing RDM medium in 1.0% agar. All bacterial colonies had the morphology of Rm1021. Bacterial colonies were counted on plates. Colony number and nodule weight was used to calculate CFU per mg of nodule tissue, and CFU per mg of nodule tissue was analyzed for significant differences among plant groups ($P < 0.05$) using analysis of variance (ANOVA).

RNA analysis.

Northern blots. Nodules and roots of hydroponic plants were separated, were placed in liquid nitrogen, and were stored at –70°C. RNA blots that contained 10 µg of total RNA per lane were prepared, hybridized, and washed at high stringency (65°C, 0.1× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 0.5% SDS) as described (Hirsch et al. 1995). *MsLEC1*, *MsLEC2* (Hirsch et al. 1995), *MsENOD40* (Asad et al. 1994), and *Msc27* (Kapros et al. 1992) probes were used. Blots were exposed to X-ray film with an intensifying screen at –70°C or to a storage phosphor screen for signal quantification with a Phosphorimager (Molecular Dynamics Inc., Sunnyvale, CA, U.S.A.). The absence of remaining signal on blots was verified before reprobing. Relative abundance of *MsENOD40* RNA was calculated from the ratio of *MsENOD40* to *Msc27* (loading control) signal. *MsENOD40* RNA accumulation was analyzed for significant differences ($P < 0.05$) using ANOVA.

In situ hybridization. Plants were grown in a Hoagland's agar medium without nitrogen, as described in Giordano and associates (2002). Nodules were excised from the roots and fixed in FAA (formalin/acetic acid/alcohol) for the in situ hybridization experiments. Dehydration and paraffin embedding steps were followed, according to McKhann and Hirsch (1993), and 8- to 10-µm sections were prepared. A 420-bp region of the *MsLEC1* gene, the same segment as used for the transgene experiments, which had been cloned into pBluescript II KS(+/–) into the multiple cloning site, was used as the source of the probe. The isolated plasmid was restricted with either *Xho*I or *Bam*HI to release the *MsLEC1* fragments. These were then transcribed using either T7 or T3 polymerase into sense or antisense RNA, respectively. Hybridization was done under high stringency conditions, as described in McKhann and Hirsch (1993).

To generate a probe specific for *MsLEC2*, a 123-bp fragment from the 3′ untranslated region was cloned into pBluescript II KS(+/–) (Stratagene, La Jolla, CA, U.S.A.). The plasmid was linearized with either *Sac*I or *Kpn*I. For *MaLEC*, a 300-bp region was cloned into pBluescript II KS(+/–) and was linearized with either *Xba*I or *Eco*RI. For both *MsLEC2* and *MaLEC*, T3 and T7 polymerases were used to transcribe sense and antisense RNA, respectively. Riboprobes were made according to manufacturer's instructions, using the digoxigenin (DIG) RNA labeling kit (Roche Diagnostics, Indianapolis, IN, U.S.A.). *MaLEC* riboprobes were hydrolyzed to 100-bp fragments by alkaline treatment prior to use. For WISH studies, white sweetclover (*Melilotus alba* U389) and alfalfa (*Medicago sativa* cv. Iroquois) were grown on ¼-strength Hoagland's agar without nitrogen (Giordano et al. 2002). For nodulation studies conducted for more than one week, plants were grown in Magenta jars containing vermiculite and perlite (2:1) and were watered with ¼-strength Hoagland's medium without nitrogen. Three days after germination, seedlings were inoculated with 10^6 to 10^7 cells of Rm1021 or were mock-inoculated with sterile water. Roots and nodules were harvested 3, 7, 14, and 21 dpi. Tissues were immediately fixed and processed for WISH, as described by Giordano and associates (2002). Hybridizations were done at 55ºC. DIG was detected using BM purple substrate (Roche Biochemical, Indianapolis, IN, U.S.A.).

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