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Steps toward Defining the Role of Lectins in Nodule Development in Legumes

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Abstract

A number of genes, of both the plant and the bacteria, are specifically induced upon the interaction between rhizobia and plants during the onset of nodulation. However, many other genes such as those encoding lectin, a protein which has been hypothesized as being involved in host recognition by rhizobia, are not specifically induced in response either to rhizobial inoculation or to Nod factor treatment. Exactly how lectin is involved in recognition between rhizobia and legume is still not well understood. To this end, we have isolated and characterized two alfalfa lectin genes from an alfalfa genomic library. Lectin mRNA-antisense alfalfa plants have been generated and the early stages of development of the transgenic plants have been studied. Differences were seen from the control plants with respect to the number of plants formed per callus. We have also transferred the soybean lectin gene into alfalfa as well as into *Lotus*, and have examined the response of the transgenic plants to inoculation with *Bradyrhizobium japonicum*, the usual symbiont for soybean. The combination of these approaches should bring us to a closer understanding of the role of lectins in nodule development.

Keywords: Lectins, alfalfa, *Lotus*, soybean, nodulation

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1. Introduction

When rhizobia encounter root hairs of legumes in the soil, a number of profound developmental events take place in the infected root. These culminate in the formation of a nitrogen-fixing root nodule, which in the case of pea, vetch, and alfalfa, is indeterminate. In contrast, nodules of *Lotus*, soybean, and common bean are determinate (see Brewin, 1991; Hirsch, 1992; Kijne, 1992 for reviews). During the earliest stages of the interaction, specific rhizobia are attracted to their legume host and subsequently, the plant root hairs deform in response to compatible rhizobia; incompatible rhizobia either elicit only a slight response or no response at all. The bacteria then induce the formation of an infection thread which penetrates the root hair cell and traverses root cell boundaries until the thread and its branches enter the cells of the nodule primordium. The nodule primordium is an assemblage of derivatives of cells stimulated to divide in response to rhizobia or rhizobial lipochitooligosaccharides. In indeterminate nodules, the nodule primordium develops within the inner root cortex and a nodule meristem is secondarily established. The nodule meristem is one of the main sources of the various tissues in the nodule. In determinate nodules, the cells of the nodule primordium, which develops from both outer and inner cortical cell derivatives, divide and then differentiate into distinct nodule cell types.

The mechanism whereby many of these initial stages comes about is still unknown. Rhizobial Nod factor – a lipochitooligosaccharide molecule with a variety of substitutions – by itself can elicit root hair deformation and cortical cell divisions (Truchet et al., 1991). Moreover, Nod factor can induce the formation of what is known as a pre-infection thread, a re-alignment of the cortical cell cytoplasms extending from outer to inner cortical cells (van Brussel et al., 1992). However, invasion of rhizobia into the root hair cell requires cell-cell contact, which suggests that some sort of receptor or recognition molecule(s) must exist on the root hair surface.

For many years, there has been considerable debate with regard to the involvement of lectins in nodulation. Bohlool and Schmidt (1974), in the lectin recognition hypothesis, proposed that the specific binding of a legume lectin to a saccharide moiety, as yet unidentified, of a compatible *Rhizobium* allows the two symbionts to recognize each other. Exact details of how such recognition occurs are unknown, however. In some versions of the lectin recognition hypothesis, lectin is proposed to be localized to the susceptible root hairs, whereas in other versions, lectin is extracellular and forms a bridge between carbohydrate ligands on the rhizobial surface and the plant cell wall (see review by Kijne et al., 1992). However, contradictory results, which no doubt arose because of the variety of assays and experimental conditions used to test the lectin recognition hypothesis,

led to a waning of interest. Moreover, the involvement of non-lectin-mediated mechanisms for rhizobial attachment, such as rhicadhesin, a small (14 kDa), rhizobial calcium-binding protein (Smit et al., 1987), focused attention away from the lectin recognition hypothesis.

Interest in the lectin recognition hypothesis was renewed when Díaz et al. (1989) demonstrated that transforming white clover with a pea lectin gene resulted in an extension of the host range of the transgenic clover plants to include *Rhizobium leguminosarum* bv. *viciae*. Site-directed mutagenesis of the lectin also showed that the carbohydrate-binding domain was important for host specificity (see Kijne et al., 1994). In spite of these results, however, it is difficult to draw broad conclusions that can be applied to other legume-*Rhizobium* interactions, in part because of the close relationship between the two rhizobial biovars that nodulate pea and clover (*R. leguminosarum* bv. *viciae* and *trifolii*, respectively). Unlike more distantly related rhizobia, *R. leguminosarum* bv. *viciae* can deform clover root hairs (Yao and Vincent, 1969).

As a first step to test whether or not lectin is directly involved with the establishment of indeterminate nodules, we isolated and characterized a full-length alfalfa lectin gene that encodes a protein that is 92% identical with and 96% homologous to MtLEC1 (Brill et al., 1995). We have also isolated and partially sequenced *Mslec2*, identified by its homology to *Mtlec2* (Bauchrowitz et al., 1992) (L.M. Brill and A.M. Hirsch, unpublished results). Our first approach to testing the lectin recognition hypothesis in alfalfa is to produce plants that express antisense constructs of the lectin genes. If lectin is involved in nodulation, plants that are reduced in lectin expression should be perturbed in their response to inoculation with *R. meliloti*. This report is a description of the methods used to obtain these plants, verification of antisense expression, and a partial listing of the phenotypes exhibited by the lectin antisense transgenic alfalfas early in their development. Mature plants will be analyzed in a forthcoming publication for a detailed description of the response of antisense and control plants to inoculation with rhizobia.

A second approach to testing the lectin recognition hypothesis is to transfer a lectin gene into a host which is distantly related to the source of the lectin gene and which is not cross-nodulated by the same rhizobial strain. This is a direct test and an extension of the experiments of Díaz et al. (1989). Alfalfa and *Lotus* were chosen as hosts because: neither are nodulated by the soybean symbiont, *Bradyrhizobium japonicum;* they form indeterminate and determinate nodules, respectively; and also they are readily transformable. We chose the soybean seed lectin (SBL) gene because it has been extensively characterized, and as in pea, this lectin appears to be the product of a single gene locus (Orf et al., 1978). However, in addition to SBL, which is a D-galactose/*N*-acetyl-D-galactosamine specific lectin, another lectin composed of 45 kDa subunits and with specificity

for 4-O-methylglucuronic acid, has also been isolated (Rutherford et al., 1986). The gene for this lectin has not yet been cloned and thus, its role in nodulation remains obscure.

We report here that transgenic *Lotus* harboring the SBL gene respond to *B. japonicum* inoculation by producing small, uninfected protrusions that resemble nodules in both their morphology and anatomy. However, transgenic alfalfa plants are unresponsive to *B. japonicum*.

2. Materials and Methods

Lotus transformation

Lotus corniculatus L. plants were transformed, following the procedure of Szabados et al. (1990), with the SBL gene (Vodkin et al., 1983) which had been cloned into pKYLX71 (Schardl et al., 1987). The transgenic plants were grown in a Conviron growth cabinet with a 16h/8h dark/light photoperiod and a 23°C/20°C day/night thermoperiod.

Alfalfa transformation

Alfalfa (*Medicago sativa* L.) cv. Regen was transformed with *Agrobacterium tumefaciens* carrying either the expression cassette in pART27 (Gleave, 1992) minus an insert, the antisense γ-fragment of *Mslec1* (positions 1280–1700), or the antisense β-fragment of *Mslec2* (positions 321–721) (Fig. 1). The binary vector pART27 uses the CaMV 35S promoter to drive transgene transcription. A separate group of transformants were made with the soybean lectin gene cloned into pKYLX71 (see Results).

Young leaves were harvested and surface-sterilized by treating them 15 sec with soapy water, 15 sec with 70% ethanol, 1 min with 20% commercial bleach/0.1% Tween-20, followed by three rinses in sterile water. Leaflet edges were removed with a sterile scissors, and the leaflets were then placed on B5h (Brown and Atanassor, 1985) agar plates. The leaflets were subsequently placed in 10 ml of a 1:10 dilution in B5h medium of an *A. tumefaciens* overnight culture carrying the control (vector alone) or the appropriate experimental construct. During the process of co-cultivation, the leaflets were further wounded with the tips of a forceps, and left in the liquid B5h medium with the *A. tumefaciens* for 30 min. The leaflets were subsequently placed on fresh B5h agar and incubated 3 days in a Conviron growth cabinet with a 16h/8h dark/light photoperiod and a 23°C/20°C day/night temperature regime. The excess *A. tumefaciens* cells were washed off by placing the leaflets in three changes of sterile water. The leaflets

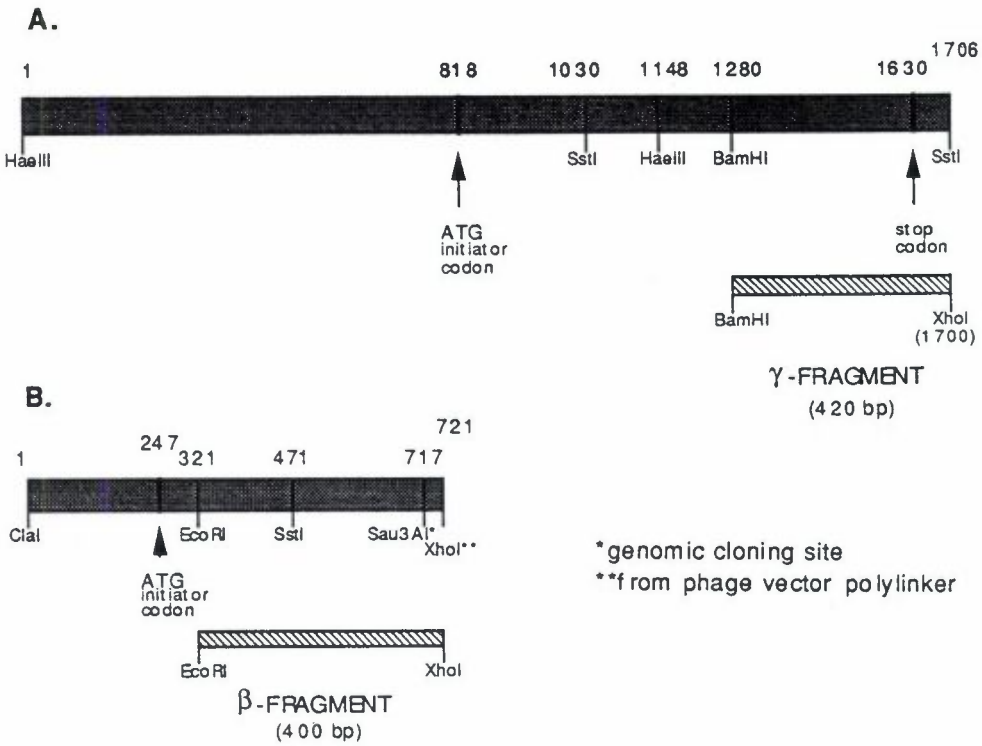

Figure 1. Restriction maps of (A) *Mslec1* and (B) the 5'-end of *Mslec2*. Not to scale.

were then transferred to fresh B5h agar plates and were incubated in the growth cabinet for an additional 4 days. After this time, the leaflets were washed as before, except that 100 μg/ml timentin was present in the last wash. The leaflets were then placed on B5h agar plates containing 100 μg/ml timentin and 25 μg/ml kanamycin. The plates were incubated in the growth chamber for 15 days. Calli were subsequently transferred to B5 agar plates containing 100 μg/ml timentin and 25 μg/ml kanamycin to induce somatic embryogenesis. After 14 days, calli and embryos were transferred to fresh B5 agar medium with antibiotics; the calli were spread over the surface of the agar with a forceps. When the embryos attained a length of 3 to 4 mm, they were removed from the calli, transferred to fresh B5 medium with antibiotics, and returned to the growth cabinet. When the embryos developed their first trifoliate leaf, they were transferred individually to Magenta jars (Magenta Corp., Chicago, IL) containing MS medium, 100 μg/ml timentin and 25 μg/ml kanamycin, and returned to the growth chamber.

Inoculations

Transgenic *Lotus* or alfalfa plants carrying the SBL gene or the vector alone were transferred to Petri dishes containing Jensen's agar minus nitrogen. To verify that the transformation did not interfere with normal nodulation, wild-type *R. loti* NZP2037 was used as inoculum for *Lotus* and *R. meliloti* 1021 was used for alfalfa. The control rhizobia, or the experimentals – *Bradyrhizobium japonicum* USDA110 or *B. japonicum* AN279 (USDA 110 *nodB*::Tn*5lacZ*) – were spot-inoculated onto several roots of individual plants following the procedure of Dudley et al. (1987).

Microscopy

Roots inoculated with various rhizobial strains were examined under a Zeiss Axiophot microscope for root hair deformation (Had), root hair curling (Hac), and cortical cell divisions (Ccd). Eight weeks after inoculation, some root segments were harvested and cleared following the procedure of Stokkermans et al. (1995). Others were fixed in glutaraldehyde:paraformaldehyde and embedded in Spurr's plastic resin as described previously (Yang et al., 1992). Sections were cut at 1-μm thickness and stained with toluidine blue (Yang et al., 1992). Lectin antisense transgenic embryos and young plants were examined using a Zeiss Stemi SV6 Dissecting Microscope. Photographs were taken with Kodak Ektachrome 160 film.

RNA analysis

Total RNA (10 μg per lane) was subjected to electrophoresis and then blotted onto Nytran as previously described (McKhann and Hirsch, 1994). Restriction fragments used as probes were labeled by random priming using α-^{32}P-dCTP (New England Nuclear, Boston, MA).

Protein analysis

Approximately 0.4 mg of fresh root or leaf tissue of transformed plants were extracted in 0.6 ml extraction buffer (PBS with 10 mM β-mercaptoethanol), and stored frozen until use. Forty μl-samples containing soluble protein were subjected to SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and electrophoretically transferred to nitrocellulose in a Transblot apparatus (Hoeffer Scientific Products, San Francisco, CA) according to standard procedures. Blots were stained with Ponceau S (Sigma Chemical Co., St. Louis, MO) to locate molecular mass markers (Sambrook et al., 1989). The blots were then probed

with a soybean seed lectin antibody (1.0 μg/ml; Sigma Chemical Co., St. Louis, MO) and the antibody-antigen complex was detected by an alkaline phosphatase reaction (Ey et al., 1986).

3. Results and Discussion

Studies on alfalfa lectin

Before alfalfa could be transformed with antisense lectin constructs, we had to determine whether we could differentiate between the two alfalfa lectin genes, *Mslec1* and *Mslec2*, that we have cloned. A BamHI-XhoI fragment of 420 bp from *Mslec1* (designated γ; Fig. 1A) was found to be significantly distinct from the comparable fragment of *Mslec2* on the basis of DNA-DNA hybridization (D. Cotter, L.M. Brill, and A.M. Hirsch, unpublished results), and a EcoRI-XhoI fragment of 400 bp of *Mslec2* (designated β; Fig. 1B) was found to be significantly distinct from the comparable fragment of *Mslec1* on the basis of DNA sequence and DNA-DNA hybridization (L.M. Brill and A.M. Hirsch, unpublished results). To evaluate lectin gene expression, we used the β and γ fragments to probe northern blots of RNAs isolated from different alfalfa tissues.

Fig. 2 represents two individual northern blots loaded with identical plant RNAs, probed with the ^{32}P-labeled γ- or β-fragments, and washed under conditions of high stringency (0.1 X SSPE, 65°C). The γ-fragment (*Mslec1*) hybridized most strongly to RNAs isolated from roots without any observable signal in stem, flower, mature leaf, and nodule RNA (Fig. 2A). The strongest hybridization was detected in uninoculated roots and in roots 1 dpi with *R. meliloti*, and decreased thereafter. Although no hybridization of the γ-fragment occurred to the RNA isolated from stems, flowers, and mature leaves following high stringency washes on this blot, we observed faint hybridization to flower RNA on other northern blots which were also washed at high stringency (data not shown).

In contrast, the β-fragment (*Mslec2*) hybridized weakly to RNA isolated from stems (lane S) and nodules (just barely detectable on other autoradiographs) and very strongly to flower (lane F) RNA (Fig. 2B). No *Mslec2* mRNAs were detected in RNA isolated from mature leaves. Signal was also detected in RNA isolated from roots 0 to 6 dpi especially in roots 1 dpi; very little expression of *Mslec2* RNA was observed in roots > 6 dpi. The fact that full-length β-probe-hybridizing transcripts were detected indicates that *Mslec2* is not a pseudogene, in contrast to *Mtlec2* (Bauchrowitz et al., 1992). A similar conclusion has been reached by Bauchrowitz et al. (1994) for the alfalfa lectin gene. Both membranes were stripped and reprobed with Msc27 (Kapros et al., 1992), a control for equal

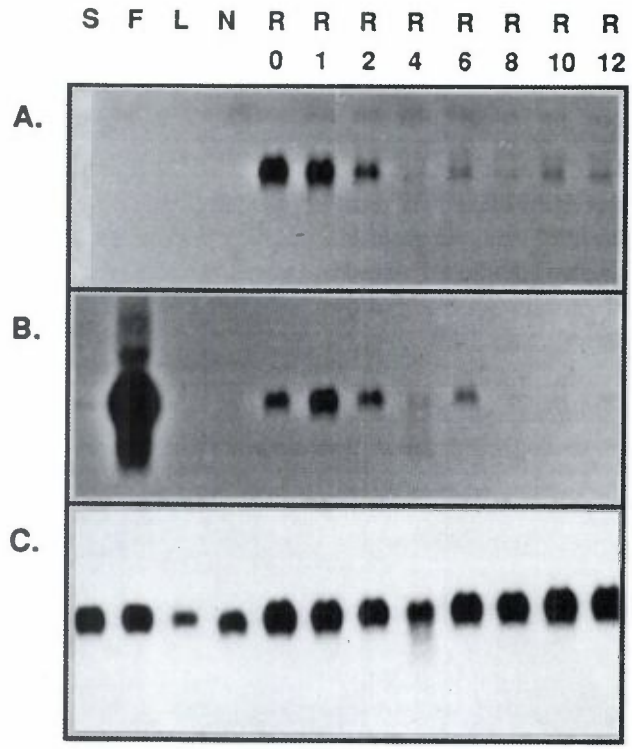

Figure 2. Northern blots of RNA isolated from different alfalfa tissues, S, stems; F, flowers, L, leaves; N, nodules; R, seedling roots 0 hours to 12 days post-inoculation (dpi) with *R. meliloti* (A) Probed with the γ-fragment of *Mslec1*. (B) A different northern blot but using the same RNAs as in (A). Probed with the β-fragment of *Mslec2*. (C) The same blot as in (A) probed with Msc27. The (B) blot probed with Msc27 was identical to this one.

loading. As both gave identical results, only one is pictured (Fig. 2C). As can be seen, the RNA from mature leaves was underloaded compared to RNAs isolated from other tissues. However, in other northern blots, there was no hybridization signal in RNA isolated from mature leaves using either the β or the γ probes (data not shown).

Based on these results, which suggest that the two fragments represented specific regions of *Mslec1* and *Mslec2*, and that *Mslec1* and *Mslec2* are differentially expressed, we cloned the γ and β-fragments each in the antisense orientation into a plant transformation vector for introduction into alfalfa.

The γ-fragment of *Mslec1* was cloned into the BamHI and XhoI sites of the primary cloning vector pART7 to produce pART7γ. The ends of the insert of pART7γ were sequenced to verify the correct orientation for antisense expression. The expression cassette of pART7γ was then cloned into the NotI site of the binary vector pART27 (Gleave, 1992) to give pART27γ. pART27γ was then electroporated into *A. tumefaciens* LBA4404, which was used to transform alfalfa plants as described in the Materials and Methods.

The β-fragment of *Mslec2* was cloned into the EcoRI and XhoI sites of pART7 to give pART7β. Sequencing of the ends confirmed the correct orientation of the fragment for the antisense experiments. Binary vector cloning, electroporation, and transformation were performed as described for the γ-fragment.

RNA was isolated from shoot tips consisting mostly of immature leaves and some stem tissue as well as from uninoculated roots of either control, γ-, or β-antisense transformed plants (Figs. 3 and 4). The RNAs were then probed with either the γ- or β-fragment to verify that the antisense mRNA was expressed and to determine whether expression of the target gene was affected by the antisense construct.

When the γ-probe was used on northern blots containing RNA isolated from shoot tips and immature leaves of γ-antisense and vector-only control lines, an antisense transcript was detected in 44 of 52 γ-antisense plants examined (RNA from 8 γ-antisense shoot tips in Fig. 3A). One transgenic plant, γ5A did not contain detectable antisense RNA (Fig. 3A, lane 3) whereas the others exhibited varying levels of antisense expression. γ-antisense transcripts were also not detected in control RNA (Fig. 3A, lane 5; Fig. 3C; lanes 6–13) or in β-antisense transgenic lines (Fig. 3C, lanes 1–5). On the other hand, a sense *Mslec1* transcript was detectable in β-antisense and control plants using the γ-probe (Fig. 3C), but not in the γ-antisense transgenic lines (Fig. 3A, lanes 1–4, 6–9).

When the β-probe was hybridized to blots containing RNA from the β-antisense lines, antisense mRNA was detected in 125 of 134 β-antisense transgenic plants (RNA isolated from five β-antisense shoot tips is shown in Fig. 3D). The Msc27-probed blots illustrated that the lanes were equally loaded (Fig. 3B, 3E).

RNA isolated from γ-antisense mature, uninoculated roots of 10 independent plant lines was subjected to northern analysis and probed with the γ-fragment (Fig. 4A). Six of 10 plant lines contained detectable antisense transcript, albeit at variable levels. Sense *Mslec1* transcripts were not detected in roots of γ-antisense plants (Fig. 4A) nor in roots of control plants using this probe (data not shown). Sense RNA was detected in all 10 plant lines, however, although at very different levels, when the β-probe was used (Fig. 4B).

When RNA from β-antisense roots of 10 different plant lines was probed with the β-fragment probe, 8 of 10 lines contained the β-antisense transcript (Fig. 4D).

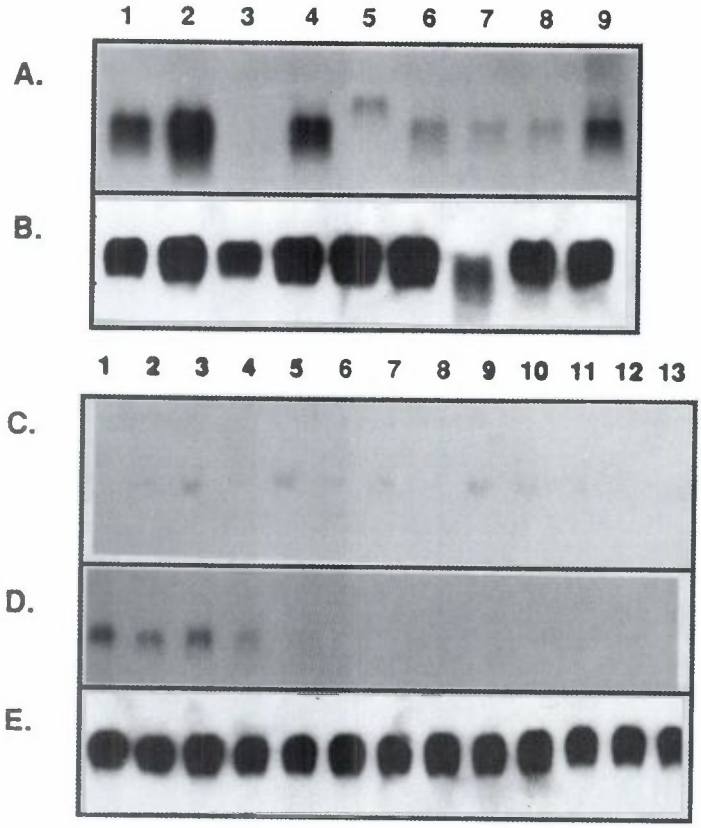

Figure 3. Northern blots of RNA isolated from shoot tips of control and lectin antisense transgenic plants. (A) RNA from γ-fragment antisense lines (lanes 1–4; 6–9) and a control plant (lane 5). Probed with the γ-fragment. The probe hybridized to the antisense transcript at ca. 700 nt, and to the sense transcript of ca. 1300 nt. (B) The same blot stripped and reprobed with Msc27. (C) RNA from β-fragment antisense (lanes 1–5) and control (lanes 6–13) plants. A faint sense signal of ca. 1300 nt is detected in most of the lanes. Probed with the γ-fragment. (D) The same blot as in (C) stripped and re-probed with the β-fragment. Bands detected were ca. 700 nt and only in the β-fragment antisense plants. No sense mRNA is found in the control lanes because the RNA was isolated from shoot tips, and *Mslec2* is not expressed in shoot tips at levels that are detectable using northern blot analysis. (E) The same blot as in (C) and (D) stripped and re-probed with Msc27.

At the same time, sense *Mslec2* mRNA was detected in RNA isolated from 7 of 10 β-antisense roots (Fig. 4D). When the blot was reprobed with the γ-fragment, no

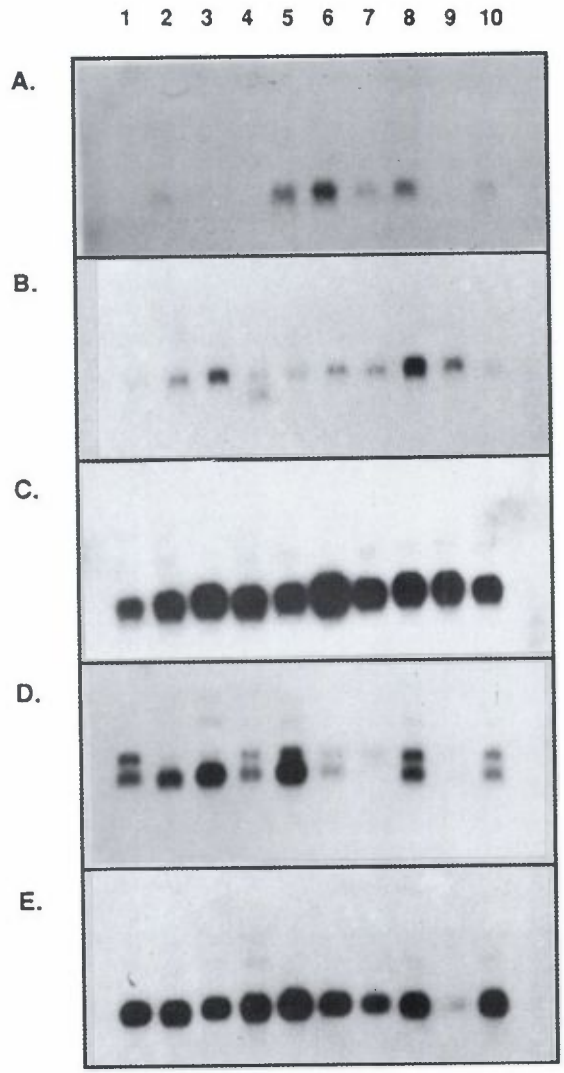

Figure 4. Northern blots of RNA isolated from roots of control and lectin antisense transgenic plants. (A) RNA isolated from uninoculated roots of 10 different γ-antisense plant lines. Probed with the γ-fragment. A ca. 700-nt antisense mRNA is detected. (B) The same blot stripped and reprobed with the β-fragment. A 1300-nt sense transcript is detected in most of the lanes. (C) The same blot as in (A) stripped and then probed with Msc27. (D) RNA isolated from uninoculated roots of 10 different β-antisense plants. Probed with the β-fragment. Both sense and antisense transcripts are detected, except for plants β8a and β24b (lanes 2, 3), which contain only antisense transcripts and plant β49b (lane 7) which contains only sense transcripts. (E) The same blot as in (D) stripped and re-probed with Msc27.

Mslec1 RNA was detected (data not shown). The Msc27-hybridized blots shown in Fig. 4C and 4E indicate that the lanes were evenly loaded, except for lane 9 in Figs. 4D, 4E.

Together with the expression patterns observed in northerns of RNA from the different alfalfa tissue types (Fig. 2), these results indicate that the β-probe is specific for *Mslec2*, which is more highly expressed than *Mslec1* in uninoculated roots from mature plants, and the γ-probe is specific for *Mslec1*, which is expressed more strongly in shoot tips than is *Mslec2*. Additionally, accumulation of the target gene's mRNA appears to be negatively affected in the γ-antisense plants and in some of the β-antisense plants.

Phenotype of lectin antisense alfalfas

There were some distinct differences among the control, γ-antisense, and β-antisense transgenic lines. Although the timing of embryo formation was slightly decreased in the antisense lines compared to the controls, the more significant difference was that overall, the antisense lines produced fewer plants per callus than the control plants (Table 1). The time until expansion of the first trifoliate leaf was slightly delayed in the γ-antisense plants compared to the controls, but the large variation seen in the individual plants did not result in a statistically significant difference among the lines (Table 1). However, fewer of the γ-antisense embryos survived to form adult plants and moreover, the stages of late embryogenesis were protracted in these plants. The antisense plants demonstrated other qualitative differences from the control plants, such as a reduction in positive geotropism of the roots of the γ-antisense plants, an increase in the number of stubby or dead roots in both sets of antisense plants compared to the controls, and also an increase in teratoma formation, especially in the β-antisense plants (L.M. Brill and A.M. Hirsch, unpublished results). However, many of the aberrant growth habits, especially those of the γ-antisense plants, were not as pronounced after the plants matured.

Transformation of alfalfa and Lotus with the soybean seed lectin gene

Both *Lotus* and alfalfa were chosen as hosts for the soybean lectin gene because these plants are representative of the two types of nodules: determinate and indeterminate. Lectin may play a number of roles in nodule development. For example, if lectin is involved in the infection process, then the type of infection thread formed may be important for the transgenic host to respond properly to the "wrong" rhizobia. Kijne (1992) suggested that rhizobial polysaccharides, such as exopolysaccharide (EPS) and capsular polysaccharide (CPS), are involved in the development of broad infection threads (typical of indeterminate nodules)

Table 1. Phenotypes of lectin antisense-expressing alfalfa transgenic plants compared to controls.

Phenotypic trait	Control	γ-antisense	β-antisense
Total number of embryos per callus ± S.D.	12.4 ± 20.6	4.1 ± 4.4	5.2 ± 6.0
Days until production of 1-mm embryos ± S.D.	32.0 ± 3.1	30.0 ± 2.6	31.0 ± 1.8
Days from oblong embryo to trifoliate leaf ± S.D.	28.5 ± 11.4	34.2 ± 13.9	30.8 ± 12.6
Total days until first trifoliate leaf ± S.D.	89.3 ± 15.2	91.1 ± 21.0	88.1 ± 18.4
Number of mature plants per callus ± S.D.	11.3 ± 20.8	2.8 ± 2.5	5.2 ± 5.5

whereas lipopolysaccharide (LPS) plays a role in the formation of narrow infection threads (typical of determinate nodules). EPS, CPS, and LPS could contain potential recognition molecules for lectin.

For the transformation of alfalfa, the plasmid containing the soybean lectin gene under the CaMV 35S promoter and the control plasmid pBI121 (Jefferson et al., 1987), were first electroporated into *A. tumefaciens* LBA4404, which was then used to infect alfalfa leaves as described in Material and Methods. During the regeneration of the transgenic plants, no remarkable differences could be detected in plants transformed with the control vector pBI121 versus those harboring the soybean lectin gene. From each plasmid, six independent transgenic lines, originating from different leaves, and which showed no differences in nodulation with *R. meliloti* compared to wild-type alfalfa plants, were chosen for further analysis.

For the transformation of *Lotus*, the plasmids were electroporated into *A. rhizogenes* A4TC24, and the resulting bacteria were used to infect young *Lotus* seedlings. Two weeks later, hairy roots appeared at the site of wounding. After another week, the transgenic hairy roots were placed on 1/2 MS medium (Murashige and Skoog, 1962) containing 100 μg/ml timentin and 25 μg/ml kanamycin; shoots spontaneously regenerated from these hairy roots. There was a difference among plants in the timing and extent of shoot formation. Although most hairy roots formed shoots in three weeks, some produced swollen, distended, and green tissue; these were discarded. From the well-developed shoots, new cuttings were made in 1/2 MS medium supplemented with 0.5

mg/ml IAA to promote rooting. Four independent lines were selected by choosing plants: (1) that exhibited normal shoot formation; (2) which, when reproduced as cuttings, retained the ability to form normal roots within three weeks after transfer; and (3) which, after inoculation with *R. loti* formed normal nitrogen-fixing root nodules within four weeks.

Alfalfa and *Lotus* transformants containing the soybean lectin gene were found to contain varying levels of lectin protein (Fig. 5). A soybean seed lectin antibody detected three cross-reacting bands of approximately 30 kDa in four transgenic alfalfa lines tested (lanes 5–8) and in two transgenic *Lotus* lines tested (lanes 2–3). The latter, represented by lane 2, may be producing a truncated lectin because the cross-reacting protein is smaller than those detected in the other transgenic lines or in an extract of soybean cotyledons (lane 10). Lectin protein, which reacted with the anti-soybean lectin antibodies, was not detected in the vector control plants in either alfalfa (lane 4) or *Lotus* (lane 1) transgenic plants.

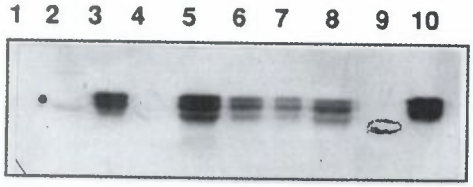

Figure 5. Immunoblot using anti-soybean lectin antiserum against protein extracts from various tissues. All except for lanes 9 and 10 are from root tissue. Lane 1, 6 μg protein from *Lotus*/vector control; lane 2, 70 μg protein from *Lotus*/lecWest; lane 3, 28 μg protein from *Lotus*/lecGR; lane 4, 16 μg protein from alfalfa/vector control; lane 5, 16 μg protein from alfalfa/lec10; lane 6, 10 μg protein from alfalfa/lec6; lane 7, 4 μg protein from alfalfa/lecB; lane 8, 24 μg protein from alfalfa/lecA; lane 9, MW markers; lane 10, 8 μg protein from cotyledon extract of *Glycine max* var. Williams.

Roots from the transgenic plants were spot-inoculated with *B. japonicum* USDA110 and examined for root hair deformation (Had), shepherd's crook formation (Hac), and cortical cell division (Ccd) events that normally take place in *Rhizobium* infection and nodule development (Table 2). All three events occurred in the transgenic *Lotus* /lecGR roots after inoculation (Table 2, Fig. 6), whereas control plants lacking the soybean lectin gene exhibited some Had, but no shepherd's crooks. Also, Ccd was not observed in control roots (data not shown). The lecWest line, which appeared to contain a faster-migrating lectin protein (Fig. 5), did not respond to *B. japonicum* inoculation.

Table 2. The effect of spot-inoculating *Bradyrhizobium japonicum* USDA110 on transgenic plants containing the soybean lectin gene

Plant	Had[a]	Hac[b]	Ccd[c]
Lotus/lecGR	+	+	+ (6/49)
Lotus/lecWest	±[d]	–	–
Lotus/lec3	+	+	+ (4/36)
Lotus/lec7	+	+	+ (2/17)
Lotus/control	±[d]	–	–
Alfalfa/lecA	+	–	–
Alfalfa/lecB	+	–	–
Alfalfa/lec6	+	–	–
Alfalfa/lec10	+	–	–
Alfalfa control	±[d]	–	–

[a]root hair deformation; [b]shepherd's crook formation; [c]cortical cell division; number of positive Ccd out of the total spots inoculated are indicated in parenthesis; [d]root hair deformation occurred but to a reduced extent.

The anatomy of the protuberances was examined in sectioned material. Cell divisions were observed in both the outer and inner cortex as would be expected for normal nodule development (Fig. 6B). However, infection threads were not present nor was there any invasion of bacteria into the newly divided cells. On the basis of structure, these protuberances appear to be true nodules, albeit uninfected. Thus, *B. japonicum* is able to elicit the formation of small, uninfected nodules on roots of *Lotus* transgenic plants carrying the soybean lectin gene. As a confirmation of this, AN279, a Nod$^-$ *B. japonicum*, does not induce Had, Hac, or Ccd on the transgenic *Lotus* plants.

However, in agreement with Kijne et al. (1994), we do not support at this time the idea that there is a direct interaction between Nod factor, the product of the *Bradyrhizobium nod* gene products, and soybean lectin. One reason for this is because we have found that alfalfa transgenic plants do not undergo Hac or Ccd in response to wild-type *B. japonicum* even though the soybean lectin gene is present; some Had takes place, however (Table 2). This suggests that in *Lotus*, lectin is probably mediating the aggregation or attachment of rhizobia such that a higher concentration of Nod factor becomes localized near the point of spot-inoculation. In the case of the transgenic *Lotus*, where the vector alone control plants exhibit Had upon inoculation with *B. japonicum*, the presence of soybean

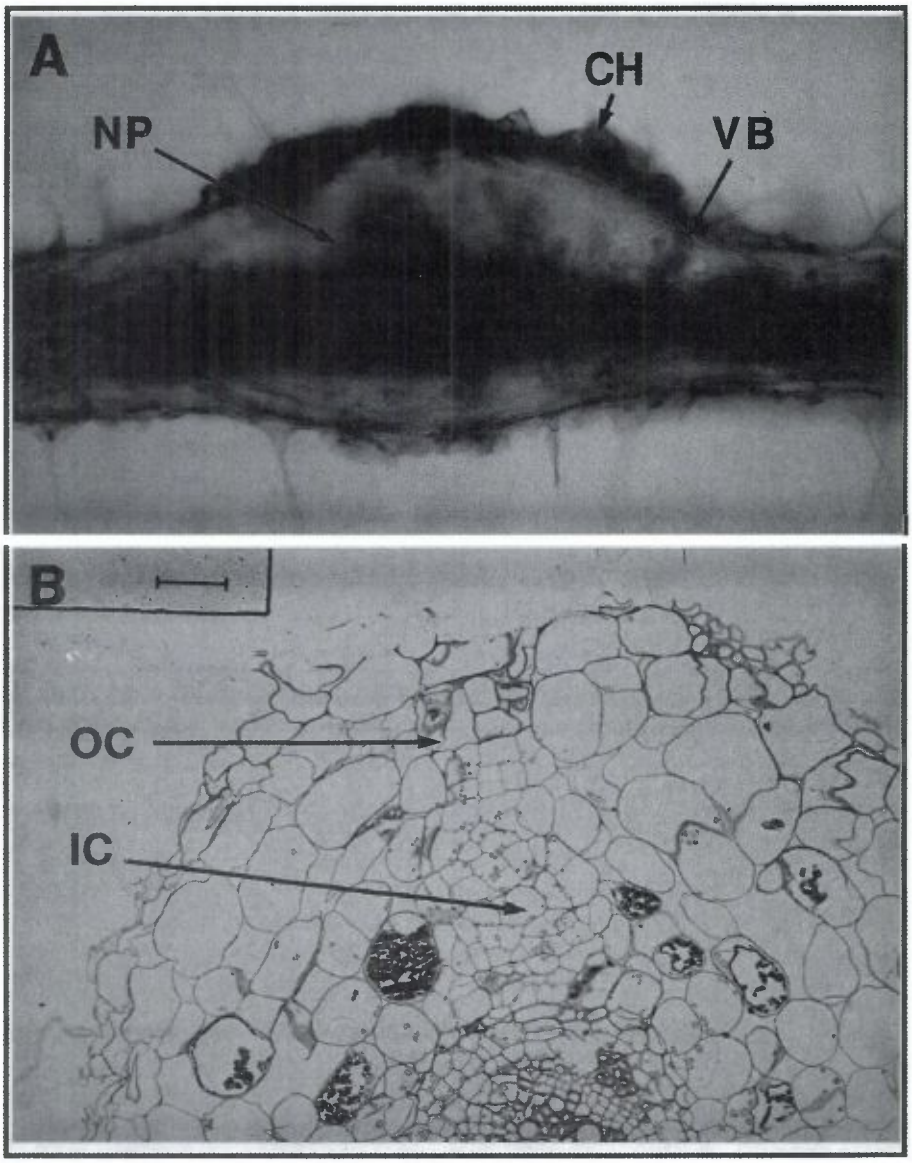

Figure 6. "Bumps" developed on transgenic *Lotus* plants carrying the soybean lectin gene after inoculation with *B. japonicum* USDA110. (A) A cleared protuberance. Vascular bundles (VB) were located at the periphery of the structure, and not in the center as would be expected for a lateral root primordium. A nodule primordium (NP) is in the center and curled root hairs (CH) are along the periphery. (B) Transverse section through a lecGR root 8 weeks after spot-inoculation with *B. japonicum* USDA 110. Cell divisions have taken place in both the outer (OC) and inner (IC) cortex.

lectin in the transgenic plants exacerbates the response due to the increased concentration of *B. japonicum* Nod factor which is partially recognized by *Lotus*. In the case of the transgenic alfalfas, there is no recognition of the *B. japonicum* Nod factor even though the density of rhizobial cells may be increased by the presence of the soybean lectin gene. Further studies are required to elucidate how lectin is involved in these early stages. The lectin antisense plants may allow us to test this idea more directly.

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