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Expression of *MsLEC1*- and *MsLEC2*-antisense genes in alfalfa plant lines causes severe embryogenic, developmental and reproductive abnormalities

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Summary

Although it has been proposed that plant lectins play a number of roles, the function of these proteins in normal plant growth and development has been unclear. To analyze the functions of putative alfalfa lectin genes, lines of transgenic alfalfa plants expressing approximately half of the open reading frame of *MsLEC1* or *MsLEC2*, in the antisense or sense orientation, were established and analyzed. The antisense plants displayed severe abnormalities in embryogenesis, and both vegetative and reproductive development were perturbed. Some differences were observed between *MsLEC1*- and *MsLEC2*-antisense plants, and abnormalities were especially severe during the early stages of development in both the primary and secondary transgenic generations. In contrast, vector-control and sense-transgene plants exhibited normal growth and development. *MsLEC1* and *MsLEC2* mRNA accumulation levels were reduced in cognate antisense plants, especially during the later stages of embryogenesis, but also tended to be low in *MsLEC1* sense-transgene plants. However, correlated with the phenotypic abnormalities observed in the *MsLEC1*-antisense plants was the specific reduction in the accumulation of a candidate MsLEC1 protein. Our results suggest that the *MsLEC1* and *MsLEC2* gene products, in addition to being important for embryogenesis, are required throughout alfalfa development.

Keywords: alfalfa, antisense-suppression, embryogenesis, lectin, plant development, plant reproduction.

Introduction

Lectins are carbohydrate-binding proteins that are ubiquitous in microbes, animals and plants (Sharon and Goldstein, 1998). The biochemistry and molecular biology of plant lectins have been extensively studied due to their widespread use as biomedical reagents and their suggested importance in plant biology (Brewin and Kardailsky, 1997; Pusztai, 1991; Sharon and Lis, 1990). However, the functions of plant lectins have remained enigmatic (Sharon and Goldstein, 1998). Proposed functions include symbiotic recognition (Bohlool and Schmidt, 1974; Díaz *et al.*, 1989; van Rhijn *et al.*, 1998), seed storage (Goldberg *et al.*, 1989), defense against predators and pathogens (Chrispeels and Raikhel, 1991), growth regulation (Howard *et al.*, 1972), and mediation of interactions between pistils and pollen (Knox *et al.*, 1976; Kovaleva *et al.*, 1999).

Lectin expression has been well characterized in large-seeded legumes such as common bean, soybean and pea (Brewin and Kardailsky, 1997; Goldberg *et al.*, 1989; Sharon and Lis, 1990), but little studied in small-seeded legumes such as alfalfa (*Medicago sativa*). The genes *MsLEC1* and *MsLEC2*, which show the highest homology to dozens of previously studied legume seed lectin genes, have been isolated and characterized from alfalfa (Brill *et al.*, 1995; L.M. Brill, unpublished results). The alfalfa genes were originally identified based on their homology to *MtLEC1* and *MtLEC2* of *Medicago truncatula* (Bauchrowitz *et al.*, 1992). *MsLEC1* encodes a putative protein that is 92% identical to MtLEC1, a presumed glucose-mannose binding protein, and *MsLEC2* is 91% identical to *MtLEC2*. Unlike *MtLEC2,* the open reading frame of *MsLEC2* is predicted to encode a full-length lectin protein. In addition, the

predicted protein products of *MsLEC1* and *MsLEC2* are 77% and 73% similar, respectively, to the full amino acid sequence of the α and β chains of the seed lectin from pea, PSL (L.M. Brill, unpublished results), a known glucose-mannose binding protein. Thus, it seems likely that both *MsLEC1* and *MsLEC2* encode lectins, although there is as yet no direct proof that the gene products are lectin proteins.

To study the function of the *MsLEC1* and *MsLEC2* genes, transgenic alfalfa plants expressing either an *MsLEC1*-antisense gene (LEC1AS plants) or an *MsLEC2*-antisense gene (LEC2AS plants) were constructed, partially characterized, and found to be stably transformed (Brill and Hirsch, 1999; Hirsch *et al.*, 1995). To test for sense-suppression induced by the *MsLEC1*- or *MsLEC2*-transgenes, LEC1ST (*MsLEC1*-sense-transgene) and LEC2ST (*MsLEC2*-sense-transgene), alfalfa plants were also constructed. Here we show that both LEC1AS and LEC2AS plants displayed a number of phenotypic abnormalities during development and reproduction whereas LEC1ST and LEC2ST plants did not significantly differ from the controls. Accumulation of *MsLEC1* and *MsLEC2* mRNAs in developing alfalfa somatic and zygotic embryos was quantitatively examined. Suppression of the accumulation of a candidate MsLEC1 protein appeared to be important for the silencing of *MsLEC1*, which led to the most severe phenotypes exhibited by the transgenic plants.

Results

MsLEC1 *and* MsLEC2 *mRNAs accumulate in alfalfa embryos*

Previously, *MsLEC1* and *MsLEC2* mRNAs were reported to accumulate to low levels in alfalfa roots and most above-ground tissues (Hirsch *et al.*, 1995), but embryonic expression was not characterized. Because lectins are highly expressed in seeds, we predicted that *MsLEC1* and *MsLEC2* would exhibit expression patterns similar to those of lectins in large-seeded legumes. We utilized gene-specific probes of both *MsLEC1* and *MsLEC2* (Hirsch *et al.*, 1995) to analyze RNA isolated from wild-type alfalfa (cv. Regen SY; Bingham, 1991) seeds.

We first determined the developmental stages of alfalfa zygotic embryos because a staging system has not been established for small-seeded legumes such as alfalfa. Regen SY plants were selfed, and the developmental stages of the embryos were described using the nomenclature of Goldberg *et al.* (1994). Embryos were at the globular stage at 8 d after pollination (dap), the transition stage at 9 dap, the heart stage at 10 dap, the torpedo stage at 11 and 12 dap, the walking stick stage at 13 dap, the maturation stage at 14–25 dap, the desiccation stage at 26–30 dap, and embryos were mature by 31 or 32 dap.

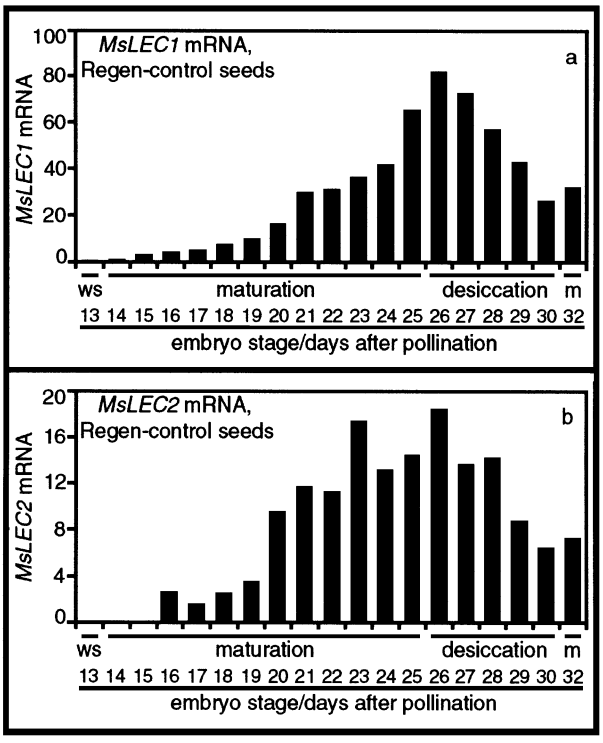

Figure 1. Accumulation of *MsLEC1* and *MsLEC2* mRNAs in whole seeds containing developing zygotic embryos of alfalfa cv. Regen SY.
RNA loading was normalized according to the constitutively expressed *Msc27* mRNA (Kapros *et al.*, 1992); vertical axes show *MsLEC1* or *MsLEC2* mRNA/*Msc27* mRNA; horizontal axes show times at which walking stick (ws) stage, maturation stage, desiccation stage and mature (m) embryos were observed in seeds at the indicated number of days after pollination. (a) Probed with a gene-specific probe of *MsLEC1*. (b) Probed with a gene-specific probe of *MsLEC2*. Data were highly reproducible.

MsLEC1 and *MsLEC2* mRNAs were first detected in alfalfa seeds when the embryos were at the walking stick and maturation stage, respectively (Figure 1a,b). High accumulation of mRNA from both genes occurred as maturation of the embryos progressed, with the maximum level at the early desiccation stage. Quantification showed that mRNA from *MsLEC1* was several-fold more abundant than mRNA from *MsLEC2* in seeds (Figure 1a,b). The mRNA from both genes was ~10- to ~100-fold more abundant in seeds than in all organs of alfalfa plants, with the exception that *MsLEC2* mRNA accumulation was highest in inflorescences (data not shown). The high level of expression of *MsLEC1* and *MsLEC2* in seeds is consistent with the proposal that these genes encode seed lectins.

MsLEC1- *and* MsLEC2*-antisense genes caused severe developmental abnormalities in the primary transgenic alfalfa lines*

Leaflets from cv. Regen SY (Regen-control) were transformed to obtain LEC1AS, LEC2AS, vector-control (Brill

and Hirsch, 1999; Hirsch *et al.*, 1995), LEC1ST and LEC2ST plant lines. Transgenic plants were regenerated by somatic embryogenesis and selected for kanamycin-resistance (kan^r). *MsLEC1* and *MsLEC2* transgenes contained approximately half of the open reading frame (Hirsch *et al.*, 1995). Cognate AS and ST plants contained the same transgene in opposite orientations, with transcription controlled by the constitutive cauliflower mosaic virus 35S promoter. Approximately 250–1300 transgenic embryos were regenerated in each experiment for each of the vector control, AS and ST plant groups.

Because somatic embryogenesis is a good developmental model for zygotic embryogenesis and is highly amenable to experimentation (Goldberg *et al.*, 1994), development of transgenic somatic embryos was examined in detail. Vector control embryos developed normally, appearing almost identical to zygotic embryos (Figure 2a,b). Somatic embryos became trifoliate-sprout plantlets when they were ~15 mm long (Figure 2c). ST embryos, which were used to test for sense suppression, also displayed normal morphology. In contrast, LEC1AS embryos exhibited vitreous tissues, proliferation of deformed organs and abnormal gravitropism (Figure 2d; compare to Figure 2b). LEC2AS abnormalities (Figure 2e) were similar to, but generally less severe than, those of the LEC1AS embryos. More than 50% of the AS somatic embryos exhibited severe developmental abnormalities.

Developmental abnormalities continued in AS plantlets. Most notably, leaves failed to expand (Figure 2f), gravitropism was abnormal and tumorigenesis was severe (not shown). Many AS plantlets died, whereas vector control and ST plantlets developed normally. Those AS plants that survived to maturity differed mildly in appearance from vector and Regen control lines. In general, the LEC1AS lines' growth habit was more prostrate and the LEC2AS lines were more upright than the controls (Figure 2g). Transgenic vector control and ST lines were visually indistinguishable from the non-transformed, Regen control plant line. We conclude that experimental introduction of *MsLEC1*- and *MsLEC2*-antisense transgenes caused morphological abnormalities, whereas the sense-transgenes had no detectable effect on alfalfa development.

Reproduction of LEC1AS and LEC2AS plant lines was severely abnormal

Experiments were performed to test the reproductive efficiency of primary transgenic lines. Four vector control, four LEC1AS and four LEC2AS lines (all stably transformed; Brill and Hirsch, 1999) were self-pollinated (selfed) in experiments designated 'A'. In experiments designated 'B', 12 vector control, 11 LEC1ST and 12 LEC2ST lines were selfed.

LEC1AS plants produced more flowers, whereas LEC2AS plants produced fewer flowers than the vector controls (Table 1, experiment A). Flower number of ST plants was similar to vector controls (Table 1, experiment B). Flower numbers for vector control plants differed between experiments A and B because the experiments were performed at different times. Thus, mean flower number is not comparable between experiments A and B. Mean dry weights of above-ground tissues of LEC1AS, LEC2AS and vector control plant groups did not significantly differ in these reproduction studies (data not shown), and therefore do not influence interpretation of flower numbers.

Successful selfing of flowers gave rise to seed pod development. Pod numbers were similar for ST and vector control plants (Table 1, experiment B). In contrast, pod number was consistently lower for AS plants than for vector controls (Table 1, experiment A), although the LEC1AS pod number was highly variable because some LEC1AS parents exhibited mild developmental abnormalities; the severity of developmental and reproductive abnormalities was correlated in AS lines.

Selfing efficiency normalized reproduction to a per flower basis. Mean percentage selfing efficiency of LEC2AS plants was low but displayed high variability (Table 1, experiment A). The LEC2AS line with the most severe developmental abnormalities exhibited 0% selfing efficiency. Mean selfing efficiency was 10-fold lower for LEC1AS plants than for vector controls. In contrast, selfing efficiency was similar among ST, vector control and Regen control plants (Table 1, experiment B and data not shown). Thus, experimental introduction of *MsLEC1*- and *MsLEC2*-antisense transgenes caused reproductive defects that were specific to the AS lines.

Second-generation transgenic LEC1AS and LEC2AS plant lines inherit severe developmental abnormalities

We previously reported that second-generation (secondary) vector and Regen control seedlings displayed normal morphology, whereas the AS seedlings showed severe deformities (Brill and Hirsch, 1999). In addition to the reported phenotypes, we commonly observed a number of other morphological irregularities, including discolored roots emerging from abnormal locations on the AS seedlings (Figure 3a), seedlings consisting of only a root and hypocotyl (Figure 3b) and vitreous, highly distorted seedlings (Figure 3c). Twenty-two per cent of the LEC1AS and 19% of the LEC2AS seedlings (out of hundreds, from eight lines) exhibited severe morphological and anatomical alterations that were not observed in vector or Regen control seedlings. However, this is probably an underestimate of the frequency of these defects because AS transgenes caused increased seed lethality (Brill and Hirsch, 1999) and decreased pod set (Table 1). The

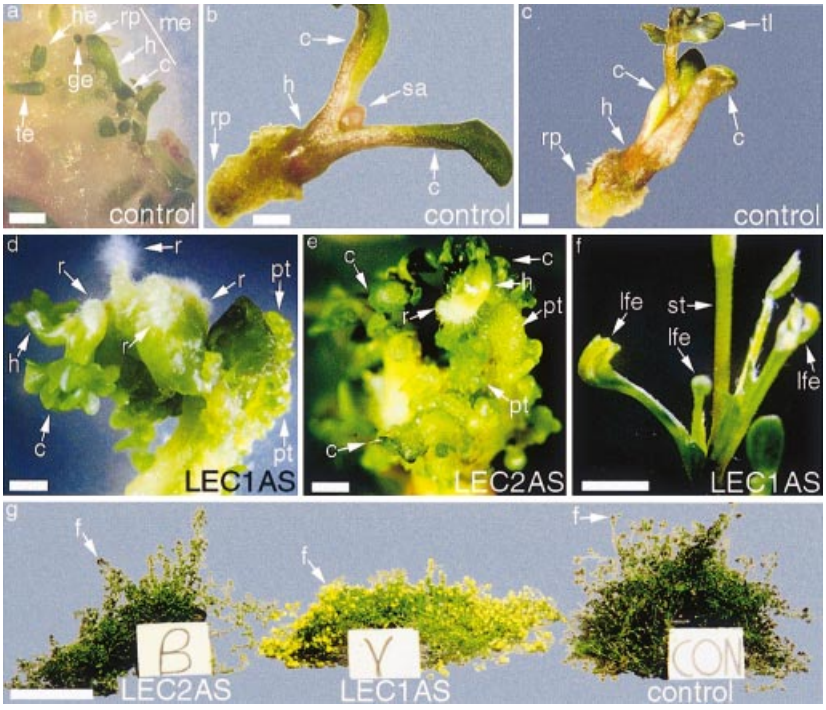

Figure 2. *MsLEC1*- and *MsLEC2*-antisense genes caused severe developmental abnormalities in first-generation (primary) transgenic alfalfa plant lines compared to control plant lines.
Plant identity is indicated at the bottom of each panel. (a) Callus (yellow mass) with vector control somatic embryos (green). (b) Developmentally advanced vector control somatic embryo. (c) Vector control trifoliate-sprout plantlet. (d) Developmentally advanced LEC1AS somatic embryo. (e) Developmentally advanced LEC2AS somatic embryo. In (a–e), scale bars represent 2 mm. Globular (ge), heart (he), torpedo (te) and maturation (me) somatic embryos; root primordium (rp); hypocotyl (h); cotyledon (c); shoot apex (sa); trifoliate leaf (tl); proliferating tissues (pt); root (r). (f) LEC1AS plantlet; scale bar represents 4 mm. Leaf failing to expand (lfe); stem (st). (g) Mature T1 plants; β, LEC2AS; γ, LEC1AS; CON, vector-control; scale bar represents 300 mm; flowers (f).

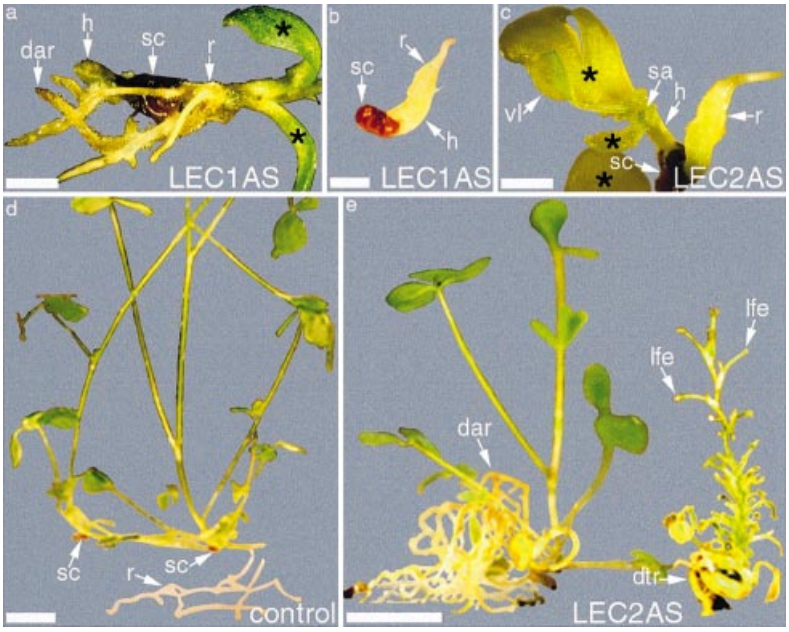

Figure 3. *MsLEC1*- and *MsLEC2*-antisense genes caused severe, heritable developmental abnormalities in many second-generation (secondary) transgenic seedlings from multiple lines of alfalfa.
All abnormal plants were kan^r, and their identity is indicated at the bottom of each panel. (a,b) LEC1AS seedlings. (c) LEC2AS seedling. Asterisks mark cotyledons (a,c); seedlings were 19 days post-imbibition (a–c); scale bars represent 5 mm (a,c) and 2 mm (b). Root (r); seed coat (sc); hypocotyl (h); discolored, aerial root (dar); vitreous leaf (vl); shoot apex (sa). (d) Vector control seedlings. Most roots were not visible due to the presence of the agar medium. (e) LEC2AS seedlings. Seedlings were 78 d post-imbibition and scale bars represent 10 mm (d,e). Discolored, tumorous roots (dtr); leaves failing to expand (lfe).

morphology of ST seedlings was normal, as determined by observing hundreds of seedlings from multiple lines in blind tests (data not shown). These tests were performed concurrently with AS, vector and Regen control lines.

Older control and ST seedlings developed normally (Figure 3d and data not shown), but AS plants were frequently stunted, some roots were aerial and/or tumorous and leaves failed to expand (Figure 3e). As found for the primary transgenics, secondary transgenic AS plants often did not survive to maturity, but the survivors exhibited mild morphological defects. We conclude that developmental abnormalities specific to secondary AS transgenic lines confirm their specificity in primary AS transgenic lines and we show that these abnormalities were heritable.

Table 1. Reproduction of alfalfa plants

Experiment A Parameter	vector-control plants	LEC1AS plants	LEC2AS plants
mean flower number/plant/selfing	977.0[b]* ± 412.7**	1828.8[c] ± 346.0	400.9[a] ± 297.7
mean pod number/plant	494.0[a] ± 278.7	87.7[ab] ± 142.3	87.8[b] ± 91.5
mean percent selfing efficiency[1]	23.9[a] ± 5.5	2.3[b] ± 3.8	9.6[ab] ± 10.1
Experiment A Parameter	vector-control plants	LEC1AS plants	LEC2AS plants
mean flower number/plant/selfing	149.6[a] ± 64.3	167.2[a] ± 114.0	173.7[a] ± 132.7
mean pod number/plant	88.0[a] ± 64.3	106.4[a] ± 69.9	118.8[a] ± 44.7
mean percent selfing efficiency[1]	26.9[a] ± 12.8	27.9[a] ± 7.9	27.0[a] ± 8.5

*Means that differed significantly in analysis of variance ($P < 0.05$) are followed by different superscripted letters, and share none of these letters.
**Standard deviation.
[1]Percentage selfing efficiency = (number of mature pods/number of flowers pollinated) × 100.

MsLEC1 *and* MsLEC2 *mRNAs accumulate in developing tissues of somatic embryos*

To test for a correlation between abnormalities of AS plants and gene expression, *MsLEC1* and *MsLEC2* mRNA accumulation was quantified. We used a gene-specific probe (Hirsch *et al.*, 1995) to demonstrate that *MsLEC1* mRNA accumulated in vector control, LEC2AS and LEC2ST embryos, with high levels of accumulation during the maturation stage of somatic embryogenesis (Figure 4a). Overall patterns and maximum accumulation of *MsLEC1* mRNA were similar in somatic embryos and developing seeds (compare Figure 1a with Figure 4a; torpedo stage somatic embryos, which lacked bending of the cotyledons, appeared to be comparable to torpedo and walking stick stage zygotic embryos). In contrast, accumulation of *MsLEC1* mRNA tended to be reduced in LEC1ST and LEC1AS somatic embryos. Although there were large standard deviations, a definite trend towards transcript reduction was evident (Figure 4a), especially during later embryogenesis where *MsLEC1* is normally highly expressed (Figures 1a, 4a).

Another gene-specific probe (Hirsch *et al.*, 1995) was used to follow *MsLEC2* mRNA accumulation throughout somatic embryogenesis (Figure 4b). In the vector control, LEC1AS and LEC1ST embryos, *MsLEC2* mRNA accumulated during embryogenesis, reaching maximum levels in large somatic embryos and trifoliate-sprout plantlets (t.s.p.). Levels of *MsLEC2* mRNA accumulation were comparable in somatic and zygotic embryos until the end of embryogenesis (Figures 1b, 4b), at which time a somatic embryo developed into a t.s.p., whereas a zygotic embryo desiccated and then entered dormancy. In the LEC2AS plants, *MsLEC2* mRNA accumulation was low during the later stages of somatic embryogenesis and the t.s.p. stage (Figure 4b). In contrast, the level of *MsLEC2* mRNA accumulation in the LEC2ST plants did not significantly differ from the controls.

These data suggest that both the LEC1AS and LEC1ST embryos exhibit lowered *MsLEC1* mRNA accumulation compared to the vector control, LEC2AS and LEC2ST lines. In contrast, the LEC2AS, but not the LEC2ST, embryos had reduced amounts of *MsLEC2* mRNA. Thus, *MsLEC2* but not *MsLEC1* mRNA accumulation levels positively correlated with normal phenotypes. The reduced level of *MsLEC1* mRNA accumulation in the LEC1ST embryos and their normal phenotypes led us to examine whether a candidate MsLEC1 protein was suppressed in plants that exhibited the altered phenotypes.

Accumulation of a candidate MsLEC1 protein was suppressed specifically in LEC1AS plant lines

Because antibodies against *Medicago* lectins are not available, antibodies against pea seed lectin (PSL; Díaz *et al.*, 1990) were used. These antibodies were expected to recognize alfalfa lectins because the predicted products of *MsLEC1* and *MsLEC2* demonstrate 77% and 73% similarity, respectively, to the full amino acid sequence of the α and β chains of PSL (L.M. Brill, unpublished results). Furthermore, legume seed lectins have been shown to demonstrate strong immunochemical cross-reactivity (Hankins *et al.*, 1979).

The anti-PSL antibodies recognized lectins in all of the legume seed extracts that were tested. PSL, phytohemagglutinins E and L (Chappell and Chrispeels, 1986), and soybean seed lectin (Gade *et al.*, 1981) were detected on Western blots (data not shown). Phytohemagglutinins E and L demonstrate 63% similarity with PSL, and soybean

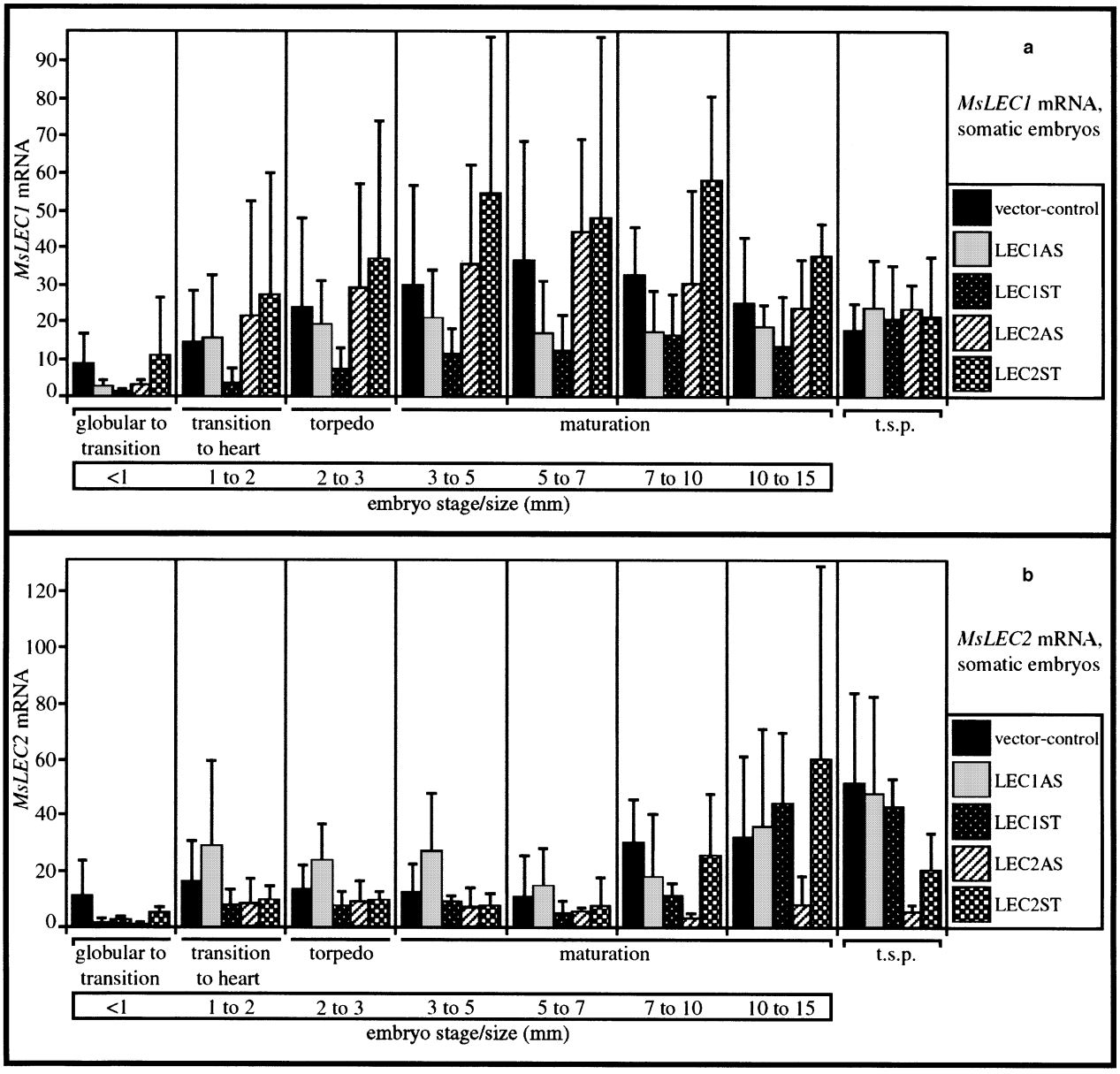

Figure 4. Accumulation of *MsLEC1* and *MsLEC2* mRNAs in developing alfalfa somatic embryos and trifoliate-sprout plantlets (t.s.p.).
RNA loading was normalized according to the constitutively expressed *Msc27* mRNA (Kapros *et al.*, 1992); vertical axes show *MsLEC1* or *MsLEC2* mRNA/*Msc27* mRNA; horizontal axes indicate that the RNA was isolated from globular stage, transition stage, heart stage, torpedo stage and maturation stage embryos (see Figure 2a,b), or t.s.p. (see Figure 2c); the sizes of the plants are also indicated on horizontal axes. Mean RNA accumulation levels and standard deviations are shown.

seed lectin is 61% similar to PSL (L.M. Brill, unpublished results). Thus, the anti-PSL antibodies recognized lectins with much less conservation to PSL than the predicted MsLEC1 and MsLEC2 proteins. A candidate lectin was recognized in alfalfa seed extracts (data not shown). The candidate alfalfa lectin was also detected in alfalfa extracts derived from roots, stems, shoot tips, leaves and flowers, and was the only reactive band on these blots (Figure 5a,b).

Extracts from vegetative shoot tips, which are composed of developing tissues that give rise to abnormal organs in LEC1AS and LEC2AS plants, were used to address the specificity of silencing of the candidate alfalfa lectin. We found that LEC1AS plants exhibited decreased accumulation of this protein compared to LEC1ST, LEC2AS, LEC2ST, vector control and Regen control lines (Figure 5b and data not shown). The antibody reacted with the candidate lectin in the extracts obtained from LEC2AS lines, but there was no decrease in the amount of this protein, even though there was a specific reduction in the accumulation of *MsLEC2* transcripts in the LEC2AS plant lines (Figures 4b,

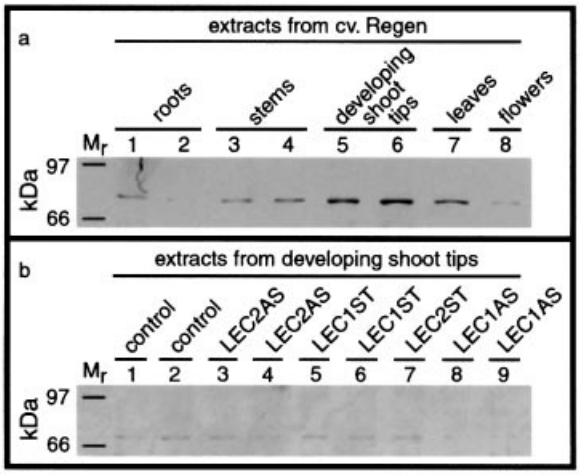

Figure 5. Western blot analysis suggested that the candidate MsLEC1 protein was specifically suppressed in LEC1AS plants.
The apparent molecular mass (M_r) of standards, in kDa, is indicated. (a) Extracts were from Regen control roots (lanes 1, 2), stems (lanes 3, 4), vegetative shoot tips (lanes 5, 6), mature leaves (lane 7) and inflorescences (lane 8). (b) Extracts of vegetative shoot tips from mature first-generation (primary) transgenic plants. An independent plant line was used for each lane. All reactive bands are shown, and staining with Ponceau-S indicated that protein loading was similar among lanes on individual blots (a,b). Because some variability was observed for the position of the reactive band relative to M_r standards among repeat blots, the M_r of these reactive bands probably did not differ significantly between (a) and (b).

5b). Thus, specific suppression in LEC1AS lines suggests that the candidate lectin could be MsLEC1, which is consistent with the more severe spectrum of phenotypic abnormalities of the LEC1AS plants.

Discussion

The functions of lectins *in planta* have been unclear, although many hypotheses have been put forth (see Pusztai, 1991; Sharon and Goldstein, 1998). To address this issue, we prepared transgenic alfalfa plants that contain stably integrated and active sense or antisense constructs of the *MsLEC1* and *MsLEC2* genes. Based on their high homology to dozens of legume seed lectin genes, it is likely that these two alfalfa genes encode lectin proteins. However, the products of *MsLEC1* and *MsLEC2* have not yet been shown to be carbohydrate-binding proteins (lectins). By examining *MsLEC1* and *MsLEC2* gene expression in zygotic embryos, we found that transcripts for the two genes accumulated at high levels in maturation stage and desiccation stage embryos, consistent with the hypothesis that they encode lectins. However, their accumulation in alfalfa seeds appears to be lower and reaches a maximum level at a developmental stage that is slightly later than lectin mRNA accumulation during embryogenesis in large legume seeds (see Goldberg *et al.*, 1989). Thus, in spite of high homology, the functions of *MsLEC1* and *MsLEC2* in embryogenesis may at least partially differ compared to seed lectin genes in large legume seeds. Very few small-seeded legumes have been examined in terms of lectin expression, so there may be, as yet, unresolved differences between these two major groups of legumes.

Based on the accumulation of *MsLEC1* and *MsLEC2* mRNAs during normal zygotic embryogenesis, as well as the developmental abnormalities observed during somatic embryogenesis of the LEC1AS and LEC2AS plants, it is likely that *MsLEC1* and *MsLEC2* are critical for alfalfa embryo development. These functions may involve growth regulation as well as storage functions, because abnormalities in tissue proliferation and organ morphology occurred in AS embryos, seedlings and plantlets. Moreover, the fact that the pod set was dramatically decreased (Table 1) and that seed lethality was increased almost threefold in LEC1AS and LEC2AS transgenic plants (Brill and Hirsch, 1999) argues strongly for the involvement of *MsLEC1* and *MsLEC2* in alfalfa embryogenesis.

Many transgenic plants display homology-dependent gene silencing upon the introduction of a sense or antisense gene construct. This often involves RNA degradation (e.g. Baulcome, 1999; Napoli *et al.*, 1990; van den Boogart *et al.*, 1998). Although the levels of cognate *MsLEC1* or *MsLEC2* mRNA in LEC1AS and LEC2AS plants were generally lower than those of the controls, decreased accumulation of *MsLEC1* mRNA was also observed in the LEC1ST lines, which did not exhibit any significant morphological deviations from the control plants. LEC1ST plants, however, showed no detectable change in accumulation of the candidate MsLEC1 protein. Thus, there appears to be a lack of functional sense-suppression in the LEC1ST lines. Taken together, the data suggest that AS-suppression of *MsLEC1* may be related to reduced levels of accumulation of the candidate MsLEC1 protein specifically in the LEC1AS lines. Van den Boogart *et al.* (1998) suggested that some cases of homology-dependent gene silencing may be RNA-mediated, whereas other cases may be protein-mediated.

The apparent molecular mass of the candidate alfalfa lectin was surprising because the predicted molecular mass of MsLEC1, as deduced from the open reading frame of the *MsLEC1* gene, is 27 kDa (Brill, 1997). However, legume seed lectins are stable proteins (Pusztai, 1991) that exist as oligomers (Hamelryck *et al.*, 1999; Sharon and Lis, 1990). Some protein oligomers that are non-covalently associated resist denaturation and demonstrate reduced mobility in SDS-PAGE (Brill *et al.*, 2000), including a portion of affinity-purified PSL (Díaz *et al.*, 1990). Furthermore, lectins are usually glycosylated, which may result in altered electrophoretic mobility. Based on the sequence of the *MsLEC1* gene, MsLEC1 is predicted to be a glycoprotein (Brill, 1997). Taken together, the specific antibody reactions, legume seed lectin conservation, specificity of homology-dependent gene silencing,

reduced accumulation in only the LEC1AS plants and the unique spectrum of phenotypes of the LEC1AS plants suggest that the ~70 kDa band may be MsLEC1.

MsLEC1 and *MsLEC2* expression displays some differences (Hirsch *et al.*, 1995; this work); likewise, LEC1AS and LEC2AS plants displayed some differing phenotypes. In the latter plants, although there was a reduction in *MsLEC2* mRNA accumulation, we were not able to identify a change in the protein that cross-reacted with the anti-PSL antibodies (Figure 5b). Because homology-dependent gene silencing is highly specific, the presence or absence of an effect of *MsLEC1* or *MsLEC2* transgenes on accumulation of the candidate alfalfa lectin can further suggest the identity of this protein. A minimum of ~80–90% nucleotide homology was proposed to be required for cross-silencing of genes in plants (Baulcome, 1999; Dangl, 1999). The coding regions of *MsLEC1* and *MsLEC2* are 80% homologous, whereas all other DNA sequences in alfalfa are less conserved (Brill, 1997). Cross-silencing of *MsLEC1* and *MsLEC2* is possible, but cross-silencing of other genes in AS or ST plants was unlikely. Although we believe it unlikely that the phenotypes observed in the LEC2AS plants were due to cross-silencing, at this time we are not certain as to the mechanism(s) whereby the LEC2AS plants exhibited altered development.

In summary, our data suggest that expression of *MsLEC1* and *MsLEC2* is important, not only for embryo and seed development, but also, based on the unusual phenotypes observed in the antisense plants, for normal vegetative development and reproduction of alfalfa. Although both LEC1AS and LEC2AS plants show a correlation between reduction in the cognate endogenous transcript and altered morphology, only the LEC1AS plants exhibit a decrease in the level of a candidate lectin protein. Thus, the mechanism(s) whereby the LEC2AS plants produce altered phenotypes remains to be elucidated. Future studies will also examine the products of the *MsLEC1* and *MsLEC2* genes and determine their sites of expression during normal growth and development. This should help us come to a better understanding of how the expression of these genes influences alfalfa vegetative and reproductive development.

Experimental procedures

Plant material

The construction of LEC1AS, LEC2AS and vector control plants has been described previously (Hirsch *et al.*, 1995).

An *Sst*I fragment of *MsLEC1* containing nucleotides 1008–1716 (Brill *et al.*, 1995) was ligated into the *Sst*I site of pUC118, which was digested with *Bam*HI, and the γ fragment of *MsLEC1* (Hirsch *et al.*, 1995) was isolated. The γ fragment was ligated into the *Bam*HI site of pART7 (Gleave, 1992), yielding pART7δ. DNA sequencing confirmed the sense-orientation of the γ fragment in pART7δ. The expression cassette of pART7δ was ligated into the *Not*I site of pART27 (Gleave, 1992), yielding pART27δ. pART27δ was electroporated into *Agrobacterium tumefaciens* LBA4404, yielding LBA4404pART27δ, which was used to generate LEC1ST transgenic somatic embryos using procedures described previously (Hirsch *et al.*, 1995).

The β fragment of *MsLEC2* (Hirsch *et al.*, 1995) was ligated into the *Eco*RI and *Bam*HI sites of pART7, yielding pART7α. DNA sequencing also confirmed that the insert in pART7α was correct. The expression cassette of pART7α was ligated into the *Not*I site of pART27, yielding pART27α. pART27α was electroporated into *A. tumefaciens* LBA4404 to yield LBA4404pART27α, which was used to generate LEC2ST somatic embryos using procedures described previously (Hirsch *et al.*, 1995).

Plantlet and plant growth, photography, reproduction and seedling analysis were as described (Brill and Hirsch, 1999; Hirsch *et al.*, 1995).

RNA blots

Somatic embryo samples were pooled by plant (transgene) group and size class. The number of embryos per sample varied from 3 to ~100, depending on their size (developmental stage). AS or ST embryos were not separated in any manner because AS-RNA accumulated in at least 85% of LEC1AS plantlets and 93% of LEC2AS plantlets (Hirsch *et al.*, 1995). Three to six independent somatic embryo samples of each size class from each plant group were used. Seed samples from selfed Regen control plants were collected from 8 to 32 d after pollination. Samples contained 5–25 seeds, depending on seed age (developmental stage).

Tissues were immediately frozen in liquid nitrogen and stored at –70°C. RNA isolation, blotting and probing was as previously described (Hirsch *et al.*, 1995), with the exception that seed RNA was isolated using a hot phenol method (Meier *et al.*, 1993). Gene-specific DNA probes (Hirsch *et al.*, 1995), with both strands randomly labeled, were used to detect endogenous mRNA, AS- and ST-RNA. RNA signal from hybridized blots was quantified with a Phosphorimager (Molecular Dynamics). Analysis of variance was used to test for significant differences in signal ($P < 0.05$).

Transgenic RNA accumulation

Transgene-encoded RNA accumulation was examined from kan^r plants to verify transgene activity. Transgenic RNAs differ in electrophoretic mobility from the endogenous *MsLEC1* and *MsLEC2* mRNAs (Brill, 1997; Hirsch *et al.*, 1995). Transgenic RNAs were present in both primary and secondary AS and ST plants, with ST-RNA signals consistently stronger than AS-RNA signals. Transgenic RNA was not detected in vector or Regen control RNAs.

Western blots

To obtain proteins, tissues were ground to a fine powder in liquid nitrogen, mixed in 10 mM Tris pH 6.8, centrifuged at 4°C for 10 min at 15 000 *g*, and supernatant (extract) protein concentrations were estimated using the Bio Rad protein assay. SDS-PAGE was performed with 20 μg of protein per lane, then proteins were electro-transferred to nitrocellulose filters (Sambrook *et al.*, 1989). Filters were incubated in Blotto (Sambrook *et al.*, 1989) for 2 h at 22°C, then for 48 h at 4°C using rabbit-anti-pea seed lectin antiserum (Díaz *et al.*, 1990) diluted 1:1000 in Blotto. Filters were washed in Blotto at 22°C and incubated overnight at 4°C with goat-anti-rabbit IgG-alkaline phosphatase conjugate (Sigma) diluted

1:1000 in Blotto. Filters were washed in Blotto followed by Tris-buffered saline (Sambrook *et al.*, 1989) at 22°C, and developed with an alkaline phosphatase color reaction.

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References

Bauchrowitz, M.A., Barker, D.G., Nadaud, I., Rougé, P. and Lescure, B. (1992) Lectin genes from the legume *Medicago truncatula*. *Plant Mol. Biol.* **19**, 1011–1017.

Baulcome, D.C. (1999) Fast forward genetics based on virus-induced gene silencing. *Curr. Opin. Plant Biol.* **2**, 109–113.

Bingham, E.T. (1991) Registration of alfalfa hybrid Regen-SY germplasm for tissue culture and transformation research. *Crop Sci.* **31**, 1098.

Bohlool, B.B. and Schmidt, E.L. (1974) Lectins: a possible basis for specificity in the *Rhizobium*-legume symbiosis. *Science*, **185**, 269–271.

Brewin, N.J. and Kardailsky, I.V. (1997) Legume lectins and nodulation by *Rhizobium*. *Trends Plant Sci.* **2**, 92–98.

Brill, L.M. (1997) Transgenic alfalfa plants expressing antisense-lectin constructs display severe developmental, reproductive, defensive, and symbiotic abnormalities. PhD Dissertation, University of California, Los Angeles.

Brill, L.M. and Hirsch, A.M. (1999) Stable transformation of T1 and T2 transgenic alfalfa with antisense-lectin constructs. *Symbiosis*, **27**, 17–32.

Brill, L.M., Konyalian, V.R. and Hirsch, A.M. (1995) A 1.7 kilobase genomic fragment of alfalfa DNA contains the lectin gene *MsLEC1*. *Plant Physiol.* **108**, 1311–1312.

Brill, L.M., Nunn, R.S., Kahn, T.W., Yeager, M. and Beachy, R.N. (2000) Recombinant tobacco mosaic virus movement protein is an RNA-binding, α-helical membrane protein. *Proc. Natl Acad. Sci. USA*, **97**, 7112–7117.

Chappell, J. and Chrispeels, M.J. (1986) Transcriptional and post-transcriptional control of phytohemagglutinin gene expression in developing cotyledons of *Phaseolus vulgaris*. *Plant Physiol.* **81**, 50–54.

Chrispeels, M.J. and Raikhel, N.V. (1991) Lectins, lectin genes, and their role in plant defense. *Plant Cell*, **3**, 1–9.

Dangl, J. (1999) Long view from a high plateau. *Nature*, **401**, 543–544.

Díaz, C.L., Hosselet, M., Logman, G.J.J., Lugtenberg, B.J.J. and Kijne, J.W. (1990) Distribution of glucose/mannose-specific isolectins in pea (*Pisum sativum* L.) seedlings. *Planta*, **181**, 451–461.

Díaz, C.L., Melchers, L.S., Hooykaas, P.J.J., Lugtenberg, B.J.J. and Kijne, J.W. (1989) Root lectin as a determinant of host plant specificity in the *Rhizobium*-legume symbiosis. *Nature*, **338**, 579–581.

Gade, W., Jack, M.A., Dahl, J.B., Schmidt, E.L. and Wold, F. (1981) The isolation and characterization of a root lectin from soybean (*Glycine max* (L) cultivar Chippewa). *J. Biol. Chem.* **256**, 12905–12910.

Gleave, A.P. (1992) A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol. Biol.* **20**, 1203–1207.

Goldberg, R.B., Barker, S.J. and Perez-Grau, L. (1989) Regulation of gene expression during plant embryogenesis. *Cell*, **56**, 149–160.

Goldberg, R.B., de Paiva, G. and Yadegari, R. (1994) Plant embryogenesis: zygote to seed. *Science*, **266**, 605–614.

Hamelryck, T.W., Loris, R., Bouchaert, J., Dao-Thi, M.H., Strecker, G., Imberty, A., Fernandez, E., Wyns, L. and Etzler, M.E. (1999) Carbohydrate binding, quaternary structure and a novel hydrophobic binding site in two legume lectin oligomers from *Dolichos biflorus*. *J. Mol. Biol.* **286**, 1161–1177.

Hankins, C.N., Kindinger, J.I. and Shannon, L.M. (1979) Legume lectins. I. Immunological cross reactions between the enzymic lectin from mung beans and other well characterized legume lectins. *Plant Physiol.* **64**, 104–107.

Hirsch, A.M., Brill, L.M., Lim, P.O., Scambray, J. and van Rhijn, P. (1995) Steps toward defining the role of lectins in nodule development in legumes. *Symbiosis*, **19**, 155–173.

Howard, I.K., Sage, H.J. and Horton, C.B. (1972) Studies on the appearance and localization of hemagglutinins from a common lentil during the lifecycle of the plant. *Arch. Biochem. Biophys.* **149**, 323–326.

Kapros, T., Bogre, L., Nemeth, K., Bako, L., Gyorgyey, H., Wu, S.C. and Dudits, D. (1992) Differential expression of histone H3 variants during cell cycle and somatic embryogenesis in alfalfa. *Plant Physiol.* **98**, 621–625.

Knox, R.B., Clarke, A., Harrison, S., Smith, P. and Marchalonis, J.J. (1976) Cell recognition in plants: determinants of the stigma surface and their pollen interactions. *Proc. Natl Acad. Sci. USA*, **73**, 2788–2792.

Kovaleva, L.V., Komarova, E.N. and Vyskrebentseva, E.I. (1999) Sporophyte–gametophyte interactions in the pollen-pistil system. 1. Cell wall lectins. *Russian J. Plant Physiol.* **46**, 83–86.

Meier, B.M., Shaw, N. and Slusarenko, A.J. (1993) Spatial and temporal accumulation of defense gene mRNAs in bean (*Phaseolus vulgaris*) leaves in relation to bacteria-induced hypersensitive cell death. *Mol. Plant-Microbe Interact.* **6**, 453–466.

Napoli, C.A., Lemieux, C. and Jorgensen, R.A. (1990) Introduction of a chimeric chalcone synthase gene into *Petunia* results in reversible co-suppression of homologous genes in trans. *Plant Cell*, **2**, 279–289.

Pusztai, A. (1991) *Plant Lectins*. Cambridge: Cambridge University Press.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular cloning. A Laboratory Manual* 2nd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Sharon, N. and Goldstein, I.J. (1998) Lectins: more than insecticides. *Science*, **282**, 1049.

Sharon, N. and Lis, H. (1990) Legume lectins-a large family of homologous proteins. *FASEB J.* **4**, 3198–3208.

van den Boogart, T., Lomonosoff, G.P. and Davies, J.W. (1998) Can we explain RNA-mediated virus resistance by homology-dependent gene silencing? *Mol. Plant Microbe Interact.* **11**, 717–723.

van Rhijn, P., Goldberg, R.B. and Hirsch, A.M. (1998) *Lotus corniculatus* nodulation specificity is changed by the presence of a soybean lectin gene. *Plant Cell*, **10**, 1233–1250.

EMBL accession number Y16754 (*MsLEC2*) and GenBank accession number U18296 (*MsLEC1*).