



The over-expression of an alfalfa RING-H2 gene induces pleiotropic effects on plant growth and development

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Abstract

The alfalfa *MsRH2-1* gene encodes a small protein with a RING-H2 motif and an N-terminal transmembrane domain. The transcript can be found in all tested plant organs, but roots and nodules show the highest levels of *RH2-1* mRNA accumulation. Promoter-GUS fusion studies demonstrate that the activity of this gene is closely correlated with development of lateral roots (in alfalfa and *Arabidopsis*) and symbiotic nodules (in alfalfa). Although antisense-expressing alfalfa plants did not show a significantly different phenotype from the control plants, by contrast, when the level of the *MsRH2-1* RNA was raised by introducing the coding part of the gene under the control of the CaMV promoter, both the transgenic alfalfa and *Arabidopsis* lines exhibited dramatic alterations in plant morphology, including shorter stature, increased apical dominance, leaf hyponasty, and inhibition of leaf venation and lateral root development. Moreover, nodulation of transgenic alfalfa roots was delayed and partially inhibited, and some of the *Arabidopsis* lines showed abnormal floral development. The nature of pleiotropic developmental phenotypes suggests a hormonal basis. The possible connection between *MsRH2-1* function and substrate specific degradation via the ubiquitin pathway involved in auxin signaling is discussed.

Introduction

The RING-finger domain, named for a Really Interesting New Gene (Hanson *et al.*, 1991), is one of the most frequently detected domains in the *Arabidopsis* proteome (Kosarev *et al.*, 2002). The amino acid RING sequence motif is a small, Cys/His-rich (C3HC/HC3), zinc-finger domain represented in two distinct variants, RING-HC and RING-H2, depending on whether Cys or His occupies the fifth position, respectively (Freemont, 2000).

RING proteins have been shown to mediate the degradation of substrate proteins required for cell cycle progression (Tyers and Jorgensen, 2000), signal transduction, and the execution of tumor suppressor

activities (Freemont, 2000). For several RING proteins, a ubiquitin ligase activity has been observed (Joazeiro and Weissman, 2000). E3 ubiquitin ligases recognize and interact with degradation substrates, and in this way are responsible for the specificity of protein degradation by the ubiquitin pathway (Hershko and Ciechanover, 1998). This pathway regulates key biological processes, such as cell division, metabolism, immune response, and apoptosis. Recent results demonstrated that the ubiquitin proteolytic system plays a central role in the auxin response pathway (Gray and Estelle, 2000).

Several RING-finger genes have been characterized in plants. The most intensively investigated plant RING-finger gene is *COPI* (Deng *et al.*, 1992), which encodes a repressor of photomorphogenesis. *COPI* acts in a multi-protein complex, associated with CIP8, a RING-H2 domain-containing protein, among oth-

The nucleotide sequence data reported will appear in the GenBank Database under the accession number AF417491 (*MsRH2-1* gene).

ers (Torii *et al.*, 1999). *RMA1*, another *Arabidopsis* gene, has been shown to function in the plant secretory pathway (Matsuda and Nakano, 1998), and recently, it has been shown that *RMA1* functions as a membrane-bound ubiquitin ligase (Matsuda *et al.*, 2001). A homologue of *RMA1*, the A-RZF gene from *Arabidopsis*, which is expressed preferentially during seed development, is proposed to form a multi-unit protein complex that is localized to the nucleus (Zou and Taylor, 1997). Finally, the *PZF* gene isolated from *Lotus japonicus* encodes a putative, nuclear-localized transcription factor (Schäuser *et al.*, 1995).

In *Arabidopsis*, a family of RING-H2 finger proteins, encoded by the *ATL* genes, has been characterized. In addition to the RING motif, all the sequences contain transmembrane domains, which are mostly located in the N-terminal end. Two members of the *ATL* gene family are early-response genes, which are rapidly induced by exposure to chitin and crude cellulase preparations. Thus, their gene products have been proposed to be involved in the early stages of the defense response (Martinez-Garcia *et al.*, 1996; Salinas-Mondragon *et al.*, 1999).

In this report, we describe the isolation and characterization of a gene from alfalfa (*Medicago sativa*) that encodes a protein containing a RING-H2 zinc finger motif. This gene is expressed predominantly during the development of roots and symbiotic nodules. When the coding region of the gene was over-expressed as a sense construct, the resulting transgenic plants were profoundly affected in both symbiosis (alfalfa) and development (alfalfa and *Arabidopsis*). Based on these results, we postulate a connection between *MsRH2-1* function and auxin signaling.

Materials and methods

Isolation of the *MsRH2-1* gene and computer analysis

The *MsRH2-1* gene was isolated as a result of a PCR-based analysis of the Nod- alfalfa mutant UMN2170 (NN-1008 [MN5863]) (Barnes *et al.*, 1988). The polymorphic PCR fragment was amplified with one primer (5'-GGGATCCAAGAGATCGACATA-3'), representing the coding part of the *MsLEC3* gene (W.M. Karłowski and A.M. Hirsch, manuscript in preparation). The conditions for the amplification were 94 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s for 30 cycles. The truncated ORF corresponding to the part of *MsRH2-1* gene was identified at one end of this fragment during sequencing.

The full cDNA clone of the *MsRH2-1* gene was isolated by using 3'- and 5'-RACE reactions with the Marathon cDNA Amplification Kit and the Advantage 2 PCR Enzyme System (Clontech Laboratories, Palo Alto, CA). Two gene-specific primers were used: forward, 5'-CAAACGCCGTTTCTCCACCGTCGTC TC-3'; reverse, 5'-ATGAGGCAGCCGCGCAAGCTC ATAAAGC-3'. The RACE reaction was carried out according to the manufacturer's instructions on poly(A)⁺ RNA isolated from 3-day old alfalfa roots.

The full genomic sequence (GenBank accession number AF417491) was identified as a result of screening an alfalfa (*Medicago sativa* cv. Chief) genomic library (Clontech Laboratories, Palo Alto, CA).

Computer-based analysis of protein domains was done with BLASTP 2.1.2 (Altschul *et al.*, 1997), DAS-Transmembrane Prediction Server (Cserzo *et al.*, 1997), Tmpred-Prediction of Transmembrane Regions and Orientation (Hofmann and Stoffel, 1993), and ProfileScan (URL: http://www.isrec.isb-sib.ch/software/PFSCAN_form.html).

Southern and northern hybridization

For Southern blot hybridizations, DNA isolated from leaves of *Medicago sativa* cv. Iroquois or *Melilotus alba* (Desr.) was extracted according to the procedure of Dellaporta *et al.* (1983). Digestion with endonucleases, gel electrophoresis, transfer onto membrane (Nytran Plus, Schleicher and Schuell, Keene, NH) and hybridization were carried out as described by Karłowski *et al.* (2000). Membranes were washed (final step) for low-stringency conditions for 20 min in 0.5 × SSC, 0.1% SDS at 55 °C, and for high-stringency conditions with 0.1 × SSC, 0.1% SDS for 20 min at 65 °C.

RNA from plant material was extracted with RNA STAT-60 reagent (Tel-Test 'B', Friendswood, TX) according to the directions of the manufacturer. The poly(A)⁺ fraction of total RNA was isolated with the Oligotex Kit (Qiagen, Valencia, CA). For northern hybridization analyses, 5 μg of poly(A)⁺ RNA were separated, transferred onto the membrane, hybridized, and washed as described by Sambrook *et al.* (1989).

Expression analysis by RT-PCR

The quality of total RNA samples was monitored by UV spectroscopy (A_{260}/A_{280} 1.85–2.0) and by agarose/formaldehyde gel electrophoresis (Sambrook *et al.*, 1989). First-strand cDNA synthesis reactions were carried out on 5 μg of total RNA

with oligo (dT)₁₈ as a primer and SuperScriptII RNase H-Reverse Transcriptase (Gibco-BRL, Carlsbad, CA) in conditions suggested by the manufacturer. Each reaction was accompanied by a negative control-RT reaction conducted on the same RNA template but without reverse transcriptase (in figures indicated with '-'). For PCR detection of the *MsRH2-1* gene, two sets of primers were used: 5'-CAAACGCCGTTCTCCACCGTCGTCTC-3'/5'-ATGAGGCAGCCGCGCAAGCTCATAAAGC-3' (reaction conditions: 95 °C for 30 s and 72 °C for 1 min; 30 cycles); and 5'-CTCCACTTCTCCGGTAACAACGCTT-3'/5'-TCTTCCCCAAATCCATTACCATTAGCTT-3' (reaction conditions: 95 °C for 30 s, 62.5 °C for 15 s and 72 °C for 40 s; 30 cycles). Additionally, on the same template, reactions with primers (5'-GGCAAAGACCAAGCTACCATCATCATGC-3'/5'-TTGTGGGAGGTTGAGGGAAAGTGGGGTTA-3') for the *Msc27* gene (Kapros *et al.*, 1992) were conducted (reaction conditions: 95 °C for 30 s, 65 °C for 15 s and 72 °C for 40 s; 30 cycles). The PCR reactions on single-stranded cDNA templates corresponding to 250 µg of total RNA were visualized on agarose gels, transferred onto a membrane, and hybridized as described for Southern hybridization.

All PCR reactions were carried out with *Taq* DNA Polymerase (Gibco-BRL) in buffers supplied by and conditions suggested by the manufacturer.

Vector construction and plant transformation

All vectors used for the plant transformation experiments were derived from pBI101 and pBI121 (Jefferson, 1987). To prepare the promoter-GUS fusion construct, a 611 bp fragment of the *MsRH2-1* gene (representing the 3'-UTR region) amplified by PCR (primers 5'-CCATTCCCgaAtTCCATACAAATTGAG-3' and 5'-GGATTTTCAGGcGcTcAAGAATGGATTA-3'; the lower-case letters in the primer sequences represent nucleotides modified to introduce restriction sites) was cloned into pBI101 vector. Next, a 2476 bp fragment (PCR-amplified with 5'-GAATTTGTGgaTCCCATTTTCGCGT-3' and 5'-GAGAAGGGTcGAcGAACTATGCATTG-3' primers), representing the 5'-non-coding end of the *MsRH2-1* gene, was introduced. As a result, a vector that contains the β-glucuronidase reporter gene coding sequence surrounded by *MsRH2-1* non-coding, putative regulatory sequences (5' end of the promoter and 3'-UTR) was obtained.

To prepare the transformation vectors for over-production and antisense expression of the *MsRH2-1* gene, the pBI121 vector with the cauliflower mosaic virus (CaMV) 35S promoter was used.

A 711 bp fragment (PCR-generated with primers 5'-ATTCGTATCGGAgctcGAGATTATGAAG-3' and 5'-AACACAAAATATTTGAggATCcTGCAAATT-3') representing the 3' end of the *MsRH2-1* coding sequence and almost the entire 3'-UTR region (minus 39 nt preceding the poly(A) part of the encoded mRNA) was cloned into the pBI121.

The vector prepared for the over-production of *MsRH2-1* RNA contained a 944 bp fragment encoding 295 amino acids of the RH2-1 protein. This fragment was generated by PCR with the following primers: 5'-TCATTATCGgagcTCCATTCTTAAACCC-3' and 5'-AAACATAGTTAGgaTcCCTGTAACAACAA-3', and then was cloned into pBI121.

Alfalfa (*Medicago sativa* L. cv. Regen) was transformed and regenerated via somatic embryogenesis as described by Hirsch *et al.* (1995). The resulting transgenic plants were generated in two independent transformation experiments. In the second experiment, a slight modification of the procedure was incorporated: the surface-sterilized young leaves were incubated on B5h agar plates for 3 to 4 days before co-cultivation with the *A. tumefaciens* strain (G. Kuleck, personal communication).

Arabidopsis thaliana ecotype Landsberg *erecta* plants were transformed with the Simplified Arabidopsis Transformation Protocol (URL: <http://www.cropsci.uiuc.edu/~abent/protocol.html>) (Clough and Bent, 1998).

Plant growth conditions and treatments

Alfalfa (*Medicago sativa* cv. Regen) and *Arabidopsis* (*Arabidopsis thaliana* ecotype Landsberg *erecta*) wild-type and transgenic plants were maintained in pots in a greenhouse. Most of the experiments were conducted in a growth chamber (Conviron, Permiana, ND) with a 16 h dark/8 h light photoperiod, and a 22 °C/20 °C day/night temperature regime.

For nodulation assays of the transgenic plants, 21 independently transformed lines over-expressing *MsRH2-1*, 5 independent antisense lines and 4 vector control lines were analyzed. Rooted cuttings of the transgenic plants were placed in sterile growth pouches (Vaughan's Seed Company, Downers Grove, IL) containing quarter-strength Hoagland's medium-N (Hoagland and Arnon, 1938). The plants were

inoculated with *Sinorhizobium meliloti* strain Rm1021 (1 ml of 10^7 cfu per ml culture per pouch). The roots were examined every 3 days. Each transgenic line (three independent cuttings) was tested in a presence of a control plant placed in the same growth pouch.

Histochemical localization of GUS activity, sectioning and microscopy

Transformed tissues were tested for GUS activity with the method described by Fang and Hirsch (1998). Some of the roots and nodules for whole-mount observations were additionally cleared with a method described by Malamy and Benfey (1997). Other blue-stained samples were either used directly for microscopic observation or embedded in paraffin via the TBA series as described by McKhann and Hirsch (1993), sectioned, and mounted onto slides for observation and photography. Photographs were taken on Kodak Ektachrome 160 film with Zeiss Axiophot or Olympus Dissecting microscopes and a Nikon camera. All photographs were prepared for publication in Adobe Photoshop 5.0 and figures were assembled in CorelDraw 7.0.

Results

Identification of a new alfalfa RING-H2 gene

This report describes a gene that was isolated during analysis of a unique polymorphic PCR fragment between UMN2170 [NN-1008 (MN5863)], a Nod⁻ alfalfa, and UMN1712 [NC-4 (MN4303)], a Nod⁺ alfalfa (Barnes *et al.*, 1988; Ehrhardt *et al.*, 1996). The DNA sequence of this 1934 bp fragment revealed on one end the presence of a truncated open reading frame, which when used for genomic library screening, allowed the isolation of the new gene, proposed to encode a RING-finger protein.

The sequence of the *MsRH2-1* gene (*Medicago sativa* RING-H2 protein-encoding gene) predicts a 295 amino acid (aa) protein (32 kDa). MsRH2-1 contains several discrete domains (Figure 1A): (1) a zinc-finger domain at the N terminus (from 83 to 124; 42 aa long), which conforms to the consensus for the RING-H2 finger class of zinc-binding protein motifs (C-X2-C-XN-C-X-H-X2-H-X2-C-XN-C-X2-C); (2) a putative transmembrane domain (from 10 to 31; length 22 aa) at the N terminus with, based on computer analysis, a 9 aa portion of the MsRH2-1 protein exposed to the outer side of the membrane; and (3) a

serine-rich region located between the transmembrane domain and the RING finger motif. No significant motifs are present at the distal C part of the protein. The amino acid sequence of MsRH2-1 is most highly homologous to the family of ATL proteins from *Arabidopsis*. Among them, ATL4 (RHX1a) (Jensen *et al.*, 1998) shows the highest level of homology (58%) and identity (46%) (Figure 1B).

Southern hybridization analysis indicates that the *MsRH2-1* is a member of a multigene family in alfalfa. High-stringency conditions resulted in a single, predominant band hybridizing to *Eco*RI-digested DNA and, in samples restricted with other enzymes, a second band of minor intensity (Figure 1C). However, less stringent conditions of hybridization resulted in numerous (6–10) hybridizing bands (Figure 1C, central panel). Interestingly, when a probe from the alfalfa *RH2-1* gene was hybridized to genomic DNA isolated from the diploid legume *Melilotus alba* (Hirsch *et al.*, 2000), only a single band was detected, even under low-stringency conditions (Figure 1C, right panel).

MsRH2-1 is expressed during lateral root development

The size and level of accumulation of *MsRH2-1* transcripts was verified by RNA hybridization analysis with a highly specific probe, representing only the 3'-coding part of the mRNA (425 bp long). A strong, visible hybridization signal was first obtained when 5 μ g of poly(A)⁺ RNA isolated from 3-day old alfalfa root tips were used (Figure 2A). The transcript of about 1.1 kb corresponded in size to the ORF identified in the *MsRH2-1* DNA sequence. Using RT-PCR analysis, we were able to detect *RH2-1* transcripts in different plant organs and tissues (Figure 2B), but to obtain the positive signals illustrated, a significant amount of RNA template had to be used (see Materials and methods). These results and those from northern blot analysis suggested that *MsRH2-1* mRNAs accumulated at very low levels in plant tissues. A specific RT-PCR band could be amplified from RNA samples isolated from roots, nodules, cotyledons, leaves, stems, flowers, and pods. Although this type of analysis is not well suited for precise quantification of transcript levels, it demonstrates that *MsRH2-1* mRNAs accumulated at the highest levels in young roots, nodules, and young flowers.

Due to the very low level of expression of *MsRH2-1* and the presence of other members belonging to the same gene family, we investigated the tissue-specific

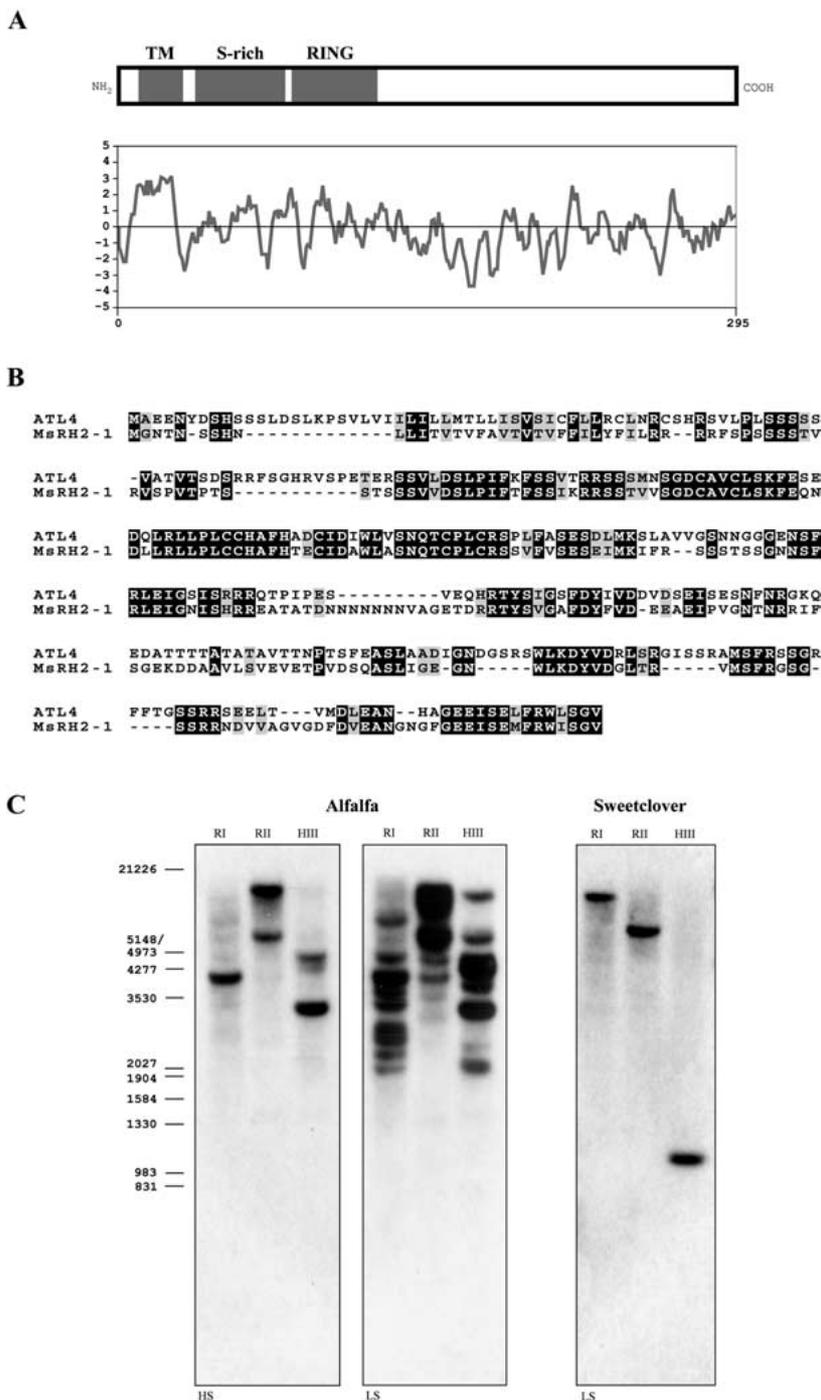


Figure 1. Analysis of the *MsRH2-1* structure and gene copy number. **A.** Schematic representation of putative domains found in the amino-acid sequence of the RH2-1 protein (top). Abbreviations: TM, transmembrane domain; S-rich, serine-rich region; RING, RINGH2 finger motif. Hydropathy plot of the RH2-1 putative protein (bottom). **B.** Amino acid sequence comparison of *MsRH2-1* and *ATL4*. Identical amino acids are marked by black and homologous amino acids by gray boxes. **C.** Southern blot hybridization of total genomic DNA isolated from alfalfa (*Medicago sativa* cv. Iroquois) and sweetclover (*Melilotus alba* U389) with a probe specific for the 3' end of the coding region of the *MsRH2-1* gene. DNA digested with *EcoRI* (RI), *EcoRII* (RII) and *HindIII* (HIII). Abbreviations: HS, high-stringency, and LS, low-stringency washing conditions (for details, see Materials and methods).

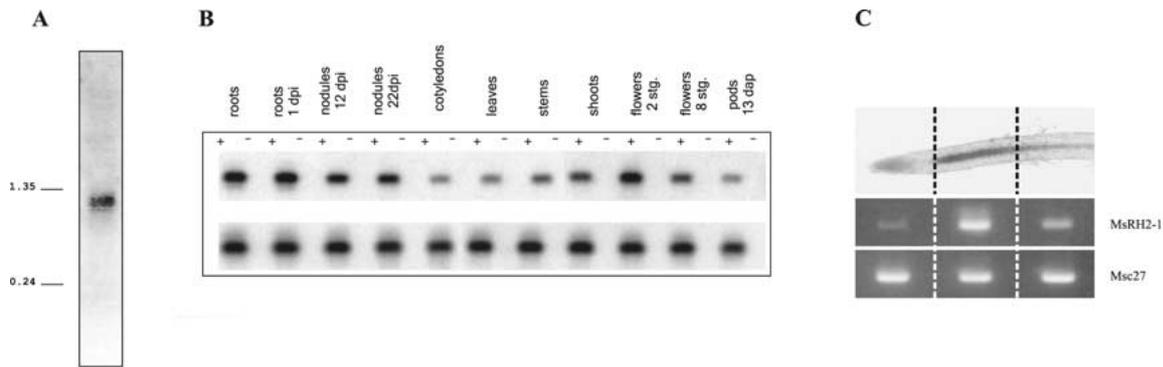


Figure 2. Analysis of expression of the *MsRH2-1* gene by hybridization and RT-PCR. A. Northern hybridization analysis of poly(A)⁺ RNA isolated from 3-day old seedling roots of alfalfa. The probe represents the 3'-coding part of the *MsRH2-1* gene. B. Identification of *MsRH2-1* mRNA by RT-PCR in different alfalfa tissues. + indicates experimental reactions with reverse transcriptase (RT) and – represents those without RT used as controls. The *Msc27*-specific band (bottom) is the loading control. C. RT-PCR-based confirmation of the localization of *MsRH2-1:GUS* promoter activity in alfalfa roots. The top picture shows stained roots of transformed alfalfa with the promoter-GUS fusion construct. *Msc27* served as the reference RNA. The RNA for the RT-PCR reaction was isolated from 40 independent wild-type alfalfa roots.

localization of *MsRH2-1* gene activity by generating transgenic alfalfa plants carrying an *MsRH2-1* promoter-reporter gene (*GUS*) translational fusion. To ensure high specificity and reproducibility of results, the coding sequence of the β -glucuronidase reporter gene was flanked by both *MsRH2-1* non-coding, putative regulatory sequences, i.e. the 5' end of the promoter (2476 bp) and the 3'-UTR (611 bp). Fourteen out of nineteen independently transformed lines exhibited the same staining pattern and were studied further. Microscopic study of whole organs revealed the highest activity of the *MsRH2-1* promoter in roots, precisely in the tissues surrounding the developing vascular bundles (Figure 3). *GUS* expression was induced as soon as the lateral roots started to develop. Strong *GUS* staining is evident at the base of a young, not yet emergent, lateral root primordium and is directly connected to the main root stele (Figure 3A). Later, when the lateral root emerged, *GUS* staining was still located at its basal end, forming a circle around the developing vascular tissue (Figure 3B). During further growth of the lateral root, *MsRH2-1* promoter activity became restricted to the vascular tissue in the differentiation zone, whereas in older tissue, the activity weakens and almost disappears (Figure 3C).

To confirm the results of the *GUS* expression analysis, RT-PCR was performed with *MsRH2-1* specific primers on mRNA isolated from 3-day old alfalfa roots. The roots were divided into three parts, representing the root tip (meristematic and elongation zones), root hair differentiation zone, and the zone of

mature root hairs. In each of the samples, the level of mRNA was established by RT-PCR and the highest level of *MsRH2-1* transcripts was detected in the root hair differentiation zone (Figure 2C). This result was full in agreement with the results of the *GUS* expression studies.

Transverse sections of roots showing the highest *GUS* signal confirmed that *MsRH2-1* promoter-*GUS* activity was localized to tissues directly connected to the root stele. In the root hair differentiation zone, the signal was located predominantly in the endodermis and the pericycle, and at lower levels, in the parenchyma of the vascular bundle (data not shown).

MsRH2-1 is active during the development of symbiotic nodules

In symbiotic nodules induced on alfalfa roots by *S. meliloti* strain Rm1021, *GUS* expression driven by the *MsRH2-1* promoter was also observed exclusively in the primordium and the tissues surrounding the developing vascular bundles. In young nodules, 6 days after inoculation (dpi), the signal was mainly restricted to cells connected to the main root stele at the nodule base (Figure 3D). In contrast to the developing root, the *GUS* signal is more extensive, making it easy to distinguish nodule from root primordia on the basis of *GUS* staining alone (Figure 3E, F). As the nodule develops, *MsRH2-1* promoter activity can be observed along the differentiating nodular vascular bundles (8 dpi; Figure 3G, H) and is no longer visible in the parent root. The *GUS* staining paralleled the position of the vascular strands, which surround the infected

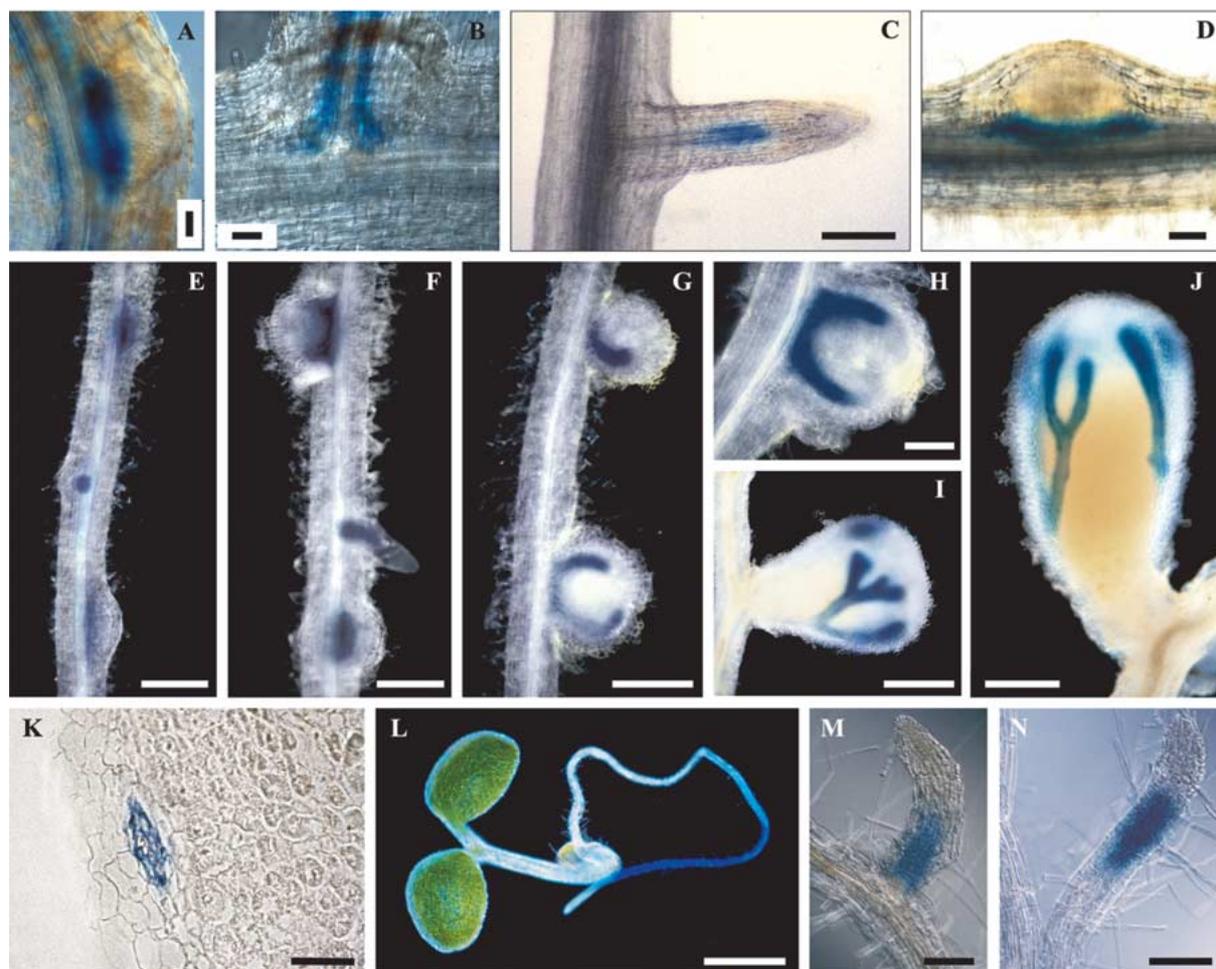


Figure 3. Expression analysis of *MsRH2-1* promoter activity in transgenic alfalfa and *Arabidopsis* plants. A. Alfalfa lateral root with strong GUS staining located at the base of a young, not yet emergent root primordium (Nomarski optics; bar, 50 μ m). B. Close-up of the base of an emergent, lateral root; blue staining is evident as a ring around the central stele (Nomarski optics; bar, 50 μ m). C. Alfalfa lateral root showing GUS expression in the differentiation zone (bar, 0.5 mm). D. Nodule primordium (6 dpi) developing on an alfalfa root. GUS staining is visible at the base of the nodule adjacent to the vascular bundles (bar, 50 μ m). E. Portion of an inoculated alfalfa root at 6 dpi. Two nodule primordia are visible (at top and bottom) and one root primordium (between the nodules). A difference in the expanse of the stained region is visible between the nodule and root primordia (bar, 1 mm). F. Older part of an inoculated alfalfa root 6 dpi (bar, 1 mm). G. Segment of an inoculated alfalfa root 8 dpi (bar, 1 mm). H. Close-up of an alfalfa nodule (8 dpi). The signal is present as a 'spur' surrounding the differentiating nodular vascular bundles (bar, 200 μ m). I and J. Two examples of alfalfa nodules at 28 dpi. GUS staining is no longer visible at the junction with the root, but is found along the differentiating vascular tissue (bars, 1 mm (I) and 0.5 mm (J)). K. Transverse section of a 28 dpi nodule showing GUS staining in the vascular tissue and surrounding cells (bar, 100 μ m). L. Transformed *Arabidopsis* seedling with strong GUS expression in the root (bar, 1 mm). M and N. Lateral roots of transformed *Arabidopsis* plants. The pattern of GUS expression is analogous to that observed in alfalfa roots (Nomarski optics; bar, 50 μ m).

central part of the symbiotic root nodule (Figure 3I, J). The level of staining was strongest in the developing vascular tissue in the proximal part of the nodule and eventually disappeared in the mature tissue, towards the distal part of the nodule. Transverse sections of the stained nodules showed a localization of *MsRH2-1* gene promoter activity that was similar to roots, i.e. in

parenchyma cells of the vascular tissue as well as the surrounding cells (Figure 3K).

The alfalfa RH2-1 gene promoter is active in Arabidopsis roots

The *MsRH2-1* promoter-GUS construct was introduced into *Arabidopsis* to examine the regulatory properties of the *MsRH2-1* gene promoter in a non-

homologous system. Five independently transformed *Arabidopsis* lines ecotype Landsberg *erecta* were propagated to the T3 generation and examined for GUS activity. All lines exhibited the same staining pattern.

In young *Arabidopsis* seedlings, the activity of *MsRH2-1* promoter can be detected in roots confirming the pattern of expression observed in *M. sativa* (Figure 3L). Also during lateral root development, the *MsRH2-1* promoter showed the highest level of activity in the root hair differentiation zone (Figure 3M, N), indicating the presence of conserved regulatory elements that drive the expression of this gene in the same plant organ and tissues as in a homologous system.

Over-expression of MsRH2-1 affects growth and development of alfalfa

Two different DNA constructs were introduced into alfalfa via somatic embryogenesis to change the level of endogenous *MsRH2-1* mRNAs as a way to elucidate the function of the *MsRH2-1* gene. The antisense DNA construct contained a short (711 bp), highly specific fragment for the *MsRH2-1* gene cloned in an antisense orientation and placed under the control of CaMV 35S promoter. The second DNA construct, which was introduced into plant tissue to raise the level of *MsRH2-1* mRNA, contained the whole coding region of this gene (a 944 bp fragment encoding 295 amino acids) placed under the direct control of the CaMV 35S promoter. This fragment was prepared so as not to contain any of the putative regulatory elements, which may have been present in the non-translated segments of this gene.

The first observed effect of introducing the antisense construct into alfalfa was the higher regeneration potential of the transformed tissue. The transgenic callus produced an increased number of somatic embryos, by ca. 70%, when compared to the vector control callus. A large number of secondary embryos also developed on the primary transformed embryos. Overall, the antisense plants grew faster under both growth chamber and greenhouse conditions, and were taller, bushier, healthier (Figures 4A and 5A), and more resistant to greenhouse pathogens (data not shown). There also appeared to be a slight increase in the number of nodules formed when the antisense plants were inoculated with symbiotic *S. meliloti* bacteria. However, the phenotypes observed in plants with a reduced level of *MsRH2-1* mRNA, which was confirmed by hybridization for five antisense lines (data not shown),

were not statistically different from that of the control plants.

When alfalfa was transformed with the construct leading to the overproduction of *MsRH2-1*, the resulting transgenic plants exhibited severe developmental abnormalities. Twelve of 21 independently transformed lines, which contained an enhanced level of *MsRH2-1* mRNA, were selected for further study. In general, growth of the plants showing an elevated level of the transgene expression was extremely inhibited; the plants were of smaller stature than the control plants and exhibited weaker branching and lateral root development (Figures 4A and 5A). Moreover, flowering in all analyzed lines was delayed by more than two months compared to the controls. We observed a direct correlation between an increased concentration of *MsRH2-1* mRNA and the severity of the abnormal phenotype (Figure 4b). The aberrant phenotype was especially manifest in leaves of the plants over-expressing *MsRH2-1*. When compared to control plants (Figure 5B), the transgenic leaves started to develop normally, but later the leaf blades did not expand. The leaves became thicker and more rigid, and frequently curled upwards, senescing at their edges. When the leaves were mature, the leaf blades were so curled as to form a 'scoop-like' shape. Transverse tissue sections of the curled leaf blades from the transgenic plants showed that they were at least 3 times thicker than the control leaves (Figure 5C compared to 5D). In addition, they had fewer veins than the control leaves (Figure 5E compared to 5F).

Over-expression of *MsRH2-1* mRNA also resulted in an inhibition of the development of symbiotic nodules. Overall, the transgenic plants developed significantly fewer nodules than the vector control lines, and nodule formation was delayed by about one week. There was a direct correlation between *MsRH2-1* mRNA levels and reduced nodulation potential. The transgenic line exhibiting the highest level of *MsRH2-1* mRNA accumulation (Figure 4B, the smallest plant on the right), even displayed a Nod⁻ phenotype 25 dpi. Other lines showing significant increases in transgene expression developed very few (1–3) nodules at the same time after inoculation.

Over-expression of alfalfa RH2-1 affects growth and development of Arabidopsis

The same DNA cassette used for over-production of *MsRH2-1* mRNA in alfalfa was introduced into *Arabidopsis* and five independently transformed lines

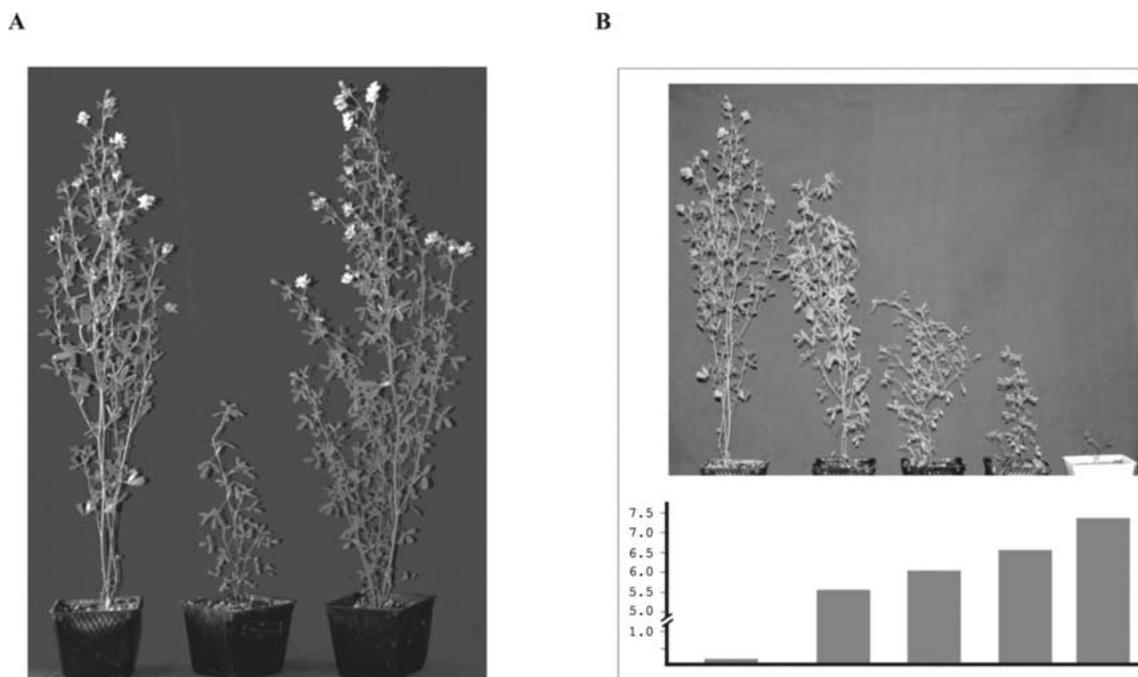


Figure 4. Alfalfa transgenic lines with altered levels of *MsRH2-1* mRNA. A. Alfalfa plants with increased (in the middle) and reduced (on the right) levels of *MsRH2-1* mRNA. The control plant is shown on the left. B. Four examples of alfalfa transgenic lines over-expressing *MsRH2-1*. The control plant is on the left. The level of *MsRH2-1* mRNA in leaves is given in arbitrary units. Similar results were obtained for RNA isolated from roots (not shown).

were selected for further analysis. These lines accumulated a range of transgene levels, and again there was a direct relationship between the amount of accumulated *MsRH2-1* mRNA and the severity of the phenotype (Figure 6A). At the same time, ectopic expression of the alfalfa gene did not result in a change in the level of RNA for the endogenous *Arabidopsis* gene, *ATLA*, which is highly homologous to *MsRH2-1* (data not shown).

The T3 generation of transgenic plants exhibited a number of severe abnormalities, which were manifest as extremely slow growth, reduced stem length, inhibition of the formation of lateral shoot branches (an extreme example is presented in Figure 6B) and delayed flowering. Moreover, an examination of the rosette leaves of the transgenic *Arabidopsis* plants revealed leaf curling similar to that observed in alfalfa (Figure 6B). However, no increase in leaf rigidity or thickness was observed.

The introduction of the *MsRH2-1* gene into *Arabidopsis* also caused changes in flower morphology, a phenotype previously not observed in the transgenic alfalfa lines. In *Arabidopsis* flowers, numerous carpels were already visible at a stage at which the wild-type

floral buds were closed (Figure 6C compared to 6D). Moreover, petal development was inhibited, with the different plant lines showing variable degrees of petal reduction. In some cases, two of the four petals were reduced in size thereby generating asymmetric flowers (Figure 6E, F). A mosaic effect was observed for entire branches of some of the transgenic plants; branches with abnormal flowers grew on the same plant as branches having wild-type flowers (Figure 6G).

Discussion

The over-expression of *MsRH2-1* in alfalfa and in *Arabidopsis thaliana* leads to dramatic alterations in plant morphology. The pleiotropic developmental phenotypes suggest defects in the ability to perceive or transmit hormonal information. The nature of the observed changes in plant structure and morphology suggests a disruption of auxin-related signaling pathway(s). Auxin is fundamentally important in plant growth and development as a regulator of root and flower development, leaf morphogenesis, vascular differentiation, stem elongation, and shoot apical dominance (Reed, 2001). Most mutations in domain II of

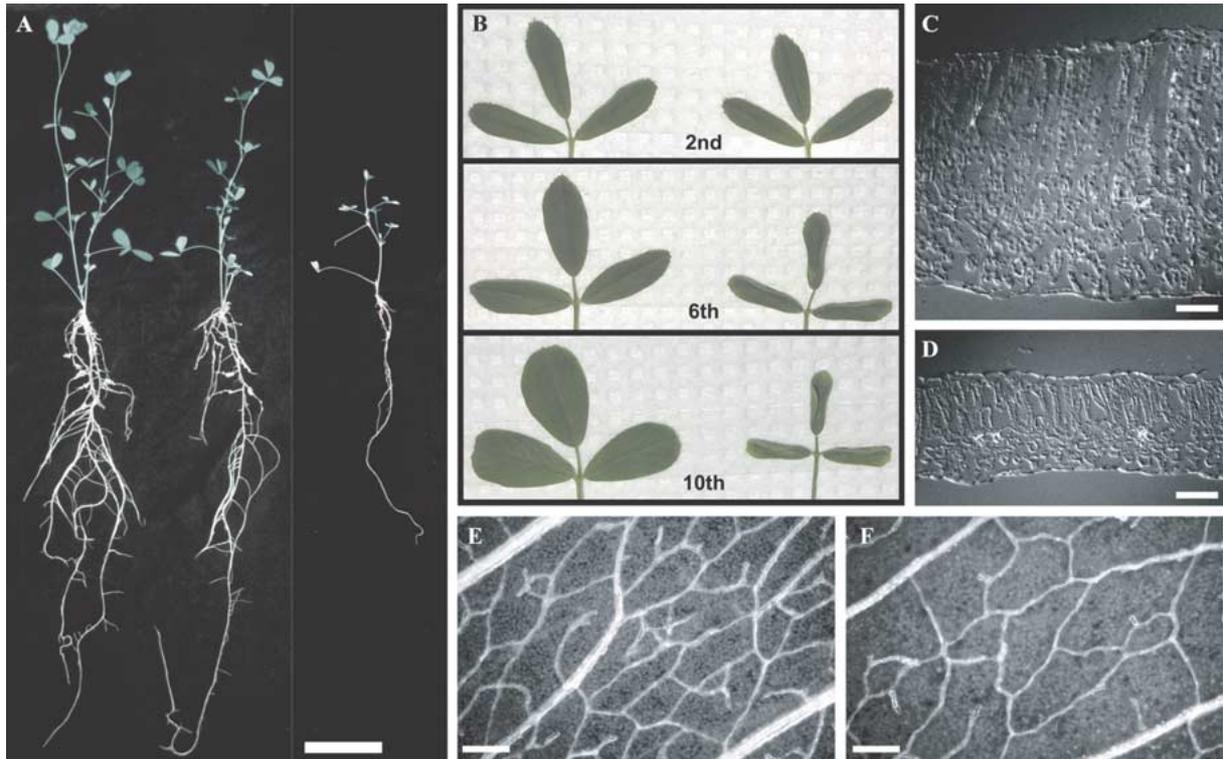


Figure 5. Analysis of alfalfa transgenic plants transformed with sense and antisense constructs. **A.** Comparison of an entire plant with a decreased (antisense) level of *MsRH2-1* mRNA (left) with an alfalfa line overproducing (sense) *MsRH2-1* mRNA (right). The vector control plant is in the middle (bar, 2.5 cm). **B.** Comparison of three developmental stages of leaves from control plants (left) and plants over-producing *MsRH2-1* RNA (right). 2nd, 6th and 10th represent plastochrons from which the leaves were collected. **C** and **D.** Transverse sections through leaves collected from transgenic, over-producing plants (**C**) and control (**D**). The sense, over-expressing leaves are at least 3 times thicker (Nomarski optics; bar, 50 μ m). **E** and **F.** Comparison of leaf venation patterns of plants overproducing *MsRH2-1* RNA (**F**) versus controls (**E**). The pictures represent corresponding parts of the leaves (bar, 0.5 mm).

different *Aux/IAA* genes cause phenotypes with hyponastic leaves (Reed, 2001). Plants with increased level of *MsRH2-1* mRNA exhibited shorter stature, increased apical dominance, inhibited leaf venation and lateral root development as well as leaf hyponasty.

Arabidopsis flowers expressing elevated levels of alfalfa *MsRH2-1* mRNA produced the most spectacular and pleiotropic phenotypes. The most consistent flower aberration was reduction of petals, which strongly resembles the phenotype of the *Arabidopsis* *PETAL LOSS* mutant (Griffith *et al.*, 1999). A similar flower morphology was observed in the *Arabidopsis* *bus1-1* mutant, which showed increased auxin content (Reintanz *et al.*, 2001). This mutant also exhibited leaf hyponasty and retardation of vascular strand formation. Reduction of petals in connection with leaf hyponasty was also reported for the *Arabidopsis* *hyl1* mutant, which is affected in responses to abscisic acid, auxin, and cytokinin (Lu and Fedoroff, 2000).

The transgenic alfalfa plants were regenerated via somatic embryogenesis. This experimental procedure made it possible to observe a high regeneration potential of tissues transformed with the antisense construct. The involvement of hormones, especially auxin, in the formation of somatic embryos in alfalfa has been previously reported in the literature (Dudits *et al.*, 1995). However, the mature antisense plants exhibited only subtle differences in phenotype from the control plants, and thus it was impossible to make any final conclusions based on their morphology. *MsRH2-1* is a member of a multi-gene family, and it is likely that plants with specifically decreased levels of this particular transcript could exhibit functional complementation by other members from within this gene family.

The expression of the *MsRH2-1* gene is correlated with the development of lateral roots and symbiotic nodules. First, the promoter is specifically activated

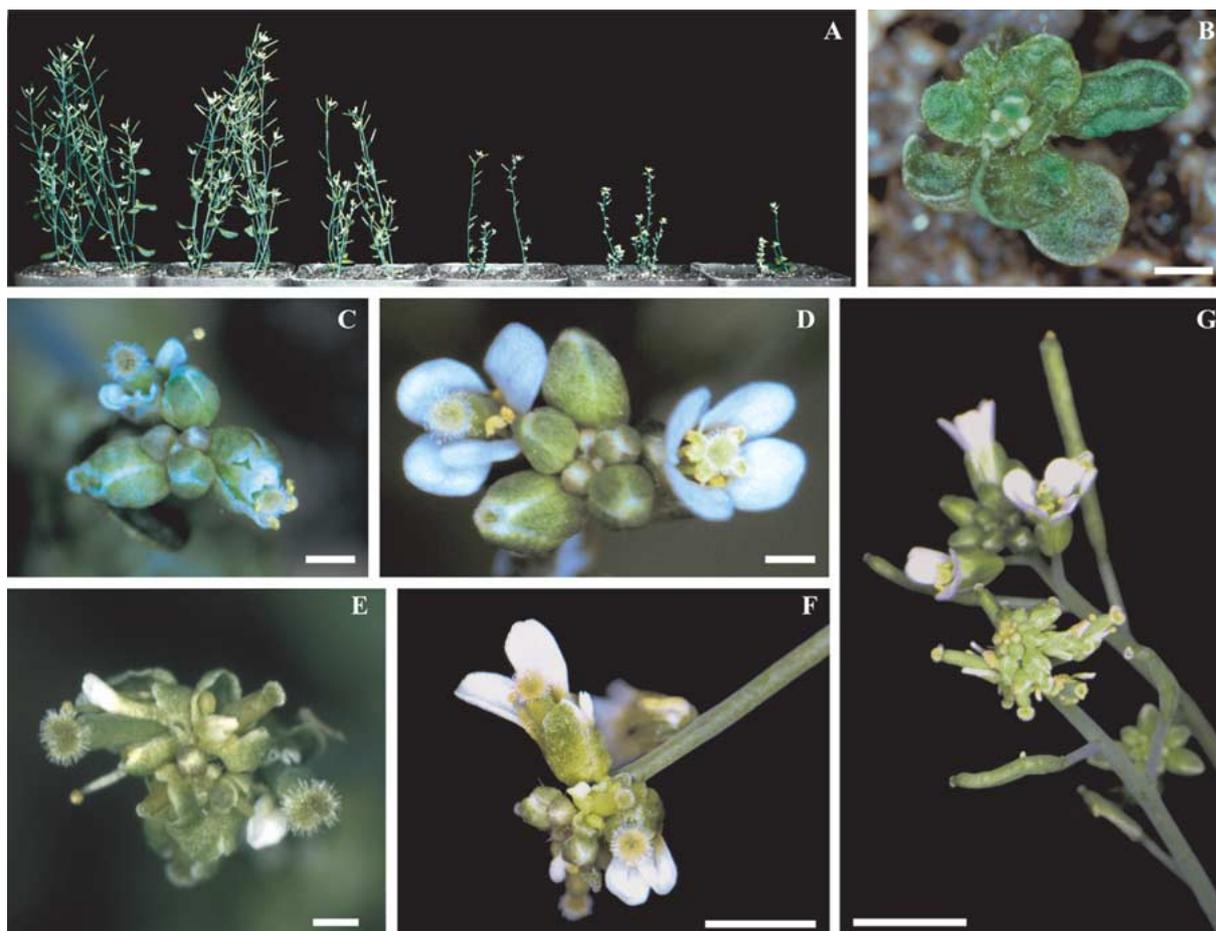


Figure 6. *Arabidopsis* plants overproducing alfalfa *RH2-1* mRNA. **A.** Five independent *Arabidopsis* transgenic lines, overexpressing alfalfa *RH2-1* mRNA, with different levels of transgene expression. The control plant is on the left. **B.** Example of an *Arabidopsis* plant expressing the alfalfa *RH2-1* gene at a very high level (bar, 1 mm). **C** and **D.** Comparison between transgenic (**C**) and control, wild-type (**D**) *Arabidopsis* flowers at the same stage of development (bar, 0.5 mm). **E** and **F.** Characteristic reduction of petals in flowers from *Arabidopsis* plants over-expressing the alfalfa *RH2-1* gene, sometimes in the form of asymmetric flowers with two reduced petals (**F**) (bars, 2 mm). **G.** Example of a 'mosaic' phenotype; two branches from the same transgenic plant have distinct flower phenotypes. The branch with reduced petals and flowers shows additional abnormalities in seed and silique formation (bar, 3 mm).

in the vascular tissue of the main root at the base of the lateral root or nodule primordia. Later, the expression is restricted to specific developmental stages of the differentiating vascular bundles. This pattern of localization of *MsRH2-1* expression suggests that this gene might be involved in auxin signaling. Expression studies on *AUX1*-like genes in *Medicago truncatula* suggest that auxin is required at two stages common to both lateral root and nodule development: development of the primordia and differentiation of the vasculature. During lateral root and nodule development, the genes are expressed in the primordia and in the regions of the developing organs where the vas-

culature arises (central position for lateral roots and peripheral region for nodules) (de Billy *et al.*, 2001).

Among numerous developmental processes in plants, the establishment of the nitrogen-fixing symbiosis between soil bacteria of the family Rhizobiaceae and leguminous plants is perhaps one of the most interesting models for investigating the mechanisms involved in formation of plant organs. Our experiments show that increased level of *MsRH2-1* transcript not only affects nodule formation, but also dramatically changes other aspects of plant development. We cannot exclude the possibility that the observed inhibition of nodulation is connected with the overall condition of the transgenic plants. On the other

hand, plant control of nodulation involves numerous genes, and many of them are involved in general plant growth and development. Therefore, it is not known at this time, whether or not *MsRH2-1* is directly involved in the development of nodules.

The *MsRH2-1* gene was isolated during the analysis of a non-nodulating alfalfa mutant (Ehrhardt *et al.*, 1996). The polymorphic fragment that differentiates the sequences from UMN2170 and UMN1712 was localized in the 5'-putative regulatory part of the *MsRH2-1* gene. Analysis of this fragment revealed the presence of conserved sequences responsible for binding of the SKN-1 transcription factor, which specifies early embryonic cell fates in *Caenorhabditis elegans* (Blackwell *et al.*, 1994). Although the characterized polymorphism seems to be unique for the mutant – we have tested several cultivars of alfalfa and only the analyzed plant was lacking the putative regulatory fragment – our co-segregation studies failed to correlate it explicitly with the Nod– mutation (data not shown).

The *MsRH2-1* gene codes for a protein with a RING-H2 domain, a type of protein that is reported to be involved in the substrate specific degradation via the ubiquitin pathway. Based on a number of RING-finger proteins found in the *Arabidopsis* genome, it seems that protein degradation regulates many processes important for plant growth and development. Unfortunately, little is known about the function of specific RING-finger proteins and their degradation substrates. Similarly, only few plant genes of this type are characterized as mutants (Kosarev *et al.*, 2002). For now, we see a strong correlation between the level of expression of *MsRH2-1* and various effects on plant development, which suggest the role of hormones, especially auxin, in the production of observed phenotypes. The pattern of expression of the *MsRH2-1* gene resembles activity of the *AUX1*-like genes from *Medicago truncatula*. However, the final answer to the question whether the *MsRH2-1* gene is involved in the development of the primordia and differentiation of the vasculature during root and nodule development via auxin signaling and targeted protein degradation will have to wait for the isolation of the RH2-1-interacting substrate(s).

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