RESEARCH COMMUNICATION

MtHAP2-1 is a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 in Medicago truncatula

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In the model legume *Medicago truncatula,* we identified a new transcription factor of the CCAAT-binding family, *MtHAP2-1,* for which RNA interference (RNAi) and in situ hybridization experiments indicate a key role during nodule development, possibly by controlling nodule meristem function. We could also show that *MtHAP2-1* is regulated by microRNA169, whose overexpression leads to the same nodule developmental block as *MtHAP2-1* RNAi constructs. The complementary expression pattern of miR169 and *MtHAP2-1* and the phenotype of miR169-resistant *MtHAP2-1* nodules strongly suggest, in addition, that the miR169-mediated restriction of *MtHAP2-1* expression to the nodule meristematic zone is essential for the differentiation of nodule cells.

Supplemental material is available at http://www.genesdev.org.

The symbiotic association between nitrogen-fixing bacteria, collectively known as rhizobia, and plants belonging to the legume family, results in the formation of specialized root organs called nodules, inside which differentiated rhizobia reduce atmospheric nitrogen to ammonium for the benefit of the plant. In the model legume *Medicago truncatula*, nodules are indeterminate; they originate from root cortical cells that are stimulated by rhizobia to re-enter mitosis, which leads to the formation of a persistent meristem, allowing the subsequent development of the nodule (Geurts et al. 2005; Stacey et al. 2006). To obtain insights into the largely unknown mechanisms regulating root nodule development, transcriptome studies were performed in *M. truncatula* to

search for transcription factors (TFs) implicated in early stages of nodulation. In this way we identified a gene, *MtHAP2-1,* whose expression is strongly up-regulated during nodule development (El Yahyaoui et al. 2004). This gene encodes an HAP2-type TF subunit, a component of the hetero-trimeric CCAAT-box-binding factor complex (CBF/NF-Y/HAP), comprising HAP2 (CBF-B, NF-YA), HAP3 (CBF-A, NF-YB), and HAP5 (CBF-C, NF-YC) subunits and characterized by its ability to bind the CCAAT motif present in many eukaryotic promoters (Maity and de Crombrugghe 1998). Interestingly, several genes coding CBF subunits have been shown to play central roles in development. This is the case for *CBF-B* genes in the mouse (Bhattacharya et al. 2003), as well as for *LEAFY COTYLEDON* (*LEC1*) and *LEAFY COTYLE-DON1-LIKE* (*L1L*), two *HAP3* genes shown to be central regulators of embryogenesis in *Arabidopsis thaliana* (Lotan et al. 1998; Kwong et al. 2003; Lee et al. 2003). Similarly, *HAP3* genes are involved in chloroplast biogenesis in rice (Miyoshi et al. 2003) and HAP5 in flowering-time control in *Arabidopsis* (Ben-Naim et al. 2006). In contrast to yeast and animals (where only one or two genes encode each CBF subunit), plants posses significantly more complex gene families. In *A. thaliana* there are 10
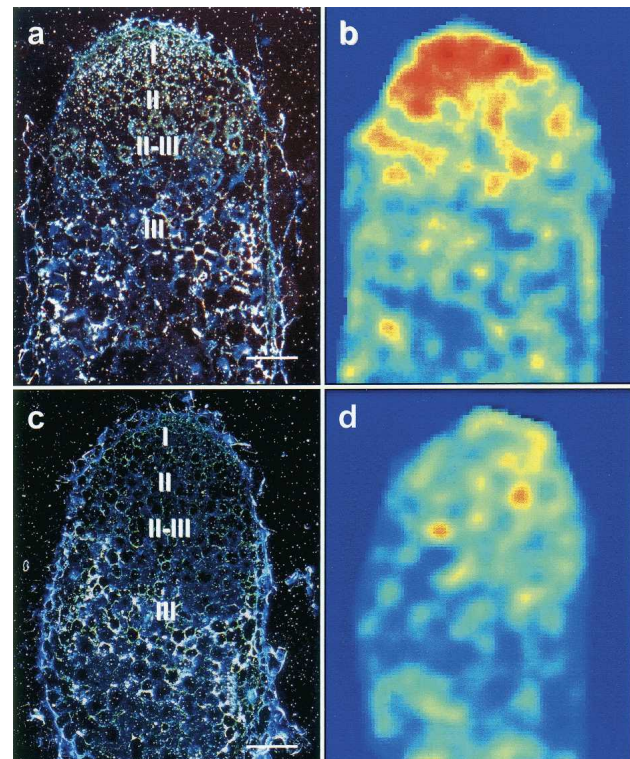
Figure 1. *MtHAP2-1* expression is nodule specific and restricted to the meristematic-zone mRNA in situ hybridization of *MtHAP2-1* in nitrogen-fixing nodules. Sections are hybridized with an antisense *MtHAP2-1* probe (*a,b*) or a control sense *MtHAP2-1* probe (*c,d*). *a* and *c* are dark-field microscopy images, to visualize silver grains corresponding to *MtHAP2-1* mRNA localization. Nodule zones are indicated according to Vasse et al. (1990) (I, meristematic zone; II, infection zone; II–III, interzone; III, nitrogen fixation zone). *b* and *d* are color density maps performed using Image-Pro plus and Diatrack softwares. Bar, 100 μm.

HAP2, 10 *HAP3*, and nine *HAP5* genes (Gusmaroli et al. 2002). Functionally specialized gene family members controlling specific developmental pathways thus appear to exist in plants, possibly because of their ability to bind to other TFs such as MADS-box TFs (Masiero et al. 2002) or nuclear regulators such as CONSTANS (Ben-Naim et al. 2006).

Results and Discussion

We therefore undertook a detailed characterization of the *MtHAP2-1* gene, which is strongly and specifically activated during symbiotic interactions (Supplementary Fig. 1). In situ hybridization showed that *MtHAP2-1* mRNA is most abundant in cells of the nodule meristematic zone, less abundant in adjacent cells of the nodule infection zone, and not detected in all other nodule tissues (Fig. 1). The symbiotic role of *MtHAP2-1* was then investigated by expressing *MtHAP2-1* RNA interference (RNAi) constructs in transgenic *M. truncatula* roots. This resulted in a reduction of *MtHAP2-1* expression (Fig. 4b, below) sufficient to significantly alter nodule development. Nodulation was delayed and nodule growth arrested at ~8–10 d post-inoculation (Fig. 2a,b). Inside the resulting spherical nodular structures, the absence of the clearly delimited zones found in wild-type nodules (I, meristem zone; II, Rhizobia infection zone; III, nitrogen-fixing zone; Fig. 2e,g) indicated an abnormal nodule developmental process. Growth arrest was often associated with the development of a closed endodermal layer surrounding the nodules (Fig. 2e). In addition, cells of the apical meristematic zone lost their typical polyhedral shape and accumulated numerous enlarged vacuoles (Fig. 2c,d). *MtHAP2-1* RNAi nodules were also unable to fix nitrogen as shown by the reduced development of plants grown in the absence of mineral nitrogen (Fig. 2h). This phenotype was confirmed by electron microscopy observations showing that in the infection zone of mature *MtHAP2-1*-RNAi nodules, bacteria were not released from infection threads (Supplementary Fig. 2). On the few occasions for which release could be observed, the corresponding rhizobia were arrested in their development and never differentiated into nitrogen-fixing type IV bacteroids (Vasse et al. 1990; data not shown). Taken together, these results suggest that *MtHAP2-1* is a novel symbiosis-specific TF playing a key role during nodule development by controlling meristem persistence and probably also symbiotic bacterial release.

MicroRNAs (miRNA) are small 21-nucleotide (nt) RNAs that play important regulatory roles in plants and metazoans by targeting mRNAs either for cleavage or translation inhibition (Jones-Rhoades et al. 2006). miRNAs have generally been shown to target genes controlling development, many of them encoding TFs (Li and Zhang 2005; Jones-Rhoades et al. 2006). Since previous studies had suggested

that HAP2 TFs are potential targets of miR169 in *A. thaliana*) (Jones-Rhoades and Bartel 2004), we searched for miR169 complementary sequences in the *MtHAP2-1* gene, and found two such sequences in the 3′ untranslated region (UTR) (Fig. 3a). An in silico approach then identified a *M. truncatula* gene susceptible to encode a miR169 precursor, called *MtmiR169-a,* and secondary structure predictions revealed the capacity of *MtmiR169-a* to form a stable stem-loop, as expected for a pre-miRNA (Fig. 3b). To investigate gene functionality we overexpressed the putative *MtmiR169-a* gene in transgenic *M. truncatula* roots under the control of a 35S
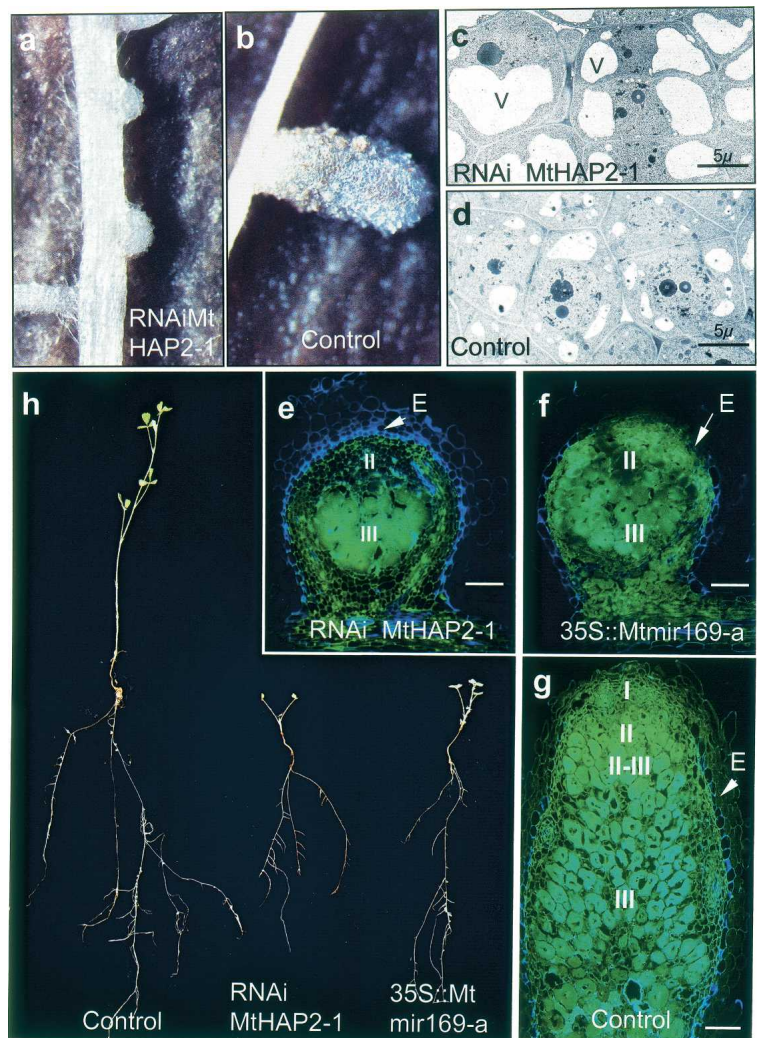
Figure 2. Altered nodulation phenotypes of *MtHAP2-1* RNAi and 35S:*MtmiR169-a* roots. (*a,b*) Nodule development on roots transformed with either an *MtHAP2-1* RNAi construct (*a*) or a control construct (empty vector) (*b*). Pictures were taken 46 d post-*Rhizobium* inoculation (d.p.i.). (*c,d*) Electron microscopy images of the meristematic zone of *MtHAP2-1* RNAi (*c*) and control (*d*) nodules. (V) Vacuoles. Cells of the meristematic region of *MtHAP2-1* RNAi nodules already start to loose meristematic characteristics at 10 d.p.i. (*c*), while they are still fully functional in control nodules at 27 d.p.i. (*d*). Bar, 5 µm. (*e–g*) Light microscopic images of longitudinal sections of 20-d-old nodules, using an overlay of two fluorescence images. Note the absence of normal nodule zonation and the abnormal closed endodermis (E) in *MtHAP2-1* RNAi (*e*) and *Mtmir169-a*-overexpressing (*f*) nodules, compared with control (empty vector) (*g*) nodules, indicating nodule developmental arrest. Bar, 100 µm. (*h*) Growth of *Rhizobium*-inoculated plants after 40 d in the absence of mineral nitrogen reveals a deficient nitrogen fixation phenotype for *MtHAP2-1* RNAi and 35S:*Mtmir169-a* plants compared with control plants.
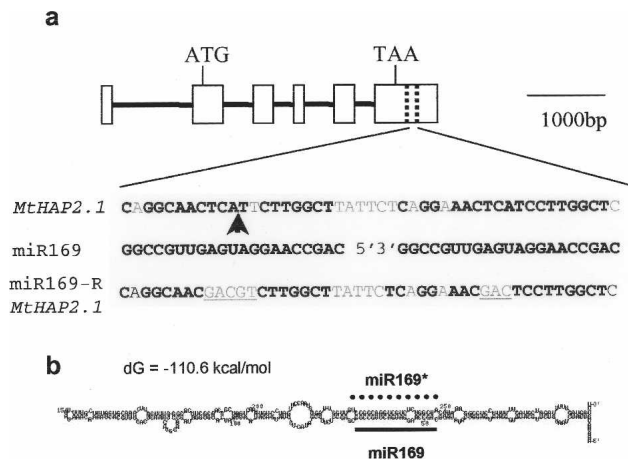
Figure 3. A *miR169-a* precursor induces cleavage of *MtHAP2-1* mRNA. (*a*) Schematic representation of the *MtHAP2-1* gene with intron (line)–exon (boxes) structure and position of the two putative miR169 recognition sites (dotted lines) in the 3′ UTR of the gene. Alignment of miR169 with the two miR169 recognition sites in *MtHAP2-1*; conserved nucleotides are indicated in bold on the *MtHAP2-1* sequence. Arrow shows predominant cleavage site revealed by 5′ RACE–PCR. Underlined nucleotides are those changed by site-directed mutagenesis in the miR169-resistant *MtHAP2-1* mutant (miR169-R *MtHAP2-1*). (*b*) Most probable stem-loop structure of *MtmiR169-a*, indicating position of mature miR169 (line) and complementary miR169* (dotted line) sequences.

promoter. This led to an increased accumulation not only of the miR169 precursor (*pre-miR169-a*) (data not shown) but also of the mature miR169 (Fig. 4a), confirming that *MtmiR169-a* encodes microRNA 169. Evidence for miR169-mediated cleavage of *MtHAP2-1* mRNA was then obtained by 5′ RACE–PCR experiments, which showed that cleavage takes place predominantly at the first miR169 recognition site (Fig. 3a). Overexpression of *MtmiR169-a* in transgenic roots led to a 3.5-fold reduction of *MtHAP2-1* transcript levels (Fig. 4b), confirming unambiguously that miR169 has a major impact on *Mt-HAP2-1* expression. Importantly, the nodulation phenotype of these roots was remarkably similar to that observed with an *MtHAP2-1* RNAi construct (Fig. 2e,f), indicating that miR169 regulation of *MtHAP2-1* is functioning in planta, and that the control of the precise level of *MtHAP2-1* mRNA is crucial for nodule development.

Real-time RT–PCR analysis showed that *MtmiR169-a* expression is undetectable in roots, expressed in young developing nodules, and maximal in 10-d-old functional nodules (Fig. 4c). This contrasts strikingly with *MtHAP2-1*, whose expression is also nodule specific but maximal in young developing nodules, and then decreases as the nodule becomes functional (Fig. 4c). A p*MtmiR169-a:GUS* fusion revealed that this miRNA is present in adjacent tissues to those expressing *MtHAP2-1* mRNA (Fig. 5a,b). Strikingly, *MtmiR169-a* transcript levels are highest in the infection zone (proximal to the meristem) (Fig. 5a,b), in which only lower levels of *MtHAP2-1* are detected (Fig. 1). Conversely, *MtmiR169-a* promoter activity could not be detected in the meristematic zone in which most *MtHAP2-1* mRNA is localized (Fig. 5b). These data are therefore consistent with a role of miR169 in restricting *MtHAP2-1* transcripts to the nodule meristematic zone.

To evaluate the functional relevance of this miR169-

mediated post-transcriptional regulation, a modified form of the *MtHAP2-1* gene, mutated in both miR169 recognition sites (miR169-resistant *MtHAP2-1*) (Fig. 3a) was expressed under the control of its own promoter, in the presence or absence of the p35S:*MtmiR169-a* construct. No difference was observed whether the p35S:*MtmiR169-a* construct was present or not (data not shown), suggesting that the mutated *MtHAP2-1* construct is indeed miR169 resistant. Nodules that developed on these roots showed no alterations in tissue differentiation or in nodule zonation (Fig. 5c) and seemed functional according to plant development in the absence of mineral nitrogen (data not shown). The miR169-resistant *MtHAP2-1* expressed under its own promoter can therefore restore nodulation, at least partly. This strongly suggests that the altered nodulation phenotype observed in p35S:*MtmiR169-a* roots is mainly mediated by *MtHAP2-1* and probably not by other putative miR169 targets, although a minor contribution cannot be excluded. The growth, of microRNA169-resistant nodules was, however, significantly reduced compared with control nodules (Fig. 5c,d). This result shows that when *MtHAP2-1* mRNA is not degraded by microRNA169 in the tissues surrounding the meristematic
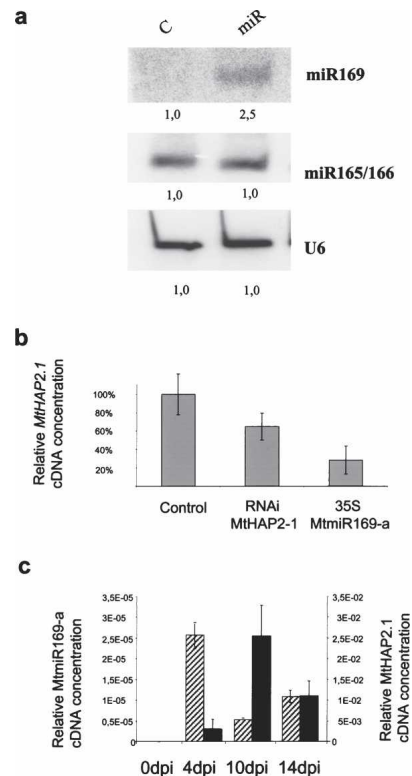
Figure 4. miR169 cleaves *MtHAP2-1* in planta and *MtHAP2-1* and the MtmiR169-a are oppositely regulated during nodulation. (*a*) Northern blot analysis of mature 21-bp miR169 expression in transgenic roots transformed with either empty vector control (C) or the 35S:*MtmiR169-a* constructs (miR). (*b*) Real-time RT–PCR analysis of *MtHAP2-1* expression in *MtHAP2-1* RNAi and 35S:*MtmiR169-a* compared with empty vector controls. Results are expressed as a percentage of the expression in control roots. (*c*) Real-time RT–PCR analysis of the expression pattern of *MtmiR169-a* (black boxes) and *MtHAP2-1* (striped boxes) during nodule development. Relative expression levels are expressed in arbitrary units; the scale for *MtmiR169-a* is on the *left* while the scale for *MtHAP2-1* is on the *right*.
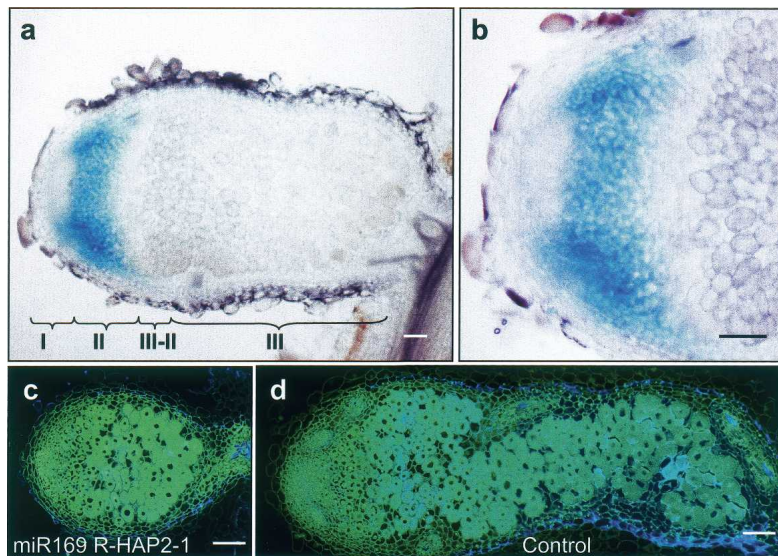
Figure 5. MtmiR169-a expression pattern in mature nodules and effects of a *MtHAP2-1* miR169-resistant construct on nodule development. (*a,b*) Expression analysis of *MtmiR169-a* in 14-d-old nodules revealed by promoter:*GUS* analysis. *b* is a magnification of the distal part of the nodule depicted in *a*, showing the expression of *MtmiR169-a* in the infection zone (II) and the absence of promoter activity in the meristematic region (I) of the nodule. Description of nodule zones is according to Vasse et al. (1990). Bar, 100 µm. (*c–d*) Images of longitudinal sections through 20-d-old nodules are an overlay of fluorescence images acquired with two different wavelengths, showing nodule development on transgenic roots expressing either a miR169-resistant *MtHAP2-1* (miR169 R-HAP2-1) gene under the control of its own promoter (*c*), or an empty vector control (control) (*d*). Bar, 100 µm.

zone, nodule elongation is affected, suggesting an inhibitory role of *MtHAP2-1* on cell differentiation and/or an indirect role on meristematic activity. This is coherent with the central role in cell proliferation and early development that has been shown for members of the CCAAT-box-binding family in other systems. Conditional CBF-B mutants in mouse cells, for example, strongly affect cell division and embryonic development (Bhattacharya et al. 2003), and *lec1* mutants are embryo lethal in *A. thaliana* (Lotan et al. 1998).

Our data show that *MtHAP2-1*, a novel symbiotically specialized CBF-B subunit, is essential for nodule meristematic persistence in *M. truncatula*, and that microRNA169 confers spatial and temporal accuracy to this developmental program. It is interesting to note that other TFs shown to specify shoot or root meristem development, such as CUC1-3 (Laufs et al. 2004), PHB, and PHV (Williams et al. 2005), or ICU4 (Ochando et al. 2006), are also the targets of microRNA mediated mRNA degradation. This additional level of regulation probably reflects the vital importance of meristems for plant development.

In conclusion, we propose that miR169-mediated regulation of *MtHAP2-1* expression leads to a critical spatial and temporal restriction of this TF to the nodule meristematic zone, thereby allowing correct tissue identity and the transition from meristematic to differentiated cells.

Materials and methods

Biological material
M. truncatula Gaertn cv. Jemalong genotype A17 was grown aeroponically as described in Journet et al. (2001), and inoculated with the *Sino-*

rhizobium meliloti strain RCR2011 pXLGD4 (GMI 6526) (Ardourel et al. 1994) as described previously (El Yahyaoui et al. 2004).

Expression analysis
mRNA in situ hybridizations were performed as described in Gamas et al. (1998). Image processing and analysis were performed using the Image-Pro plus software (Media Cybernetics) and the Diatrack software (Semasopht). For real-time RT–PCR experiments, RNA was isolated using the SV total RNA extraction kit (Promega), and quality checked using a Bioanalyser (Agilent). Three micrograms of total RNA were reverse-transcribed using the SuperScript II enzyme from Invitrogen. Real-time PCR was then performed on a LightCycler (Roche), using Syber-Green and specific primers. For *MtHAP2-1*, we used *MtHAP2-1R* and *MtHAP2-1F* (see Supplementary Table 1) while expression of the *pre-MtmiR169-a* was analyzed using miR169F and miR169R that amplify the stem-loop structure (249 base pairs [bp]) (Fig. 3b). Data were normalized using the *EF1-α* gene as described in El Yahyaoui et al. (2004). The specificity of primer pairs was confirmed by sequencing of PCR amplicons and the analysis of dissociation curves. Histochemical GUS staining was performed as described in Journet et al. (1994).

5′ RACE–PCR experiment and Northern blot analysis of small RNAs
5′ RACE–PCR was performed as described in Hirsch et al. (2006) using primers *MtHAP2-1-3′* (adapter), CBFrace-miR1 (inner), and CBF-race-miR2 (outer) (see Supplementary Table 1).

For Northern blots of small RNAs, extractions were performed using the TRIzol reagent (Invitrogen). Twenty micrograms of each RNA were subjected to electrophoresis on a 17% polyacrylamide denaturing gel and electroblotted onto Hybond N⁺ membranes (GE Healthcare) using a Miniprotean II system (Bio-Rad). Blots were hybridized with miR169, miR166, or U6 probes as described in Hirsch et al. (2006).

Root transformation
Root transformation using *Agrobacterium rhizogenes* was performed as described in Boisson-Dernier et al. (2005). Composite plants were subsequently transferred to growth pouches supplemented with nitrogen-deprived medium as in Gallusci et al. (1991) and inoculated with *S. meliloti* strain RCR2011 pXLGD4 (Ardourel et al. 1994).

Constructions and vectors
All primers are listed in Supplementary Table 1.

For all root transformation experiments, we used a Pgreen (http://www.pgreen.ac.uk)-based vector with a modified polylinker, pPex (L. Sauviac, unpubl.).

For GUS fusion constructs, pPEX was modified by adding a GUS cassette from the pBS–GUS vector (Vernoud et al. 1999).

A 2.4-kb sequence upstream of the stem-loop structure of MtmiR169-a (Fig. 3b) was amplified using Pfx polymerase (Invitrogen) and primers MtmiR169-5′ and Pre-miR169-3′ and cloned into pPEX–GUS.

For RNAi experiments, the pPex vector was modified by introducing the intron from the pRNAi vector (Limpens et al. 2003) in addition to the cloning of the *DsRED* gene (under the control of the ubiquitin promoter) from the Predroot (Limpens et al. 2003) vector. A 298-bp region of the 3′ UTR of the *MtHAP2-1* gene showing no significant homology with other known *MtHAP2* genes was amplified using primers CBF RNAi-5′ and CBFRNAi-3′ and cloned into the pPexRNAi vector.

For *MtmiR169-a* overexpression, a fragment located just outside of the stem-loop structure (Fig. 3b) was amplified with Pfx polymerase (Invitrogen) using miR169-5′ and miR169-3′ primers, and subsequently cloned into pPex.

For the miRNA-resistant version of *MtHAP2-1*, the gene including 2.3 kb of the upstream sequence was amplified with Pfx polymerase (Invitrogen) using the primers *ProMtHAP2-1-5′* and *MtHAP2-1-3′*, and then cloned into pPex. Mutagenesis of the two miR169 recognition sites was

performed by PCR amplification of this plasmid using Pfx polymerase (Invitrogen) and the following primers: first mutated miR169 recognition site (Fig. 3a): miR1muta-5′ and miR1muta-3′; second miR169 recognition site (Fig. 3a): miR2muta-5′ and miR2muta-3′. DpnI digestion was used to eliminate nonmutagenized plasmid prior to cloning.

Accession numbers: *MtHAP2-1*: MtC10582 (MENS database; http://medicago.toulouse.inra.fr/Mt/EST) and TC95981(TIGR database; http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago).

Pre-MtmiR169-a: BAC AC148485.10 (NCBI databases; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed).

Acknowledgments

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