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.

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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 COTYLEDON1-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 possess significantly more complex gene families. In A. thaliana there are 10...
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 RNAi 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. 2c–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. 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), a finding consistent with 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.

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
This led to an increased accumulation not only of the MtHAP2-1 mRNA. This increased accumulation was confirmed by Northern blot analysis of mature 21-bp miR169 expression in transgenic roots, showing 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 MtHAP2-1 expression. Importantly, the nodulation phenotype of these roots was remarkably similar to that observed with an MtHAP2-1 RNAi construct (Fig. 4c), indicating that miR169 regulates MtHAP2-1 function 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. 4d). 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. 4e). A pMtmiR169-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 MtmiR169 in restricting MtHAP2-1 transcripts to the nodule meristemetic 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 meristemetic zone, the MtHAP2-1 gene is functional according to plant development in the absence of mineral nitrogen.
microRNA control of nodule development

Expression analysis
mRNA in situ hybridizations were performed as described in Gamasa et al. (1998). Image processing and analyses were performed using the Image-Pro plus software [Media Cybernetics] and the DIAtract software [Se-masoph]. For real-time RT–PCR experiments, RNA was isolated using the SV total RNA extraction kit (Promega), and quality checked using a Bioanalyzer [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 mr169F and miR169R that amplify the stem-loop structure [249 base pairs [bp]] [Fig. 3b]. Data were normalized using the EF-1α 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).

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′-primer 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 fragment of the DsRED gene was fused at the EcoR1 site of the pPex vector. Expression of the construct in transgenic plants was confirmed by sequencing of PCR amplicons and the analysis of dissociation curves. Northern blot analysis of small RNAs Northern blot experiments were performed as described in Hirsch et al. (2006) using primers MtHAP2-1-3′-adapter, CBFRace-miR1 (inner), and CBFRace-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 electropholotted onto Hybond N+ membranes [GE Healthcare] using a MiniProtein II system [Bio-Rad]. Blots were hybridized with miR169, miR166, or U6 probes as described in Hirsch et al. (2006).

Materials and methods Biological material M. truncatula Gaertn cv. Jemalong genotype A17 was grown aeropically as described in Journet et al. (2001), and inoculated with the Sinorhizobium meliloti strain RCR2011 pXLGD4 [GMI 6526] [Ardourel et al. 1994] as described previously [El Yahyaoui et al. 2004].

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 CBFRace-miR2 (outer) [see Supplementary Table 1].

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

Accession numbers: MrHAP2-1: Mct10582 (MENs database; http://medicago.toulouse.inra.fr/Mct/EST and Tc95981 (TIGR database; http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago). Pre-MtrR169-a: RAC144845.10 (NCBI databases; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed).

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