miR172 Regulates Soybean Nodulation

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Micro-RNAs (miRNAs) play a pivotal role in the control of gene expression and regulate plant developmental processes. miRNA 172 (miR172) is a conserved miRNA in plants reported to control the expression of genes involved in developmental phase transition, floral organ identity, and flowering time. However, the specific role of miR172 in legume nodulation is undefined. Ectopic expression of soybean miR172 resulted in an increase in nodule numbers in transgenic roots and an increase in the expression of both symbiotic leghemoglobin and nonsymbiotic hemoglobin. These nodules showed higher levels of nitrogenase activity. Further analysis revealed a complex regulatory circuit in which miR156 regulates miR172 expression and controls the level of an AP2 transcription factor. The latter, either directly or indirectly, controls the expression of nonsymbiotic hemoglobin, which is essential for regulating the levels of nodulation.

The rhizobium–legume nitrogen-fixing symbiosis is agronomically and ecologically important. This symbiosis results in the formation of a new root organ, the nodule, in which the rhizobia establish an intracellular symbiosis (Murray 2011). Rhizobial infection of legumes occurs primarily through root hairs, single-cell extensions of the root epidermis. The rhizobia infect the root hairs and gain access to the nodule cells through a tubular infection thread, specifically induced in response to rhizobial signals. The infecting rhizobia induce cortical cell divisions leading to nodule development. The rhizobia are ultimately released from the infection threads through an endocytotic process and colonize cells within a membrane-bound vacuolar-like compartment, termed a symbiosome (Roth et al. 1988). The symbiosome is the functional unit of biological nitrogen fixation (Kereszt et al. 2011).

Rhizobia fix atmospheric nitrogen via the nitrogenase enzyme (Kim and Rees 1994). Although cleavage of the N₂ triple bond is exergonic, considerable activation energy is required that is provided by 16 mol of ATP per N₂ reduced (Ljones and

Burris 1972). The nitrogenase enzyme is extremely oxygen labile and, hence, needs to be protected from free oxygen. However, rhizobia are obligate aerobes. In the nodule, the rhizobia induce the expression of high-affinity, terminal oxidases, while the plant produces leghemoglobin (Lb) in high amount, which effectively binds all free oxygen. These adaptations allow the rhizobia to fix nitrogen aerobically in an environment in which nitrogenase is protected (Cutting and Schulman 1971; Wittenberg et al. 1972).

In legumes, there are two main types of hemoglobin, including symbiotic Lb and nonsymbiotic hemoglobin (Hb) (Anderson et al. 1996). Hb was originally found in the only nonleguminous plant known to be nodulated by rhizobia, a *Parasponia* sp. (Ulmaceae) (Appleby et al. 1983). Later, Hb was identified in a large variety of nonnodulated plants (Hardison 1996). Hb was also identified to function in nodulation (Shimoda et al. 2009). In *Lotus* spp. ectopic expression of Hb was found to increase nodule numbers with a concomitant increase in nitrogenase activity (Shimoda et al. 2009).

In recent years, many studies demonstrated an important role for micro-RNA (miRNAs) during plant development (Rubio-Somoza and Weigel 2011; Zhang et al. 2006). For instance, miR172 is a conserved miRNA in plants, present in all angiosperm lineages. In *Arabidopsis*, miR172 associates with AGO1 or AGO10 (Ji et al. 2011) and acts to silence specific target gene expression. For example, *Arabidopsis* miR172 targets the transcription factor gene *APETALA2* (AP2), which regulates juvenile to adult-phase transition, floral organ identity, and flowering time (Aukerman and Sakai 2003; Chen 2004; Lauter et al. 2005; Nair et al. 2010). *Arabidopsis* miR156 is a negative regulator of miR172 expression (Wu et al. 2009). miR156 targets SPL transcription factors, which directly bind to the miR172 promoter and positively regulate miR172 expression. In legumes, miR172 accumulates to high levels in mature nodules, including those formed on *Medicago truncatula*, *Lotus japonicus*, common bean, and soybean, relative to the levels in other tissues, such as leaves and roots (De Luis et al. 2012; Lelandais-Briere et al. 2009; Valdes-Lopez et al. 2010; Wang et al. 2009). However, a direct, functional role for miR172 in nodulation has not demonstrated.

Here, we report data from soybean suggesting a complex regulatory circuit in which the levels of miR156 negatively control miR172 expression, which targets the mRNA of the AP2-2 transcription factor. The latter, either directly or indirectly, regulates the expression of Hb, which is essential for regulating the levels of nodulation. The net effect is that the level of nodulation is controlled by the interaction of miR156 and miR172, which impacts the expression of their downstream target genes.

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RESULTS

miR172 regulates soybean nodulation.

Previous studies demonstrated that the level of miR172 accumulated in mature nodules in different legume species, including soybean (De Luis et al. 2012; Lelandais-Briere et al. 2009; Valdes-Lopez et al. 2010; Wang et al. 2009). In order to examine the expression activity of miR172 in soybean nodule development, we measured the levels of miR172 in different stages of soybean nodules using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). We selected soybean nodules according to their relative symbiotic nitrogen-fixation activity (Pessi et al. 2007) and harvested them 10, 20, and 30 days after rhizobial inoculation (DAI). At 10 DAI, the nodules can be clearly distinguished but are still developing; at approximately 20 DAI, the relative symbiotic nitrogen fixation activities, under our growth conditions, were the highest and, therefore, representing the mature, fully functioning nodules; at approximately 30 DAI, the relative symbiotic nitrogen fixation activities decrease to 61%, representing nodules that have already begun to senescent. Compared with 10-DAI nodules, the expression of miR172 was increased significantly 20 DAI. However, miR172 expression was slightly decreased at 30 DAI (Fig. 1).

In order to investigate the role of miR172 during soybean nodulation, the miR172 precursor gene was ectopically expressed from the strong *Cassava mosaic virus* (CvMV) promoter in the binary vector pCAMGFP-CvMV:GWOX, which expresses a constitutive green fluorescent protein (GFP) cassette (Li et al. 2010). Ectopic expression of miR172 resulted in a 2.4-fold increase in nodule numbers (Fig. 2). The qRT-PCR analysis showed that the levels of miR172 were increased 9.3-fold by expression from the CvMV promoter, relative to transgenic roots transformed with the empty vector (Fig. 3).

miR172 expression affects nitrogenase activity and hemoglobin accumulation.

Given the strong effects of miR172 expression on nodule formation, we also measured nodule nitrogenase activity using the acetylene-reduction assay. Compared with the empty vector control, ectopic expression of miR172 resulted in a significant increase in nitrogenase activity (Fig. 4). According to previous reports, the levels of both symbiotic hemoglobin and Hb can

Fig. 1. Relative expression level of micro-RNA 172 (miR172) and miR156 between 10 to 30 days after *Bradyrhizobium japonicum* inoculation (DAI). The expression of miR156 and miR172 was measured by quantitative reverse-transcriptase polymerase chain reaction 10, 20, and 30 DAI nodules. Three biological replications were performed. The relative expressions of miR156 and miR172 were calculated by the expression of reference genes *snoR1* and *5.8S*. Error bar indicates $\pm$ standard error.

affect nitrogenase activity (Dakora 1995; Shimoda et al. 2009). A sequence-based search of the soybean genome sequence identified six hemoglobin genes, including four Lb genes (*Lba*, *Lbc1*, *Lbc2*, and *Lbc3*) and two Hb genes (*Hb-1* and *Hb-2*). We designed specific primers for each of these genes and used them to measure gene expression by qRT-PCR. In the CvMV::miR172 tissues, mRNA levels of one Lb gene, Glyma10g34280 (*Lbc1*), and the two Hb genes, Glyma11g12960 (*Hb-1*) and Glyma11g12980 (*Hb-2*), were significantly increased compared with the empty vector control (Fig. 5). There were no significant changes in the expression levels of *Lba*, *Lbc2*, and *Lbc3* in the CvMV::miR172 tissues (data not shown).

An AP2 transcription factor regulates nodulation.

According to previous reports, miR172 targets the plant-specific transcription factor gene *AP2* in different plant species (Aukerman and Sakai 2003). According to target prediction (Joshi et al. 2010), we predicted 11 *AP2* genes as targets for soybean miR172. Target gene qRT-PCR analysis revealed that the levels of 3 of these 11 *AP2* transcription factors were decreased significantly in CvMV::miR172 tissues (Fig. 3), including Glyma03g33470 (*AP2-1*), Glyma11g15650 (*AP2-2*), and Glyma19g36200 (*AP2-3*). The other eight *AP2* expression levels were not affected by miR172 (data not shown). In order to investigate the possible role of these *AP2* genes in nodulation, we designed specific RNAi silencing constructs for each *AP2* gene and expressed these in transgenic soybean roots (Fig. 2A). Compared with transgenic roots expressing a β -glucuronidase-RNAi construct, there was no significant change in nodule numbers in roots expressing either *AP2-1* RNAi or *AP2-3* RNAi. However, expression of the *AP2-2* RNAi resulted in a significant increase in nodule numbers (Fig. 2A), with no effect on the expression of *AP2-1* and *AP2-3* (Supplementary Fig. S1).

AP2-2 represses nodulation and negatively regulates Hb-1 accumulation in soybean.

Given the results above, we focused specifically on the role of *AP2-2* in nodulation. We constructed an *AP2-2* overexpression vector and transformed soybean roots. A significant reduction in nodule formation was seen in roots ectopically expressing *AP2-2* relative to transgenic roots transformed with the empty vector (Fig. 2A and B). Given the results that miR172 affects hemoglobin levels, we also measured hemoglobin levels in nodules formed on the CvMV::AP2-2 roots. Ectopic expression of *AP2-2* significantly decreased *Hb-1* expression levels (Fig. 6). Contrary to the results found with roots transformed with the CvMV::miR172 construct, there were no apparent differences in the levels of *Lbc1* and *Hb-2* expression in the *AP2-2*-expressing roots. We assume that the effects on *Lbc1* and *Hb-2* expression are likely mediated by another, unknown transcription factor that may also be targeted by miR172. We also measured *Hb-1* expression levels in *AP2-2* RNAi tissues. Consistent with the results obtained by ectopic expression of *AP2-2*, RNAi silencing of *AP2-2* expression resulted in increased expression of *Hb-1*. However, given the variation in these experiments, the effect was not statistically significant (Supplementary Fig. S2).

Interestingly, in CvMV::AP2-2 transgenic tissues, the expression of miR172 was decreased significantly compared with the control. However, the expression of miR156 remained unaffected. Therefore, although the levels of miR172 and miR156 showed a negative correlation in CvMV::AP2-2 transgenic tissues, the data would suggest that *AP2-2* does not suppress miR172 expression by elevating levels of miR156 (Fig. 6).

Ecotopic expression of Hb-1 enhances soybean nodulation.

Previously, Shimoda and associates (2009) reported that ectopic expression of Hb in *L. japonicas* roots resulted in a

significant increase in nodule numbers upon rhizobial inoculation and a strong enhancement of nodule nitrogenase activity (Shimoda et al. 2009). These results suggested the possibility that the increase in nodule formation seen on roots ectopically expressing miR172 could be due to a reduction in *AP2* expression, thereby relieving the repressive effect of this transcription factor on *Hb-1* expression (Fig. 5). In order to test this hypothesis, we transformed soybean transgenic roots with a *CvMV::Hb-1* construct and compared nodulation with roots transformed with the empty vector. Consistent with the hypothesis, those roots expressing *Hb-1* produced significantly more nodules than the control roots (Fig. 2A and B). We also measured the nitrogenase activity of nodules formed on the *CvMV::Hb-1* roots. As expected, the *CvMV::Hb-1* roots showed significantly higher nitrogenase activity (Fig. 4).

miR156 is a negative regulator of miR172 expression during soybean nodulation.

According to a previous study in *Arabidopsis*, miR156 represses miR172 expression by targeting an SPL transcription factor, which is required for transcription of the miR172 pre-mRNA (Wu et al. 2009). Therefore, we also measured the

expression of soybean miR156 in nodules formed 10, 20, and 30 DAI. The expression of miR156 was negatively correlated with miR172 levels at all time points measured (Fig. 1).

We also analyzed the nodulation phenotype of soybean transgenic roots transformed with a *CvMV::miR156* construct. These roots showed a marked reduction in nodulation (40% reduction) compared with roots transformed with the empty vector (Fig. 2). These results are consistent with the notion that, similar to *Arabidopsis*, miR156 represses expression of miR172. Consistent with this hypothesis, the expression of miR172 was decreased by 44% in the *CvMV::miR156*-transformed roots. We also measured the expression of the predicted mRNA targets of miR156, which were predicted in our previous article (Joshi et al. 2010). miRNA156 targets SPL transcription factors. Indeed, the mRNA levels of two SPL transcription factors, Glyma08g01450 (*SPL 1*) and Glyma12g27330 (*SPL 2*), were significantly decreased in the *CvMV::miR156*-transformed roots (Fig. 7). We also observed miR172 targets (*AP 2-1*, *AP 2-2*, and *AP 2-3*) and *Hb 1* expression in the *CvMV::miR156*-transformed tissues. As expected, we found that miR172 target expression was increased significantly, while *Hb 1* decreased (Fig. 7). Moreover, the *CvMV::miR156*

Fig. 2. Soybean nodule numbers were affected by micro-RNA 172 (miR172), miR156, *AP2-2*, and *Hb-1*. **A**, Relative nodule numbers formed on soybean transgenic roots either ectopically expressing miR172, miR156, *AP2-2*, and *Hb-1* or in which the expression of the *AP2-1*, *AP2-2*, or *AP2-3* genes was silenced using RNAi. Nodule numbers were counted 6 weeks after inoculation. Relative nodule numbers formed on soybean transgenic hairy roots were calculated by the ratio change between treatments with control roots, which were transformed with the empty vector. Error bar indicates $\pm$ standard error. The *t* test was performed using the null hypothesis that the fold-change difference between the experimental and control was equal to one; *, **, and *** indicate significance at the 0.05, 0.01, and 0.005 probability level, respectively. **B**, Images of soybean transgenic roots ectopically expressing miR156, miR172, *AP2-2*, or *Hb-1* from the strong *CvMV* promoter. Representative photos showing nodulation. Top: fluorescent image showing transgenic expression of green fluorescent protein (GFP). Bottom: corresponding light micrographs. Fluorescence arises from constitutive expression of GFP in *Agrobacterium rhizogenes* transformed roots. Control: transformed with empty vector pCAMGFP-*CvMV*-GWOX.

roots also showed a significant decrease in nitrogenase activity (Fig. 4). The ectopic expression of either miR156 or miR172 did not affect nodule morphology (Supplementary Fig. S3). Collectively, the results suggest a model in which miR156 negatively regulates miR172 expression, thereby controlling hemoglobin expression, nitrogenase activity, and soybean nodule number.

DISCUSSION

miRNAs play pivotal roles in the control of gene expression and regulate plant developmental processes. miR172 is a conserved miRNA in plants. Levels of miR172 were shown to

Fig. 3. Relative expression levels of micro-RNA 172 (miR172) and its predicted target genes in soybean transgenic roots ectopically expressing miR172 from the strong CvMV promoter. The expression level of miR172 and *AP2-1*, *AP2-2*, and *AP2-3* were measured in CvMV::miR172 transgenic tissues. The relative expression was calculated by dividing the experimental value and the control value. Three replications were performed. Error bar indicates $\pm$ standard error; * and *** indicate significance at the 0.05 and 0.005 probability level, respectively.

Fig. 4. Relative nitrogenase activity of nodules formed on transgenic roots expressing micro-RNA 172 (miR172), *Hb-1*, or miR156 from the strong CvMV promoter. Nodule nitrogenase activity on roots expressing miR172, CvMV::Hb-1, or miR156 was measured using the acetylene reduction assay and compared with roots transformed with the empty vector. Nodules were harvested 6 weeks after inoculation. The relative nitrogenase activity was calculated by dividing the experimental value and the control value. Two replications were performed. Error bar indicates $\pm$ standard error; * indicates significance at the 0.05 probability level.

affect *Arabidopsis* growth-phase transition and flower development (Aukerman et al. 2003; Chen 2004; Lauter et al. 2005; Nair et al. 2010) and the expression of miR172 promotes *Arabidopsis* adult identity (Wu et al. 2009). The data presented here indicate that soybean miR172 is a regulator of nodule development. Transgenic soybean roots ectopically expressing miR172 from the strong, constitutive CvMV promoter produced significantly more nodules than transgenic roots transformed with the empty vector. Further analysis showed that these transgenic roots also showed an increase in the expression of both symbiotic Lb and Hb. The nodules of CvMV::miR172 also showed higher levels of nitrogenase activity. Comparing miR172 expression in nodules in different development stages, miR172 levels were higher at 20 DAI compared with 10 and 30 DAI. As previously reported (Pessi et al. 2007), nodules at 10, 20, and 30 DAI exhibit 9, 100, and 61%

Fig. 5. Relative expression of *Lbc1*, *Hb-1*, and *Hb-2* in transgenic roots expressing micro-RNA 172 (miR172) from the strong CvMV promoter. Expression levels of *Lbc1*, *Hb-1*, and *Hb-2* were measured in CvMV::miR172 transgenic tissues. The relative expression was calculated by dividing the experimental value and the control value. Three replications were measured. Error bar indicates $\pm$ standard error; * and ** indicate significance at the 0.05 and 0.01 probability level, respectively.

Fig. 6. Relative expression of hemoglobin, micro-RNA 156 (miR156), and miR172 in CvMV::AP2-2 transgenic nodules. Expression levels of AP2-2, hemoglobin genes (*Lbc1*, *Hb-1*, and *Hb-2*), miR156, and miR172 were compared between control plants transformed with empty vector and CvMV::AP2-2 transgenic tissues. The relative expression was calculated by dividing the experimental value and the control value. Three replications were measured. Error bar indicates $\pm$ standard error; * and ** indicate significance at the 0.05 and 0.01 probability level, respectively.

relative nitrogen fixation (i.e., acetylene reduction) activities, respectively. These levels are roughly consistent with the miR172 expression pattern in nodules, suggesting either a direct or indirect role for miR172 in controlling nodule nitrogenase activity.

Symbiotic Lb facilitates oxygen transport to rhizobia in nodules to support energy production, while also limiting free oxygen concentration in the nodules to protect the oxygen-sensitive nitrogenase enzyme (O'Brian et al. 1987). In *L. japonicus*, the expression of Hb increased nitrogenase activity and improved nodulation (Shimoda et al. 2009). In this study, we showed that, similar to the studies in *L. japonicus*, strong ectopic expression of Hb in soybean transgenic roots significantly increased nodule numbers and nitrogenase activity but without obvious morphological differences.

AP2 transcription factors are involved in a variety of plant processes, including phase transition (Lauter et al. 2005), floral organ identity (Chen 2004), cleistogamy (Nair et al. 2010), flowering time (Aukerman and Sakai 2003), stem cell niche (Wurschum et al. 2006), seed mass (Ohto et al. 2005), and plant resistance to necrotrophic pathogens (Zhao et al. 2012). For example, AP2 members, such as *AP2-like ethylene-responsive transcription factor (SMZ)*, act as repressors of flowering during *Arabidopsis* development. Distinct SMZ binding sites were identified in the promoters of *FLOWERING LOCUS T (FT)*. SMZ binding to these sites represses *FT* expression and, therefore, strongly affects flowering (Mathieu et al. 2009).

In soybean, the data indicate that miR172 targets an AP2-2 transcription factor, which either directly or indirectly represses *Hb* gene expression. This is consistent with previous reports showing that CPP1, a cysteine-rich repeat protein, is induced during nodulation and binds to the *Lbc3* promoter and represses *Lbc3* expression (Cvitanich et al. 2000). ChIP-seq analysis of the *Arabidopsis* genome identified thousands of AP2-binding loci, including an *Arabidopsis* hemoglobin gene (Yant et al. 2010). Although the ability of AP2-2 to directly bind to the *Hb-1* promoter was not tested, the data presented argue that AP2-2 does play a role in repression of *Hb-1* expression.

Fig. 7. Relative expression of micro-RNA 156 (miR156), miR172, *AP2*, and *Hb-1* genes in nodules ectopically expressing miR156. Expression levels of miR156, miR172, *AP2-1*, *AP2-2*, *AP2-3*, and *Hb-1* were measured by quantitative reverse-transcriptase polymerase chain reaction in CvMV::miR156 transgenic tissues and compared with roots transformed with the empty vector. The relative expression was calculated by dividing the experimental value and the control value. Three replications were measured. Error bar indicates $\pm$ standard error; * and ** indicate significance at the 0.05 and 0.01 probability level, respectively.

Previous research in *Arabidopsis* showed that miR156 is a negative regulator of miR172 expression (Wu et al. 2009). miR156 targets SPL transcription factors, which directly bind to the miR172 gene promoter and positively regulate miR172 expression. In addition to *Arabidopsis*, miR156 was also shown to negatively regulate miR172 expression in maize (Chuck et al. 2007) and *Populus × canadensis* (Wang et al. 2011). In both *Arabidopsis* and maize, miR156 and miR172 play a role in developmental phase transition. In the case of soybean nodulation, the data are also consistent with a role for miR156 in the negative regulation of miR172 expression. This was seen in transgenic plants ectopically expressing miR156, which resulted in reduction in miR172 levels, with a concomitant increase in *AP2-2* expression and a reduction in nodule formation. We also found a strong negative correlation between miR156 and miR172 levels during a time course of nodule formation (10 to 30 DAI) (Fig. 1).

The results presented, when taken as a whole, suggest the model shown in Figure 8. This postulated regulatory circuit suggests that soybean nodule formation is controlled by the opposing effects of miR156 and miR172 accumulation. miR156 represses the expression of its targets, SPL transcription factors, which regulate miR172 expression; miR172 controls *AP2-2* expression and directly or indirectly affects *Hb-1* levels. The level of *Hb-1* positively affects soybean nodulation. What is not clear and will require further research is how *Hb-1* levels are regulated and how *Hb-1*, either directly or indirectly, controls the number of nodules formed and their relative nitrogenase activity.

MATERIALS AND METHODS

Plant materials.

Methods for plant culture were modified from Malik and associates (1987). Soybean seed were surface sterilized with 30% bleach, rinsed several times with autoclaved double-distilled H₂O (ddiH₂O), once with 0.01 M HCl for 10 min, and then several times with autoclaved ddiH₂O. Sterilized seed were planted on plates contained nitrogen-free Broughton and Dilworth agar medium (Broughton and Dilworth 1971) and germinated in a growth chamber in the dark at 27°C and 80% humidity. After 3 days, soybean seedlings were transferred to plastic growth pouches. The paper wick within each plant pouch was wetted with nitrogen-free plant nutrient solution (Subramanian et al. 2004). Seedlings were grown in an environmentally controlled chamber maintained at a 14-h photoperiod with temperature maintained at 28°C during the day and 26°C during the night. Soybean roots were inoculated with *Bradyrhizobium japonicum* USDA110. Nodules were collected 10, 20, and 30 DAI.

Total RNA isolation and RT-PCR.

Total RNA was purified using Trizol Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, U.S.A.) and subsequently purified using chloroform extraction.

Fig. 8. Model of micro-RNA 156 (miR156) and miR172 regulation of soybean nodulation.

Genomic DNA (gDNA)-free RNA (1 µg) was reverse transcribed using Superscript III (Invitrogen). Specific primers were designed for each candidate gene (Supplementary Table S1C) and qRT-PCR was performed as described by Libault and associates (2008). *Con4* and *Con15* were used as reference genes to normalize gene expression.

MicroRNA qRT-PCR analysis.

Quantification of mature miRNA abundance was performed by qRT-PCR following the protocol described by Varkonyi-Gasic and associates (2007). gDNA-free RNA (1 µg) was reverse transcribed using Superscript III (Invitrogen) using a stem-loop-specific RT primer, which contained six nucleotide-specific sequences from the complementary 3' end sequence of each miRNA. qRT-PCR was performed using SYBR Green PCR mix (ABI enzyme) using an miRNA-specific forward primer and a universal reverse primer (Varkonyi-Gasic et al. 2007). The reference genes *snoR1* and *5.8S* were selected to calculate the relative expression of each miRNA.

Plasmid construction and soybean hairy root transformation.

Agrobacterium rhizogenes-dependent hairy root transformation was used to construct transgenic soybean roots ectopically expressing the selected miRNAs. The vector pCAMGFP-CvMV-GWOX (Li et al. 2010) was used, which allowed cloning of the precursor mRNA of each of the miRNA behind the strong CvMV promoter. The T-DNA of this vector also contains a constitutively expressed GFP cassette, which enables identification of transformed roots by GFP fluorescence (Li et al. 2010). The nodulation phenotypes of only the transformed roots were measured and compared with empty vector controls.

To clone the miRNA precursors, miR172j and miR156o were selected. The regions of DNA spanning 200 bp upstream and downstream of each mature miRNA region were amplified from soybean gDNA using specific primers. The amplified precursors were cloned in the pDONR-Zeo vector (Invitrogen) and sequenced. For *AP2-2* and *Hb-1* overexpression cloning, the fragments containing the coding region of *AP2-2* or *Hb-1* was amplified by PCR and cloned in the pDONR-Zeo vector. The resulting plasmids were recombined into the pCAMGFP-CvMV-GWOX binary vector using the Gateway LR Clonase II enzyme mixes (Invitrogen). Similar to overexpression cloning, for the target gene RNAi construct, an approximately 150-bp specific sequence of each gene was amplified and cloned into the pDONR-Zeo vector. The resulting positive plasmids were recombined into the pCAMGFP-CvMV-GWi (Graham et al. 2007) binary vector using the Gateway LR Clonase II enzyme mixes (Invitrogen). The resulting plasmids were introduced by electroporation into *A. rhizogenes* K599 and used for hairy root transformation, as previously described (Libault et al. 2009). Briefly, apical stem sections were excised from 14-day-old soybean seedlings and infected by *A. rhizogenes* K599 harboring either empty vector as a control or the miRNA precursor expression constructs. After 24 days of incubation, the soybean hairy roots were inoculated with *B. japonicum* USDA110. Transgenic roots were identified 6 weeks postinoculation based on their strong GFP fluorescence. Hairy roots were generated on 12 plants per construct, and the transformation was repeated six times under the same culture conditions. For each replicate, the ratio change of nodule numbers formed on miRNA-expressing roots were compared with control roots transformed with the empty vector and quantified as the relative nodule number of the control (= 1). In order to see whether such differences were statistically significant, a *t* test was performed using the null hypothesis that the fold-change difference between the experimental and control was equal to one.

Acetylene-reduction activity assay.

The acetylene reduction assays were performed essentially as described (Hardy et al. 1968). Briefly, root samples with approximately 5 to 20 nodules were placed into 20-ml glass vials, each fitted with a rubber stoppers. Acetylene (2 ml) was injected into the vial to create a 10% acetylene atmosphere. These vials were incubated at room temperature. After the given assay time, 2 ml of the head gas from each vial was removed to analyze for ethylene by gas chromatography. Nitrogenase activity was recorded as micromoles of ethylene produced per milligram of nodule fresh weight per hour. The levels of the experimental and control samples were compared with two replications using a *t* test.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

miRBase, the microRNA database: mirbase.org
The Phytozome database: www.phytozome.net