The Micro-RNA172c-APETALA2-1 Node as a Key Regulator of the Common Bean-*Rhizobium etli* Nitrogen Fixation Symbiosis[1][OPEN]

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Micro-RNAs are recognized as important posttranscriptional regulators in plants. The relevance of micro-RNAs as regulators of the legume-rhizobia nitrogen-fixing symbiosis is emerging. The objective of this work was to functionally characterize the role of micro-RNA172 (miR172) and its conserved target APETALA2 (AP2) transcription factor in the common bean (*Phaseolus vulgaris*)-*Rhizobium etli* symbiosis. Our expression analysis revealed that mature miR172c increased upon rhizobial infection and continued increasing during nodule development, reaching its maximum in mature nodules and decaying in senescent nodules. The expression of *AP2-1* target showed a negative correlation with miR172c expression. A drastic decrease in miR172c and high *AP2-1* mRNA levels were observed in ineffective nodules. Phenotypic analysis of composite bean plants with transgenic roots overexpressing miR172c or a mutated *AP2-1* insensitive to miR172c cleavage demonstrated the pivotal regulatory role of the miR172 node in the common bean-rhizobia symbiosis. Increased miR172 resulted in improved root growth, increased rhizobial infection, increased expression of early nodulation and autoregulation of nodulation genes, and improved nodulation and nitrogen fixation. In addition, these plants showed decreased sensitivity to nitrate inhibition of nodulation. Through transcriptome analysis, we identified 114 common bean genes that coexpressed with *AP2-1* and proposed these as being targets for transcriptional activation by *AP2-1*. Several of these genes are related to nodule senescence, and we propose that they have to be silenced, through miR172c-induced *AP2-1* cleavage, in active mature nodules. Our work sets the basis for exploring the miR172-mediated improvement of symbiotic nitrogen fixation in common bean, the most important grain legume for human consumption.

The symbiotic nitrogen fixation (SNF) occurring in the legume-rhizobia symbiosis takes place in root-developed specialized organs called nodules. Nodulation is a complex process that involves communication between rhizobia and legumes through molecular signals, including rhizobial lipochitin-oligosaccharide symbiotic signals known as nodulation factors (NFs), that triggers a root-signaling cascade essential for rhizobia infection (for review, see Crespi and Frugier, 2008; Oldroyd and Downie, 2008; Kouchi et al., 2010; Murray, 2011; Oldroyd, 2013). Nuclear Ca^{2+} oscillations, or calcium spiking, is one of the earliest NF-induced responses in legume root hairs. Perception and transduction of the calcium-spiking signal involves Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE (CCaMK), which interacts with the nuclear protein CYCLOPS, and other downstream components, such as the transcriptional regulators NODULATION SIGNALING PATHWAY (NSP1)/NSP2, NUCLEAR FACTOR YA1 (NF-YA1)/YA2, ETHYLENE-RESPONSIVE FACTOR REQUIRED FOR NODULATION1, and NODULE INCEPTION (NIN), which, in turn, control the expression of early nodulation genes.

Legumes strictly regulate the number of developing nodules in response to internal and external cues. An important internal cue is the systemic feedback regulatory mechanism called autoregulation of nodulation (AON), which consists of root-derived and shoot-derived long-distance signals. AON is initiated in response to rhizobial NF during nodule primordium formation by the root production of CLAVATA3/Embryo-Surrounding Region Protein-related (CLE) peptides (Reid et al., 2011a). Some CLE peptides are predicted, although not proven, to act as the ligand for a shoot CLAVATA1-like Leu-rich

[1] This work was supported by the Dirección General de Asuntos del Personal Académico/Universidad Nacional Autónoma de México (grant nos. PAPIIT: IN209710 and IN210814) and the Consejo Nacional de Ciencia y Tecnología, México (studentship nos. 351615 and 340334 to B.N.-F. and L.P.I., respectively).

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www.plantphysiol.org/cgi/doi/10.1104/pp.114.255547

repeat receptor kinase (Okamoto et al., 2009). Activation of this receptor is proposed to initiate the production of a shoot-derived inhibitor that is transported to the root, where it inhibits further nodule formation (for review, see Magori and Kawaguchi, 2009; Ferguson et al., 2010; Kouchi et al., 2010; Reid et al., 2011b). Soil nitrogen is an important external cue for the control of nodulation (Streeter and Wong, 1988). Recent work indicates that nitrate inhibition of nodulation may function via an up-regulation of a nitrate-induced CLE peptide that is perceived by a Leu-rich repeat receptor kinase in the root (Okamoto et al., 2009; Reid et al., 2011a).

In recent years, microRNAs (miRNAs), a class of noncoding RNA 21 to 24 nucleotides in length, have been identified as central regulators of gene expression in plants, controlling fundamental processes such as stress response, phytohormone regulation, organ morphogenesis, and development (Rogers and Chen, 2013). The plant miRNA precursors, generally transcribed by RNA polymerase II, adopt stem-loop structures that are processed by several enzymes and generate mature miRNAs that are exported to the cytosol. The role of miRNAs in posttranscriptional regulation is mediated by the almost perfect complementarity with their target mRNAs, thereby causing their degradation or their translational inhibition (Zhang et al., 2006; Rogers and Chen, 2013).

Progress in high-throughput sequencing technologies has facilitated the genome-wide identification of large miRNA populations and their target mRNAs in different legumes (for review, see Simon et al., 2009; Bazin et al., 2012; Bustos-Sanmamed et al., 2013). Conserved and legume-specific miRNA families differentially expressed during nodule organogenesis have been reported for *Medicago truncatula*, soybean (*Glycine max*), and *Lotus japonicus* (Subramanian et al., 2008; Lelandais-Brière et al., 2009; De Luis et al., 2012; Turner et al., 2012; Dong et al., 2013). Recently, Formey et al. (2014) identified miRNAs from *M. truncatula* roots that respond to treatments with purified NF. However, evidence for the functional involvement of miRNAs in rhizobial infection and the functionality of nodules has only been obtained for a small number of candidates. The involvement of *M. truncatula* microRNA166 (miR166), miR169, and miR164 in nodule development has been reported. miR169 controls nodule meristem maintenance through the repression of *NF-YA1* (previously called *HAEM ACTIVATOR PROTEIN2-1*), a nodule-responsive transcription factor (TF; Combier et al., 2006), while miR166 and its target gene, *HOMEODOMAIN-LEUCINE ZIPPER protein of class III* TF, regulate meristem activity and vascular differentiation in roots and nodules (Boualem et al., 2008). The overexpression of miR164, a conserved miRNA targeting *NAC1* (for no apical meristem [NAM], Arabidopsis transcription activation factor [ATAF1-2], and cup-shaped cotyledon [CUC2] domain1) TF in roots, affected nodule organogenesis presumably through the deregulation of auxin responses (D'haeseleer et al., 2011). In soybean, the overexpression of miR482, miR1512, and miR1515 results in increased nodule numbers without affecting root development or the number of nodule primordia (Li et al., 2010). Recently, Turner et al. (2013) reported that the overexpression of soybean miR160, which targets a set of repressor auxin response factors, resulted in an enhanced sensitivity to auxin and inhibition of nodule development, apparently through a reduction in cytokinin sensitivity. Likewise, the overexpression of *M. truncatula* miR160 affected root gravitropism and nodule number (Bustos-Sanmamed et al., 2013). Specific variants of *L. japonicus* and *M. truncatula* miR171 target the GRAS-family *NSP2* TF, a key regulator of the common symbiotic pathway for rhizobial and arbuscular mychorrizal symbioses (Ariel et al., 2012; De Luis et al., 2012; Lauressergues at al., 2012). *M. truncatula* roots overexpressing miR171h showed deceased arbuscular mychorrizal colonization (Lauressergues at al., 2012), while in *L. japonicus*, miR171c regulates the maintenance and establishment of the nodule but not the bacterial infection (De Luis et al., 2012). In addition, the role of *L. japonicus* miR397 in nodule copper homeostasis, through the regulation of a member of the laccase copper protein family, has been documented (De Luis et al., 2012).

Common bean (*Phaseolus vulgaris*) is the most important crop legume for human consumption and the main source of proteins for people in African and Central/South American countries (Broughton et al., 2003). Our research is focused on identifying and functionally characterizing common bean miRNAs. High-throughput sequencing of small RNAs generated from different organs of common bean let us identify more than 100 conserved miRNAs and to predict novel miRNAs (Peláez et al., 2012). Common bean miRNAs that respond to drought, salinity, nutrient deficiencies, or metal toxicity stresses have been identified, and their target genes have been predicted or validated (Arenas-Huertero et al., 2009; Valdés-López et al., 2010; Contreras-Cubas et al., 2012). The roles of miR399 in the common bean root response to phosphorus deficiency (Valdés-López et al., 2008) and of miR398 in the regulation of copper homeostasis and response to biotic interactions (Naya et al., 2014) have been demonstrated. In this work, we analyzed the role of miR172 in common bean roots and nodules.

miR172 is conserved in all angiosperm lineages; its conserved targets are TFs from the *APETALA2* (*AP2*) family. The miR172 node that involves the miR156 node is one of the best-understood networks that regulate developmental timing in Arabidopsis (*Arabidopsis thaliana*) and other plants (Rubio-Somoza and Weigel, 2011). Aukerman and Sakai (2003) first described that miR172 promotes flowering by repressing *AP2* genes, primarily through translation inhibition (Chen, 2004) but also through mRNA cleavage (Kasschau et al., 2003; Jung et al., 2007). In addition, the miR172 node regulates the juvenile-to-adult phase transition during shoot development (Wu et al., 2009; Huijser and Schmid, 2011). Such developmental transitions are coordinated by the antagonistic activities of the miR156 and miR172 nodes. miR156 targets a subset of SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE (SPL) TFs that bind to the miR712 promoter and directly promote its transcription, resulting in *AP2* silencing (Wu et al., 2009).

miR172 has been identified in the legumes *M. truncatula*, *L. japonicus*, soybean, and common bean; it is highly accumulated in mature nodules relative to other plant tissues (Lelandais-Brière et al., 2009; Wang et al., 2009; Valdes-López et al., 2010; De Luis et al., 2012). Yan et al. (2013) reported the regulation of soybean nodulation by miR172 that involves a complex regulatory circuit in which miR156 regulates miR172 expression, which, in turn, controls the level of its *AP2* target gene. They propose that *AP2*, directly or indirectly, controls the expression of nonsymbiotic hemoglobin, which is essential for regulating the levels of nodulation and nitrogenase activity (Yan et al., 2013). Very recently, Wang et al. (2014) demonstrated that soybean miR172c modulates rhizobial infection and nodule organogenesis. They showed that miR172c regulates nodule formation by repressing its target gene *NODULE NUMBER CONTROL1* (*NNC1*), an AP2 TF, which directly targets and represses the early nodulin gene *ENOD40* that plays a key role in nodulation. However, it is not clear whether miR172c controls early responses that are critical to establish a functional symbiosis between legumes and rhizobia.

The aim of this work was to analyze the role of the miR172 node in the common bean-rhizobia symbiosis. We determined an increased expression of miR172c upon rhizobial infection and during nodulation, showing a negative correlation with *AP2-1* expression. We achieved the overexpression of miR172c and of a mutagenized *AP2-1* insensitive to miR172 cleavage in composite common bean plants. Common bean plants with increased miR172c levels showed an improved symbiotic phenotype as well as lower sensitivity to nitrate inhibition of nodulation. We explored the possible role of AP2-1 as a transcriptional activator and/or repressor. Candidate target genes for downstream transcriptional activation by AP2-1 were identified; these could be relevant in the nodule senescence process. Our work extends the knowledge of miR172 function in the nodulation of common bean, an agronomically important legume.

RESULTS

Common Bean miR172 Isoforms and Target Genes

The Arabidopsis genome contains five loci that generate miR172 isoforms miR172a to miR172e, while 12 miR172 isoforms are reported for soybean (www.mirbase.org, version 20). The high-throughput small RNA sequencing analysis by Peláez et al. (2012) led us to identify four isoforms of common bean miR172.

In this work, we analyzed the recently published (Schmutz et al., 2014; www.phytozome.net/commonbean.php, v1.0) common bean genome sequence and identified six *MIR172* loci that map in different common bean chromosomes. The most stable secondary structure of the miR172 precursors was predicted, and these showed the expected stem-loop structure. The six isoforms of mature common bean miR172, 20 or 21 nucleotides long, were designated miR172a to miR172f (Supplemental Fig. S1). The nucleotide sequences of miR172a, miR172b, and miR172c isoforms differ. However, although encoded by different loci, the sequences of miR172e and miR172f are identical to miR172a, while miR172d is identical to miR172c (Supplemental Fig. S1).

The conserved targets for miR172 in different plants are transcripts that encode TFs from the *AP2* superfamily. In Arabidopsis, six *AP2* TF genes, *AP2*, *TARGET OF EARLY ACTIVATION TAGGED1* (*TOE1*), *TOE2*, *TOE3*, *SCHLAFMUTZE*, and *SCHNARCHZAPFEN*, which act as floral repressors, are targeted by miR172 and silenced through translation inhibition or cleavage (Aukerman and Sakai, 2003; Schmid et al., 2003; Chen, 2004; Jung et al., 2007). In soybean, 10 *AP2* TF genes have been proposed as miR172 targets (Song et al., 2011). A recent analysis of the common bean transcriptome by RNA sequencing (RNA-seq) combined with available gene calls (O'Rourke et al., 2014) identified 202 transcripts encoding AP2-type TFs. The phylogenetic tree generated using sequence alignments of the common bean AP2 proteins is depicted in Figure 1A. From the whole set (202), we identified eight *AP2* transcripts, encoded by six loci, with putative miR172 binding sites within their coding regions. The six predicted *AP2* target genes showed base pairing with the three different miR172 isoforms (Fig. 1B). In each case, the penalty score for miRNA:mRNA (Jones-Rhoades and Bartel, 2004) was low; the highest score was observed in Phvul.007G240200, with the three miR172 isoforms. From the predicted *AP2* targets, Phvul.005G138300, hereafter denominated as *AP2-1*, has been experimentally validated as a target of common bean miR172 (Arenas-Huertero et al., 2009). In addition, Phvul.011G071100 was identified as a target in a common bean degradome analysis (D. Formey, L.P. Íñiguez, P. Peláez, Y.F. Li, R. Sunkar, F. Sánchez, J.L. Reyes, and G. Hernández, unpublished data). Interestingly, the transcripts of *AP2* predicted targets were organized in a single clade of the phylogenetic tree (Fig. 1A). However, this clade also includes the Phvul.008G185400.1 transcript, which has an AP2 domain (http://www.phytozome.net/commonbean.php) but lacks a detectable miR172 binding site and, therefore, is not proposed as a target (Fig. 1A). The recently published *Phaseolus vulgaris* Gene Expression Atlas (*Pv* GEA; O'Rourke et al., 2014) showed a very low expression of this *AP2* gene in all the tissues reported (reads per kilobase per million = 6, highest values in leaves and pods). Therefore, Phvul.008G185400 *AP2* is perhaps highly expressed in tissue-, development-, or environment-specific conditions not yet analyzed and its transcript level could be regulated by factors other than miR172. In addition, we could not detect a Phvul.008G185400.1 ortholog in the soybean genome sequence, perhaps indicating that it could be a pseudogene.

Differential Expression of miR172, Predicted *AP2* Target Genes, miR156, and *SPL6* in Plant Tissues

The differential expression of miR172 isoforms in plant organs/tissues at different developmental stages has been reported for Arabidopsis and soybean (Aukerman

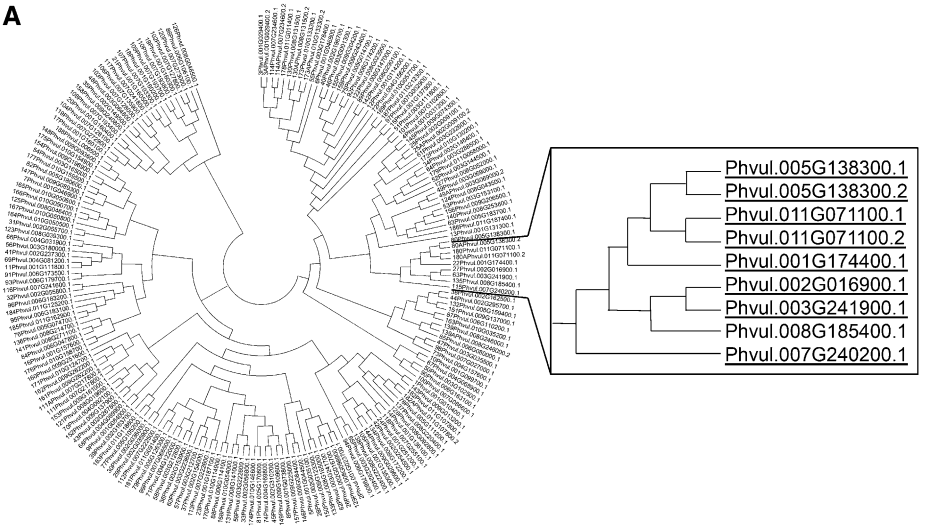

Figure 1. Common bean *AP2* transcripts with predicted miR172 binding sites. A, Neighbor-joining tree of AP2 proteins retrieved from the common bean genome sequence (http://www.phytozome.net/commonbean.php, v1.0). The clade including *AP2* transcripts with miR172 binding sites (underlined) is shown in the inset. B, Pairing of the three different miR172 isoforms (a–c) with the predicted binding sites of the six different AP2 transcripts highlighted in A. Watson-Crick base pairing is indicated by lines, G:U base pairing is indicated by circles, and dashes indicate mismatches. Penalty scores, shown in parentheses, were calculated as described by Jones-Rhoades and Bartel (2004).

B

	miR172a	miR172b	miR172c
	miRNA:mRNA pairing (penalty score)		
Phvul.005G138300	**5'**IIIIIIIIIIIII°IIIIII**3'** (0.5)	**5'**IIIIIIIIIIIIII°IIIIII**3'** (1.0)	**5'**IIIIIIIIIII°IIIIII°**3'** (1.0)
Phvul.011G071100	**5'**IIIIIIIIIIIII°IIIIII**3'** (0.5)	**5'**IIIIIIIIIIIIII°IIIIII**3'** (1.0)	**5'**IIIIIIIIIIIII°IIIII°**3'** (1.0)
Phvul.001G174400	**5'**IIIIIIIIIIIII°IIIII-**3'** (1.5)	**5'**IIIIIIIIIIIIII°IIIII-**3'** (1.5)	**5'**IIIIIIIIIIIII°IIIIII**3'** (0.5)
Phvul.002G016900	**5'**IIIIIIIIIIIII°IIIII-**3'** (1.5)	**5'**IIIIIIIIIIIIII°IIIII-**3'** (1.5)	**5'**IIIIIIIIIIIII°IIIIII**3'** (0.5)
Phvul.003G241900	**5'**IIIIIIIIIIIII°IIIII-**3'** (1.5)	**5'**IIIIIIIIIIIIII°IIIII-**3'** (1.5)	**5'**IIIIIIIIIIIII°IIIIII**3'** (0.5)
Phvul.007G240200	**5'**-IIIIIIIIIIII°IIIII-**3'** (2.5)	**5'**-IIIIIIIIIIIII°IIIII-**3'** (2.5)	**5'**-IIIIIIIIIIII°IIIII-**3'** (2.5)

and Sakai, 2003; Wu et al., 2009; Yan et al., 2013; Wang et al., 2014). In this work, we performed expression analyses of the miR172 isoforms and their putative *AP2* target genes (Fig. 1) in different tissues of SNF common bean plants 18 d post inoculation (dpi) with *Rhizobium etli* (Fig. 2).

Northern-blot analysis revealed that mature miR172 transcripts were most abundant in nodules followed by flowers (Fig. 2A). The miR172a probe was used for blot hybridization, but the quantified signals might reflect the combined levels of miR172 isoforms whose sequences differ only in two nucleotides (Supplemental Fig. S1). For real-time quantitative reverse transcription (qRT)-PCR expression analysis, specific primers were synthesized for each of the miR172 isoforms (a, b, and c; Supplemental Fig. S1; Supplemental Table S1). The data obtained by northern-blot and qRT-PCR expression analyses showed similar trends regarding the highest levels in nodules and flowers and the lowest levels in roots and leaves (Fig. 2, A and B). The observed variation in the levels of cumulative miR172 expression for each tissue may be attributable to the different sensitivities of the two methods. In addition, qRT-PCR analysis revealed differential expression of the miR172 isoforms among tissues, especially in those tissues with higher cumulative levels. Nodules showed the highest level of miR172c and very low amounts of miR172a and miR172b, while flowers showed the highest level of miR172b, followed by miR172c and a low amount of miR172a (Fig. 2B).

The transcript levels of each of the *AP2* TF genes proposed as miR172 targets (Fig. 1) were determined by qRT-PCR (Fig. 2C) in tissues from SNF bean plants (Fig. 2C). Cumulative *AP2* transcript levels were very high in roots and very low in nodules, thus showing a negative correlation with cumulative miR172 levels in these tissues (Fig. 2, B and C). In roots, the most highly expressed of the *AP2* genes were Phvul.005G138300 (*AP2-1*) and Phvul.011G071100; *AP2-1* was also highly expressed in embryonic leaves (Fig. 2C). However, a distinct pattern was observed in flowers, where the expression of these two genes was negligible and Phvul.001G174400, Phvul.003G241900, and Phvul.002G16900 were highly expressed.

The miR156 node has been implicated in upstream negative regulation of the miR172 node (Rubio-Somoza and Weigel, 2011). Arabidopsis miR156 represses miR172 expression by targeting members of the *SPL* family of TFs that directly bind to the *MIR172* promoter and positively regulate its expression (Wu et al., 2009). Here,

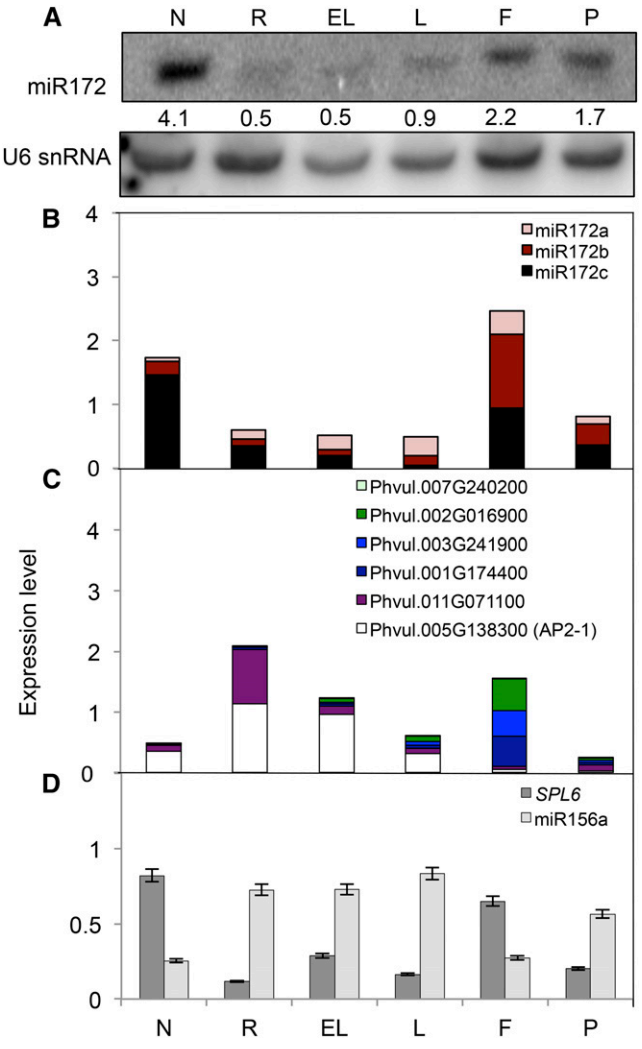

Figure 2. Expression analysis of miR172, *AP2* target genes, miR156a, and its *SPL* transcription factor target gene in different tissues of *R. etli* CE3-inoculated common bean plants (18 dpi). A, Mature miR172 levels detected by northern-blot analysis using U6 small nuclear RNA (snRNA) as a loading control for normalization. Signal intensities of the miR172 and U6 hybridization bands for each tissue were determined to calculate normalized expression levels. Numbers in each lane indicate normalized values of miR172 signal intensity. N, Nodules; R, roots; EL, embryonic leaves; L, leaves; F, flowers; P, pods. B to D, Transcript levels of mature miR172 isoforms (B; Supplemental Fig. S1), predicted *AP2* target genes (C; Fig. 1), and mature miR156a and its target gene *SPL6* (D) determined by qRT-PCR. Expression level refers to gene expression, based on threshold cycle (C_t) value, normalized with the expression of the housekeeping miR159 or *UBIQUITIN CONJUGATING ENZYME9* (*UBC9*) gene.

we determined the levels of mature miR156a (Peláez et al., 2012) in common bean tissues. The expression of miR156a was elevated in roots and leaves but low in nodules and flowers, showing an opposite trend of the cumulative expression of miR172 (Fig. 2D). We identified 32 *SPL* genes in the common bean genome, and 14 of these showed putative miR156 binding sites, including Phvul.009G165100, which was validated as a common bean miR156a target (Arenas-Huertero et al., 2009). Comparative sequence analysis with the Arabidopsis *SPL* gene family indicated that the common bean Phvul.009G165100 *SPL* gene is an ortholog to Arabidopsis *SPL6*. We analyzed the expression of the validated miR156a target *SPL6* gene in different common bean organs. As shown in Figure 2D, common bean *SPL6* was highly expressed in nodules and flowers while it was decreased in roots and leaves, thus showing a negative correlation with miR156 expression. We also searched for SPL transcription factor binding sites (TFBS) in the 5′ (promoter) region of each of the six *MIR172* loci mapped in the genome, but we could not identify any, while 35 other TFBS were present in one or more of these loci (Supplemental Table S2).

Our data (Fig. 2) showed miR172c as the isoform with the highest expression in nodules and low expression in roots. Its expression pattern is opposite that of *AP2-1* (Phvul.005G138300), the experimentally validated target (Arenas-Huertero et al., 2009) that showed the highest expression in roots. Therefore, we then focused our analysis on miR172c and *AP2-1* in common bean plants interacting with *R. etli*.

Expression Analysis of miR172 and *AP2-1* during Symbiosis

To assess a possible role of miR172c/*AP2-1* in the common bean-*R. etli* symbiosis, we determined their expression in inoculated roots (Fig. 3) and in effective nodules at different developmental stages (Fig. 4A).

To analyze miR172c/*AP2-1* regulation at early stages of the symbiosis, common bean plantlets were grown in plastic square bioassay dishes and *R. etli* inoculum was applied directly to the roots. For gene expression analysis, only the responsive root zone, where initial bacteria-host recognition takes place, was collected at the initial time (0 h) and at 3, 6, 12, 24, and 48 h post inoculation (hpi). The mature miR172c level increased significantly after 6 h, whereas the high expression level of the *AP2-1* target gene decreased significantly after 12 h; both transcript levels persisted until 48 h (Fig. 3A).

To investigate if miR172c up-regulation correlated with relevant events in the rhizobial infection process, we determined the expression of early nodulation genes in inoculated roots (Fig. 3, B–G). O'Rourke et al. (2014) identified common bean nodulation genes that were highly expressed in young and/or mature nodules and that are homologous to cognate nodulation genes previously identified in other legume species. From these, we selected six early nodulation genes for expression analysis: the TF genes *NF-YA1* (Phvul.001G196800.1), *NSP2* (Phvul.009G122700.1), and *NIN* (Phvul.009G115800); *CYCLOPS* (Phvul.002G128600.1), coding for a nuclear protein that interacts with CCaMK; *FLOTILLIN-LIKE2* (*FLOT2*; Phvul.009G090700.1), coding for a lipid raft component; and *ENOD40* (Phvul.008G291800), which lacks an open reading frame but encodes two small peptides and may function as a cell-cell signaling molecule for nodulation (Crespi and Frugier, 2008; Oldroyd and

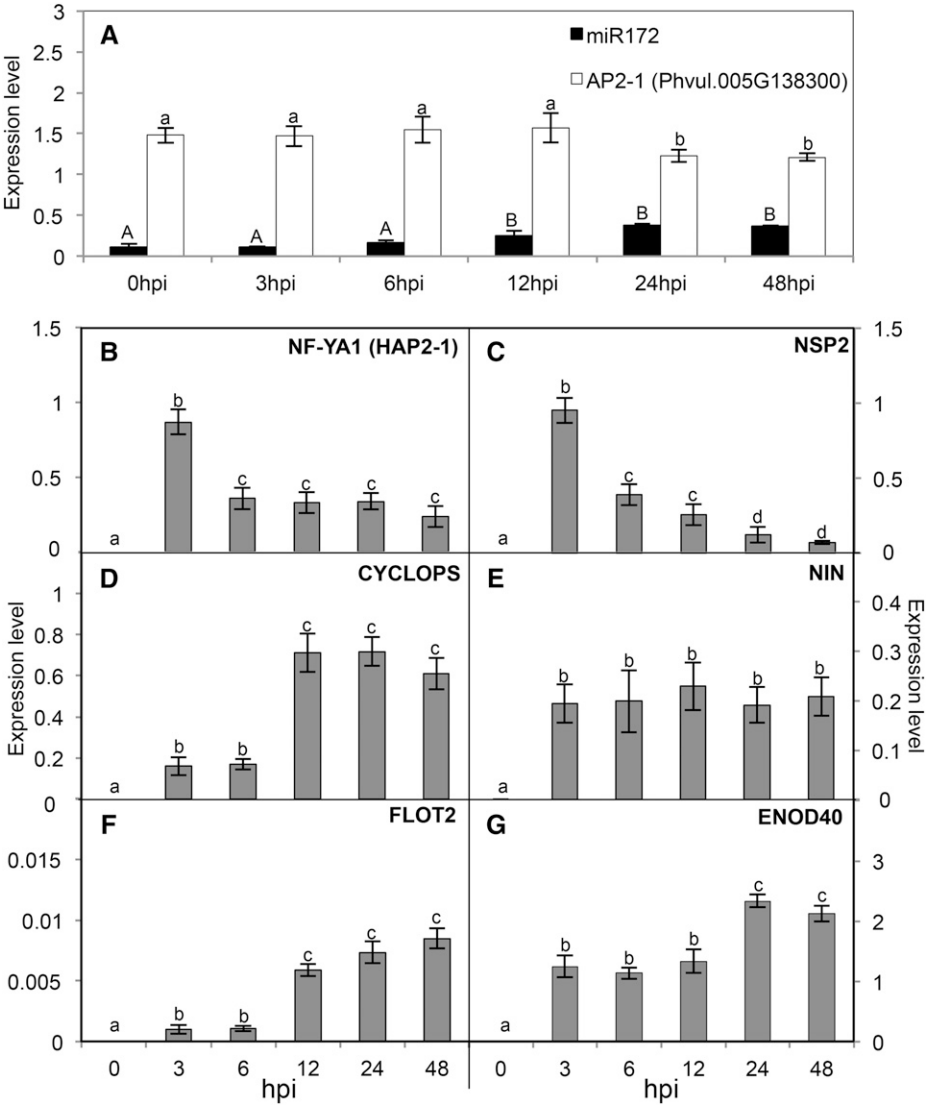

Figure 3. Increased expression of miR172c and of early nodulation genes upon rhizobial infection. Expression levels of mature miR172c, *AP2-1* (Phvul.005G138300; A), and early nodulation genes (B–G) were determined in roots inoculated with *R. etli* CE3 at the initial time (0) and after the indicated hpi. The common bean early nodulation genes were identified in the *Pv* GEA (O'Rourke et al., 2014): *NF-YA1*, Phvul.001G196800 (B); *NSP2*, Phvul.009G122700 (C); *CYCLOPS*, Phvul.002G128600 (D); *NIN*, Phvul.009G115800 (E); *FLOT2*, Phvul.009G090700 (F); and *ENOD40*, Phvul.002G064200 (G). Values represent averages ± SD from three biological replicates and two technical replicates each. Expression level refers to gene expression, based on C_t value, normalized with the expression of the housekeeping miR159 or *UBC9* gene. Different lowercase letters indicate statistically different groups (ANOVA, $P < 0.001$); in A, lowercase and uppercase letters were used for *AP2-1* and miR172c values, respectively.

Downie, 2008; Kouchi et al., 2010; Murray, 2011; Oldroyd, 2013). Figure 3, B to G, shows the expression levels of the early nodulation genes in the responsive zone of *R. etli*-inoculated roots. The expression of all the genes tested increased significantly after the initial time (3 h). Highest levels of *NF-YA1* and *NSP2* decreased gradually after 3 h. Increased *NIN* expression persisted, whereas *CYCLOPS* and *FLOT2* transcripts increased further after 6 h and persisted until 48 h. The *ENOD40* transcript level increased significantly after 12 h and persisted until 48 h.

We then analyzed the regulation of the miR172 node during the development of effective nodules elicited by the *R. etli* CE3 wild-type strain (Fig. 4A). Nodules from inoculated common bean plants were harvested at different developmental stages, as defined by the differential expression of nodule development marker genes (Ramírez et al., 2005; Van de Velde et al., 2006; Supplemental Table S3). Immature, prefixing, 13-dpi nodules showed the highest *ENOD55* expression and low (19.6%) nitrogenase activity. At 18 dpi, the nodules were fully developed and showed the highest nitrogenase activity and expression of the *PHOSPHOENOLPYRUVATE CARBOXYLASE* (*PEPc*) gene, essential for carbon assimilation in mature nodules (Ramírez et al., 2005). By 35 dpi, nodules had low nitrogenase activity (11%) and high *CYSTEINE PROTEINASE* (*CP*) gene expression, described as being specific for nodule senescence (Van de Velde et al., 2006; Supplemental Table S3). As shown in Figure 4A, the increased expression level of miR172c observed at 2 dpi (or 48 h in Fig. 3A) persisted in immature, prefixing nodules (13 dpi). In contrast, miR172c increased significantly to its highest level in mature, fully active nodules (18 dpi). Afterward, a drastic decrease in miR172c level was observed; it remained barely detectable until nodule senescence (35 dpi). Slightly decreased levels of *AP2-1* transcripts persisted in immature nodules (13 dpi), a further decrease was observed in mature 18-dpi nodules, and afterward, the level of *AP2-1* transcripts gradually increased. The lowest level of *AP2-1* correlated with the highest level of miR172c in mature nodules (18 dpi).

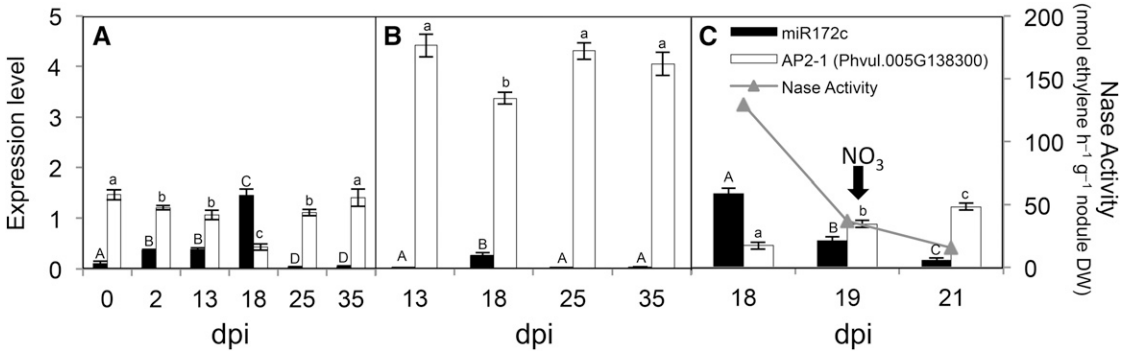

Figure 4. miR172c and *AP2-1* are differentially regulated in effective versus ineffective *R. etli* symbioses. Transcript levels were determined by qRT-PCR in inoculated roots or nodules harvested at the indicated dpi. A, Plants inoculated with the CE3 wild-type strain; determinations at 0 and 2 dpi were done in inoculated roots. B, Plants inoculated with the *fix*$^-$ *R. etli nifA*$^-$ mutant. C, Plants inoculated with the CE3 wild-type strain were grown for 18 d and watered with nitrogen-free nutrient solution. Subsequently, these plants were watered with nutrient solution supplemented with 10 mM KNO_3 (black arrow). Nitrogenase (Nase) activity and transcript levels were determined at 18 dpi and at 1 or 3 d after nitrate addition (19 or 21 dpi). Values represent averages ± SD from three biological replicates and two technical replicates each. Expression level refers to gene expression, based on C_t value, normalized with the expression of the housekeeping miR159 or *UBC9* gene. Different letters indicate statistically different groups (ANOVA, $P < 0.001$); lowercase and uppercase letters were used for *AP2-1* and miR172c values, respectively.

Taken together, these data suggest that miR172c is involved in rhizobial infection and nodule development/function.

Altered Expression of miR172c and *AP2-1* in Ineffective Symbioses

Because the maximum level of miR172c expression and *AP2-1* silencing correlated with the peak of SNF (Fig. 4A; Supplemental Table S2), we assessed the regulation of the miR172 node in ineffective, nonfixing common bean-*R. etli* symbioses.

The nitrogen fixation genes regulator A (NifA)/RNA polymerase sigma factor complex is a master regulator of the N_2 fixation genes in rhizobia. Transcriptional analysis of the *R. etli nifA*$^-$ (CFNX247) mutant strain demonstrated the *nifA* dependency of symbiotic genes on the symbiotic plasmid (Girard et al., 1996). The symbiotic phenotype of common bean plants inoculated with the *R. etli nifA*$^-$ mutant strain was drastically altered, as evidenced by a diminished amount of early-senescent nodules with few infected cells having bacteroids and devoid of nitrogenase activity and with symptoms characteristic of nitrogen deprivation in the leaves (Supplemental Fig. S2; Supplemental Table S3). As shown in Figure 4B, the ineffective nodules elicited by *R. etli nifA*$^-$ had nearly undetectable levels of miR172c, although a minor, but significant, increase was observed in 18-dpi ineffective nodules. Meanwhile, the *AP2-1* target gene was highly induced at the different developmental stages of ineffective nodules; these values were even higher (approximately 2-fold) than those observed during effective symbiosis (Fig. 4, A and B). A slight but significant decrease in *AP2-1* transcript was observed in 18-dpi ineffective nodules, when the miR172c level increased (Fig. 4B).

A similar effect was observed when the abolishment of SNF was achieved by adding nitrate to effective *R. etli*-elicited nodules (Fig. 4C), a well-known phenomenon in the legume-rhizobia symbiosis (Streeter and Wong, 1988). A short time (1 and 3 d) after nitrate addition, nitrogenase activity decreased drastically and nodules senesced (Fig. 4C; Supplemental Table S3). The latter correlated with the drastic decrease in mature miR172c and a concomitant increase of *AP2-1* transcript level in the ineffective nodules (Fig. 4C).

Taken together, these data indicate a contrasting regulation of miR172c/*AP2-1* expression in effective versus ineffective symbioses.

Effect of miR172c Overexpression on Root Development and Rhizobial SNF

To further investigate the role of miR172c and its target gene *AP2-1* in SNF, we aimed to modulate their expression in common bean composite plants with transgenic roots and untransformed aerial organs, generated through *Agrobacterium rhizogenes*-mediated genetic transformation. This protocol has been used as an alternative method for stable transformation in common bean and other recalcitrant species (Estrada-Navarrete et al., 2007). The construct for miR172 overexpression (OE172) contained the 35S cauliflower mosaic virus promoter fused to the miR172c precursor. The OEAP2m plasmid contained a mutagenized *AP2-1* gene that is insensitive to miR172 cleavage due to nucleotide substitutions in the miR172 binding site. Both constructs as well as the control empty vector (EV) contain the tdTomato (red fluorescent protein) reporter gene (Supplemental Fig. S3). We obtained several composite plants and determined the level of transgene expression for each plant (Supplemental Fig. S4). The OE172 composite plants showed very high levels of mature miR172c in both nodules and roots as well as a decreased level of *AP2-1* transcript. Roots and nodules of OEAP2m plants showed very high levels of *AP2-1*. The

variation in the degree of overexpression between individual transgenic roots is because each results from an independent transformation event.

We first assessed if miR172 overexpression affected the root phenotype of fertilized (noninoculated) common bean plants as compared with those inoculated with *R. etli*. As shown in Figure 5, roots with high miR172c showed increased biomass and density of secondary roots, both in fertilized and SNF composite plants. The opposite phenotype was observed in OEAP2m composite plants. These data indicate that miR172 had a positive effect on root biomass/architecture independent of the presence of rhizobia.

To analyze if the positive effect of miR172 on root development (Fig. 5) also affects rhizobial infection and SNF, we investigated the response of composite plants altered in miR172 content to *R. etli* infection, early symbiotic stages, and nodule development/function.

Figure 6 shows data for the analysis of rhizobial infection and early nodulation gene expression. For these experiments, the plastic square bioassay dish system was used for the inoculation and growth of OE172, EV, or OEAP2m composite plants. Notably, the amount of deformed root hairs was significantly higher in 48-hpi inoculated roots that overexpress miR172, while the opposite effect was observed in OEAP2m roots (Fig. 6A; Supplemental Fig. S5). A correlation of altered root hair deformation and the expression of early nodulation genes essential for rhizobial infection was observed after determining the transcript level of selected genes (*NF-YA1*, *NSP2*, *CYCLOPS*, *ENOD40*, *FLOT2*, and *NIN*) in the responsive root zone from 0 to 48 hpi (Fig. 6, B–G). All the genes tested showed increased expression in OE172 inoculated roots; *NIN* expression was increased only as compared with OEAP2m inoculated roots (Fig. 6, B–G).

Nodule number and nitrogenase activity as well as histological analysis of nodules stained with SYTO13 (a fluorescent dye binding nucleic acids) from composite plants overexpressing miR172 or *AP2m* and control (EV) plants are presented in Figures 7 and 8. At 14 and 21 dpi, OE172 plants showed increased nodule number and nitrogenase activity that correlated with higher *PEPc* and reduced *CP* expression (Fig. 7; Supplemental Table S4). In addition, OE172 plants showed accelerated nodule development: nodule primordia and well-formed nodules (approximately 50 per root) were easily observed at 5 and 7 dpi, respectively, while only some unorganized primordia and very few tiny nodules were seen in EV plants. In addition, young (7 and 14 dpi) nodules from OE172 plants were larger (increased perimeter; Fig. 8, A and B). In contrast, OEAP2m plants at 21 dpi had fewer nodules with diminished nitrogenase activity and higher *CP* expression (Fig. 7; Supplemental Table S4). At all time points analyzed, the OEAP2m nodules had significantly reduced perimeters (Fig. 8, A and B). Similar values for SYTO13 intensity per nodule area were obtained for EV, OE172, and OEAP2m nodules, indicating similar bacteroid densities in infected cells (Fig. 8C).

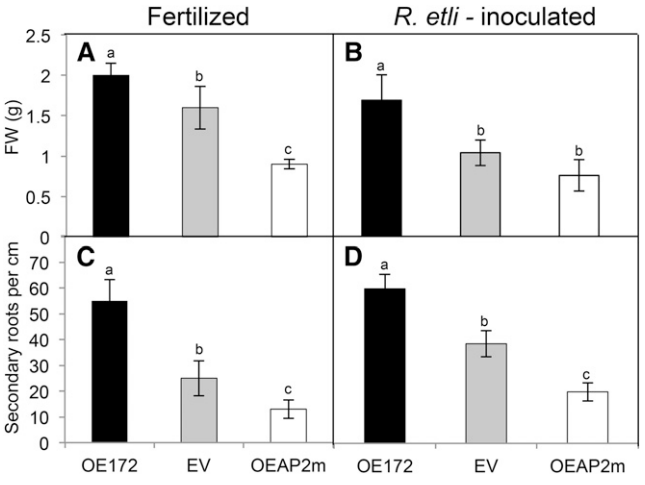

Figure 5. miR172c and *AP2-1* control the root development of fertilized and *R. etli*-inoculated common bean plants. Root fresh weight (FW; A and B) and density of secondary roots (C and D) were determined in composite bean plants with transgenic roots overexpressing miR172c (OE172) or a mutated insensitive *AP2-1* target gene (OEAP2m) as compared with control EV transformed roots. A set of composite plants was fertilized with full-nutrient solution for 10 d (A and C), and another set was inoculated with *R. etli* and watered with nitrogen-free nutrient solution for 21 dpi (B and D). Values represent averages ± SD from roots of eight independent composite plants each. Different lowercase letters indicate statistically different groups (ANOVA, $P < 0.001$).

We assessed if increased nodulation in OE172 common bean plants could be related to alterations in the AON. In soybean, AON involves long-distance signaling requiring the interaction of RHIZOBIA-INDUCED CLE peptides (RIC1/RIC2), with NODULE AUTOREGULATION RECEPTOR KINASE (NARK) in the leaf and the subsequent inhibition of nodulation via the production of a shoot-derived inhibitor. For local nitrate inhibition, the nitrate-induced CLE peptide (NIC1) interacts with NARK in the root, leading to a nitrate-induced inhibitor (Reid et al., 2011a). The homologous common bean *RIC* and *NIC* genes *RIC1* (Phvul.005G096900), *RIC2* (Phvul.011G135900), and *NIC1* (Phvul.005G097000) were identified from the *Pv* GEA (O'Rourke et al., 2014). As in soybean (Reid et al., 2011a), the common bean *RIC1* genes were expressed in inoculated common roots at early stages of rhizobial infection, while *RIC2* was expressed at later time points in prefixing and mature nodules. Figure 6, H and I, shows the expression levels of *RIC1* and *NIC1* genes in 48-hpi inoculated roots from OE172, EV, and OEAP2m plants. *RIC1* expression in control (EV) transgenic roots indicates the rhizobial induction of CLE-derived peptides for AON. Interestingly, the level of *RIC1* was decreased significantly in OE172 inoculated roots that showed increased nodulation, while it was increased significantly in OEAP2m roots with diminished nodulation (Fig. 6H). As expected, the expression of *NIC1* was low in the transgenic inoculated roots (Fig. 6I) under nitrogen-free conditions. These data point to the involvement of a common bean AON mechanism in the miR172 control of nodulation.

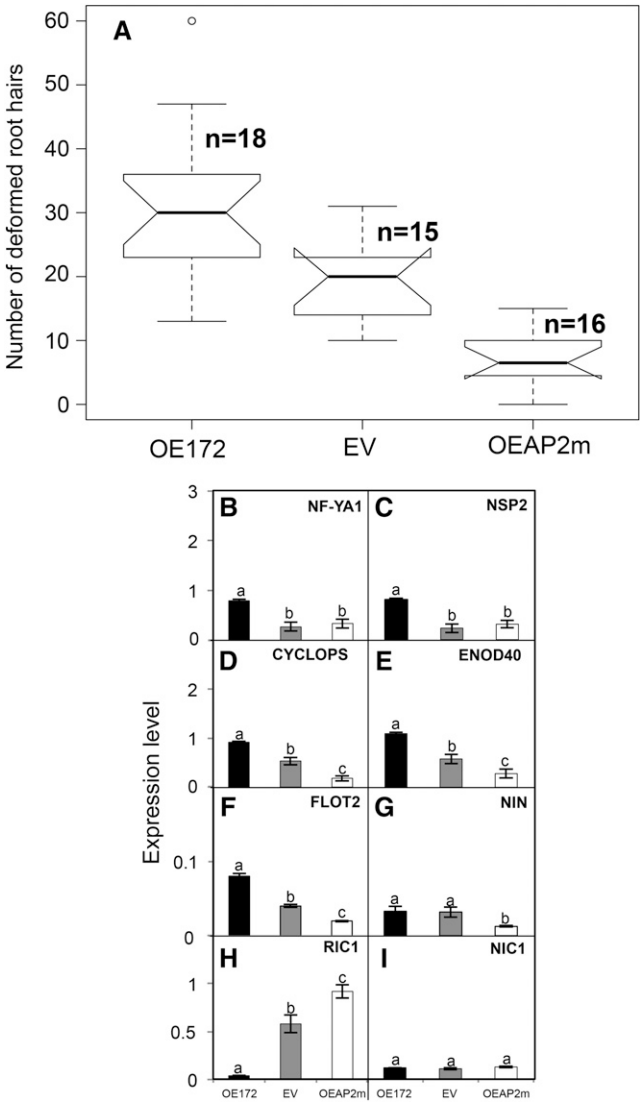

Figure 6. miR172c and *AP2-1* control the rhizobial infection of common bean roots. Roots from OE172, EV, or OEAP2m composite plants were inoculated with *R. etli* CE3, and at 48 hpi, the root responsive zones were harvested for analysis. A, Quantification of the number of deformed root hairs (branched and swollen root hair) per 0.5 cm; each box plot indicates the number of the transgenic roots analyzed for each construct. B to I, Expression analysis of selected early nodulation genes. Most gene identifiers are indicated in the legend to Figure 3; *RIC1*, Phvul.005G096900; and *NIC1*, Phvul005G097000. Values represent averages ± SD from three biological replicates and two technical replicates each. Expression level refers to gene expression, based on C_t value, normalized with the expression of the housekeeping *UBC9* gene. Different lowercase letters indicate statistically different groups (ANOVA, $P < 0.001$).

miR172c Overexpression Decreases the Sensitivity to Nitrate Inhibition of Rhizobial Symbiosis

Nitrogen (nitrate or ammonia) in the soil perceived by legume plants is an important external stimulus that inhibits nodulation as part of the AON mechanism. Considering the improved rhizobial infection and nodule development/function in common bean plants with increased miR172c (Figs. 6–8), we assessed if these alterations could be related to a decreased sensitivity to external nitrate inhibition of *R. etli* nodulation (Fig. 9). For this experiment, we applied a low nitrate concentration (1 mM) to *R. etli*-inoculated OE172, EV, and OEAP2m composite plants. The nodulation of inoculated OEAP2m plants, with low miR172c, was completely abolished when low nitrate was added. For this reason, we analyzed plants overexpressing miR172c as compared with control (EV) plants (Fig. 9). As expected for nitrate inhibition of the rhizobial infection process, the expression level of most early nodulation genes was reduced in EV-inoculated plants in the presence of nitrate (Fig. 9, A–F) as compared with the nitrogen-free condition (Fig. 6, B–G). Notably, in the presence of nitrate, the early nodulation genes *NF-YA1*, *NSP2*, *CYCLOPS*, *ENOD40*, and *FLOT2* showed significantly increased expression in OE172 inoculated roots (Fig. 9, A–F). In fact, the expression level of early nodulation genes in OE172 inoculated roots was similar in the absence (Fig. 6, B–G) or presence (Fig. 9, A–F) of nitrate. As expected, while the *RIC1* gene was barely detectable, *NIC1* was expressed in the responsive root zone of EV plants inoculated in the presence of nitrate, and it was increased in OE172 roots (Fig. 9, G–H).

Nitrate inhibition of nodulation was evident in control (EV) plants that presented delayed and diminished nodulation (Fig. 9I). These plants also showed decreased nitrogenase activity and *PEPc* expression and increased *ENOD55* (Fig. 9J; Supplemental Table S4) as compared with nitrogen-free inoculated plants (Fig. 7B). Notably, inoculated OE172 plants in the presence of nitrate showed few active nodules at 14 dpi, while at 21 dpi they had a similar number of mature nodules with slightly higher nitrogenase activity as compared with plants inoculated without nitrogen (Figs. 7 and 9, I and J; Supplemental Table S4). In addition, we observed that a higher nitrate concentration (3 mM) totally blocked the nodulation of EV plants, while OE172 plants were able to form active nodules (approximately 100 per root) at 21 dpi.

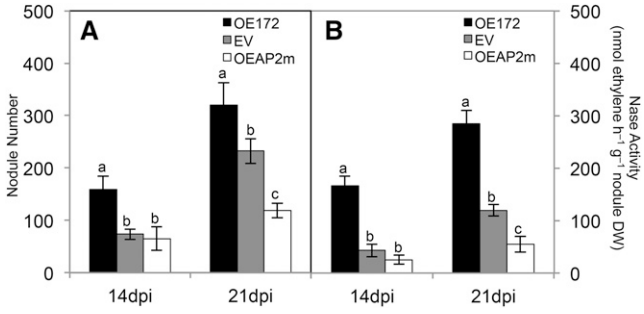

Figure 7. miR172c and *AP2-1* control nodule number (A) and nitrogenase (Nase) activity (B) of SNF common bean. Plants were inoculated with *R. etli* CE3 for the indicated dpi. Values represent averages ± SD from five replicate samples per time point. Different lowercase letters from each set of values at different dpi indicate statistically different groups (ANOVA, $P < 0.001$).

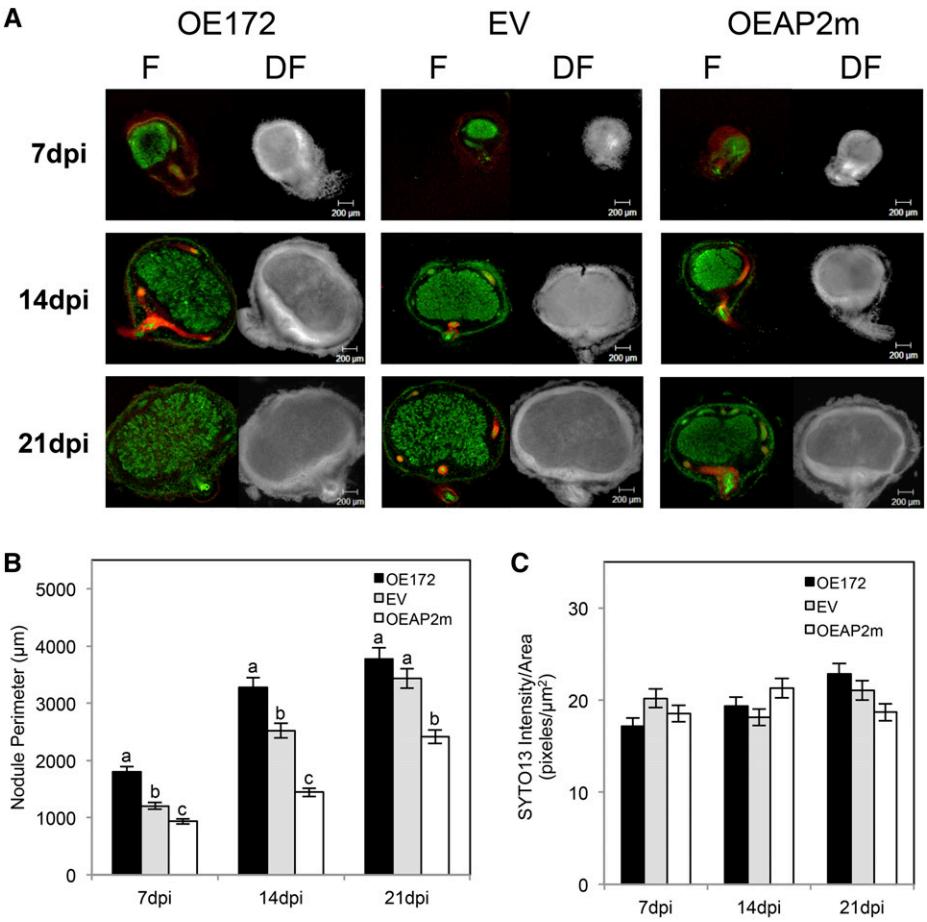

Figure 8. Alterations in the nodule development of OE172 and OEAP2m *R. etli*-inoculated composite bean plants. A, Fluorescent (F; left) and corresponding dark-field (DF; right) micrographs of central sections of nodules harvested at the indicated dpi. Red fluorescence from the tdTomato reporter gene expressed in *A. rhizogenes* transformed roots and green fluorescence from SYTO13 staining were observed. Magnification = 5×. B and C, Nodule perimeter (B) and SYTO13 fluorescence intensity per infection area (C) were calculated using the ImageJ program. Values represent averages ± SD from 10 replicate nodule images per condition. Different lowercase letters in B indicate statistically different groups (ANOVA, $P < 0.001$); values from C were not statistically different.

Exploring the Downstream *AP2-1* Regulation in SNF Plants

TFs from the *AP2* superfamily are widespread in plants and control diverse developmental programs and stress responses. Different *AP2* family members have been classified as activators or repressors of specific target genes (Licausi et al., 2013). In this work, we aimed to predict target genes for AP2-1 transcriptional activation or repression by analyzing data from the root and nodule libraries reported in the *Pv* GEA (O'Rourke et al., 2014), especially those from young roots and mature effective nodules that were derived from common bean tissues similar to those analyzed in this work. However, a caveat of this analysis is that the *Pv* GEA does not include libraries from roots inoculated with rhizobia for short periods, so we could not predict AP2-1 targets that would be regulated during the rhizobial infection process.

As shown in Figures 2 and 4, *AP2-1* showed high expression in common bean roots as opposed to mature nodules. We hypothesized that genes with an expression pattern similar to *AP2-1* are likely to be involved in root function/development and to be positively regulated by *AP2-1*, thus providing information on a possible mechanism of action of miR172/*AP2-1*. We identified 114 genes that had an expression pattern similar to *AP2-1*, designated *AP2-1* coexpressed genes (Supplemental Fig. S6). In order to support the latter contention, we searched for TFBS in the 5′ promoter region of *AP2-1* coexpressed genes. Besides WRKY, the most statistically overrepresented ($P = 0$ and 0.001) TFBS were ETHYLENE-RESPONSIVE FACTOR2 (ERF2) and DEHYDRATION-RESPONSIVE ELEMENT BINDING 1B (DREB1B), which belong to the AP2 superfamily. These TFBS were identified in 82% of *AP2-1* coexpressed genes (Supplemental Table S5).

Sixty-seven of the 114 *AP2-1* coexpressed genes could be assigned to a Gene Ontology (GO) category (Table I; Supplemental Table S5). The most statistically significant ($P = 0.006$) assigned GO category, which included 19 coexpressed genes, is GO:0004672, associated with protein kinase activity (Table I). We validated by qRT-PCR the expression of protein kinase activity genes in roots and nodules of control (EV) plants and also of plants overexpressing *AP2-1*. We hypothesized that those genes positively targeted by *AP2-1*, with high expression in roots and low expression in nodules from wild-type or control EV plants (Supplemental Fig. S6), would show a different expression pattern, higher and/or similar in roots and nodules, in OEAP2m plants that have constitutively enhanced expression of the *AP2-1* transcriptional regulator. Table II shows six *AP2-1* coexpressed genes assigned to the protein kinase activity category (GO:0004672) whose expression levels agree with our hypothesis. In control

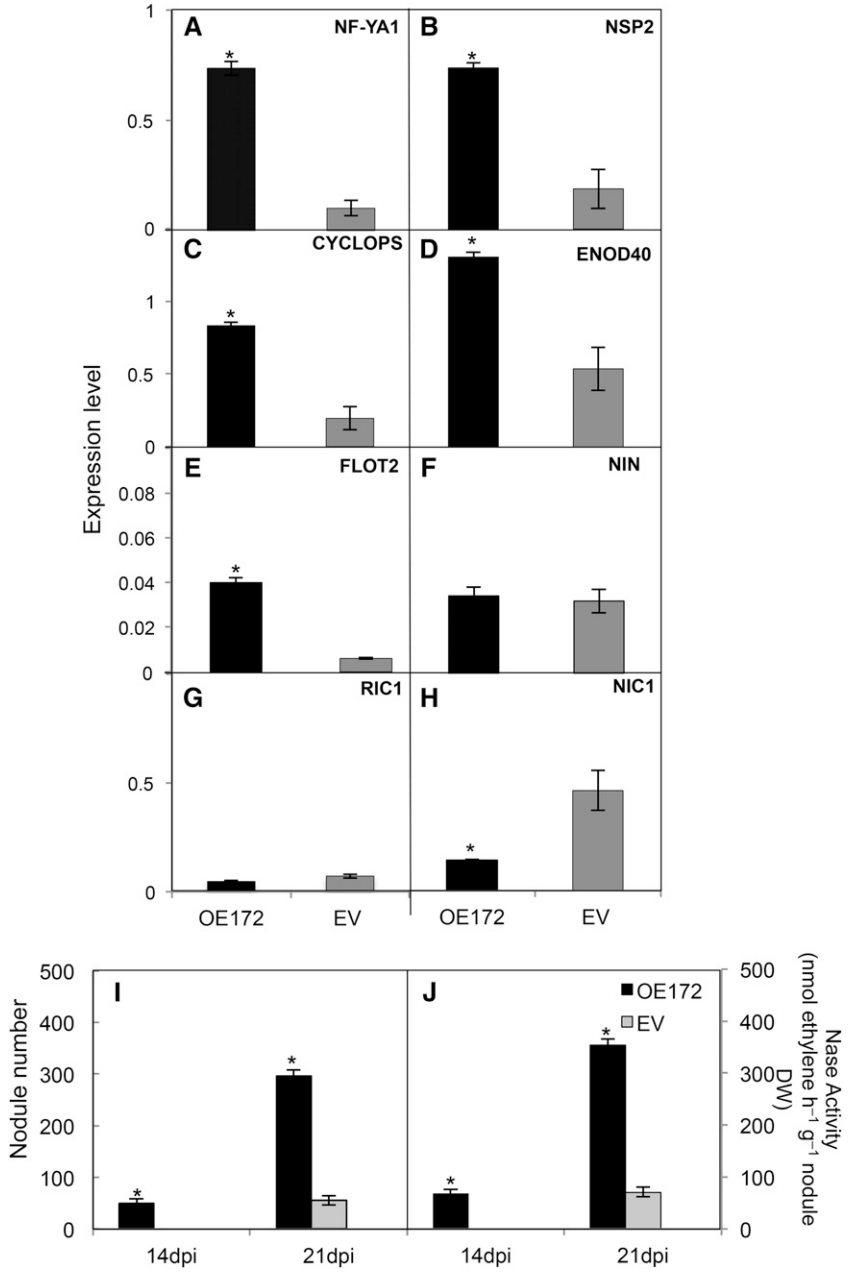

Figure 9. miR172c controls the sensitivity to nitrate inhibition of rhizobial symbiosis. To analyze the effect of nitrate in the symbiosis of *R. etli*-inoculated OE172 or EV composite plants, 1 mM KNO_3 was added to the nutrient solution used to water each set of plants daily. A to H, Expression analysis of selected early nodulation genes determined at 48 hpi in the root responsive zone. Gene identifiers are indicated in the legends to Figures 3 and 6. Values represent averages ± SD from three biological replicates and two technical replicates each. Expression level refers to gene expression, based on C_t value, normalized with the expression of the housekeeping *UBC9* gene. I and J, Nodules were counted (I) and nitrogenase (Nase) activity (J) was determined at the indicated dpi. Values represent averages ± SD from five replicate samples per time point. Asterisks indicate the level of statistically significant difference, if any, among values from OE172 and EV roots (Student's *t* test, $P < 0.01$).

(EV) plants, these genes were highly expressed in roots as compared with nodules, similar to *AP2-1*, thus validating the *Pv* GEA data (O'Rourke et al., 2014; Supplemental Fig. S6), while they showed an increased and/or similar expression in both tissues from OEAP2m plants (Table II). Interestingly, these genes shared the DREB, ERF, or both TFBS in their promoter regions (Table II; Supplemental Table S5). Transcriptomic analyses of *M. truncatula* nodule senescence have shown that protein kinases genes are one of the highly induced gene classes at different stages of this process (Van de Velde et al., 2006; Pérez Guerra et al., 2010). On this basis, we asked if the some of the *AP2-1* coexpressed genes from the protein kinase activity GO category might be related to nodule senescence in common bean. As shown in Table II, four of the protein kinase activity genes analyzed (Phvul.002G326600, Phvul.007G049500, Phvul.007G049000, and Phvul.006G174500) were induced in senescent (35 dpi) as compared with mature (18 dpi) nodules, thus indicating their possible relation to common bean nodule senescence.

The root/nodule expression pattern of *AP2-1* was opposite that of miR172c, which showed highest expression in mature effective nodules and very low expression in young roots (Figs. 2 and 4). O'Rourke et al. (2014) have described a set of 402 common bean genes highly expressed in mature effective nodules as compared with all other tissues analyzed in the *Pv* GEA. These genes are likely involved in the establishment of symbiosis and SNF as supported by their assigned GO categories (O'Rourke et al., 2014). Here, we hypothesized

Table I. *GO categories statistically overrepresented for genes coexpressed with AP2-1 in roots*

GO Identifier	Description (Molecular Function)	*P*
GO:0004672	Protein kinase activity	0.006
GO:0008703	5-Amino-6-(5-phosphoribosylamino)uracil reductase activity	0.016
GO:0005516	Calmodulin binding	0.018
GO:0005471	ATP:ADP antiporter activity	0.024
GO:0004674	Protein Ser/Thr kinase activity	0.036
GO:0004351	Glu decarboxylase activity	0.055
GO:0004435	Phosphatidylinositol phospholipase C activity	0.055

that these nodule-enhanced genes, lowly expressed in young roots with increased *AP2-1* (Figs. 2 and 4), are candidates for AP2-1 negative regulation. To this end, we searched for TFBS in the 5′ promoter region of nodule-enhanced genes, but we did not find a statistical overrepresentation of AP2 TFBS.

DISCUSSION

The key role of the miR172 node in Arabidopsis flowering time and phase transition is well known; similar roles have also been documented in maize (*Zea mays*), rice (*Oryza sativa*), and barley (*Hordeum vulgare*; Zhu and Helliwell, 2011). Besides conserved roles, specialized/particular species-specific functions of miR172, such as the induction of tuberization in potato (*Solanum tuberosum*), have been reported (Martin et al., 2009). In legumes, conserved roles of the miR172 node have been documented for *L. japonicus* (control of flowering time; Yamashino et al., 2013) and soybean (control of juvenile-to-adult phase transition; Yoshikawa et al., 2013). In addition, the control of nodulation during the rhizobia symbiosis has been proposed as a family-specific acquired function of miR172 in different legumes and has been demonstrated for soybean (Yan et al., 2013; Wang et al., 2014). In this work, we identified the miR172 node as a relevant regulator of rhizobial infection and nodulation in common bean.

We propose that different miR172 isoforms regulate different processes: miR172b is involved in flowering, while miR172c mainly regulates nodulation. Our data indicate that these miR172 isoforms exert their effects by silencing different target genes from the AP2 TF superfamily. Transcripts from two *AP2* genes (Phvul.005G138300 and Phvul.011G071100) are likely to function in roots and are cleaved by miR172c in nodules. Three other *AP2* genes (Phvul.003G241900, Phvul.002G16900, and Phvul.001G174400) are likely to function in young flowers, which showed a high level of these transcripts as well as of miR172, thus suggesting that, in flowers, the *AP2* target genes are silenced by miR172-induced translation inhibition, similar to Arabidopsis (Chen, 2004). Our work focused on the analysis of the miR172c/*AP2-1* (Phvul.005G138300) node in the common bean-rhizobia nitrogen-fixing symbiosis, and our proposed regulatory model is summarized in Figure 10.

In Arabidopsis, miR156 represses miR172 expression by targeting *SPL* TFs that directly bind to the *MIR172*

Table II. *Selected genes coexpressed with AP2-1 from the statistically overrepresented GO:0004672 category: protein kinase activity*

Expression level was determined by qRT-PCR from 21-dpi mature nodules (N) and roots (R) of EV and OEAP2m *R. etli*-inoculated composite plants and from 18 dpi mature or 35 dpi senescent nodules (N) from *R. etli*-inoculated wild-type plants. Values represent averages ± SD from three independent biological replicates and two technical replicates. TFBS for ERF and DREB (subfamilies of the AP2 TF family) were identified as statistically overrepresented in the promoter sequence of each gene as indicated.

Gene Identifier[a]	Annotation[a]	TFBS	Expression Level					
			21 dpi				Wild Type	
			EV		OEAP2m		18 dpi	35 dpi
			R	N	R	N	N	N
Phvul.002G326600	Aminocyclopropane carboxylate oxidase	DREB	0.76 ± 0.08	0.35 ± 0.13	1.2 ± 0.06	1.03 ± 0.07	0.11 ± 0.01	1.03 ± 0.07
Phvul.007G049500	Ser/Thr protein kinase	ERF	0.61 ± 0.09	0.25 ± 0.03	0.93 ± 0.08	0.97 ± 0.09	0.10 ± 0.02	0.53 ± 0.05
Phvul.007G049000	Ser/Thr protein kinase	DREB	0.10 ± 0.007	0.043 ± 0.01	0.02 ± 0.003	0.03 ± 0.004	0.07 ± 0.009	0.21± 0.03
Phvul.006G174500	Glycogen synthase kinase-3α	DREB	0.45 ± 0.06	0.24 ± 0.03	0.71 ± 0.07	0.57 ± 0.06	0.16 ± 0.02	0.37 ± 0.03
Phvul.011G169600	Ser/Thr protein kinase	DREB	0.35 ± 0.02	0.18 ± 0.02	0.52 ± 0.03	0.47 ± 0.03	0.33 ± 0.03	0.36 ± 0.02
Phvul.008G263900	Ser/Thr protein kinase	ERF and DREB	1.2 ± 0.06	0.15 ± 0.04	0.57 ± 0.02	0.50 ± 0.09	0.0023 ± 0.0003	0.0019 ± 0.0002

[a]From http://www.phytozome.net/commonbean.php.

promoter and positively regulate its expression (Wu et al., 2009). Transgenic soybean roots overexpressing miR156 showed a reduction in nodulation, decreased miR172 level, and decreased expression of two *SPL* genes proposed as miR156 targets, although evidence for the binding of SPL to *MIR172* promoters for transcription activation was not provided (Yan et al., 2013). Recently, Wang et al. (2015) reported that the overexpression of miR156 affects several aspects of plant architecture in *L. japonicus*, including underdeveloped roots and reduced nodulation, which correlate with the repression of several early symbiotic genes. However, the authors did not analyze a possible regulation of miR172 by miR156, which may be related to the miR156 effects in nodulation that they showed (Wang et al., 2015). In common bean, we observed opposite levels of mature miR156a as compared with miR172 and also a negative correlation between miR156a and its validated target *SPL6* gene (Phvul.009G165100). However, we could not identify SPL TFBS in any of the promoter regions of the six *MIR172* loci from the common bean genome, so it is difficult to propose SPL as a direct transcriptional regulator of miR172. However, the binding of SPL proteins to yet unknown sequence motifs present in *MIR172* promoters cannot be ruled out. Alternatively, miR156a may exert its negative regulation over common bean miR172 through other target genes not yet identified. For example, transcripts coding for tryptophan-aspartic acid repeats protein domain proteins, which may be involved in microtubule organization, protein-protein and protein-DNA interactions, or chromatin conformation, have been validated as miR156 targets in *M. truncatula* and *L. japonicus*, but their specific regulatory function has not been analyzed (Naya et al., 2010; Wang et al., 2015).

In this work, we showed that miR172c has a positive effect on root development independent of rhizobium infection. In addition, miR172c is relevant for the control of rhizobial infection. This miRNA increased after 6 h in *R. etli*-inoculated roots, when infection threads are formed, and this is related to the increase in root hair deformation observed in plants that overexpress miR172c. Preliminary data indicate that the roots overexpressing AP2m induce irregular infection threads (B. Nova-Franco, O. Valdés-López, and G. Hernández, unpublished data). Together, these data indicate a regulatory/signaling role of miR172c in the rhizobial infection of common bean (Fig. 10). Up-regulation of miR172c was concomitant with that of early nodulation genes, mainly expressed in the cortical cells, that are involved in infection thread initiation/progression (i.e. *FLOT2* and *ENOD40*) and act downstream of *NSP2*, *NIN*, and *NF-YA1* (Murray, 2011; Oldroyd, 2013), whose expression was highest after 3 h of rhizobial inoculation of common bean roots. Therefore, we propose that miR172c-mediated control of rhizobial infection is exerted at the level of cortical cell division downstream of NF perception, Ca^{2+} spiking, CCaMK, NSP2, and NIN (Fig. 10). In addition, our data on the repression of *RIC1* in roots overexpressing miR172c indicate the involvement of this miRNA in the AON at early stages of the common bean symbiosis. Soyano et al. (2014) reported that the AON *L. japonicus CLE* root signal genes *CLE-RS1* and *CLE-RS2*, which are orthologous to soybean *RIC1* and *RIC2*, are directly transcribed by NIN, the essential inducer for nodule primordium formation. This constitutes a complex regulatory circuit with a long-distance feedback loop involved in the homeostatic regulation of nodule organ production in *L. japonicus* (Soyano et al., 2014). In soybean, Wang et al. (2014a) recently reported that NARK negatively regulates miR172 transcription during nodule primordium formation to prevent excess nodulation. In this work, we showed that both *NIN* and *RIC1* are expressed at early stages of rhizobial infection in common bean roots and that OEAP2 roots with decreased nodulation showed increased *RIC1* levels. Taken together, these data would indicate a positive regulation of *NIN* and *AP2-1* to *RIC1*, thus leading to reduced nodulation through AON in common bean (Fig. 10). Sequence analysis of the *RIC1* promoter region led us to identify NIN- and DREB/ERF-enriched regions, something that supports the latter contention. However, further work is required to fully demonstrate which TFs activate *RIC1* expression in common bean.

The regulation of nodulation through AON signaling is also relevant for the inhibition of nodulation that occurs when nitrate is present in the rhizosphere. For local nitrate inhibition, the nitrate-induced CLE peptide in soybean (NIC) interacts with NARK in the root, leading to a nitrate-induced inhibition of nodulation in soybean (Reid et al., 2011a). Our data indicate that common bean miR172c is a signaling component of the nitrate-induced AON (Fig. 10). In the presence of nitrate, rhizobia-inoculated roots that overexpress miR172c developed more active nodules and showed very low expression of *NIC1* that correlates with the up-regulation of *NF-YA1*, *NSP2*, *CYCLOPS*, *ENOD40*, and *FLOT2* early symbiotic genes. The expression of *NIN* was similar in EV and OE172 roots inoculated in the absence or presence of nitrate, which is in agreement with data from *L. japonicus* reported by Soyano et al. (2015). The legume-rhizobia symbiosis with increased resistance to soil nitrate is relevant for improving plant growth and crop production. Our better understanding of the elements involved in the control of this phenomenon, such as miR172c in common bean, opens the possibility to exploit it for the future improvement of symbiosis.

The effect of miR172c in rhizobial infection and nodulation of common bean is likely to be directly exerted by its target gene, the AP2-1 transcriptional regulator. TFs from the AP2 superfamily may function as repressors or as activators of transcription (Licausi et al., 2013). Work recently published by Yan et al. (2013) and Wang et al. (2014) about the mechanism of action of AP2 in soybean nodulation points to the repressor role of AP2. In this work, we explored the possible role of *AP2-1* as a transcriptional activator and/or repressor of genes relevant for common bean rhizobium infection and nodulation. Recently, Soyano et al. (2015) reported that in *L. japonicus*, the NIN TF could repress or activate transcription in different scenarios of rhizobial infection in the presence or

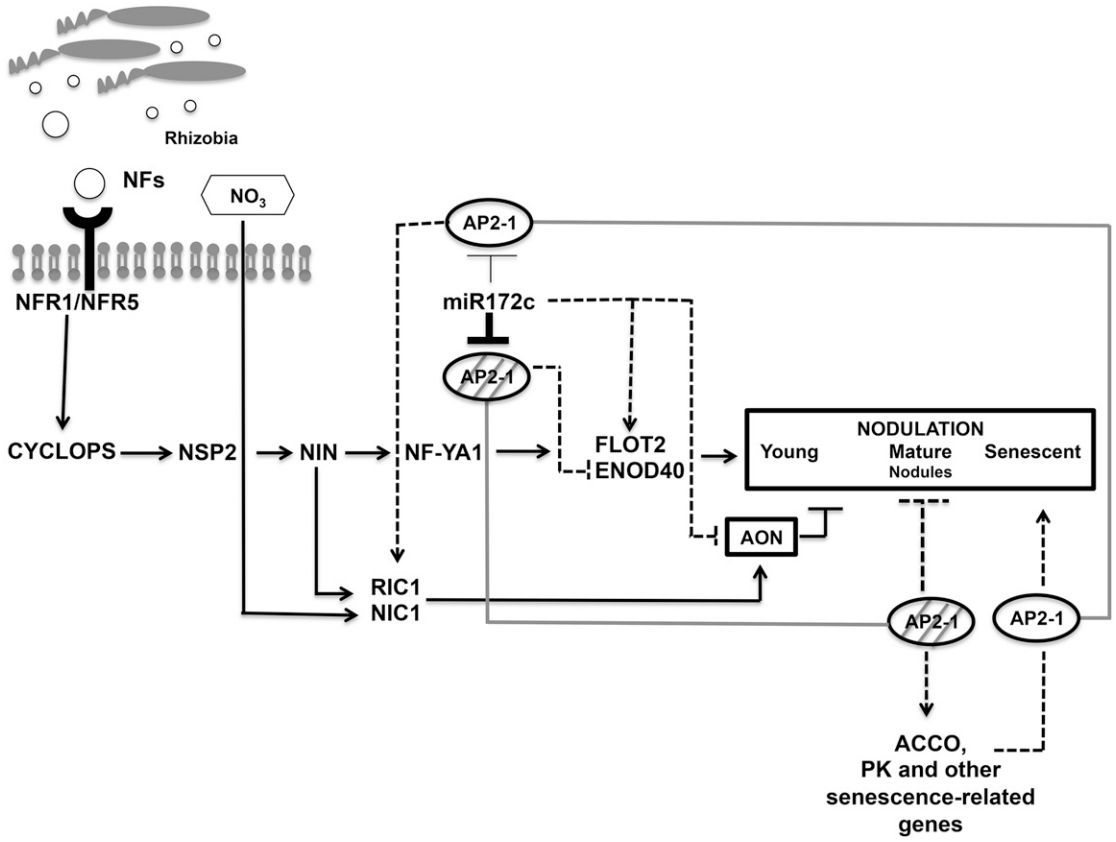

Figure 10. Model of miR172 node regulation in common bean-rhizobia symbiosis. Positive regulation is represented with arrows and negative regulation with lines. The root signaling cascade, triggered by rhizobial NF, is essential for rhizobia infection and nodule development in different legumes. Regulation of common bean rhizobial infection and nodulation by miR172c and AP2-1 are represented by dashed arrows or lines. A high level of miR172c (thick line) induces AP2-1 degradation (hatched circle), while active AP2-1 (circle) is present when the miR172c level is very low (thin line). miR172c positively regulates early nodulation gene expression and rhizobial infection, silencing AP2-1 that may repress *ENOD40* expression. Nodule number is positively regulated by miR172c; AON decreases through low RIC1/NIC1 expression, perhaps regulated by AP2-1. In mature nodules, abundant miR172c silences AP2-1, an activator of senescence-related genes that are further required during nodule senescence when AP2-1 levels are recovered. ACCO, Aminocyclopropane carboxylase oxidase.

absence of nitrate. They postulated that such dual regulatory functions might depend on specific coactivator or corepressor molecules that would interact with the same NIN TF in different scenarios.

We identified genes that significantly coexpress with *AP2-1* and are candidates for transcriptional activation by this TF (Fig. 10). Several of these genes were assigned to the protein kinase activity GO category. The best candidates are four protein kinases that are repressed in mature nodules and induced in roots and in senescent nodules of control plants, whereas they show high and/or constitutive expression in roots and nodules of AP2m overexpressing plants. This group includes the Phvul.007G049500 gene annotated (www.phytozome.net/commonbean.php) as a Ser/Thr protein kinase with a Cys-rich receptor-like protein kinase, Domain of Unknown Function26 (transmembrane), and Ser/Thr protein kinase domains. We found that this common bean kinase gene is similar (48%) to the Arabidopsis *CYSTEINE-RICH RECEPTOR-LIKE PROTEIN KINASE29* (*CRK29*) gene and to *M. truncatula SymCRK* (52% similarity) and has domains characteristic of the Cys-rich kinase family. Several members of this family are induced during *M. truncatula* nodule senescence (Van de Velde et al., 2006; Pérez Guerra et al., 2010). Specifically, SymCRK is involved in senescence and defense-like reactions during the *M. truncatula-Sinorhizobium meliloti* symbiosis (Berrabah et al., 2014). Another gene from this group encodes the aminocyclopropane carboxylase oxidase, the ethylene-forming enzyme. This and other genes encoding enzymes from the ethylene biosynthetic pathway are up-regulated during *M. truncatula* nodule senescence (Van de Velde et al., 2006). Ethylene plays a positive role in nodule senescence and also a significant inhibitory role in rhizobial infection and nodule formation (Van de Velde et al., 2006; Murray, 2011). Our interpretation of these results is that *AP2-1* transcriptional regulation is important in common bean roots but that this TF needs to be silenced for an adequate nodule function (SNF), something that is achieved

by posttranscriptional target cleavage mediated by miR172c. *AP2-1* silencing in effective mature nodules would maintain functionality by avoiding senescence through the down-regulation of nodule senescence genes activated by this TF (Fig. 10). Ineffective nodules where miR172c is not induced and AP2-1 remains elevated, as well as OEAP2 nodules, showed early senescence. Alternatively, other protein kinases, proposed as *AP2-1* targets, may participate in signaling pathways important for root development or nodule senescence. Nodule-specific protein kinases essential for signaling pathways during initial stages of nodulation are known (Oldroyd and Downie, 2008; Kouchi et al., 2010; Murray, 2011; Oldroyd, 2013).

Regarding AP2 transcriptional repression, Wang et al. (2014) recently reported that soybean NNC1, a miR172 target, represses *ENOD40* expression, which results in negative regulation of early stages of rhizobial symbiosis. In this work, we showed that the expression of common bean *ENOD40* is decreased in rhizobia-inoculated roots that overexpress AP2m. In addition, we identified ERF- and DREB-enriched regions in the *ENOD40* 5′ promoter region. So it is conceivable that, as in soybean, common bean *ENOD40* expression is repressed by AP2-1 (Fig. 10). However, it is important to consider that soybean *NNC1* is not the ortholog of common bean *AP2-1*. We identified *NNC1* (glyma12g07800) as the ortholog (84% similarity) of the Phvul.011G071100 *AP2* gene, identified as a miR172 target in a common bean degradome analysis (D. Formey, L.P. Íñiguez, P. Peláez, Y.F. Li, R. Sunkar, F. Sánchez, J.L. Reyes, and G. Hernández, unpublished data) but not further analyzed in this work; while common bean *AP2-1* was identified as the ortholog (93% similarity) of the soybean *AP2* gene glyma15g04930 that was predicted but not validated as the miR172 target (Wang et al., 2014). Therefore, different miR172 target genes from the AP2 family analyzed in soybean and in common bean may have different mechanisms for transcriptional regulation.

In addition, Yan et al. (2013) postulated that the regulation of soybean nodulation by miR172 is explained by the *AP2* repression of nonsymbiotic hemoglobin (*Hb*) gene expression that is essential for regulating the level of nodulation; however, the authors did not provide evidence demonstrating AP2 binding and direct transcriptional repression of *Hb* genes. To explore if this circuit is functioning in common bean, we first identified *Hb* genes encoded by the common bean genome: five symbiotic leg-hemoglobin (*Lb*) genes having greatly increased expression in effective nodules and four nonsymbiotic *Hb* genes (O'Rourke et al., 2014; Supplemental Fig. S7). From the latter, Phvul011G048600 and Phvul.011G048700 were identified as orthologs of the nonsymbiotic *Hb-1* and *Hb-2* genes in soybean, respectively. In common bean, these *Hb* genes showed similar low expression in roots and nodules of wild-type plants and also in composite plants that overexpress *AP2-1* or that have very low AP2-1 resulting from miR172 overexpression (Supplemental Fig. S7). Therefore, our data differ from those of Yan et al. (2013) and lead us to conclude that *AP2-1* repression of *Hb-1* genes is not relevant for common bean nodulation. Our exploration of other common bean symbiotic genes repressed by *AP2-1* included the identification of TFBS statistically overrepresented in the promoter regions of 402 common bean genes reported by O'Rourke et al. (2014) as nodule-enhanced genes. The expression pattern of these genes is similar to that of miR172c and opposite to that of *AP2-1*, which shows low expression in mature nodules and high expression in roots, suggesting these as candidates for transcriptional repression by AP2-1. However, AP2 (ERF and DREB) TFBS were not overrepresented in these genes. The latter is different from our data from *AP2-1* coexpressed genes proposed as being activated by this TF; these genes did show overrepresentation of ERF/DREB TFBS in their promoter regions. Further work is required to demonstrate the direct transcription repression, if any, of the miR172c target gene *AP2-1* in common bean.

Legume crops with increased nodulation/decreased nitrate inhibition of nodulation would be relevant for sustainable agriculture. This work sets the basis for further exploration, through genetic/genomic approaches, for common bean cultivars with improved traits resulting from increased miR172 in roots and nodules.

MATERIALS AND METHODS

Identification and Analysis of miR172 Precursor Genes, Isoforms, and Target Genes

The common bean (*Phaseolus vulgaris*) genome sequence recently published (Schmutz et al., 2014; http://www.phytozome.net/commonbean.php, v1.0) was analyzed, and six miR172 isoforms (a–f) were identified. Of these, four isoforms were described previously through RNA-seq analysis of common bean small RNAs by Peláez et al. (2012). The secondary RNA structure of each miR172 isoform was predicted using mfold software (Zuker, 2003) available at http://mfold.ma.albany.edu, and only the lowest energy structure generated for each sequence was chosen (Supplemental Fig. S1).

Since the only targets identified for miR172 from different plant species belong to the *AP2*-type TF family, we focused our analysis on identifying common bean miR172 targets within this gene family. We performed target prediction analysis for all the common bean *AP2* gene transcripts identified in the *Pv* GEA (O'Rourke, et al., 2014) using the Web server psRNATarget (http://plantgrn.noble.org/psRNATarget/; Dai and Zhao, 2011). Stringent criteria were used to predict targets; that is, an alignment spanning at least 18 bp with maximum penalty score of 3. Score calculation considered 0.5 points for each G:U wobble, one point for each non-G:U mismatch, and two points for each bulged nucleotide in either RNA strand (Jones-Rhoades and Bartel., 2004). In addition, we constructed a phylogenetic cladogram from the amino acid sequences of common bean *AP2* genes; these were aligned using ClustalX version 2.1 (Larkin et al., 2007). The sequence-aligned file was used to construct the bootstrapped neighbor-joining tree using the NJ clustering algorithm and Phylip output format (.phb). The reliability of the phylogenetic analysis was estimated from 1,000 bootstrap resamplings, and the tree was viewed using the program MEGA version 5.2.1 (Tamura et al., 2011).

Plant Material and Growth Conditions

Common bean seeds from the Mesoamerican cv Negro Jamapa 81 were surface sterilized and germinated for 2 d at 26°C to 28°C in darkness. Plants were grown in a hydroponic system under controlled environmental conditions as described previously (Valdés-López et al., 2010). The hydroponic trays contained 8 L of Franco and Munns (1982) nutrient solution. The volume and pH (6.5) of the trays were controlled throughout the experiment. For SNF conditions, plantlets adapted by growth for 7 d in the hydroponic system with

nitrogen-free nutrient solution were inoculated with 200 mL of a saturated liquid culture of the *Rhizobium etli* CE3 wild-type strain or the *R. etli fix*$^-$ mutant strain CFNX247 (Δ*nifA*::ΩSp/Sm; Girard et al., 1996). Plants were harvested at different times (dpi) for analysis; tissues for RNA isolation were collected directly into liquid nitrogen and stored at −80°C.

To analyze the initial events of rhizobial infection, 2-d-old seedlings were placed in plastic square bioassay dishes (24 × 24 cm; Corning) with solidified nitrogen-free Fähreus medium (Vincent, 1970). Plates containing common bean seedlings were incubated in a growth chamber at 25°C with a 16-h photoperiod. After 2 d, seedlings were inoculated by applying 1 mL of *R. etli* CE3 saturated liquid culture directly to the root and were further incubated at various times (3–48 h). After specific incubation times, the root responsive zones were detached, frozen in liquid nitrogen, and stored at −80°C until used.

Plasmid Construction, Plant Transformation, and Production of Composite Plants

To generate a plasmid to overexpress the pre-miR172 in common bean transgenic roots, a 217-bp PCR product was obtained using common bean nodule complementary DNA (cDNA) as template and the specific primers Fw-pre172 (5′-CACCCCAGTCACTGTTTGCCGGTGGAG-3′) and R-pre172 (5′-AAAAACCTCCTTTGCTCTGAGCGT-3′), based on the Phvul.001G233200 sequence. The PCR product was cloned by T-A annealing into pCR 2.1-TOPO (Invitrogen) and sequenced. To construct the OE172 plasmid, the pre-miR172 region was excised using the *Xho*I and *Bam*HI sites of the vector and cloned into the pTDTO plasmid that carries the reporter tdTomato (red fluorescent protein) gene (Aparicio-Fabre et al., 2013; Supplemental Fig. S3).

The complete cDNA clone of common bean *AP2-1* was obtained by PCR amplification using cDNA from roots and the specific primers Fw-AP2 (5′-CAGCTACCTTTCCGCCAAATGC-3′) and Rv-AP2 (5′-TAGGCTGGGATGGTGTCTGCAG-3′), based on the Phvul.005G138300 sequence. The 1,127-bp product was cloned by T-A annealing into pCR 2.1 TOPO (Invitrogen) and analyzed by sequencing. Mutations of the putative miR172 cleavage site of *AP2-1* were introduced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The *Pst*I site present in the wild-type miR172 cleavage site (5′-CTGCAGCATCATCAGGATTCT-3′) was eliminated by changing the A to a C at the 3′ position of the recognition site for this enzyme. Additionally, the nucleotides at positions 9, 10, and 11 of the miR172 cleavage site were modified by introducing a *Bgl*II site. The primers used were forward (5′-TTCTCTACTGCCGCCAGATCTGGATTCTCAATT-3′) and reverse (5′-AATTGAGAATCCAGATCTGGCGGCAGTAGAGAA-3′). The changes were checked by sequencing, and the modified AP2 was cloned into plasmid pTDTO to obtain plasmid OEAP2m. The nucleotide changes in AP2m introduced an amino acid substitution (Arg for Ser), but this does not seem to affect AP2-1 function (Supplemental Fig. S3).

Common bean composite plants with transformed root system and untransformed aerial system were generated as described (Estrada-Navarrete et al., 2007; Aparicio-Fabre et al., 2013). For plant transformation, *Agrobacterium rhizogenes* K599 strains bearing the EV, OE172, or OEAP2m plasmids were used. Selected composite plants were grown under controlled environmental conditions in pots with vermiculite and watered daily with B&D nutrient solution (Broughton and Dilworth, 1971), either nitrogen free for the symbiotic condition or with 10 mM potassium nitrate for the full-nutrient condition. SNF plants were adapted by growing in pots for 7 d and then inoculated with *R. etli* CE3. For experiments designed to analyze the effect of nitrate on symbiosis, B&D nutrient solution supplemented with 1 or 3 mM KNO_3 was used to water the inoculated plants daily from 1 dpi. Composite plants were analyzed phenotypically at different dpi; transgenic roots and nodules were collected in liquid nitrogen and stored at −80°C.

To analyze the initial events of rhizobial infection in transgenic roots, plastic square bioassay dishes were used to grow selected composite plants under the same conditions described above. Plates containing composite plants were sealed with Parafilm, and the root zone was covered with aluminum foil. After 2 d, composite plants were inoculated by applying 1 mL of *R. etli* CE3 saturated liquid culture directly to the root. At 48 hpi, the root responsive zones were detached and stored at −80°C until used or collected into phosphate-buffered saline (PBS) buffer for microscopic analyses.

Phenotypic Analysis

Nitrogenase activity was determined in detached nodulated roots by the acetylene reduction assay essentially as described by Hardy et al. (1968). Specific activity is expressed as nmol ethylene h^{-1} g^{-1} nodule dry weight.

The root fresh weight and the number of secondary roots per plant were determined in composite plants grown under full-nutrient (10 d) or symbiotic (21 dpi) conditions.

Microscopic analysis was performed on transgenic nodules at different developmental stages from EV, OE172, and OEAP2m composite plants. The protocol described by Haynes et al. (2004) was used for tissue staining with the nucleic acid-binding dye SYTO13. Nodule sections were stained with SYTO13 (1 μL mL^{-1}) in 80 mM PIPES, pH 7, for 5 min, then mounted in 1% (v/v) PBS/50% (v/v) glycerol and analyzed. Images were obtained using the Zeiss LSM 510 laser scanning microscope attached to an Axiovert 200 M. SYTO13 excitation was obtained at 488 nm using an argon laser and an HFT UV 488/543/633-nm dual dichroic excitation mirror with an LP 560 emission filter for detection. Sequentially, red fluorescence from the reporter gene was observed by exciting at 543 nm with a helium/neon laser, with the same dual dichroic excitation mirror and a BP 500-530 IR emission filter. Images were processed using the LSM 510 version 4.2 5P1 software (Carl Zeiss Micro-Imaging). For the determination of nodule perimeter and SYTO13 intensity per infected area, 10 images from individual nodule replicates from each condition were analyzed using the ImageJ program.

Statistical analyses of symbiotic parameters (root biomass/architecture, nodulation, and nitrogenase activity) were performed using one-way ANOVA and multiple paired Student's *t* tests ($P < 0.001$).

For analyses of root hair deformation and infection thread induction by rhizobial infection, the root responsive zones from inoculated composite plants grown in plastic square bioassay dishes, as described above, were collected at 48 hpi into PBS buffer. Responsive zone root samples were stained with 0.01% (w/v) Methylene Blue for 1 h and washed three times with double-distilled water; infection events were observed in an optical microscope.

RNA Isolation and Analysis

Total RNA was isolated from 100 mg of frozen nodules, 250 mg of frozen roots, or 200 mg of other frozen tissues from wild-type or composite plants grown under similar conditions, using Trizol reagent (Life Technologies) following the manufacturer's instructions. These samples were preserved at −80°C until tested. Genomic DNA removal, cDNA synthesis, and quality verification for qRT-PCR were performed as reported (Hernández et al., 2007).

RNA preparations were used to detect mature miR172 in different plant tissues by low-molecular-weight RNA-gel hybridization using 15 μg of total RNA, as reported (Naya et al., 2014). Synthetic DNA oligonucleotides with antisense sequence corresponding to miR172 (5′-ATGCAGCATCATCAAGATTCT-3′) and to U6 snRNA (5′-CCAATTTTATCGGATGTCCCCG-3′) were used as probes after radioactive labeling. Hybridization of U6 snRNA was used as a loading control. The signal intensities of miR172 and U6 hybridization bands were determined using ImageQuant 5.2 software (Molecular Dynamics). Normalized miR172 expression levels were calculated related to U6 snRNA.

For the quantification of transcript levels of mature miRNAs, cDNA was synthesized from 1 μg of total RNA using the NCode miRNA First-Strand cDNA Synthesis Kit (Invitrogen) or the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) for transcripts of selected genes. Resulting cDNAs were then diluted and used to perform qRT-PCR assays using SYBR Green PCR Master Mix (Applied Biosystems), following the manufacturer's instructions. The sequences of oligonucleotide primers used for qRT-PCR amplification of each gene are provided in Supplemental Table S1. Reactions were analyzed in a real-time thermocycler (Eco Illumina Real-Time PCR System; Illumina) with settings of 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 57°C for 60 s. Relative expression for each sample was calculated with the comparative C_t method. The C_t value obtained after each reaction was normalized with the C_t value of miR159 for miRNA levels or with the C_t value of *UBC* (Phvul.006G110100) for expression levels of miRNAs and transcripts, respectively.

Statistical analyses of gene expression (miR172c, *AP2-1*, and early symbiotic genes) from wild-type and composite SNF plants were performed using one-way ANOVA and multiple paired Student's *t* tests ($P < 0.001$).

Identification of Root-Enhanced Genes Coexpressed with *AP2-1*

To identify common bean genes with an expression pattern similar to that of the *AP2-1* target gene, the Euclidian distance between Z scores for each gene was determined in RNA-seq samples from the *Pv* GEA (O'Rourke et al., 2014). The tissue samples analyzed were as follows: young roots, prefixing effective

(5 dpi) nodules, effective (21 dpi) nodules, ineffective (21 dpi) nodules, roots from nonsymbiotic plants grown in full-nutrient solution, nodule-detached roots (5 dpi), effective nodule-detached roots (21 dpi), and ineffective nodule-detached roots (21 dpi). A threshold Euclidian distance of 0.9 was established as significant. A total of 114 genes within the threshold were identified as genes coexpressed with *AP2-1* (Supplemental Table S5).

Gene Sequence Analysis for the Identification of TFBS

The CLOVER program (Frith et al., 2004) was used to identify TFBS in 5′ promoter regions of *AP2-1* coexpressed genes (Supplemental Table S5) and of genes highly expressed in mature effective nodules described previously by O'Rourke et al. (2014). For this analysis, a 2,000-bp sequence from the region immediately upstream of the transcription start site of each gene was retrieved from the common bean genome sequence (Schmutz et al., 2014; http://www.phytozome.net/commonbean.php, v1.0).

Promoter regions (2,000-bp sequence) of selected early nodulation genes were tested for DREB/ERF or NIN binding sites using http://plants.rsat.eu/. A Markov order of 2 was used for predicting cis-regulatory element-enriched regions with default parameters. The cis-regulatory element-enriched regions were also searched in the promoter region of each locus encoding an miR172 isoform; 1,000 bp upstream of the transcription start site of isoforms a and c and 1,500 bp upstream of the precursors of isoforms b, d, e, and f were analyzed (Supplemental Table S2).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. miR172 isoforms encoded in the common bean genome and the most stable secondary structures predicted for their precursors.

Supplemental Figure S2. Symbiotic phenotypes of common bean plants inoculated with the *R. etli nifA*$^-$ mutant strain as compared with the CE3 wild-type strain.

Supplemental Figure S3. Schematic representation of plasmids used for miR172c or *AP2-1m* overexpression.

Supplemental Figure S4. Overexpression of miR172c and *AP2m* in transgenic roots and nodules of composite bean plants.

Supplemental Figure S5. miR172 and *AP2-1* control rhizobial infection in common bean roots.

Supplemental Figure S6. Expression pattern of *AP2-1* coexpressed genes.

Supplemental Figure S7. Expression analysis of common bean symbiotic (*Lb*) and nonsymbiotic (*Hb*) hemoglobin genes.

Supplemental Table S1. Primer sequences for qRT-PCR.

Supplemental Table S2. TFBS identified in the 5′ promoter region of each *MIR172* gene.

Supplemental Table S3. Nitrogenase activity and expression analysis of marker genes for nodule development.

Supplemental Table S4. Expression analysis of marker genes for nodule development in transgenic nodules of OE172, EV, or OEAP2m plants.

Supplemental Table S5. *AP2-1* coexpressed genes: assigned GO categories and identified TFBS.

ACKNOWLEDGMENTS

We thank Dr. Jesús Arellano (Universidad Autónoma del Estado de Morelos [UAEM]) and María del Socorro Sánchez Correa (Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México) for technical assistance in plant transformation, Dr. Ramón Suárez (UAEM) for cooperation in qRT-PCR determinations, Darla Boydston (Noble Foundation) for helping in the graphic design of the figures, and Dr. Michael Dunn (Centro de Ciencias Genómicas-Universidad Nacional Autónoma de México) for critically reviewing the article.

Received December 12, 2014; accepted March 2, 2015; published March 4, 2015.

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