The MicroRNA390/TAS3 Pathway Mediates Symbiotic Nodulation and Lateral Root Growth

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Legume roots form two types of postembryonic organs, lateral roots and symbiotic nodules. Nodule formation is the result of the interaction of legumes with rhizobia and requires the mitotic activation and differentiation of root cells as well as an independent, but coordinated, program that allows infection by rhizobia. MicroRNA390 (miR390) is an evolutionarily conserved microRNA that targets the Trans-Acting Short Interference RNA3 (TAS3) transcript. Cleavage of TAS3 by ARGONAUTE7 results in the production of trans-acting small interference RNAs, which target mRNAs encoding AUXIN RESPONSE FACTOR2 (ARF2), ARF3, and ARF4. Here, we show that activation of the miR390/TAS3 regulatory module by overexpression of miR390 in Medicago truncatula promotes lateral root growth but prevents nodule organogenesis, rhizobial infection, and the induction of two key nodulation genes, Nodulation Signaling Pathway1 (NSP1) and NSP2. Accordingly, inactivation of the miR390/TAS3 module, either by expression of a miR390 target mimicry construct or mutations in the NSP1 promoter, enhances nodule formation and rhizobial infection, alters the spatial distribution of the nodules, and increases the percentage of nodules with multiple meristems. Our results revealed a key role of the miR390/TAS3 pathway in legumes as a modulator of lateral root organs, playing opposite roles in lateral root and nodule development.

Plants adapt their root architecture in response to changes in their environmental conditions, mainly water and nutrient availability. Legume roots form two types of postembryonic organs, lateral roots and symbiotic nodules, which participate in water/nutrients uptake and nitrogen fixation, respectively. Both organs have considerable impact on plant growth; thus, understanding the mechanisms underlying the development of lateral roots and nodules is crucial to improve agronomical traits in legumes. Lateral roots originate from a small number of pericycle cells of the primary root that differentiate into lateral root primordia, emerge from the primary root surface, and continue to elongate (De Smet, 2012; De Smet et al., 2012). On the other hand, root nodules are the result of the interaction of legumes with soil bacteria, collectively known as rhizobia, which infect root cells and are hosted intracellularly as endosymbionts. Nodule formation requires the mitotic activation and reprogramming of differentiated root cells as well as the development of an independent genetic program that allows infection by rhizobia to complete the formation of a functional nitrogen-fixing organ (Oldroyd et al., 2011; Oldroyd, 2013). Typically, infection by rhizobia takes place through plant-made tubular structures formed in growing root hairs, referred to as infection threads (ITs). These ITs grow, ramify, and reach the dividing cortical cells, where bacteria are released, forming organelle-like structures called symbiosomes (Murray, 2011). Depending on the legume species, nodules can be either determinate or indeterminate (Oldroyd et al., 2011). Determinate nodules, such as those

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developed in *Lotus japonicus* and *Glycine max*, are formed as a consequence of transient cell divisions in the outer cortex, and then the nodule grows by cell enlargement rather than by cell division, resulting in spherical nodules. On the other hand, indeterminate nodules, such as those formed in roots of *Medicago truncatula*, contain an active and persistent meristem at their apices that continues adding cells to the nodule tissues throughout their lifetime. These nodules are cylindrically shaped and contain distinct zones that represent different stages of nodule development: zone I is the meristematic tissue with undifferentiated cells; zone II corresponds to the infection zone, where bacteria are released from ITs and begin to differentiate; and zone III is the nitrogen fixation zone, which consists of cells that contain symbiosomes with fully differentiated bacteroids. After several weeks, nodules also develop a senescent zone (zone IV), where nitrogen fixation ceases and both symbiosomes and host cells start to senesce. A recent cytological study precisely described the ontogeny of different nodule tissues in *M. truncatula*, which originated from a combination of endodermis, pericycle, and cortical cell divisions (Xiao et al., 2014). The nodule meristem is derived from the middle cortex, whereas the inner cortex gives rise to fully infected cells at the base of the central tissue of the nodule. Endodermis and pericycle cell divisions give rise to the uninfected vascular bundles and peripheral tissues of the nodule.

Nodule development and rhizobial infection, as well as the coordination between the two genetic programs, depend on a signaling pathway that involves the perception of rhizobial signals by plasma membrane receptor-like kinases with extracellular Lys motifs (LysM), referred to as Nod Factor Perception and the LysM Receptor Kinase3 in *M. truncatula* (Amor et al., 2003; Smit et al., 2007). These two receptors, the Leu-rich repeat receptor-like kinase, known as Does Not Make Infection2 (DMl2) in *M. truncatula* (Catoire et al., 2000), the potassium-permeable channel DMl1 (Ané et al., 2004; Peiter et al., 2007), and three cyclic nucleotide-gated channels located at the nuclear envelope (Charpentier et al., 2016) are required for the activation of calcium oscillations within and around the nucleus (Ehrhardt et al., 1996; Wais et al., 2000; Sieberer et al., 2009). Calcium oscillations are decoded by a calcium- and calmodulin-dependent protein kinase (CcamK; Lévy et al., 2004; Mitra et al., 2004), which interacts with and phosphorylates transcription factor designated CYCLOPS in *L. japonicus* or Interacting Protein with DMl3 (IPD3) in *M. truncatula* (Yano et al., 2008; Ovchinnikova et al., 2011). In *L. japonicus*, CYCLOPS transactivates in a phosphorylation-dependent manner the Nodule Inception (NIN) gene, a central regulator of nodulation (Schauer et al., 1999; Marsh et al., 2007; Singh et al., 2014). More recently, Vernié et al. (2015) reported that NIN can directly suppress the expression of early nodulation genes (ENODs), such as ENOD11, in the root epidermis but directly activates the transcription of the Cytokinin Receptor1 (CRE1) in the cortex of *M. truncatula* roots. A number of additional transcription factors belonging to the GRAS (Kaló et al., 2005; Smit et al., 2005; Hirsch et al., 2009; Battaglia et al., 2014), Ethylene Response Factor (ERF)/APETALA2 (Andriankaja et al., 2007; Middleton et al., 2007; Cerri et al., 2012), Nuclear factor Y (NF-Y; Zanetti et al., 2010; Laloum et al., 2014; Baudin et al., 2015), and NAC (D’haeseleer et al., 2011) families also are required for nodulation. The expression of some of these transcription factors, such as NF-YA1, Nodulation Signaling Pathway2 (NSP2), and NAC1, is regulated by the action of small regulatory RNAs (sRNAs; Combier et al., 2006; D’haeseleer et al., 2011; De Luis et al., 2012; Laressergues et al., 2012; Hofferek et al., 2014). Among them, microRNAs (miRNAs) and trans-acting small interference RNAs (tasiRNAs) are endogenous sRNAs of 20 to 22 nucleotides that act as negative regulators of gene expression either by cleaving or inhibiting the translation of their mRNA targets; therefore, they can control developmental processes and the adaptation to changes in environmental conditions. A number of miRNAs differentially accumulate at different stages of the root nodule symbiosis (Subramanian et al., 2008; Lelandais-Brière et al., 2009; Li et al., 2010; Reynoso et al., 2013; Formey et al., 2014). However, the roles played by these miRNAs in the control of nodulation have been investigated only for a few of them. In *M. truncatula*, overexpression of miR160 (Bustos-Sanmamed et al., 2013), miR164 (D’haeseleer et al., 2011), miR166 (Boualem et al., 2008), miR169 (Combier et al., 2006), and miR171h (Hofferek et al., 2014), which target the AUXIN RESPONSE FACTOR (ARF)10/16/17, NAC1, HD-Zip, NF-YA1, and NSP2 transcription factors, respectively, negatively affected nodule number and/or development.

miR390 is an evolutionarily conserved 21-nucleotide miRNA that targets the *Trans-Acting Short Interference RNA3 (TAS3)* transcript in two different sites, a mode of action referred to as the two-hit model and illustrated in Figure 1A (Allen et al., 2005). miR390-directed cleavage of TAS3 by ARGONAUTE7 (AGO7) at the 3' end most proximal site results in the production of TAS3-derived tasiRNAs (Montgomery et al., 2008). The production of tasiRNAs also requires components of the siRNA biogenesis pathway, including SUPPRESSOR OF GENE SILENCING3 (SGS3), RNA-DEPENDENT RNA POLYMERASE6 (RDR6), and DICER-LIKE4 (DCL4; Xie et al., 2005). In turn, TAS3-derived tasiRNAs direct the cleavage of complementary mRNAs encoding ARF2, ARF3, and ARF4; therefore, they are also referred to as tasiARFs (Marin et al., 2010). ARFs are transcriptional regulators that bind with high specificity to auxin response elements present in the promoters of primary auxin-response genes (Tiwari et al., 2003). ARFs belong to a large gene family with 12 to 39 members, depending on the species (Finet et al., 2013). In *M. truncatula*, this gene family is composed of 24 members (Shen et al., 2015).

The miR390/TAS3 pathway plays key roles in plant development. tasiARFs suppress the juvenile-to-adult phase transition in Arabidopsis (*Arabidopsis thaliana*) and are required for leaf patterning and leaf polarity in different plant species, including the two model
leguminous plants *L. japonicus* and *M. truncatula* (Adenot et al., 2006; Fahlgren et al., 2006; Garcia et al., 2006; Liu et al., 2007; Nagasaki et al., 2007; Yan et al., 2010; Yifhar et al., 2012; Zhou et al., 2013). In *M. truncatula*, loss-of-function mutations in the *AGO7/LOBED LEAFLET1* (hereafter *AGO7*) gene affected leaf margin development and lateral organ separation, suggesting that the miR390/TAS3 pathway functions as a repressor of *ARF2/3/4* genes during the formation of boundaries of organs or tissues (Zhou et al., 2013). The miR390/TAS3 pathway also defines a network that quantitatively controls lateral root growth in Arabidopsis (Marin et al., 2010). Activation tagging or overexpression of the non-coding *TAS3* gene increased the average length of lateral roots but did not affect primary root length and lateral root density, whereas *tas3* mutant plants exhibited shorter lateral roots. Consistently, a mutation in the *MIR390a* gene, which impairs the accumulation of miR390 and tasiARFs in the roots, also reduced lateral root length (Marin et al., 2010). More recently, the miR390/TAS3 pathway has been implicated in the interaction of Arabidopsis plants with the parasitic root-knot nematodes. The use of tasiARF sensor and tasiARF-resistant *ARF3* lines revealed that this regulatory module is required for nematode-induced gall formation in Arabidopsis roots (Cabrera et al., 2016). In addition, the miR390/TAS3 pathway has been linked to root nodule symbiosis in *L. japonicus*. De Luis et al. (2012) reported that miR390 exhibited higher relative abundance in mature nodules as compared with roots. Later, Li et al. (2014) showed that *rel3/ago7* mutants produced fewer nodules and had reduced infection frequency in *L. japonicus*.

Our previous analysis of the selective recruitment of mRNAs and sRNAs to polyribosomes at early stages of the interaction between *M. truncatula* and *Sinorhizobium meliloti* revealed that tasiARF levels decreased in total and polyribosome-associated RNA samples upon rhizobia inoculation (Reynoso et al., 2013). Concomitantly, the levels of *ARF2*, *ARF3*, and *ARF4* transcripts, which have been validated experimentally as targets of tasiARFs in *M. truncatula* (Jagadeeswaran et al., 2009; Zhou et al., 2013), increased in response to rhizobial infection. However, the function of the miR390/TAS3 pathway during rhizobial infection or the initiation and development of root nodules remains to be elucidated.

Figure 1. Expression analysis of miR390/TAS3 pathway components in roots and nodules of *M. truncatula* at different stages of the root nodule symbiosis. A, Schematic representation of the miR390/TAS3 pathway. miR390 interacts specifically with *AGO7* and binds to two different target sites within the TAS3 transcript. AGO7 bound to miR390 cleaves the TAS3 RNA at the most 3’ end proximal target site. The 5’ end cleavage product is converted into a double-stranded RNA by the action of RDR6 and SGS3 and then processed into 21-nucleotide tasiARFs by DCL4. tasiARFs target *ARF2*, *ARF3*, and *ARF4* mRNAs at either one or two target sites. B, Expression levels of mature miR390a/b, tasiARFs, and TAS3, *ARF2*, *ARF3*, and *ARF4* transcripts in mock (inoculated with water; white bars) or *S. meliloti*-inoculated (*Sm*; blue bars) roots at 48 h post inoculation (hpi). C, Expression levels of mature miR390a/b, tasiARFs, and TAS3, *ARF2*, *ARF3*, and *ARF4* transcripts in noninoculated roots of 7 d post germination and in nodules (*Nod*) at 10 and 21 dpi with *S. meliloti*. In B and C, miR390 and tasiARF levels were determined by stem-loop RT-qPCR, and expression values were normalized to *U6* transcript. Values are expressed relative to the mock-inoculated sample (B) or the root sample (C). Error bars represent se of three independent biological replicates. Asterisks denote statistically significant differences in an unpaired two-tailed Student’s *t* test (*, *P* < 0.05 and **, *P* < 0.01) with the mock-inoculated sample (B) or the root sample (C).
development of indeterminate nodules has not been investigated. As the nodule developmental program has been proposed to derive from that existing in lateral root formation (Desbrosses and Stougaard, 2011) and the miR390/TAS3 pathway regulates lateral root growth in Arabidopsis (Marin et al., 2010), we questioned whether this pathway has been recruited or modified to control the development of both lateral root organs (lateral roots and nodules) in *M. truncatula*. Here, we show that ectopic activation of the miR390/TAS3 pathway prevents the organogenesis of indeterminate nodules, rhizobial infection, and the induction of key nodulation genes, although it promotes the growth of emerged lateral roots. Accordingly, inactivation of this pathway enhances rhizobial infection and nodulation and alters the spatial distribution/positioning and morphology of the nodules within the infection zone. These results revealed a common key role of the miR390/TAS3 pathway as a modulator of lateral root organs, playing opposite roles in lateral root and nodule development.

RESULTS

Expression of miR390/TAS3 Pathway Components during Root Nodule Symbiosis

The miR390a of *M. truncatula* was described previously by Jagadeeswaran et al. (2009) and annotated in the miRBase database (Kozomara and Griffiths-Jones, 2014). However, a BLASTN search using the 21-nucleotide sequence of the *M. truncatula* miR390a identified two loci in the *M. truncatula* genome (Medtr4g014400 and Medtr3g031300) that were named as MIR390a and MIR390b, respectively. The precursors of miR390 produced by both genes (premiR390a and premiR390b) form thermodynamically stable stem-loop secondary structures according to RNA fold (Gruber et al., 2008) and produce mature miRNAs that are indistinguishable at the nucleotide level (Supplemental Fig. S1). Thus, quantification of the mature miR390a accounts for miRNAs processed from both precursors. RT-qPCR revealed that, at early stages of the interaction, levels of miR390a/b and tasiARFs were markedly lower, whereas levels of TAS3, ARF2, ARF3, and ARF4 were substantially higher, in roots inoculated with *S. meliloti* than in mock-inoculated roots (treated with water; Fig. 1B). The expression of miR390a/b and other components of the pathway also was analyzed in noninoculated roots at 7 d after germination and in nodules at 10 and 21 d post inoculation (dpi) with rhizobia. Levels of miR390a/b, TAS3, and tasiARFs were significantly higher in nodules at 10 dpi than in noninoculated root tissue or in nodules at 21 dpi, whereas the three ARF transcripts exhibited substantially lower levels in nodules at 10 dpi than in noninoculated roots (Fig. 1C). In addition, transcript levels of ARF3, but not of ARF2 and ARF4, also were significantly lower in nodules at 21 dpi than in noninoculated roots. These results are consistent with previous RNA sequencing data of roots and nodules formed by *S. meliloti* described by Roux et al. (2014; Supplemental Fig. S2A). Examination of TAS3, ARF2, ARF3, and ARF4 transcript levels in different nodule sections obtained by laser-capture microdissection (Roux et al., 2014) revealed an enrichment of these transcripts in the meristematic zone and, to a lesser extent, in the distal infection zone of the nodule (Supplemental Fig. S2B). The exception was ARF4, which also exhibited a relatively high mRNA accumulation in the proximal infection zone (Supplemental Fig. S2B). Altogether, these results indicate that the miR390/TAS3 pathway is repressed at early stages of symbiosis (48 hpi with *S. meliloti*), releasing ARF repression; but once nodules are formed (10–15 dpi), the expression of ARF2, ARF3, and ARF4 transcripts decreases and is restricted mainly to the nodule meristematic and infection zones.

To study the tissue-specific expression pattern of MIR390a and MIR390b genes in roots, nodule promoter: GUS-GFP constructs were introduced into *M. truncatula* roots by *Agrobacterium rhizogenes*-mediated transformation. Histochemical GUS staining revealed that pMIR390a is expressed in the vascular tissue of primary and lateral roots as well as in the meristem of lateral roots (Fig. 2, A and D). Transverse cross sections revealed that this promoter is active in the pericycle, phloem, and xylem but not in the endodermis (Fig. 2, B and C). On the other hand, pMIR390b was active in the vasculature of primary roots, particularly in the pericycle (Fig. 2, F–H), as well as in the vasculature of lateral roots, but not in the lateral root meristem (Fig. 2I). GUS staining and longitudinal cross sections of mature nodules showed that pMIR390a was active in the nodule apex, including the meristematic and distal infection zones (Figs. 2E and 3A, 28 dpi), whereas activity of the pMIR390b was restricted to the nodule meristem (Figs. 2J and 3A, 28 dpi). Altogether, these results showed that both MIR390a and MIR390b are expressed in the vasculature of primary and lateral roots as well as in the apical region of mature nodules.

The spatial and temporal expression patterns of MIR390 genes also were analyzed at different time points after rhizobial infection by confocal fluorescence microscopy and histological GUS staining of roots and nodules inoculated with an *S. meliloti* strain that constitutively expresses the red fluorescent protein (RFP; Tian et al., 2012). Activity of pMIR390a was detected only at late stages of the interaction: in the peripheral vasculature bundles of nodules at 21 dpi and in the meristematic and distal infection zones of nodules at 28 dpi. On the other hand, activity of pMIR390b was undetected at 2 dpi; however, at 6 dpi, when ITs ramified and reached the cortex, strong activity was detected in the infected root hair, the adjacent epidermal cells reached by the ramifying ITs, and the dividing cortical cells beneath the site of infection. In nodules at 10 and 21 dpi, activity of pMIR390b was found in those cells of the infection zone that are reached by ITs and in noninfected cells of the fixation zone, but not in those cells completely filled with bacteria. In nodules at 28 dpi, pMIR390b was active in the nodule meristem. These results indicate that MIR390b is a rhizobia-responsive gene and that its expression is associated
miR390 Promotes Lateral Root Growth But Prevents Nodulation and Infection by *S. meliloti*

The expression pattern observed for miR390b suggests a function at early and/or late stages of the root nodule symbiosis. Thus, premiR390b was ectopically overexpressed in *M. truncatula* roots (OX390). OX390 roots accumulated significantly higher levels of mature miR390a/b (5-fold) and tasiARFs (4-fold) as compared with roots transformed with the empty vector (EV; Fig. 4, A and B). Considering that the miR390/TAS3 pathway controls lateral root growth in Arabidopsis and that miR390 is highly conserved among flowering plants (Cuperus et al., 2011), we investigated the architecture of OX390 roots. Neither the primary root length nor the density of emerged lateral roots (i.e. the number of emerged lateral roots per centimeter of primary root) was affected in OX390 roots (Fig. 4, C and D). However, the average length of lateral roots was significantly higher in OX390 than in EV roots (Fig. 4, E and F). No obvious phenotype was observed in the lateral root tip; however, the use of the synthetic *DR5* promoter fused to the monomeric RFP (*DR5:mRFP*) auxin reporter indicated an increase of auxin signaling and/or response in the lateral root tips of OX390 roots as compared with EV roots (Fig. 4G). Hence, activation of the miR390/TAS3 pathway promotes the growth of emerged lateral roots, presumably by increasing sensitivity to auxin signaling or response. Exogenous auxin treatment inhibited the primary root growth to a greater extent in OX390 than in EV roots, either at 5 or 10 μM indole-3-acetic acid (IAA; Fig. 4H), supporting the idea that OX390 roots are more sensitive to auxin.

Then, we explored a role in the formation and/or development of root nodules. OX390 and EV transgenic roots were inoculated with *S. meliloti*, and the number of plants with nodules was monitored over a time course. At 16 dpi with *S. meliloti*, the percentage of plants with nodules was 22% lower in OX390 than in EV roots (Table I). In addition, the average number of nodules per root was significantly lower (40%) in OX390 than in EV roots at 13 and 16 dpi (Fig. 5A), indicating that deregulation of the miR390/TAS3 pathway negatively affects nodule formation. This negative effect on nodulation also was reflected in a 20% reduction in the shoot dry weight of OX390 composite

Figure 2. Tissue-specific expression analysis of MiR390a and MiR390b in roots and nodules. A and F, Expression of the GUS reporter gene in primary roots transformed with the pMIR390a:GUS-GFP (A) or pMIR390b:GUS-GFP (F) construct. GUS staining was observed in the vasculature of primary roots. B and G, Transverse cross section of GUS-stained primary roots of pMIR390a:GUS-GFP (B) or pMIR390b:GUS-GFP (G) composite plants. C and H, Magnification of B and G, respectively. GUS staining was observed in the pericycle, phloem, and xylem of pMIR390a:GUS-GFP primary roots and in the pericycle of pMIR390b:GUS-GFP primary roots. D and I, Expression of the GUS reporter gene in lateral roots of pMIR390a:GUS-GFP (D) and pMIR390b:GUS-GFP (I) transgenic roots. GUS staining was observed in the vasculature of lateral roots. E and J, Expression of the GUS reporter gene in mature nodules (28 dpi) formed in pMIR390a:GUS-GFP (E) or pMIR390b:GUS-GFP (J) transgenic roots. Black arrows point to the nodule apex zone, where GUS staining was observed. The fixation zone, revealed by the pink color of the leghemoglobin, is marked with asterisks. c, Cortex; ed, endodermis; ep, epidermis; pc, pericycle; ph, phloem; xy, xylem. Bars = 100 μm in A, D, F, and I, 50 μm in B and G, 25 μm in C and H, and 200 μm in E and J.

with cells of roots and young nodules that are reached by actively growing ITs, whereas in mature nodules, its expression is restricted to the nodule meristem.

In order to assess whether the expression of MiR390a or MiR390b spatially and temporally overlaps with one of the miR390/TAS3 targets, transgenic roots expressing a pARF4:GUS-GFP reporter were generated. At 2 and 6 dpi, pARF4 activity was detected in the root epidermis, including noninfected root hairs and those containing elongated ITs (Fig. 3B). In nodules at 10 dpi, ARF4 expression was detected in cells reached by ITs or in noninfected cells surrounding the infected cells (Fig. 3B), reminiscent of that observed for pMiR390b (Fig. 3A). Later (at 28 dpi), longitudinal cross sections of GUS-stained nodules revealed that ARF4 was active in the meristematic and infection zones, in agreement with the RNA sequencing data from nodule sections reported by Roux et al. (2014) and presented in Supplemental Figure S2B. This analysis indicates that the expression of MiR390b and ARF4, one target of the miR390/TAS3 pathway, partially overlaps during rhizobial infection (6 dpi) as well as in young and mature nodules (10 and 28 dpi, respectively).
plants as compared with EV plants (Fig. 5B). In addition, the size of nodules was reduced 27% by OX390 expression (0.73 ± 0.03 mm versus 1.04 ± 0.06 mm in OX390 and EV roots, respectively; n > 25), and these nodules were spherical and pale, in contrast with the characteristic cylindrically shaped and pink nodules formed in EV roots (Fig. 5, C and D), suggesting that miR390/TAS3 also control nodule development and/or infection. Optical microscopy of semi-thin sections of nodules at 28 dpi with *S. meliloti* revealed that OX390 nodules presented all zones characteristic of indeterminate nodules, but the fixation zone contained a very low number of infected cells as compared with control EV nodules (Fig. 5, E and F). Transmission electron microscopy (TEM) showed that infected cells in OX390 nodules presented normal symbiosomes with fully differentiated bacteroids (Fig. 5, G and H), indicating that OX390 did not affect differentiation of the bacteria once symbiosomes were formed.

Considering the low number of infected cells observed in OX390 nodules, we questioned whether infection by rhizobia was affected in the OX390 roots. Infection events were visualized and quantified in transgenic hairy roots at 5 dpi with the *S. meliloti* strain constitutively expressing RFP (Tian et al., 2012). The density of infection events was greatly reduced (greater than 50%) in OX390 as compared with EV roots (Fig. 5I). Progression of ITs was unaffected by overexpression of miR390b (Fig. 5J), indicating that miR390 negatively regulates initiation, rather than elongation, of ITs.

In summary, the phenotypic analysis of OX390 composite plants indicates that the miR390/TAS3 pathway plays a central role in the control of both lateral root and nodule developmental programs in *M. truncatula*, acting as a positive modulator of lateral root growth but negatively affecting both nodule organogenesis and the initiation of infection events during symbiosis.

**OX390 Affects the Rhizobia-Induced Accumulation of ARF2/3/4 and NSP1/2 Transcripts**

We then explored whether activation of the miR390/TAS3 pathway affected the expression of genes required for nodulation and/or rhizobial infection. RT-qPCR experiments verified that OX390 roots accumulated higher levels of mature miR390a/b either in the presence or absence of rhizobia (Supplemental Fig. S4A); consequently, transcript levels of TAS3 (the direct target of miR390), ARF2, ARF3, and ARF4 in these roots were significantly lower in OX390 than in EV roots and did not increase at 48 hpi with rhizobia (Supplemental Fig. S4B). The set of selected genes required for nodulation included those encoding NIN (Marsh et al., 2007), Required for Nodulation1 (ERN1; Andriankaja et al., 2007; Middleton et al., 2007; Cerri et al., 2012), NSP1 (Smit et al., 2005), and NSP2 (Kaló et al., 2005).
miR390 in Lateral Root Growth and Nodulation

Disruption of tasiARF Production Promotes Nodulation and Alters the Spatial Distribution of Nodules

To further understand how the miR390/TAS3 pathway operates during nodulation, we expressed MIM390, which sequesters and partially blocks the action of miR390a/b on TAS3 transcripts. This effect is accompanied in several cases by degradation of the targeted miRNA (Franco-Zorrilla et al., 2007). Indeed, the expression of MIM390 effectively reduced (75%) the levels of the mature miR390a/b and the production of tasiARFs (Fig. 7, A and B). MIM390 roots exhibited a slight increase (20%) in mRNA levels of two of the tasiARFs targets, ARF2 and ARF4, as compared with EV roots (Supplemental Fig. S5). Neither the primary root length nor the lateral root length and density was significantly affected in OX390 roots. On the other hand, ENOD40 showed higher levels in mock-treated OX390 than in EV roots; however, upon inoculation with S. meliloti, similar expression levels were detected in EV and OX390 roots. Interestingly, up-regulation of both NSP1 and NSP2 in response to rhizobia was not observed in OX390 roots (Fig. 6), indicating that miR390 might control the rhizobial induction of NSP1 and NSP2 genes, which are strictly required for nodulation in M. truncatula (Kaló et al., 2005; Smit et al., 2005). This result might explain the OX390 phenotype and reveals a new link between miR390/TAS3 and the nodulation signaling pathway.

Figure 4. Overexpression of the miR390b precursor affects lateral root elongation. A. Scheme predicting how overexpression of the miR390b precursor (OX390) impacts the production of tasiARFs and the levels of ARF2, ARF3, and ARF4 transcripts. B, Expression levels of mature miR390 and tasiARFs in control roots transformed with the EV or with a construct for the overexpression of the miR390b precursor (OX390) as determined by RT-qPCR. Values were normalized to U6 transcript levels and are expressed relative to the mock-inoculated sample. Error bars represent s of three biological replicates. In each biological replicate, root tissue from at least six composite plants containing three to four transgenic hairy roots each were pooled. The asterisk denotes a statistically significant difference in an unpaired two-tailed Student’s t test (***, P < 0.001). Results are representative of three independent biological replicates performed with more than 30 roots in each experiment. F, Images of EV and OX390 composite plants illustrating the longer lateral roots observed in OX390 roots. Diagrams of EV and OX390 composite plants drawn from the photographs are presented at right, where primary roots (PR) and lateral roots (LR) in the hairy root systems are indicated. Roots that emerged directly from the sectioned A. rhizogenes-inoculated radicle (usually three to four) were considered primary roots, whereas roots that emerged from these primary roots were considered lateral roots in the hairy root system. Bars = 1 cm. G, Confocal microscopy images of lateral root tips of DR5:mRFP plants transformed with the EV or the OX390 construct. The GFP protein is encoded in the vector used for root transformation and used as a screenable marker for the selection of transgenic roots. Images of green and red fluorescence channels were merged. Bars = 100 μm. H, Primary root length was measured in EV and OX390 roots upon exogenous application of 5 μM (light blue bars) or 10 μM (dark blue bars) IAA or water as a control (white bars). Error bars represent s of two biological replicates with at least 10 transgenic roots per biological replicate. Different letters above the bars indicate statistically significant differences between samples in an unpaired two-tailed Student’s t test with P < 0.05.
MIM390 roots were grouped in clusters along the susceptible zone of the root (Fig. 7D), and over 40% (n > 40) of them were multilobed, possibly as a consequence of multiple nodule meristems, whereas control EV roots presented only 14% (n > 40) of multilobed nodules. Nodules formed in MIM390 roots were elongated and pink, indicating that MIM390 did not affect the expression of leghemoglobin-encoding genes. TEM of ultrathin sections of MIM390 nodules revealed that blocking the action of miR390a/b does not prevent the infection of nodules or the differentiation of bacteria (Fig. 7E). Moreover, the density of ITs was slightly higher in MIM390 than in EV roots (Fig. 7F), whereas their progression was not affected (Fig. 7G). Hence, inhibition of miR390 action in roots led to an opposite nodulation phenotype of that observed in OX390 roots.

To further support the role of the miR390/TAS3 pathway in legumes, we investigated the expression pattern and function of AGO7, a specific component of the miR390/TAS3 pathway, in contrast to SGS3, RDR6,

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Numbers in parentheses indicate the number of plants with nodules among the total number of plants analyzed. Two independent biological replicates performed on different days were included. MIM390, Target mimicry of miR390.
and DCL4, which also participate in the biogenesis of other small interference RNAs (Xie et al., 2005; Montgomery et al., 2008; Bustos-Sanmamed et al., 2014). AGO7 transcripts were detected in roots and nodules of different stages of development (from 4 to 28 dpi; Benedetto et al., 2008) and, like miR390, TAS3, ARF2, ARF3, and ARF4, accumulated at high levels in the meristematic zone (Supplemental Fig. S7). RDR6, SG53, and DCL4 mRNAs also were detected in roots and nodules (Supplemental Fig. S8, A–C); however, their distribution along the different nodule sections differed from that of AGO7 (Supplemental Fig. S8, D–F). To explore the function of AGO7 in root architecture and root nodule symbiosis, three Tnt1 insertion lines (ago7-1, ago7-2, and ago7-3; Tadege et al., 2008; Zhou et al., 2013; Cheng et al., 2014) were characterized. AGO7 transcripts were undetectable in roots of ago7 homozygous plants based on semiquantitative reverse transcription-PCR (Supplemental Fig. S9A). As expected, roots of ago7 plants, which produced null levels of tasiARFs (Zhou et al., 2013), showed significantly higher levels of ARF2, ARF3, and ARF4 transcript than wild-type roots (Fig. 8A; Supplemental Fig. S9B), in agreement with that observed in the aerial part (Zhou et al., 2013). The three ago7 lines exhibited shorter primary roots than wild-type plants (Supplemental Fig. S10), most likely as a consequence of the pleiotropic phenotypes observed in different organs of these plants, which also affected plant height (Zhou et al., 2013). Consistent with the high number of nodules observed in MIM390 roots, ago7 mutants exhibited enhanced nodulation as compared with wild-type plants (Fig. 8B) and an altered spatial positioning of the nodules, which appeared in clusters along the susceptible zone (Fig. 8C). In addition, a high proportion (53%; n > 40) of the nodules formed in ago7-1, ago7-2, or ago7-3 mutants was multilobed as compared with wild-type plants, which only exhibited 7% (n > 25) multilobed nodules. The number of nodules developed in the wild type and ago7 lines was analyzed also in the presence or absence of 5 μM IAA. Although ago7 roots exhibited more nodules than wild-type roots (13 versus six nodules on average), nodulation was completely abolished by this concentration of IAA in both genetic backgrounds, in agreement with that reported previously by van Noorden et al. (2006). TEM of an ultrathin section of the infection zone of nodules reported previously by van Noorden et al. (2006). TEM of an ultrathin section of the infection zone of nodules revealed the presence of symbiosomes, which were indistinguishable from those observed in wild-type plants (Fig. 8D). At early stages of the interaction, a higher density of ITs was observed in the ago7-1 and ago7-3 mutants as compared with the wild type (Fig. 8, E and F). Taken together, the results obtained using MIM390 roots and ago7 mutants demonstrate a function of the miR390/TAS3 pathway not only in the control of nodule number and positioning but also in the frequency of infection events initiated in M. truncatula roots.

Disruption of the miR390/TAS3 Pathway Enhances the Expression of NSP1 and NSP2 Transcripts

We next explored whether the disruption of the miR390/TAS3 pathway impacted the expression of genes required for nodulation, namely ERN1, NIN, CRE1, ENOD40, NSP1, and NSP2. In the absence of rhizobia, MIM390 roots exhibited higher levels of all these transcripts as compared with EV roots (Fig. 9A, white bars), indicating that alterations in the miR390/TAS3 pathway impacted the expression of nodulation signaling genes prior to rhizobial infection. Remarkably, upon inoculation with S. meliloti, only NSP1 and NSP2 mRNAs accumulated at significantly higher levels in

Figure 6. Accumulation of NSP1 and NSP2 transcripts after inoculation with rhizobia is impaired in OX390 roots. Transcript levels of the early nodulation transcripts NIN, CRE1, ERN1, ENOD40, NSP1, and NSP2 in EV and OX390 roots at 48 hpi with water (Mock; white bars) or with S. meliloti Sm; blue bars) were determined by RT-qPCR. Expression values were normalized to HIS3L and are expressed relative to the mock EV sample. Error bars represent s. Root tissue from at least six composite plants containing three to four transgenic hairy roots each was pooled. Different letters above the bars indicate statistically significant differences between samples in an unpaired two-tailed Student’s t test with P < 0.05.
**MIM390 increases nodulation and alters the spatial distribution of the nodules.**

A. Scheme predicting how the expression of MIM390 impacts the production of tasiARFs and the levels of ARF2, ARF3, and ARF4 transcripts. B. Expression levels of miR390a/b and tasiARFs in EV and MIM390 roots as determined by RT-qPCR. Expression values were normalized to U6 and are expressed relative to the EV sample. Error bars represent SE of three biological replicates. In each biological replicate, root tissue from at least six composite plants containing three to four transgenic hairy roots each was pooled. C. Time course of nodule formation in EV and MIM390 roots at 6, 9, 13, and 16 dpi with **S. meliloti**. Data are representative of two biological replicates. At least 20 roots were used for IT quantification in each biological replicate. G. Percentage of ITs in EV and MIM390 roots. ITs were classified as ITs that end in the root hair, in the epidermal cell layer, or that reach the cortex at 6 dpi, and each category is as the percentage of total ITs. ITs from at least 20 roots were classified. The asterisks in B, C, and F indicate statistically significant differences in an unpaired two-tailed Student’s t test with $P < 0.05$.

**DISCUSSION**

In this study, we showed that the miR390/TAS3 pathway plays key functions in the development of lateral root organs in *M. truncatula*, acting as a positive modulator of lateral root growth and a negative regulator of nodulation. Null *ago7* mutants exhibited a higher frequency of ITs and more nodules than wild-type plants; moreover, these nodules were grouped in clusters within the susceptible zone of the root. Consistent with the idea of a negative regulatory role in nodulation, the pathway is transiently repressed at early stages of the interaction with **S. meliloti**, releasing the tasiARF-mediated repression of ARF2/3/4. This transient repression of the pathway seems to be required for rhizobial infection and the proper activation of specific genes of the nodulation signaling pathway, since constitutive expression of premiR390b leads to a reduction in the number of infection events as well as to impaired induction of NSP1 and NSP2. Supporting this idea, inactivation of the pathway either by expression of MIM390 or mutations in *AGO7* had an opposite effect: increased frequency of infection events and higher accumulation of **NSP1** and **NSP2** transcripts in rhizobia-inoculated roots. At later stages of root nodule symbiosis, the miR390/TAS3 regulatory pathway acts mainly in the meristematic and distal infection zones of nodules. This tissue-specific expression pattern might be associated with a role in maintaining a single-nodule meristem, since disruption of tasiARF production in MIM390 or *ago7* roots led to the formation of a high proportion of multilobed nodules with more than one meristem.

**Role of the miR390/TAS3 Pathway in the Elongation of Postemerged Lateral Roots**

Lateral roots are initiated at the location of auxin maxima, where the lateral root primordia will form. In Arabidopsis, miR390, TAS3, and ARF2/3/4 form an
Figure 8. Mutations in the AGO7 gene enhance nodulation and alter nodule distribution. A, Scheme predicting how mutations in AGO7 impact the production of tasiARFs and the levels of ARF2, ARF3, and ARF4 transcripts. B, Average number of nodules per plant developed in the wild type (WT) or three independent ago7 mutants (ago7-1, ago7-2, and ago7-3) at 28 dpi with S. meliloti. Error bars represent SD of two biological replicates. At least five plants of each genotype were used for nodule quantification in each biological replicate. Asterisks denote statistically significant differences in an unpaired two-tailed Student’s t test between the wild type and ago7 mutants with P < 0.01. C, Images illustrating nodule morphology and distribution in the wild type, ago7-1, ago7-2, and ago7-3 mutants at 28 dpi with S. meliloti. Multilobed nodules and clusters containing multiple nodules were more frequently observed in ago7 mutants than in wild-type plants. Bars = 1 mm. D, TEM of ultrathin nodule sections of the wild type, ago7-1, ago7-2, and ago7-3 mutants. Sections correspond to the infection zone of 28-dpi nodules. BT, Bacteroids. Bars = 2 μm. E, IT density in the wild type, ago7-1, and ago7-3 at 6 dpi with S. meliloti expressing RFP. Error bars represent SE. At least five plants of each genotype were used. Asterisks denote statistically significant differences in Student’s t test between the wild type and ago7 mutants (*, P < 0.05 and **, P < 0.01). F, Images illustrating IT distribution in the wild type, ago7-1, and ago7-3 at 6 dpi with S. meliloti expressing RFP in nodule primordia. Multiple ITs are observed in nodule primordia of ago7 mutants. Arrows point to ITs. Arrowheads indicate microcolonies. Dashed lines mark the root epidermis. Bars = 50 μm.

Auxin-responsive regulatory network controlling lateral root growth. Activation tagging or overexpression of the TAS3 gene promotes the growth of emerged lateral roots (Marin et al., 2010). However, the effect of miR390 overexpression in root architecture has not been reported yet. In this study, we showed that overexpression of miR390 enhanced auxin sensitivity, increased auxin signaling/response at the lateral root tip, and promoted lateral root growth in M. truncatula. In this legume, the expression of MIR390a and MIR390b was detected in the vascular cylinder of both primary and emerged lateral roots, in agreement with that described previously in Arabidopsis, where MIR390a promoter activity was found in the central cylinder, the pericycle, and the flanks of the developing lateral roots (Marin et al., 2010). Due to the conservation of this pathway in flowering plants (Cuperus et al., 2011), the up-regulation of miR390 in roots may open interesting perspectives for the manipulation of root growth in agriculture.

Role of the miR390/TAS3 Pathway in Rhizobial Infection and Nodulation

Auxin also is an important regulator of root nodule symbiosis, acting at early stages of infection, as well as in nodule development (Mathesius, 2008; Breaksppear et al., 2014). Exogenous auxin application inhibited nodule formation in M. truncatula roots (van Noorden et al., 2006), whereas application of auxin transport inhibitors to legume roots resulted in the formation of nodule-like structures in the absence of rhizobia (Allen et al., 1953), indicating that changes in auxin distribution are required for nodule formation. More recently, Roy et al. (2017) reported that the auxin influx inhibitors 1-naphthoxyacetic acid (1-NOA) and 2-NOA or mutations in the LAX2 auxin influx transporter reduced nodulation in M. truncatula roots, suggesting that localized auxin influx at the sites of rhizobial infection is required for nodule formation. On the other hand, roots with reduced sensitivity to auxin exhibited enhanced nodulation (Kuppusamy et al., 2009). In this work, we found that OX390 roots exhibited a reduced frequency of infection events and decreased nodulation, which correlate with an enhanced sensitivity to auxin. This is in agreement with a recent report showing that mutations in ARF16, a transcriptional repressor of auxin-responsive genes, enhanced auxin sensitivity and reduced the frequency of infection events (Breaksppear et al., 2014). In that report, the authors analyzed the transcriptome of infected root hair in M. truncatula, revealing that the transcriptional activation of the auxin-response genes GH3, SAUR1, and ARF16 is restricted to the infection site at early stages of the symbiotic association. Our results showed that ARF4 is also expressed in the infection site at 2 and 6 dpi with S. meliloti and, moreover, that the constitutive expression of pre-miR390b, which prevents the accumulation of ARF2, ARF3, and ARF4, also significantly decreased the frequency of ITs. Both our findings and those reported by Breaksppear et al. (2014) highlighted the importance of auxin signaling/response in rhizobial infection. Consistently, ago7 roots, which accumulated higher levels of ARF2, ARF3, and ARF4, exhibited an enhanced
Figure 9. Expression of NSP1 and NSP2 transcripts in roots is enhanced by the disruption of tasiARF production. A, Transcript levels of the early nodulation transcripts NIN, CRE1, ERN1, ENOD40, NSP1, and NSP2 in EV and MIM390 roots at 48 hpi with water (Mock; white bars) or with S. meliloti (Sm; blue bars) determined by RT-qPCR. Values were normalized to HIS3L mRNA and are expressed relative to the mock EV sample. Error bars represent SE of two biological replicates.

B, Transcript levels of NSP1 and NSP2 in wild-type (WT) and ago7-3 roots at 48 hpi with water (white bars) or with S. meliloti (blue bars) determined by RT-qPCR. Values were normalized to HIS3L mRNA and are expressed relative to the wild-type sample. Error bars represent SE of two biological replicates.

The expression and phenotypic analyses suggest that the miR390/TAS3 pathway is repressed at early stages of the symbiotic interaction, allowing rhizobial infection and the initiation of nodule formation. At later stages of the symbiotic interaction (10 dpi or later), this pathway seems to be required to control the number and spatial frequency of ITs. It is notable that levels of both miR390 and tasiARFs decreased at the initiation of infection events (48 hpi), with a concomitant increase in ARF2, ARF3, and ARF4 mRNA accumulation. Thus, enhanced IT initiation correlates with high levels of ARF2, ARF3, and ARF4 transcripts, reinforcing the link between auxin signaling/response and rhizobial infection. We have shown here that alteration of the miR390/TAS3 pathway also affected the number of nodules (i.e. activation of the pathway by OX390 delayed and reduced nodule formation). This might be a likely consequence of the reduction of the frequency of infection events, since not only the number of nodules in OX390 roots was reduced but also the number of infected cells in the nodule.

Increased auxin signaling occurs at the site of nodule formation in both determinate and indeterminate nodules and, in the latter case, also in the meristematic region of the nodule (Mathesius et al., 1998; Suzaki et al., 2012). In mature elongated nodules of M. truncatula, expression of the auxin-responsive genes GH3, SAUR1, and ARF16 is limited to the apical region, including the nodule meristem and the infection zone (Breakspear et al., 2014). Here, the expression of ARF2, ARF3, and ARF4 also was found in the meristematic and distal infection zones of mature nodules. TAS3 and AGO7 transcripts, which are involved specifically in the production of tasiARFs, was also accumulated in the nodule apex. MIR390a and MIR390b promoters were active in the apical region of mature nodules, indicating that most of the components of the miR390/TAS3 pathway are coexpressed in the nodule meristem. In Arabidopsis, miR390 is responsive to auxin, and its expression is connected by a positive feedback loop with ARF2/ARF3 and by a negative feedback loop with ARF4 (Marin et al., 2010). The positive feedback, referred to as the homeostatic model for miR390/ARF function, might help to tightly regulate the expression levels of miR390/TAS3/ARFs in nodules, explaining the coincident expression pattern observed for MIR390a/b genes, TAS3, and the ARF2/ARF3 transcripts in mature nodules. As ARF4 might function as a negative regulator of MIR390 genes and the expression of ARF4 is relatively higher in the proximal infection zone, this suggests that ARF4 could restrict the expression of MIR390a/b to the meristematic and distal infection zones of the nodules. On the other hand, tasiARFs might act in a non-cell-autonomous way, moving to act as an instructive signal during development (Chitwood et al., 2009; Schwab et al., 2009). This leads us to speculate that tasiARFs produced by the action of miR390 on the TAS3 transcript in the nodule meristematic region might act systemically to repress the expression of ARF2 and ARF3 transcripts in the proximal infection and fixation zones of the nodules.
distribution of the nodules as well as the maintenance of a single-nodule meristem. Thus, the miR390/TAS3 pathway acts in two waves during early and relatively late symbiosis, a resemblance with the well-known mechanism of autoregulation of nodulation linked to altered auxin transport (Oka-Kira and Kawaguchi, 2006; van Noorden et al., 2006; Jin et al., 2012).

Cross Talk between the miR390/TAS3 Module and the Nodulation Signaling Pathway

Aiming to establish a connection between miR390 and the nodulation signaling pathway, we identified an interesting link between the miR390/TAS3 pathway and two transcription factors that are essential for nodulation, NSP1 and NSP2. Ox390 prevented the induction of NSP1 and NSP2 in response to rhizobial infection. In agreement, roots expressing the MIM390 or ago7 mutants showed elevated levels of NSP1 and NSP2 in S. meliloti-inoculated roots. The correlation between NSP1/NSP2 and ARF2/3/4 levels might explain, at least in part, the nodulation and infection phenotypes observed in ox390, mim390, and ago7 roots. Overexpression of miR171h, which targets and effectively reduces roots. Overexpression of miR171h, which targets and effectively reduces phenotypes observed in OX390, MIM390, and ago7 roots. Although both NSP1 and NSP2 are direct targets of ARF2, ARF3, and/or ARF4. Indeed, examination of the 2 kb upstream of the translation initiation codon of NSP1 and NSP2 indicated that both promoters contain auxin response elements. Although ARF2/3/4 have been proposed to act as transcriptional repressors of primary auxin-responsive genes (tiwari et al., 2003), we cannot exclude a role as transcriptional activators of other genes, acting either alone or in combination with other transcription factors. The lack of induction of NSP1 and NSP2 observed in ox390 roots might be a consequence of a reduction in the frequency of infection events. However, this seems to be unlikely, since induction of other transcripts linked to rhizobial infection, such as NIN or ERN1, occurs normally in ox390 roots. Although both NIN and ERN1 are direct targets of NSP1/NSP2 (hirsch et al., 2009), they are transcriptionally activated also by IPD3 (singh et al., 2014; cerri et al., 2017) and, in the case of ERN1, by the heterotrimeric NF-Y complex (laloum et al., 2014; baudin et al., 2015). Thus, the induction of ERN1 and NIN in ox390 roots might be a consequence of the activation of the branch that involves NF-Ys and IPD3 in the nodulation signaling pathway (Fig. 10).

Role of miRNAs and ARFs in the Developmental Programs of Determinate and Indeterminate Nodules

An increasing number of sRNAs associated with the nodulation process have been identified at different stages of nodule development in several legume species, including M. truncatula, L. japonicus, and G. max (for review, see bustos-sannamed et al., 2013; lelandais-brière et al., 2016). Targets of some of these miRNAs encode proteins involved in auxin signaling and response. For example, miR160 targets ARF10, ARF16, and ARF17. G. max roots ectopically expressing miR160 exhibited hypersensitivity to auxin and reduced nodulation; however, IT formation and progression were not affected (turner et al., 2013). A more recent report showed that disruption of miR160 function using a short tandem

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target mimicry resulted in reduced sensitivity to auxin and increased nodulation (Nizampatnam et al., 2015). On the other hand, overexpression of miR160 in M. truncatula roots led to a reduction in nodule formation (Bustos-Sannamed et al., 2013), indicating that miR160 acts in both determinate and indeterminate nodules. Our results indicate that the miR390/TAS3 pathway, which targets a different set of putative transcriptional regulators involved in auxin signaling and response (ARF2/3/4), negatively regulates both nodulation and rhizobial infection in M. truncatula. ago7 mutants, which had increased levels of ARF2/3/4, exhibited enhanced infection and nodulation. A contrasting scenario has been observed in L. japonicus, a legume forming determinate types of nodules (Li et al., 2014). rel3/ago7 mutants, which exhibited reduced levels of tasiARFs and increased levels of ARF3/4 and ARF4, produced fewer nitrogen-fixing nodules and showed lower frequency of ITs. The contrasting nodulation phenotypes observed in M. truncatula ago7 and L. japonicus rel3 mutants suggest that blocking the miR390/TAS3 pathway might have different effects on root nodule symbiosis depending on the legume species. Differences between L. japonicus rel3 and M. truncatula ago7 mutants were already observed in compound leaf patterning, leading to the speculation that distinct determination mechanisms of leaf development might result in distinct responses to the loss of tasiARFs in each species (Yan et al., 2010; Zhou et al., 2013). Similarly, the mechanisms governing the organogenesis of determinate and indeterminate types of nodules might exhibit different responses to the lack of tasiARF production.

Taken together, our work shows that deregulation of the miR390/TAS3 pathway, which is involved in lateral root growth, also affects nodule proliferation and the maintenance of single-nodule meristems as well as the initiation of infection events and the induction of two key transcription factors (i.e. NSP1 and NSP2) required for nodulation in M. truncatula. This pathway might be part of the cross talk between lateral root growth and the formation of nitrogen-fixing nodules. We propose that the miR390/TAS3 pathway, which works in lateral root growth, has been recruited and subsequently modified to act in an opposite way in root nodule symbiosis.

**MATERIALS AND METHODS**

**Biological Material**

Medicago truncatula Jemalong A17 seeds were obtained from the Institut National de la Recherche Agronomique. nsp1 and nsp2-1 mutants were described previously (O’Drovyd and Long, 2003; Kaló et al., 2005; Smit et al., 2005). M. truncatula ago7-1, ago7-2, and ago7-3 Tnt-1 insertion mutant seeds, described previously by Zhou et al. (2013), were obtained from the Samuel Roberts Noble Foundation. M. truncatula DR5-mRFP seeds (Couriós et al., 2013, 2016) were provided by Pascal Ratet and Jean Malo Couzigou. Sinorhizobium meliloti strain 1021 was described previously (Meade and Signer, 1977), and the S. meliloti strain constitutively expressing GFP (Tian et al., 2012) was provided by Jacques Batut. Agrobacterium rhizogenes Arqua1 used for hairy root transformations was described previously by Quandt et al. (1993).

**Vectors and Hairy Root Transformation**

The OX390 construct was generated by PCR amplification of premiR390b using genomic DNA from M. truncatula as a template, Mtprre-miR390b F and Mtprre-miR390b R primers (Supplemental Table S1), and pfu DNA polymerase (Promega). The amplified fragment was cloned into the pENTR/D-TOPO vector (Invitrogen) and then recombined into the Gateway-compatible binary vector pK7WG2D.1 (Karimi et al., 2002), in which the expression of premiR390b is driven by the cauliflower mosaic virus 35S promoter. To generate the MIM399 construct, the INDUCED BY PHOSPHATE STARVATION1 (IPS1) gene was engineered to replace the sequence of MIM399 with the sequence of MIM399 by PCR amplification using the mim390 F and mim390 R primers (Supplemental Table S1), as described previously (Franco-Zorrilla et al., 2007). The sequence of the IPS1 gene with MIM399 was amplified by PCR using IPS1F and IPS1R primers (Supplemental Table S1) and pfu DNA polymerase (Promega), cloned into the pENTR/D-TOPO vector, and then recombined into pK7WG2D1.1 (Karimi et al., 2002). This vector contains the rolD:gfp screenable gene for the detection of transgenic roots; therefore, only roots with detectable GFP fluorescence (more than 80% of the roots) were taken into account for expression and phenotypic analyses. The EV pK7WG2D1 was used as a control. For RNA extraction and qPCR experiments, root tissue from at least six composite plants with three to four independent hairy roots per plant was collected at 48 hpi with S. meliloti, frozen in liquid N2, and stored at −80°C. To generate the pMIR390a:GUS-GFP and pMIR390b:GUS-GFP constructs, the genomic regions upstream of premiR390a or premiR390b precursors (1,568 and 1,492 bp, respectively) were amplified by PCR from genomic DNA using the MTrpmiR390a F/MTrpmiR390a R or MTrpmiR390b F/MTrpmiR390b R primer pair, respectively (Supplemental Table S1). The parF4:GUS-GFP construct was generated by amplifying the 1.816-bp region upstream of the translational initiation codon of ARF4 using pMPARF4F and pMPARF4R primers (Supplemental Table S1). The pMIR390a, pMIR390b, and parF4 DNA fragments were cloned into pENTR/D-TOPO vector and then recombined into the Gateway-compatible binary vector pKGWPF70 (Karimi et al., 2002). All constructs were verified by sequencing. Binary vectors were introduced into A. rhizogenes Arqua1 (Quandt et al., 1993) by electroporation. A. rhizogenes-mediated transformation of M. truncatula root was performed essentially as described previously (Boisson-Dernier et al., 2001).

**Growth of M. truncatula and Rhizobia Inoculation**

Seeds were surface sterilized and germinated on 10% (w/v) water-agar plates at 25°C in the dark for 24 h. Germinated seedlings of wild-type and ago7 genotypes were transferred to pots containing a perlite:sand (3:1) mixed substrate. Seedlings were grown at 25°C with a 16/8-h day/night cycle and 60% humidity and irrigated with Faehraeus medium (Fahrnaeus, 1957) free of nitrogen. Roots of 5-d-old seedlings were inoculated with 20 mL of a 1:1,000 dilution of an S. meliloti 1021 culture grown in liquid TY medium until the OD600 was 0.8, as described previously (Reynoso et al., 2013). Alternatively, germinated wild-type seedlings were transferred to square petri dishes (12 cm × 12 cm) containing slanted agar-Faehraeus medium free of nitrogen covered with sterile filter paper. Seedlings were grown at 25°C with a 16/8-h day/night cycle. Five days after transplantation to square petri dishes, seedlings were inoculated with 10 mL of the S. meliloti suspension grown as described above or with water (mock). Root and nodule tissue was harvested from at least five plants, frozen in liquid N2, and stored at −80°C.

Composite plants (consisting of a wild-type shoot and transgenic hairy roots) transformed with the EV, OX390, and MIM390 were selected for 7 d in agar-Faehraeus medium supplemented with 8 μM KNO3 and 12.5 μg/mL kanamycin. Seedlings that developed hairy roots were transferred to square petri dishes (12 cm × 12 cm) containing slanted agar-Faehraeus medium free of nitrogen covered with sterile filter paper and grown at 25°C with a 16/8-h day/night cycle. Five days after transplantation, seedlings were inoculated with 10 mL of a suspension of S. meliloti 1021 or with water (mock) as described above.

**Root and Nodulation Phenotypic Analyses**

For root architecture analysis, wild-type, ago7 mutant, and composite plants generated by A. rhizogenes-mediated transformation were transferred to slanted boxes containing agar-Faehraeus medium supplemented with 8 μM KNO3. The number of lateral roots per centimeter of primary root and the length of primary and lateral roots were determined 15 d after transplantation. Each root that emerged directly from the sectioned radicle transformed with A. rhizogenes was
considered a primary root in the hairy root system. Usually, *M. truncatula* develops three to four independent primary roots per composite plant. Roots that emerged from these primary roots were considered lateral roots. Three biological replicates were performed, and a minimum of 30 independent roots per construct were analyzed in each biological replicate.

To evaluate auxin sensitivity, OX390 and EV composite plants were transferred to square petri dishes containing agar-Fahraeus medium supplemented with 5 mM NO3, 100 μM IAA or without IAA as a control. Plants were grown at 25°C with a 16/8-h day/night cycle. Ten days after transplantation, the primary root length was determined. Two biological replicates were performed, and a minimum of 10 independent roots per construct were analyzed in each biological replicate.

For nodulation analysis, composite plants were transferred to slanted boxes containing nitrogen-free Fahraeus medium and inoculated with *S. meliloti* 1021. 5. After transplantation of roots, as described above. The number of plants with nodules and the number of nodules per hairy root were counted at 6, 9, 13, and 16 dpi using three biological replicates and a minimum of 78 independent roots per construct in each biological replicate. The size of nodules was measured from digital images using Photoshop CS6 at 16 dpi. For the determination of shoot dry weight, the aerial part of 16-dpi individual EV or OX390 composite plants was dried at 80°C for 24 h and weighed using an analytical balance. For nodulation analysis of the wild type or ago7-1, ago7-2, and ago7-3, germinated seeds were transferred to pots and inoculated with *S. meliloti* as described above, and the number of nodules per plant was measured at 28 dpi.

For IT analysis, composite plants were transferred to petri dishes containing agar-Fahraeus medium free of nitrogen. Five days after transplantation, roots were inoculated with the *S. meliloti* strain expressing RFP (Tian et al., 2012) grown as described above. FITC was visualized, quantified, and imaged at 6 dpi in an Olympus IX71 inverted microscope. FITC was classified as ending in the root hair, in the epidermis, or reaching the cortical cells as described previously (Zanetti et al., 2010). Two biological replicates were performed for each experiment, with a minimum of 20 independent roots per construct.

The statistical significance of the differences for each parameter was determined by unpaired two-tailed Student's *t*-tests for each construct or for the wild type and mutant lines at each time point.

**RNA Extraction and RT-qPCR**

Total RNA extraction was performed with Trizol according to the manufacturer's instructions (Invitrogen). RNA concentration was determined by measuring absorbance using a Nanodrop ND-1000 (Nanodrop Technologies), and RNA integrity was evaluated by electrophoresis on 1.2% (w/v) agarose gels. Total RNA was treated with DNase I following the manufacturer's instructions (Promega). Semiquantitative reverse transcription-PCR to detect AGOT transcripts was performed as described previously (Reynoso et al., 2013) using MIGOT F and MIGOT G primers. RT-qPCR was performed as described above (Reynoso et al., 2013). For each primer pair (Supplemental Table S1), the presence of a unique product of the expected size was verified on 1.2% (w/v) agarose gels. In all cases, negative controls without template or without reverse transcription were included. Expression values were normalized to *H3F3A*, which was validated by qPCR software (Vandesompele et al., 2002), as reported previously by Ariel et al. (2010) and Reynoso et al. (2013). Quantification of miR390 in individual samples was performed using the stem-loop RT-qPCR methods described by Varkonyi-Gasic and Hellens (2011). The MtmiR390, MttaisiARFs, and MtU6 stem-loop reverse transcription primers listed in Supplemental Table S1 were used in the reverse transcription reactions. MtmiR390 F, MttaisiARFs F, or MtU6 F were used in combination with the common primer R in the quantitative PCR (Supplemental Table S1) for quantification of the mature miR390a/b, the taisiARFs, or U6 transcript, respectively. PCR products were cloned and sequenced to confirm their identity. Statistical significance between samples was determined by unpaired two-tailed Student's *t*-test comparing means ± SE of at least two biological replicates for each data point.

**GUS Staining**

For GUS staining of roots, pMT390a:GUS-GFP, pMT390b:GUS-GFP, or pARF4:GUS-GFP plants were transferred to square petri dishes (12 cm × 12 cm) containing slanted agar-Fahraeus medium. For GUS staining of nodules, plants were transferred to pots containing perlite:sand (3:1) mixed substrate, irrigated with Fahraeus medium free of nitrogen, and inoculated with *S. meliloti*. Histochmical GUS staining was performed in roots and nodules at 28 dpi according to Jefferson et al. (1987). Cross sections (70 μm) of roots and nodules were obtained from samples embedded in 4% agarose using an EMS 5000 Tissue Slicer (Electron Microscopy Science). GUS-stained tissue was visualized and photographed under white light using an inverted microscope (Olympus IX51).

**Microscopy**

For optical and electronic microscopy, individual nodules were excised from wild-type and ago7 mutant plants grown in pots containing perlite:sand (3:1) at 28 dpi with *S. meliloti* 1021 or from OX390, MIM390, and EV hairy roots grown on square petri dishes containing slanted agar-Fahraeus medium. Nodules were fixed in 50% potassium phosphate buffer, pH 7.4, containing 2% (w/v) paraformaldehyde during 2 h at 4°C. After fixation, samples were subjected to short pulses of gentle vacuum until they sank. Nodules were postfixed in 50% potassium phosphate buffer, pH 7.4, containing 1% (w/v) osmium tetroxide for 1 h at 48°C, rinsed three times in the same buffer, dehydrated by passing through a series of graded ethanol, and embedded in epoxy resin. Ultrathin sections (70 nm) were obtained with a microtome, stained with uranyl acetate and lead citrate, and observed in a JEM 1200 EX II (JEOL, USA) transmission electron microscope. Semithin longitudinal nodule sections (1–2 mm) of the same samples were stained with 0.04% (w/v) Toluidine Blue and observed under white light in an inverted microscope (Olympus IX51). Imaging of IT formation by the RFP-labeled *S. meliloti* strain was performed with an Olympus IX51 inverted microscope using white and UV light with appropriate filters for GFP and RFP. Confocal microscopy was performed on pMiR390a:GUS-GFP, pHMR390a:GUS-GFP, and pARF4:GUS-GFP roots at 48 hpi and on nodules at 6, 10, and 21 dpi with an S. meliloti strain expressing RFP (Tian et al., 2012) and on DR5::RFP roots transformed with the OX390 or the EV construct using an inverted S5p confocal microscope (Leica Microsystems) with a 20× objective. GFP and RFP were excited using 488- and 543-nm lasers, and emissions were collected from 498 to 552 nm and from 578 to 626 nm, respectively. Images were processed with the LAS Image Analysis software (Leica Microsystems).

**Accession Numbers**

Sequence data from this article can be found in the National Center for Bio-technology Information GenBank, M46401, or miRBase databases under the following accession numbers: miR390a/b (MI0005880), tasiARFs (Medtr2g03380), premiR390a (Medtr3g03380), premiR390b (Medtr3g03380), ARF2 (Medtr6g003050), ARF3 (Medtr2g04770), ARF4 (Medtr4g06440), NSP1 (Medtr2g020840), NSP2 (Medtr3g072710), NIN (Medtr5g099060), ERF1 (Medtr7g085810), CREI (Medtr8g106150), and ERF4 (X80264).

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Stem-loop structures of premiR390a and premiR390b.

**Supplemental Figure S2.** Expression analysis of TAS3, ARF2, ARF3, and ARF4 in *M. truncatula* roots and nodules.

**Supplemental Figure S3.** Classification of ITs.

**Supplemental Figure S4.** Expression levels of miR390, TAS3, ARF2, ARF3, and ARF4 transcripts in EV and OX390 roots at 48 hpi with water or *S. meliloti*.

**Supplemental Figure S5.** Expression levels of ARF3, ARF4, and ARF4 mRNAs in EV and MIM390 roots.

**Supplemental Figure S6.** Root architecture analysis in EV and MIM390 plants.

**Supplemental Figure S7.** Expression analysis of AGOT in *M. truncatula* roots and nodules.

**Supplemental Figure S8.** Expression analysis of DRED, SGS3, and DCL4 in *M. truncatula* roots and nodules.

**Supplemental Figure S9.** Expression analysis of AGOT transcript in ago7 mutant roots.

**Supplemental Figure S10.** Root architecture analysis in the wild type and ago7 mutants.

miR390 in Lateral Root Growth and Nodulation


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LITERATURE CITED

Baudin M, Laloum T, Lepage A, Dias Batut from LIPM, Institut National de la Recherche Agronomique-Centre National de la Recherche Scientifique, for providing the S. meliloti strain expressing RFP; Andres Niebel for fruitful discussions; and Claudio Mazo, Silvana Tongiani, and Paula Giménez from the Instituto de Biotecnología y Biología Molecular-CONICET for technical assistance.

Couzigou JM, Mondy S, Sahli L, Gourion B, Ratet P (2013) To be or not to be: evolutionary tinkering for symbiotic organ identity. Plant Signal Behav 8: e24969

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