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Selective recruitment of mRNAs and miRNAs to polyribosomes in response to rhizobia infection in *Medicago truncatula*

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SUMMARY

Translation of mRNAs is a key regulatory step that contributes to the coordination and modulation of eukaryotic gene expression during development or adaptation to the environment. mRNA stability or translatability can be regulated by the action of small regulatory RNAs (sRNAs), which control diverse biological processes. Under low nitrogen conditions, leguminous plants associate with soil bacteria and develop a new organ specialized in nitrogen fixation, the nodule. To gain insight into the translational regulation of mRNAs during nodule formation, the association of mRNAs and sRNAs to polysomes was characterized in roots of the model legume *Medicago truncatula* during the symbiotic interaction with *Sinorhizobium meliloti.* Quantitative comparison of steady-state and polysomal mRNAs for fifteen genes involved in nodulation identified a group of transcripts with slight or no change in total cellular abundance that were significantly up-regulated at the level of association with polysomes in response to rhizobia. This group included mRNAs encoding receptors like kinases required either for nodule organogenesis, bacterial infection or both and transcripts encoding GRAS and NF-Y transcription factors (TFs). Quantitative analysis of sRNAs in total and polysomal RNA samples revealed that mature microRNAs (miRNAs) were associated with the translational machinery, notably, miR169 and miR172, which target the *NF-YA/HAP2* and AP2 TFs, respectively. Upon inoculation, levels of miR169 pronouncedly decreased in polysomal complexes, concomitant with increased accumulation of NF-YA/HAP2 protein. These results indicate that both mRNAs and miRNAs are subjected to a differential recruitment to polysomes and expose the importance of selective mRNA translation during root nodule symbiosis.

INTRODUCTION

Translation of mRNAs plays a crucial role in the regulation of gene expression during development or adaptation to changing environmental conditions in eukaryotic organisms. Despite a few examples showing selective inhibition of elongation or termination phases of translation in higher plants (Butler *et al.,* 1990, Mittler *et al.,* 1998, Onouchi *et al.,* 2005) the recruitment of ribosomes at the initiation phase is the main step of regulation of polypeptide synthesis (Kawaguchi and Bailey-Serres 2002). Examples of differential translation of individual mRNAs during development include flowering and photomorphogenesis (Jiao and Meyerowitz 2010, Liu *et al.,* 2012). Additionally, numerous environmental stimuli were found to adjust the translation status of transcripts, including heat and salt stress, dehydration, oxygen deprivation and light–dark transitions (reviewed in Bailey-Serres *et al.,* 2009, Juntawong and Bailey-Serres 2012). A consequence of inhibition of initiation or re-initiation steps is the reduction of ribosome abundance on an mRNA, which will shift from polyribosomes (polysomes) into non-polysomal cytosolic messenger ribonucleoprotein (mRNPs) complexes, where it can be stabilized or degraded (Bailey-Serres *et al.,* 2009).

Plant roots are able to adapt to the local environment to maximize water and nutrient acquisition by adjusting their developmental programs (e.g. forming new lateral roots). Under low nitrogen availability, roots of leguminous plants establish a symbiotic association with soil bacteria called rhizobia, which results in the formation of a highly specialized lateral root organ, the nodule. Within this organ, the bacteria differentiate into bacteroids and fix atmospheric nitrogen into reduced forms useful for the host plant. The formation of functional nitrogen fixing nodules depends on a developmental process, the nodule organogenesis, and the suppression of plant defense responses that allow the bacteria to infect the root tissue and reach the developing nodule

(Oldroyd *et al.,* 2011). Nodule organogenesis is initiated by activation of cortical cell divisions that form the nodule primordium, whereas bacterial infection occurs predominantly through root hairs, which curl and entrap the bacteria forming an infection focus. This intracellular mechanism of infection involves the formation of a plant-derived tubular structure, referred to as the infection thread, which guides bacteria to the developing nodule. These morphological events are triggered by the exchange of signals between the host plant and the bacterial symbiont. Nod factors (NF) are lipochito-oligosaccharide signals produced by rhizobia and likely perceived by the receptor-like kinases (RLK) with LysM motifs in the extracellular domain, named NFP (Nod Factor Perception) and LYK3 (LysM domain–containing RLK 3) in *Medicago truncatula* (Limpens *et al.,* 2003, Arrighi *et al.,* 2006). Perception of rhizobia triggers a so-called common symbiotic pathway that includes a RLK with extracellular leucine rich repeats (DMI2, Does not Make Infections 2), a putative ion channel (DMI1) and various nucleoporins (Endre *et al.,* 2002, Ane *et al.,* 2004, Kanamori *et al.,* 2006, Saito *et al.,* 2007, Groth *et al.,* 2010). These components of the cascade are required for the activation of calcium spiking in and around the nucleus, which acts as a secondary messenger in the symbiotic pathway (Ehrhardt *et al.,* 1996, Sieberer *et al.,* 2009). Calcium spiking is thought to be decoded by a nuclear complex formed by a calcium/calmodulin-dependent protein kinase (DMI3) and a protein with coiled-coil motif (IPD3, Interacting Protein with DMI3) (Lévy *et al.,* 2004, Mitra *et al.,* 2004, Messinese *et al.,* 2007, Limpens *et al.,* 2011, Horváth *et al.,* 2011). These events activate a set of transcription factors (TFs) that control the expression of early nodulation genes (ENODs) and triggers later steps of the rhizobial symbiotic interaction. These TFs include nodule inception (NIN) (Marsh *et al.,* 2007), the Ethylene response factor Required for Nodulation (ERN1) (Middleton *et al.,* 2007), two GRAS domain transcriptional regulators, NSP1 and NSP2 (Middleton *et al.,* 2007,

Hirsch *et al.,* 2009) and two subunits of the nuclear factor Y (NF-Y) family, NF-YA/HAP2 and NF-YC/HAP5, which are required for both nodule organogenesis and rhizobial infection (Combier *et al.,* 2006, Zanetti *et al.,* 2010). In addition, E3 ubiquitin ligases, such as LUMPY INFECTION (LIN1), are also required for bacterial infection (Kiss *et al.,* 2009). It was proposed that these proteins might act by regulating the amount and cellular location of the NF receptor, even though they also might target other proteins of the nodulation pathway (Oldroyd *et al.,* 2011). Finally, genes involved in phytohormone perception were identified to regulate nodule formation, such as the cytokinin receptor CRE1 of *M. truncatula* (Gonzalez-Rizzo *et al.,* 2006). To date, studies on the symbiotic process have assessed steady-state levels of mRNAs at different stages of the interaction (Lohar *et al.,* 2006, Benedito *et al.,* 2008, Maunoury *et al.,* 2010, Moreau *et al*., 2011). However, these data do not distinguish mRNAs that are targeted for degradation, sequestered in mRNPs or being actively translated on polysomes. In addition, mRNA stability and/or translatability are also affected by the action of short interfering (siRNAs) and micro RNAs (miRNAs), which have emerged as versatile regulators of eukaryotic gene expression (Bartel 2009). Although plant miRNAs were proposed to primarily control mRNA cleavage and degradation, several studies have shown that some miRNAs do not affect the abundance of their target mRNAs, but significantly reduce the levels of the encoded proteins (Aukerman and Sakai 2003, Chen 2004, Gandikota *et al.,* 2007). Subsequent forward genetic screenings identified miRNA-action deficient (*mad*) and *suo* mutants in Arabidopsis, which were defective in miRNA mediated translation repression, suggesting that the action of miRNA in plants entails a combination of RNA cleavage and translational repression (Brodersen *et al.,* 2008, Yang *et al.,* 2012). The finding that miRNAs and the Argonaute 1 (AGO1) protein are detected in sucrose density gradient purified polysomal complexes of Arabidopsis seedlings

provided additional support to the conclusion that miRNAs might mediate translational repression in plants (Lanet *et al.,* 2009). In legumes, certain miRNAs have been involved in nodule development, such as miR169 and miR166 of *M. truncatula* (Combier *et al.,* 2006, Boualem *et al.,* 2008) and miR482, miR1512, and miR1515 of *Glycine max* (Li *et al.,* 2010). In addition, mi/siRNA diversity was analyzed in various legume species (Lelandais-Briere *et al.,* 2009, Jagadeeswaran *et al.*, 2009, Zhai *et al.,* 2011) revealing that legumes can synthesize phasiRNAs, siRNAs triggered in a phased register by miRNAs of 22 nt on specific mRNAs. Nevertheless, the interaction of the plant miRNA machinery with translation complexes in specific biological contexts has not been yet examined.

In this study, we characterized the association of diverse mRNAs and mi/siRNAs to polysomal complexes in roots of the model legume *M. truncatula* during the symbiotic interaction with *Sinorhizobium meliloti*. By expressing an epitope-tagged ribosomal protein, polysomes were immunopurified and the modulation of mRNA levels in total and polysomal RNA samples was analyzed for a set of genes involved in the nodulation signaling pathway at 48 hours post-inoculation (hpi), the time of root hair curling and the initiation of cortical cell divisions (Timmers *et al.,* 1999, Lohar *et al.,* 2006). Several mRNAs encoding key proteins of the nodulation signalling pathway were selectively recruited into polysomes without any change in transcript levels upon infection. Furthermore, mi/siRNAs were found associated with immunopurified polysomes. Notably, miR169, which targets the NF-YA/HAP2 family of TFs, significantly decreased its abundance in polysomal complexes concomitant with increased accumulation of NF-YA/HAP2 protein upon inoculation with rhizobia. Taken together, our results suggested that differential translation of mRNAs significantly contribute to regulation of gene expression during nodule formation.

RESULTS

Analysis of *M. truncatula* composite plants expressing a FLAG-tagged version of the ribosomal protein L18 (RPL18) and immunopurification of polysomes

Although sucrose density gradient fractionation is considered a powerful tool to separate actively translating mRNAs from other mRNP complexes, polysomal fractions might be contaminated with high sedimentation coefficient complexes such as pseudo-polysomes, processing (P) bodies, storage granules or other mRNPs (Fleischer *et al.,* 2006, Thermann and Hentze 2007, Halbeisen and Gerber 2009). Hence, we expressed a FLAG-tagged version of the large subunit ribosomal protein 18 (RPL18) in *M. truncatula* roots. This ribosomal protein has a solvent N-terminus, allowing the efficient immunopurification of polysomal complexes from Arabidopsis leaf and root tissue (Spahn *et al*., 2001; Zanetti *et al.* , 2005, Branco-Price *et al.*, 2008, Mustroph *et al.*, 2009a). A construct designed to express the *M. truncatula* RPL18 fused to the FLAG epitope under the control of the CaMV35S promoter (p35S:FLAG-RPL18) or the empty vector (EV) p35S:FLAG were introduced into *M. truncatula* roots by *Agrobacterium rhizogenes* transformation. Expression of the FLAG-RPL18 protein in hairy roots of composite plants (which consist on a wild type shoot and transgenic roots) was evaluated using an anti-FLAG antibody. As expected, a polypeptide of 25 kDa was detected in 35S:FLAG-RPL18 hairy roots, but not in roots transformed with the EV (Figure 1a). Plants expressing FLAG-RPL18 did not show any obvious phenotype in terms of root and shoot length, number of leaves (Figure S1) or the number of nodules developed at 15 days post-inoculation (dpi) (Figure 1b). To evaluate whether 35S:FLAG-RPL18 roots responded to rhizobia inoculation, we quantified the accumulation of *ENOD11*, *ENOD12, ENOD40*, and the cytokinin receptor *CRE1b* transcripts by

qRT-PCR at different time points after inoculation (Table 1). These transcripts accumulated at higher levels in inoculated than non-inoculated 35S:FLAG-RPL18 roots, consistent with that previously described in wild type roots (Lohar *et al.*, 2006; Moreau *et al.*, 2011). Next, we examined whether the FLAG-tagged version of RPL18 was incorporated into ribosomal complexes. The presence of FLAG-RPL18 was detected by immunoblot in the whole cell extract (S-16), the ribosomal pellet (P-170) and in the fraction containing large polysomes (LP, mRNAs bound to 5 or more ribosomes), indicating that the FLAG-RPL18 protein was incorporated into ribosomes that are engaged in the active translation of mRNAs (Figure 1c). An antibody that recognizes the 40S ribosomal protein S6 (RPS6) from various plant species (Williams *et al.*, 2003, Zanetti *et al*., 2005) was used as a marker of ribosomal and polysomal fractions. As expected, RPS6 was also detected in the S-16, P-170 and LP fractions along with the FLAG-tagged version of RPL18, but not in the post-ribosomal supernatant (S-170).

Polysomal complexes were immunopurified (IP) from *M. truncatula* root cell extracts (S-16) as previously described (Zanetti *et al.,* 2005; Mustroph *et al.*, 2009a). These complexes contained the FLAG-RPL18 protein (Figure 2a) and polypeptides with similar electrophoretic mobility and relative intensity to those detected in the P-170 fraction (Figure 2b). Some polypeptides detected exclusively in the P-170 sample might be components of other complexes that co-sediment with ribosomes during ultracentrifugation. The specificity of the IP was confirmed by the limited detection of polypeptides in the IP sample from roots transformed with the EV. In addition, the 25S and 18S rRNAs were specifically detected in the IP samples from 35S:FLAG-RPL18 roots (Figure 2c). The yield of RNA in the IP sample was 400-500 nanograms per ml of pulverized tissue, which is comparable to that reported for IP of polysomal RNA from Arabidopsis root tissue (Mustroph *et al.,* 2009a). Semiquantitative RT-PCR analyses of a highly abundant

transcript, *ACTIN11* (*ACT11*), were conducted to confirm the presence of mRNAs in the IP sample. Primers that spanned in two different exons of the *ACT11* gene were used to verify the presence of mRNA and the absence of genomic DNA contamination. A single band of the expected size was detected in total RNA samples from EV and 35S:FLAG-RPL18 roots, and in the IP RNA sample from the 35S:FLAG-RPL18 roots. On the contrary, no amplification was detected in the IP sample from EV roots (Figure 2d). These data confirmed that mRNAs were present in the IP sample obtained from 35S:FLAG-RPL18 roots and demonstrate that the ribosome IP system developed in Arabidopsis can be transferred to other plants. We concluded that expression of FLAG-RPL18 in *M. truncatula* roots allowed IP of polysomal complexes and associated mRNAs, without affecting plant growth, nodule formation or the induction of early nodulation markers.

Inoculation with *S. meliloti* did not alter the global polysome status at early stages of the interaction

Exposure of plants to certain environmental conditions may impose a global inhibition on mRNA translation (Bailey-Serres *et al.,* 2009). Hence, we investigated whether infection of *M. truncatula* roots by *S. meliloti* affected the levels of polysomes engaged in the energy demanding process of mRNA translation. The absorbance profiles of ribosomal complexes obtained by fractionation in sucrose density gradients are presented in Figure 3a. A quantitative analysis of the percentage of RNA associated to three regions of the gradient showed that the percentage of RNA associated with small and large polysomes in mock-inoculated roots was ~78 % (Figure 3b), which is very close to the polysomal RNA content previously observed in Arabidopsis seedlings (Branco-Price *et al.,* 2008). In *S. meliloti* inoculated roots, the percentage of RNA in

small and large polysomes was not significantly different than those of mock-inoculated roots (Figure 3b), indicating that *S. meliloti* infection did not alter the global polysome status in *M. truncatula* roots 48 hpi. Reference transcripts, such as *ACT11* or *histone like 3* (*HISL3*), showed a high percentage (>99%) of association with polysomes that did not significantly change upon inoculation with rhizobia (Figure 3c).

Selective recruitment of mRNA to polysomes in response to *S. meliloti* inoculation

The level of fifteen individual mRNAs involved in the root nodule signaling pathway was quantified by qRT-PCR in total and IP polysomal RNA fractions of mock and inoculated roots. The fold change in total and IP mRNA abundance in response to *S. meliloti* for each mRNA is presented in Figure 4. To quantitatively assess the translational regulation independently of the mRNA steady-state level, we calculated the ratio between the fold change in IP and the fold change in total RNA samples for individual transcripts. Based on this analysis, we classified mRNA species into three categories: transcripts with ratios of fold change over 2.0, between 0.5 and 2.0 or below 0.5. The first class is composed of mRNAs that show evidence of up-regulation at the translational level, and includes transcripts encoding the RLKs NFP, DMI2 and CRE1, as well as the TFs NSP1, NSP2, HAP2-1 and HAP5b (Figure 4a). Interestingly, the GRAS TFs NSP1 and NSP2, which form a heterodimer (Hirsch *et al.,* 2009), showed highly similar ratios of 3.8 and 4.0, respectively. Similar ratios were also observed for HAP2-1 and HAP5b (2.9 and 2.6, respectively), which are part of an heterotrimeric transcriptional complex in eukaryotes (Mantovani 1999, Hackenberg *et al.,* 2011). The second category includes genes that do not show evidence of regulation at the translational level, such as those encoding the NIN and ERN TFs and ENOD40, whose mRNA levels exhibited similar change in abundance in the total and

IP mRNA samples (Figure 4b). Also included in this non-translationally regulated group were mRNAs that encode the LYK3 receptor, the E3 ligase U box protein LIN1, the CCaMK DMI3 and its interacting protein IPD3 (Figure 4b). The third category is represented only by *DMI1*, which showed evidence of translational down-regulation in response to infection (Figure 4c). The ratios used to estimate the translational regulation in two biological replicates were well correlated ($r^2 = 0.84$), verifying the reproducibility of the method used to monitor the translational response to *S. meliloti* (Figure 4d). Based on this analysis, we conclude that differential loading of mRNA into polysomes significantly contributes to the regulation of selected mRNAs that are critical for nodule formation, bacterial infection or both.

To validate the results obtained from IP RNA samples, polysomes were conventionally purified (CP) using sucrose density gradients. Fractions containing mRNAs associated with two or more ribosomes were pooled to obtain CP polysomal RNA samples from mock and *S. meliloti* inoculated roots (see Figure 3a). Fold changes in response to *S. meliloti* of individual mRNA species in the CP sample (Figure 5) were consistent with those of IP samples for thirteen of the fifteen transcripts analyzed. Exceptions were represented by *NFP* and *HAP5b*, which increased in response to rhizobial infection in the IP but not in the CP polysomal sample. The discrepancies in the abundance of these transcripts might be due to contamination of CP polysomes with co-sedimenting high molecular weight mRNP complexes that are excluded by IP of polysomes. Alternatively, the experimental procedure and/or the high centrifugal force used for sucrose density fractionation might cause differential dissociation of specific mRNAs from polysomes or their partial degradation.

Inoculation with *S. meliloti* changed the abundance of mature miRNAs associated with polysomal complexes

In plants, previous studies suggest that miRNAs can function by limiting the translation of their target mRNAs (Brodersen *et al.,* 2008, Lanet *et al.,* 2009, Yang *et al.,* 2012). This prompted us to investigate whether miRNAs were present in the IP polysomal samples from 35S:FLAG-RPL18 *M. truncatula* roots. Our analysis targeted miRNAs (Table 2) of 21 nucleotides (nt) previously shown to play a role in nodulation, such as miR166, miR169 and indirectly miR171, which targets the NSP2 TF (Boualem *et al.*, 2008; Combier *et al*., 2006; Devers *et al*., 2011). Other selected miRNAs were detected as up-regulated (miR160, miR172 and miR2609) or down-regulated (miR396) in nodules as compared to roots by next generation sequencing in *M. truncatula* (Lelandais-Briere *et al*., 2009). The analysis also included the 22 nt species miR1509 and miR2118, which have been identified in *M. truncatula* as highly abundant miRNAs that lead to the production of phasiRNAs and the trans-acting small interference RNA (tasiRNA) tasiARFs derived from the *TAS3* precursor (Zhai *et al*., 2011). Abundance of mature mi/tasiRNAs in total and IP samples was determined by qPCR using the miScript system and mi/tasiRNA specific primers (Table S1). This highly sensitive method reduces the non-specific hybridization signals normally encountered with Northern blot analysis and proved to consistently quantify mature miRNAs in polysomal RNA samples isolated from human cells (Lee *et al*., 2007; Janas *et al*., 2012) or from *M. truncatula* roots (see Figure S2).

Individual mature small RNAs (sRNA) were detected in both total and IP polysomal RNA samples from mock and *S. meliloti* inoculated roots. In the total RNA sample, inoculation with *S. meliloti* did not significantly alter the abundance of the majority of the sRNAs, at least at the time point sampled (Figure 6a). The exceptions were the miR1509 and the tasiARFs, which

showed reductions of 40% and 25% in the total RNA sample in response to rhizobia, respectively. Consistently, the abundance of the tasiARFs targets *ARF2*, *ARF3* and *ARF4* (Jagadeeswaran *et al.*, 2009) increased in the total RNA at 48 hpi with *S. meliloti* (Figure 6b). The proportion of each sRNA associated with polysomes in mock-inoculated roots ranged between 0.01 and 0.42 (Table 2). Upon inoculation with *S. meliloti*, the abundance of miR166 and miR396 in the IP sample slightly increased by 1.7 and 1.5 fold, respectively, whereas miR169 showed a pronounced decrease (~ 60 %) in the IP polysomal sample (Figure 6a). This result indicated that a significant fraction of miR169 dissociates from polysomal complexes in response to rhizobial infection. Interestingly, a target of miR169 is *HAP2-1* whose mRNA contains two target sites located in the 3`-UTR of the mRNA (Combier *et al.,* 2006, Devers *et al.,* 2011), a characteristic feature of miRNAs functioning in translational regulation in animals (Bartel 2009). Even though rapid amplification of 5` complementary DNA ends (5`RACE) experiments and degradome analysis showed cleavage of *HAP2-1* predominantly at the first miR169 recognition site (Combier *et al.,* 2006, Devers *et al.,* 2011), *HAP2-1* was one the mRNAs shown to be translationally up-regulated (Figures 4a and 5a), prompting us to further explore whether HAP2-1 protein levels were modified in *M. truncatula* roots at 48 hpi with rhizobia. Immunoblot using anti-HAP2-1 antisera revealed that HAP2-1 protein accumulated at higher levels (~35 %) in *S. meliloti* inoculated than in mock-inoculated roots (Figure 6c). The fact that the abundance of miR169 decreased in the IP polysomal sample upon rhizobia inoculation suggests that, in addition to mRNA cleavage, miR169d/l might also contribute to the translational repression of *HAP2-1* prior to inoculation.

DISCUSSION

This study showed that differential translation of mRNAs significantly contributes to the regulation of gene expression at early stages of the legume-rhizobia symbiosis. Analysis of the translational status of mRNAs identified a group of transcripts required either for bacterial infection, nodule organogenesis or both, that showed slight or no changes in total cellular abundance, but significantly up-regulated at the translational level in response to *S. meliloti*. This implies that these mRNAs are sequestered as mRNPs within the cells of non-inoculated roots and, upon stimulation with rhizobia, are selectively recruited to the translational machinery. This group of genes included three RLKs (*NFP*, *DMI2* and *CRE1*) and four TFs (*HAP2-1*, *HAP5b*, *NSP1* and *NSP2*). A previous report showed that *HAP2-1* mRNA levels increased (2-5 folds) in roots at early time points after inoculation with *S. meliloti* strain 2011 or treatment with purified Nod Factor (Moreau *et al.*, 2011). However, we did not detect an increase in *HAP2* mRNA abundance in the total RNA sample upon rhizobia inoculation (Figure 4a). The discrepancy between the studies might be explained by differences in plant age or growth conditions, the use of a different *S. meliloti* strain (1021 in this study) or a distinct amount of inoculum. A second group of genes exhibited increases in total mRNA abundance in response to rhizobia, which was accompanied by similar increases in the amount of these mRNAs associated with the polysomes (*NIN*, *ERN1* and *ENOD40*). This is most likely the result of a coordinated and highly balanced transcriptional and translational response of these genes during nodule formation, in which 5´capped and polyadenylated mRNAs exported to the cytoplasm are rapidly recruited into polysomes. Only one of the selected genes, *DMI1*, was classified as down-regulated at the translational level. Transcripts of this gene showed a significant increase in rhizobia inoculated roots as compared to mock-inoculated roots; however, this increase was accompanied by a decrease rather than an increase in the polysomal fraction. This results support the idea that

increases in transcript abundance in response to an environmental stimulus do not necessarily correlate with an increase in the synthesis of the encoded protein. This type of translational regulation was previously reported for a set of genes of Arabidopsis seedlings exposed to a short period of oxygen deprivation or in response to gibberellins (Branco-Price *et al.,* 2008, Ribeiro *et al.*, 2012). On the other hand, *DMI3* and its partner *IPD3* were not regulated at transcriptional or translational level, at least at 48 hpi with *S. meliloti*. The same was true for the E3 ubiquitin ligase *LIN1* and LYK3. Nevertheless, all the genes analyzed in this study had detectable transcripts prior to and following inoculation that were engaged with polysomes, although to different extents, in roots grown in the absence of nitrogen. This is consistent with their role in the perception of the rhizobia and the downstream signaling required for the formation of nitrogen fixing nodules. It is interesting to note that three of the receptors required for nodulation are positively regulated at the translational level. It has been proposed that after perception of a signal, receptors might be internalized and degraded by the ubiquitin-proteasome pathway (Oldroyd *et al.,* 2011), and thus be subjected to active turnover. NF perception is required not only for initial events such as root hair curling and formation of infection foci, but also for the progression of the infection thread that will reach the developing nodule (Arrighi *et al.,* 2006). In this context, the NF receptor might be *de novo* synthesized, which is consistent with the higher levels of *NFP* mRNAs associated with polysome observed in rhizobia inoculated roots. The second group of genes that increase their levels of mRNAs in the polysomal fraction is composed of four TFs. In mammals; it was found that the levels of NF-YA/HAP2 subunit of the heterotrimeric TF are modulated by the ubiquitin-proteasome pathway, influencing cell proliferation (Manni *et al.,* 2008). Thus, there may be highly regulated mechanisms of protein synthesis and degradation that tightly modulate the abundance and function of receptors and TFs

during bacterial infection and/or nodule development. The fact that the E3 ubiquitin ligase LIN1 and nsRING are required for bacterial infection (Shimomura *et al.,* 2006, Kiss *et al.,* 2009) suggests that ubiquitination may be the pathway for degradation of these components of the nodulation signaling cascade.

We also have shown that miRNAs, as well as the tasiARFs, are associated to some extent with polysomes. The fraction of sRNAs associated with polysomes is in the range of that described in polysomes of Arabidopsis seedlings purified by ultracentrifugation procedures (Lanet *et al.,* 2009). As previously hypothesized, the association of sRNAs with polysomes might depend on the translatability of the target mRNA (Lanet *et al.,* 2009). Recruitment of mRNAs to the translational machinery is an actively dynamic process and mRNAs may be partitioned between different cytoplasmic compartments including 43S pre-initiation complexes, ribosomes and polysomes, P bodies, stress granules and other mRNP complexes involved in inter- and intra-cellular trafficking of mRNAs (Bailey-Serres *et al.,* 2009). In our study, *HAP2-1* was positively regulated at the translational level in response to *S. meliloti*; this was accompanied by increased accumulation of HAP2-1 protein in inoculated roots. On the other hand, miR169, which targets the NF-Y/HAP2 family of TFs, decreased in the polysomal fraction upon infection with *S. meliloti*. This leads to the suggestion that *HAP2-1* mRNA and miR169 might be subjected to a dynamic partitioning between translational complexes and other mRNPs. P-bodies are cytoplasmic RNP complexes that contain mRNA decay factors, translational repressors and untranslated mRNAs (Balagopal and Parker 2009), which might participate in such partitioning. In animals, GW182 is a P-body localized protein that contains GW repeats and promotes miRNA mediated translational repression and mRNA degradation via its interaction with Argonaute proteins (Ding and Han 2007, Eulalio *et al.,* 2009). A recent report identified a GW-repeat

protein from Arabidopsis, named SUO, which co-localized with the P-body component DCP1 (Decapping enzyme 1) and is proposed to function in miRNA mediated translational repression (Yang *et al.,* 2012). In addition, Brodersen *et al.,* (2008) showed that the P-body component VARICOSE (VCS) is required for slicing independent miRNA mediated gene silencing. It would be interesting to test whether *SUO* and *VARICOSE* proteins might function in the dynamic cytoplasmic partitioning proposed for *HAP2-1* and miR169. However, regulation of *HAP2-1* gene is not attributable solely to the action of miR169. Expression of *HAP2-1* is also subject to trans-regulation by a small peptide encoded in an upstream open reading frame (uORF), which is produced by alternative splicing (Combier *et al.,* 2008). The alternative spliced form of *HAP2-1* mRNA, which accumulates at higher levels in non-inoculated roots, retains a long intron (865 bp) in the 5' leader sequence (LS) that contains three uORFs. Transcripts containing uORFs are translated at lower levels than mRNAs with no uORFs (Kawaguchi and Bailey-Serres 2005); therefore, the long 5´ LS and the presence of uORF in *HAP2-1* might be also the cause of the poor translation in non-inoculated roots.

Experiments using GUS promoter fusions and *in situ* hybridization have established that miR169 spatially restricts expression of *HAP2-1* transcript to the meristem of nodules by a mechanism of RNA cleavage (Combier *et al.,* 2006). However, increased levels of the HAP2-1 protein with no change in total mRNA abundance were observed in roots at 48 hpi with *S. meliloti*. Voinnet (2009) proposed a model for the action of miRNAs in plants with two layers of regulation, translational repression and mRNA stability, not necessarily coinciding spatially or temporally. In the first regulatory mode of action, miRNAs would predominantly operate through transcript cleavage, which would produce irreversible switches required to establish permanent cell-fates during development, for example in the establishment of new meristems. In the second mode of

action, miRNAs would mainly repress translation of target mRNAs in a reversible manner, which could regulate cell-fate decisions, but also contribute to the coordination and modulation of gene expression during specific responses to environmental cues. In this study, we propose that the decrease of miR169 in polysomes might contribute to the translational derepression of *HAP2-1* mRNA at early time points after inoculation with *S. meliloti*. In this scenario, it can be speculated that translational derepression of *HAP2-1* might be an early response to rhizobia, which is followed by a miR169-guided RNA cleavage mechanism that restricts the expression of *HAP2-1* to the nodule meristems to preserve cell identity (Combier *et al.,* 2006). The extent of the contribution of miR169 to the translational control of *HAP2-1* mRNA or its stability requires further investigation. Nevertheless, the quantitative analysis presented in this study revealed that several mature miRNAs are associated with polysomes of inoculated and non inoculated *M. truncatula* roots, supporting the notion that miRNAs can act at the level of translational repression in plants.

Finally, this study shows that IP of polysomes and associated mRNAs and sRNAs can be achieved in *M. truncatula* roots by expression of the FLAG-tagged version of RPL18. Quantification of individual transcript from two independent biological replicates indicated that the IP of polysomes is reproducible. The extension of polysome IP to *M. truncatula* constitutes a significant step forward to access the translational regulation of gene expression in two agronomical important associations between plant and microorganisms, the root nodule and the mycorrhizal symbiosis. This methodology combined with RNA sequencing technologies will allow a genome wide evaluation of mRNA and miRNAs associated with translational complexes. Furthermore, the use of cell- or tissue-specific promoters to drive the expression the FLAG-RPL18 (Mustroph *et al.,* 2009b, Jiao and Meyerowitz 2010) may be envisioned as a tool

to access the population of polysome associated mRNA within root specific cell types, in particular in those involved in bacterial infection and nodule development.

EXPERIMENTAL PROCEDURES

Biological material and vectors

M. truncatula Jemalong A17 seeds were obtained from INRA Montpellier, France. The p35S:FLAG-RPL18 construct was generated by PCR amplification of the open reading frame (ORF) of *M. truncatula* RPL18 (Gene ID: Medtr1g083460.1, assembly version Mt3.5) using primers MtRPL18 ORF-F and MtRPL18 ORF-R (see Supplemental Table 1) and cDNA from roots as template. The PCR product was introduced into the pENTR/D-TOPO vector (Invitrogen, http://www.invitrogen.com.ar) and recombined into the destination vector p35S:FLAG-GATA (Mustroph *et al.,* 2010). The expression cassette was sequenced on both strands. The empty vector p35S:FLAG (Zanetti *et al.,* 2005) was used as a control. Binary vectors were introduced in *A. rhizogenes* ARqua1 (Quandt 1993) by electroporation.

Plant Growth and rhizobia inoculation

Seeds were surface sterilized and germinated on 10% H_2O-agar plates at 20 °C in the dark for 24 hours. Germinated seedlings were transferred to square petri dishes containing slanted agar Fahraeus media free of nitrogen (Fahraeus 1957) covered with sterile filter paper. Seedlings were grown at 25 °C with a 16/8h day/night cycle and 50 % humidity. Roots of five-day old seedlings were inoculated with 10 ml of a 1:1000 dilution of a *S. meliloti* 1021 (Meade 1977) culture grown in liquid TY media until the OD_{600} was 0.8 or with water as a control (mock). After 1 hour, the excess of liquid was discarded and seedlings were incubated vertically under the

growth conditions described above. For ribosome isolation, root tissue was harvested, frozen in liquid N_2 and stored at -80 °C.

Hairy roots transformation and phenotypic analysis

Root transformation was performed as described (Boisson-Dernier *et al.,* 2001). Plants that developed hairy roots (~70%) were transferred to slanted boxes containing Fahraeus media. Five days after transplantation, roots were inoculated with *S. meliloti* as described above, Root tissue of at least 50 composite plants per replicate and condition were harvested for IP or CP of polysomes. For phenotypic analysis, leaf number and root and shoot length were determined in individual 3 week-old composite plants transformed with the EV or the p35S:FLAG-RPL18 construct. For nodulation assays, 3 week-old composite plants were transferred to a 4:1 mixture of perlite and sand, watered with Fahraeus, and nodules were quantified 15 dpi. Two biological replicates were performed and a minimum of 30 independent roots per construct and condition were analyzed.

Isolation of polysomes by ultracentrifugation or immunopurification procedures

Conventional isolation of ribosomes was performed according to Zanetti *et al.* (2005). Fractionation of ribosomes through 20% to 60% (v/v) sucrose density gradients was performed as described (Kawaguchi *et al.,* 2004). The percentage of RNA present in non-polysomal (mRNPs, ribosome subunits and monosomes), small polysomal (from 2 to 5 ribosomes per mRNA) and large polysomal (more than 5 ribosomes per mRNA) was calculated by integration of the area of each fraction divided by the total area. For RNA extraction and qRT-PCR analysis, fractions with two or more polysomes were pooled and are referred as CP polysomes. For

immunoblots, the fractions containing more than 5 ribosomes were pooled and proteins were precipitated by addition of two volumes of 100% (v/v) ethanol, incubation over night at 4º C, centrifugation at 16,000g for 15 min, and finally resuspended in 50 µl of SDS-loading buffer.

IP of polysomes was accomplished as previously described (Zanetti *et al.,* 2005, Mustroph *et al.,* 2009a). Five ml of packed frozen root tissue was used for each sample. IP material was subjected to RNA extraction, or stored at -80 ºC prior to analyses by SDS-PAGE and immunoblot.

SDS-PAGE and immunoblots

Proteins were separated on 15%(w/v) SDS-PAGE and detected by silver staining, Ponceau S red or by immunoblot as described (Zanetti *et al.,* 2005) using an anti-FLAG-horseradish-peroxidase (HRP)-conjugated monoclonal antibody (1:500; Sigma-Aldrich, www.sigmaaldrich.com), a polyclonal antiserum against *Zea mays* RPS6 (1:5,000; (Williams *et al.,* 2003) or a polyclonal antiserum against HAP2.1 (1:2,000; (Combier *et al.,* 2008). Secondary antibody was HRP-conjugated goat anti-rabbit IgG (1:10,000; Amersham, http://www.gehealthcare.com). Densitometry was performed using Image J 1.42q (http://rsb.info.nih.gov/ij/).

RNA extraction and qRT-PCR

RNA extractions from total cellular extracts (S-16) and IP material were performed with Trizol (Invitrogen). RNA concentration was determined by measuring absorbance at 260 nm in a Nanodrop ND-1000 (Nanodrop Technologies Inc, http://www.nandrop.com) and integrity was evaluated by electrophoresis in agarose gels. Total RNA was subjected to DNAse I digestion (Promega, http://www.promega.com) following manufacture's instructions. First-strand cDNA synthesis and qPCR reactions were performed as previously described (Peltzer Meschini *et al.,*

2008). For each primer pair (Table S1), the presence of a unique product of the expected size was verified on agarose gels. In all cases, negative controls without template or with RNA were included. GNORM software (Vandesompele *et al.,* 2002) indicated that *HIS3L* could be used for normalization as previously reported (Ariel *et al.,* 2010). sRNAs quantification was performed using the miScript Reverse Transcription Kit (Qiagen, http://www.qiagen.com) and mi/siRNA primers (Table S1). Annealing temperatures were 56 ºC for miR169d/l, 50 ºC for miR171h, tasiARFs and miR2118a/b/c and 52 ºC for all other sRNAs. PCR products were cloned and sequenced to confirm their identity.

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SUPPORTING INFORMATION

Figure S1. *M. truncatula* composite plants expressing 35S:FLAG-RPL18 do not show phenotypic alterations.

Figure S2. Representative results of qRT-PCR of mature miRNAs using the miScript system.

Table S1. List of primers used in this study.

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Table 1. Relative induction early nodulation markers in 35S:FLAG-RPL18 transgenic roots compared to wild type roots.

Gene ID[a]	Gene description	Time point	Fold Change	
		(hpi)	35S:FLAG-RPL18 roots[b]	Wild type roots[c]
X80264	*ENOD40*	12	1.31 ± 0.10	1.3
		24	1.44 ± 0.20	1.3
		48	2.30 ± 0.68	2.4
AJ297721.1	*ENOD11*	48	146.76 ± 20.08	224.2
X68032.1	*ENOD12*	48	2.82 ± 0.08	3.7
Medtr8g106150.1	*CRE1*	48	1.20 ± 0.19	1.21

[a] Based on *M. truncatula* genome release 3.5 or GenBank Acc. No.

[b] Mean fold change ± SD of mRNA abundance in roots inoculated with *S. meliloti* 1021 relative to mock-inoculated roots measured by qRT-PCR in 35S:FLAG-RPL18 hairy roots; data were normalized using *HIS3L*.

[c] Microarray data obtained from Lohar *et al.,* 2006. *ENOD11* qRT-PCR data obtained from Moreau *et al.*, 2011.

Table 2. *M. truncatula* sRNAs selected and their association with IP polysomes in 35S:FLAG-RPL18 roots

miRNA	*Length (nt)*	*Ratio IP/total*	*Targeted gene family*	*Reference*
miR160	21	0.07	ARF8/16/17	(Lelandais-Briere *et al.,* 2009)
miR166	21	0.01	HD-Zip class III	(Boualem *et al.,* 2008)
miR167	21	0.05	ARF2/3/4	(Lelandais-Briere *et al.,* 2009)
miR169	21	0.20	HAP2/NF-YA	(Combier *et al.,* 2006; Devers *et al.,* 2011)
miR171	21	0.02	GRAS TF (NSP2)	(Devers *et al.,* 2011)
miR172	21	0.42	AP2 TF	(Lelandais-Briere *et al.,* 2009)
miR396	21	0.02	GRF1/2/4	(Lelandais-Briere *et al.,* 2009)
miR1509	22	0.16	Predicted TF	(Zhai *et al.,* 2011)
miR2118	22	0.05	NBS-LRRs	(Zhai *et al.,* 2011)
miR2609	21	0.27	Disease resistant protein	(Lelandais-Briere *et al.,* 2009)
tasiARFs	21	0.02	ARF2/3/4	(Zhai *et al.,* 2011)

Figure legends

Figure 1. FLAG-tagged RPL18 is incorporated into polysomal complexes of *M. truncatula* roots.

(a) Protein extracts from 35S:FLAG (EV) and 35S:FLAG-RPL18 (F-L18) roots were analyzed by immunoblot with an anti-FLAG antibody (α-FLAG). The band corresponding to the expected mobility of FLAG-RPL18 is indicated with an arrow.

(b) Number of nodules per root formed in EV or 35S:FLAG-RPL18 (F-L18) composite plants at 15 days post-inoculation (dpi) with *S. meliloti*. Data represent mean ± SE (n>30) of three biological replicates.

(c) Ribosomes were pelleted by ultracentrifugation of whole extracts (S-16) of 35S:FLAG-RPL18 roots through a 1.7 M sucrose cushion to obtain the post-ribosomal supernatant (S-170) and the ribosome pellet (P-170). The P-170 was fractionated by ultracentrifugation through a 20% to 60% (w/v) sucrose density gradient and the absorbance at 254 nm was recorded. Positions of monosomes (80S) and large polysomes (LP, ≥5 ribosomes) are indicated. Fractions corresponding to LP were pooled. Aliquots of each fraction were analyzed by immunoblot with α-FLAG or α- RPS6. Molecular mass markers in kDa are indicated on the left.

Figure 2. Immunopurification of ribosomes from *M. truncatula* roots.

(a) Total, unbound (UB) and immunopurified (IP) fractions prepared from roots transformed with the EV or 35S:FLAG-RPL18 (F-L18) were analyzed by immunoblot using the α-FLAG antibody. Lane samples correspond to approximately 1% (Total and UB) and 10% (IP) of the total volume. Migration of the molecular mass marker is indicated on the left.

(b) Proteins co-immunopurified with FLAG-RPL18 (IP FLAG) and ribosomal proteins isolated by conventional ultracentrifugation procedures (P-170) were separated by 15% (w/v) SDS-PAGE and visualized by silver staining. An equal volume of IP samples from EV or 35S:FLAG-L18 (F-L18) roots were loaded in lanes 1 and 2. Asterisks on the right indicate bands that are present only in the P-170 fraction. Migration of the molecular mass marker is indicated on the left.

(c) Total and IP RNA from of 35S:FLAG-RPL18 (F-L18) or EV composite plants were resolved in an agarose. Migration of 25S and 18S rRNAs are indicated on the left.

(d) Total and IP RNAs from 35S:FLAG-RPL18 (F-L18) or EV roots were subjected to semiquantitative RT-PCR for amplification of *ACT11* mRNA. First lane is a control of amplification without DNA (w/o DNA). The molecular mass marker is indicated on the left.

Figure 3. Polysome profile is not altered by inoculation with *S. meliloti.*

(a) Ribosomes from roots 48 hpi with *S. meliloti* or water (mock) were fractioned on sucrose density gradients. The absorbance profile was recorded at 254 nm. The positions of the ribosomal subunits (40S, 60S), monosomes (80S), small (SP, 2 to 5 ribosomes per mRNA) and large polysomes (LP, more than 5 ribosome per mRNA) are indicated.

(b) Percentage of RNA in non-polysomal complexes (NP), SP and LP from mock or *S. meliloti* inoculated roots quantified by integration of each area divided by the total (NP + SP+ LP) area of each profile. Data are mean ± SD of two independent biological replicates.

(c) Percentage of *ACT11* and *HIS3L* mRNAs in the polysomal fraction (P= SP + LP, ≥2 ribosomes per mRNA) in *S. meliloti* and mock-inoculated roots. Abundance of mRNAs was quantified by qRT-PCR in the NP and P fractions and normalized to account for differences in

the RNA yield. Values are expressed as percentage in the P fraction divided by NP + P fractions. Data are mean ± SD of three technical replicates and are representative of two biological experiments.

Figure 4. Selective recruitment of mRNAs to polysomes in response to *S. meliloti* inoculation. Levels of selected mRNAs in total and IP samples from *M. truncatula* roots 48 hpi with *S. meliloti* (grey bars) or mock-inoculated (black bars) were analyzed by qRT-PCR. Transcripts were classified into three categories based on the ratio of fold change in IP RNA relative to the fold change in total RNA, which is indicated in parentheses following the name of each transcript: (a) translational up-regulated, (b) non-regulated and (c) down-regulated. Data are mean ± SD from three technical replicates normalized to *HIS3L* mRNA and expressed as relative to mock-inoculated samples. Asterisks indicate that values of *S. meliloti* samples are significantly different from mock samples in an unpaired two-tailed t-test with $0.01<P<0.05$ (*) and $0.001<P<0.01$ (**). Results are representative of three independent biological replicates. (d) Scatter-plot comparing the ratio of fold induction in IP RNA relative to the fold induction in total RNA upon *S. meliloti* inoculation in two independent biological replicates. The correlation coefficient obtained by linear regression analysis is indicated.

Figure 5. Selective recruitment of mRNAs to polysomes conventionally purified (CP) by sucrose-density gradient fractionation**.** Levels of selected mRNAs in CP polysomal fractions (≥2 ribosomes per mRNA) from roots 48 hpi with *S. meliloti* (grey bars) or mock-inoculated (black bars) were analyzed by qRT-PCR. Transcripts were classified as (a) up-regulated, (b) non-regulated and (c) down-regulated based on the fold change in the CP fraction. Data are mean ±

SD from three technical replicates normalized to *HIS3L* mRNA and expressed as relative to the mock-inoculated sample. Asterisks indicate that values in *S. meliloti* samples are significantly different from mock samples in an unpaired two-tailed t-test with 0.01<P<0.05 (*), 0.001<P<0.01 (**) and P<0.001 (***).

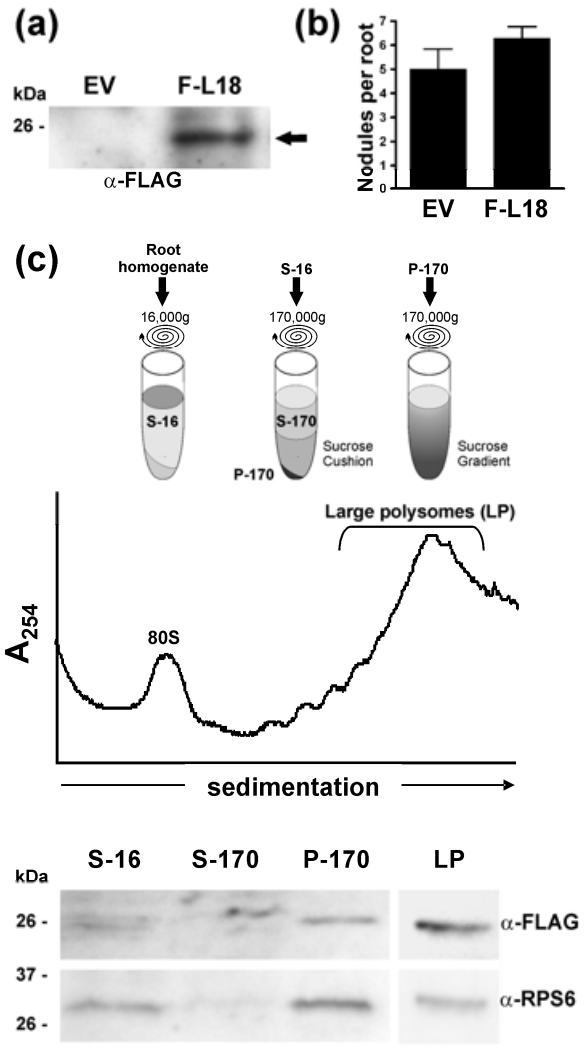
Figure 6. Recruitment of sRNA to polysomes in mock and inoculated roots.

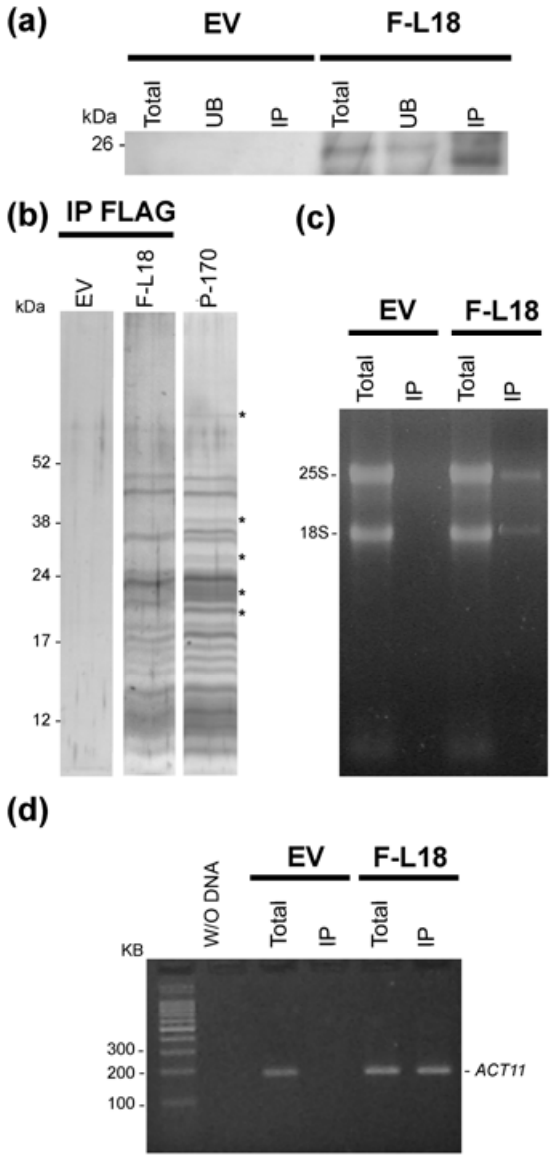
(a) sRNA levels in total and IP samples from roots 48 hpi with *S. meliloti* (grey bars) or mock-inoculated (black bars) were quantitatively analyzed by qRT-PCR.

(b) mRNA levels of tasiARFs target transcripts *ARF2*, *ARF3 a*nd *ARF4*, were analyzed by qRT-PCR.

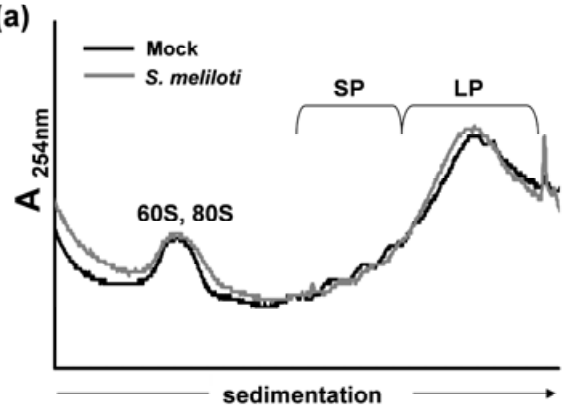
In (a) and (b) data are mean ± SD from three technical replicates normalized to *HIS3L* mRNA and expressed as relative to the non-inoculated sample. Asterisks indicate that values in *S. meliloti* samples are significantly different from mock samples in an unpaired two-tailed t-test with 0.01<P<0.05 (*), 0.001<P<0.01 (**) and P<0.001 (***). Results are representative of three independent biological replicates.

(c) Proteins extracted from hairy roots expressing 35S:FLAG-RPL18 (F-L18) 48 hpi with *S. meliloti* (S.me.) or mock-inoculated were analyzed by immunoblot using the α-HAP2.1 antibody (upper panel) or by Ponceau staining (lower panel).The arrow indicates the HAP2.1 protein and the asterisk indicates a non-specific band used as loading control.





(a)



(b)

	NP	SP	LP
Mock	21.7 ± 0.9	20.8 ± 0.1	57.5 ± 0.8
S. meliloti	21.9 ± 0.4	20.4 ± 0.1	57.7 ± 1.0

(c)