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Insights into post-transcriptional regulation during legume-rhizobia symbiosis

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During the past ten years, changes in the transcriptome have been assessed at different stages of the legume-rhizobia association by the use of DNA microarrays and, more recently, by RNA sequencing technologies. These studies allowed the identification of hundred or thousand of genes whose steady-state mRNA levels increase or decrease upon bacterial infection or in nodules as compared with uninfected roots.[1-7] However, transcriptome based-approaches do not distinguish between mRNAs that are being actively translated, stored as messenger ribonucleoproteins (mRNPs) or targeted for degradation. Despite that the increase in steady-state levels of an mRNA does not necessarily correlate with an increase in abundance or activity of the encoded protein, this information has been commonly used to select genes that are candidates to play a role during nodule organogenesis or bacterial infection. Such criterion does not take into account the post-transcriptional mechanisms that contribute to the regulation of gene expression. One of such mechanisms, which has significant impact on gene expression, is the selective recruitment of mRNAs to the translational machinery. Here, we review the post-transcriptional mechanisms that contribute to the regulation of gene expression in the context of the ecological and agronomical important symbiotic interaction established between roots of legumes and the nitrogen fixing bacteria collectively known as rhizobia.[8] In addition, we discuss how the development of new technologies that allow the assessment of these regulatory layers would help to understand the genetic network governing legume rhizobia symbiosis.

Post-Transcriptional Regulation of Gene Expression in Plants

Plants respond to environmental constrains by regulating gene expression, a process that can occurs at transcriptional, post-transcriptional or a combination of both levels. In eukaryotes, transcription of mRNAs is performed by the DNA dependant RNA polymerase II, followed by a maturation process that includes 5' capping, intron removal and 3' cleavage and polyadenilation (**Fig. 1**). During this processing, mRNAs associate with nuclear localized RNA binding proteins to form messenger ribonucleoprotein complexes (mRNPs) that are transported through the nuclear pore to the cytoplasm, where mRNAs are subjected to a quality control check -also known as mRNA surveillance- that distinguish and target aberrant mRNAs for degradation.[9] Cytoplasmic mRNAs can be recruited by the translational machinery, stored as mRNPs or sequestered into the so called processing bodies (P-bodies), i.e., cytoplasmic foci involved in mRNA turnover that contain decapping enzymes, decapping promoting enzymes, RNA helicases and exonucleases. The activity of P-bodies has been associated to general and non-sense mediated mRNA decay and microRNA (miRNAs) mediated mRNA silencing.[10] However, not all eukaryotic mRNAs that are recruited to P-bodies are subjected to degradation. In yeast and mammals, it has been reported that some mRNAs can exit P-bodies and re-initiate translation.[11,12] In plants, several decapping enzymes and exonucleases were found to localize into cytoplasmic mRNP foci,[13-17] but it is not known whether plant mRNAs sequestered in these foci can return to translation. Afterward, translation might be regulated at each of the three phases: initiation, elongation or termination. The initiation step is a highly selective process and the most frequently controlled on cases of translation regulation.[18] Initiation of translation requires the formation of the 43S pre-initiation complex, which binds to the 5' cap and scans the mRNA in the 5' to 3' direction until it encounters an initiation codon (usually AUG) with the appropriate sequence context, where the 60S subunit is joined to form an elongation-competent 80S ribosome. The sequential recruitment of ribosomes to the mRNA results in the formation of polysomes (mRNA bound with two or more ribosomes).

Gene expression is also regulated post-transcriptionally by miRNAs, endogenous small RNAs, which can act by mRNA cleavage, inhibition of mRNA translation or accelerated mRNA decay.[19] miRNAs are synthesized as long RNA precursor, which are further processed to produce a mature miRNA duplex (miRNA/miRNA*). In the cytoplasm, a single strand of the mature miRNAs is incorporated into the RNA induced silencing complex (RISC), whose main component is a member of the ARGONAUTE (AGO) protein family (**Fig. 1**).[20] Although RNA cleavage was considered the predominant mechanisms of action of plant miRNA, AGO1 as well as a number of miRNAs were found associated with polysomes in plants, leading to the suggestion

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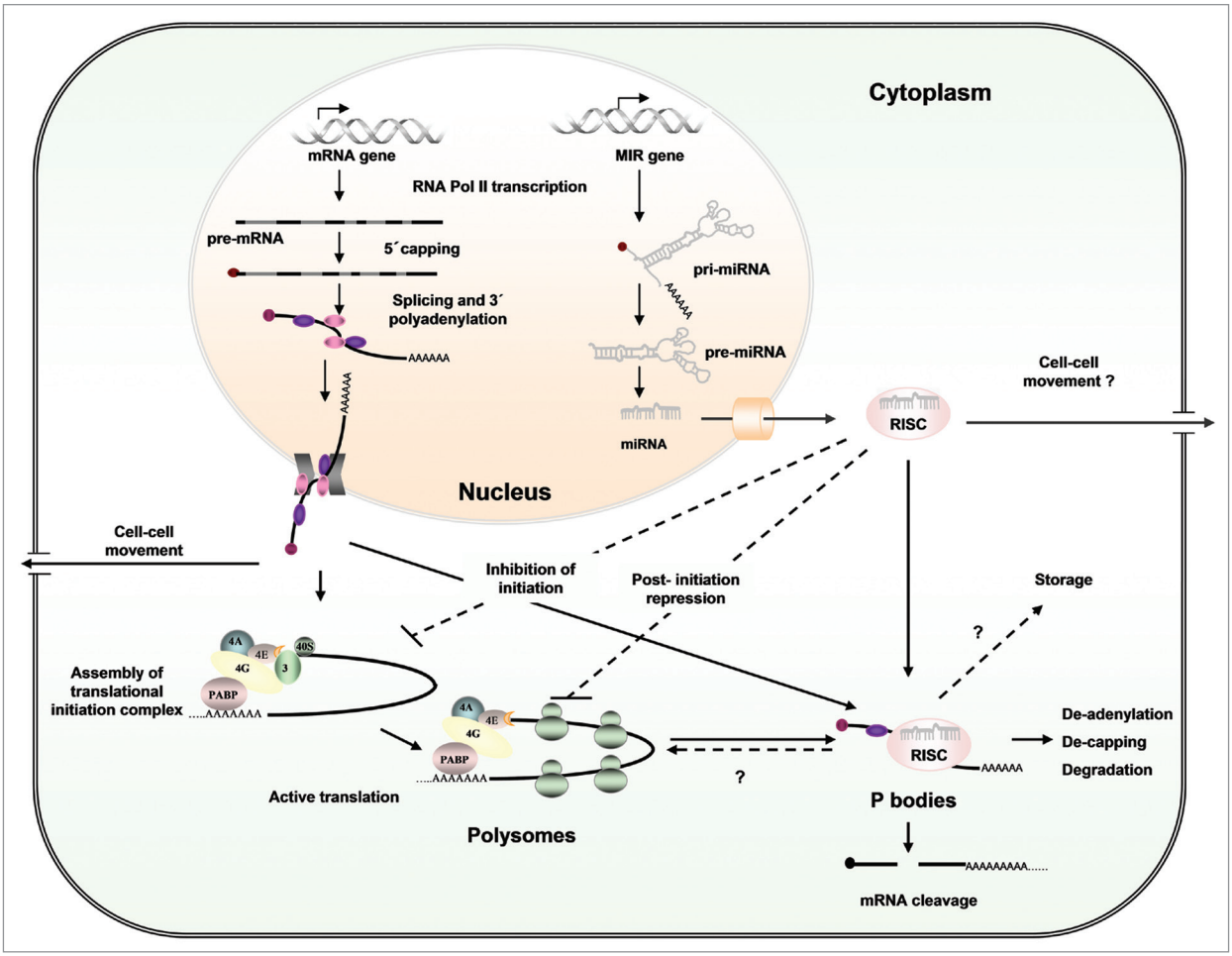

Figure 1. Overview of post-transcriptional regulatory events in plants. Plant mRNAs are transcribed in the nucleus by the DNA dependant RNA polymerase II (RNA Pol II) as primary mRNAs (pre-mRNAs) that undergo a series of processing events that include the addition of a m7GpppN to the 5'end (5' capping), removal of introns (RNA splicing), 3' cleavage and polyadenylation and the association with several nuclear RNA binding proteins before export to the cytoplasm through the nuclear pore. Cytoplasmic mRNAs associate to the 43S translation pre-inititaion complex, which is composed of the 40S ribosomal subunit, several eukaryotic initiation factors (eIF4E, eIF4G, eIF3 and eiF4A) and the polyA binding protein (PABP). Subsequently, mRNA undergo active translation in polysomes (mRNAs bound to several ribosomes). Interaction between eIF4G and PABP promotes circularization of the mRNP complex, which facilitate ribosome recycling and translational re-initiation. On the other hand, some mRNAs can move to adjacent cells trough plasmodesmata, be targeted to P-bodies for decapping, deadenylation and degradation, or stored in translational repressed mRNPs complexes. MIR genes are transcribed by RNA Pol II to produce long 5' capped and polyadenylated primary miRNAs (pri-miRNAs) that form imperfect fold back-structures. The pri-miRNAs are first processed into stem-loop precursors called pre-miRNAs, and then into short mature RNA duplexes (miRNAs/miRNAs*). miRNAs are exported to the cytoplasm and the single-strand of mature miRNAs is incorporated into the RNA induced silencing complex (RISC), which could act by RNA cleavage or inhibition of translation at initiation or post-initiations stages.

that miRNA can also act at the level of translational inhibition in plants.[21] Moreover, the identification of *mad* (*microRNA action deficient*) class III mutants in *Arabidopsis thaliana* provided additional evidences supporting that plant miRNAs can act inhibiting mRNA translation.[22] More recently, the GW repeat-containing protein SUO was identified in *A. thaliana* as required for miRNA-mediated translational repression and has shown to colocalize with the P-body component DCP1.[23] Altogether, these evidences strongly support the hypothesis that the mode of action of plant miRNA entails a combination of mRNA cleavage and translational repression. Whether miRNAs inhibit translation at initiation or post-initiation steps (elongation, ribosome drop-off or termination) is still controversial in metazoan, as well as in plants.[24-26]

Thus, expression of protein-coding genes involves several layers of regulation that can act during mRNA synthesis and processing, export to the cytoplasm, partitioning of mRNAs between different cytoplasmic mRNPs involved in mRNA translation, storage or degradation, as well as during protein synthesis or post-translational modifications. These regulatory mechanisms provide the flexibility and versatility required to orchestrate the adaption of plant cells to changing environmental conditions. However, some aspects of gene expression have received less attention than others due to technical limitation or

the high cost and time required to evaluate the contribution of each level of regulation.

Affinity Purification of mRNPs Complexes to Characterize the Dynamic Partitioning of mRNAs in the Cytoplasm

Cytoplasmic mRNPs can be captured by affinity purification of associated proteins that participate in translation, storage or degradation of mRNAs. A seminal affinity purification-based approach for large scale analysis of mRNPs complexes, called ribonomics,[27] was reported 12 y ago by Tenenbaum et al.[28] In that study, the authors described the direct isolation and identification of subsets of mRNAs contained in endogenous mRNP complexes of P19 carcinoma cells by differential co-immunopurification of RNA binding proteins (e.g., the HuB protein, the poly(A)-binding protein or the eukaryotic initiator factor 4E). Later on, a similar approach combined with the DNA microarray technology was applied in *C. elegans* to identify muscle,[29] ciliated sensory neurons expressed genes[30] and more recently, for single cell analysis of taste sensory neuron cell pairs.[31] In addition, an efficient method for affinity purification of polysomes was developed in the model plant *A thaliana*,[32] and later on in yeast[33] and mammals.[34,35] This approach, referred as translating ribosome affinity purification (TRAP), consists on the expression of a ribosomal protein fused to an epitope (e.g., HA, FLAG, c-myc, HIS_6, etc), which is incorporated into ribosomes and polysomes, providing a mean to capture these RNP complexes by affinity purification with either agarose or magnetic beads. In *A. thaliana*, transgenic plants expressing a FLAG tagged version of the RPL18 (ribosomal protein large subunit 18) under the control of constitutive or developmentally regulated promoters were generated and used to characterize changes in the translatome (i.e., the mRNA population associated with polysomes) in root and shoot cells after exposure to a short period of oxygen deprivation,[36,37] during flower development,[38] light-dark transitions[39] and photomorphogenesis.[40] These studies exposed the importance of translational regulation during development or adaptation of plants to changing environmental conditions.

Translational Regulation of the Root Nodule Symbiotic Pathway

In a recent report, the TRAP technology was applied in the model legume *Medicago truncatula* to characterize the translational level of regulation in the context of the relevant and fascinating biological process of root nodule symbiosis.[8] The symbiotic interaction between legumes and bacteria from the genus *Rhizobia* is responsible for most of the nitrogen incorporated biologically into ecosystems. Most legumes allocate bacteria within a new organ developed in their root systems, the nodule, where nitrogen fixation takes place. Root nodule symbiosis requires the activation of two highly coordinated morphogenetic programs: the re-activation of cell divisions in the root cortex to form a nodule primordium and the bacterial infection (for a review see refs[41,42]). Penetration of bacteria occurs predominantly by a sophisticated intracellular mechanism that requires the attachment of the bacteria to actively growing root hairs and the formation of a tubular structure called the infection thread (IT) that guides bacteria from the epidermis to the nodule primordia. Once the IT reaches the developing nodule, bacteria are released into organelle-like structures called symbiosomes, where they differentiate to nitrogen fixing bacteroids.

The nodulation signaling pathway has been characterized by a combination of forward genetic and cell biology approaches (for a detailed review see refs[42]). The pathway is initiated by the recognition of the Nod factor -a lipochitoligosacharide produced by rhizobia- by LysM receptor-like kinases (RLKs) present in the plant plasma membrane (NFP and LYK3 in *M. truncatula*),[43,44] activating a signaling pathway that includes an RLK with extracellular leucine-rich repeats (DMI2[45]), a nucleus localized ion channel (DMI1[46]) and nuclear pore proteins.[47-49] These components of the pathway are required to trigger oscillations in the cytoplasmic and nuclear concentration of calcium, which are presumably decoded by a nucleus-localized calcium/calmodulin- dependant protein kinase (CCaMK, referred as DMI3 in *M. truncatula*).[50] This kinase interacts with a coiled-coil domain containing protein of unknown function (IPD3).[51] Decoding of calcium oscillations is required for the activation of several transcriptional regulators, including particular members of the GRAS,[52] ERF[53] and NF-Y[54] families of transcription factors, which are directly or indirectly involved in the transcriptional induction of early nodulation genes (ENODs). In addition, CRE1, a cytokinin receptor present in cortical cells, is required for nodule organogenesis.[55]

Translational regulation of genes involved in this cascade was analyzed at the time of IT formation and activation of cortical cell division in the model legume *M. truncatula*[8] and the results are summarized in **Figure 2**. This analysis revealed that genes encoding the RLKs NFP, DMI2 and CRE1 were significantly upregulated at translational level in response to rhizobia, despite that slight or no changes were observed at the steady-state level of mRNA. Another group that showed significant upregulation at translational level includes genes that encode GRAS and NF-Y transcription factors. It is noteworthy the strong translational co-regulation exhibited by *NSP1* and *NSP2,* whose products form an heterodimer that binds and activates the expression of the *ENOD11* promoter, and by *NF-YA* and *NF-YC*, which encode components of the same transcriptional complex. Promoters bound by the NF-Y complex are still unknown in legumes; however, it has been shown that they activate directly or indirectly the expression of genes involved in the G2/M transition of the cell cycle.[56] Other genes, such as *LYK3*, *DMI3, IPD3* and *LIN* (which encodes an ubiquitin E3 ligase) did not show any evidence of upregulation either at transcriptional or translational level. In spite of this, analysis of mutants has demonstrated that these genes play crucial roles during nodule formation and/or bacterial infection. A most likely scenario is that the functional products encoded by *LYK3*, *DMI3, IPD3* and *LIN* are regulated at post-translational level. Consistent with this hypothesis, a recent report has shown that autophosphorylation of the LYK3 receptor is required for its functionality in nodule formation.[57] Interestingly, there was a group of transcripts, such as *ENOD40* and those encoding the ERN1 and NIN TFs, which showed similar increases in total RNA abundance and in their association with polysomes in response to

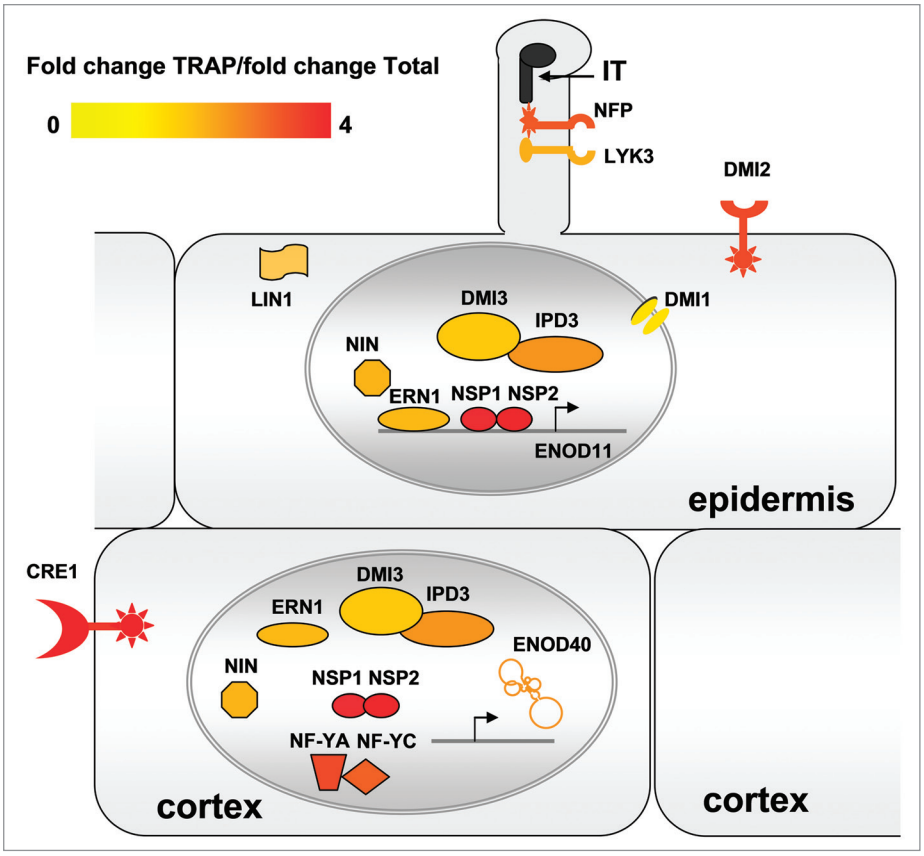

Figure 2. Translational regulation of genes involved in the root nodule symbiotic pathway. Transcripts of 15 genes involved in epidermal and cortical cells responses to rhizobia were classified based on the ratio of the fold-change in the TRAP RNA sample relative to the fold change in total RNA sample, which is indicated in a color scale defined from 0 (yellow) to 4 (red). Transcripts that exhibited ratios of fold change in TRAP/ fold change in total > 2 were considered upregulated at translational level and are presented in dark orange and red. This category includes transcripts encoding three RLKs (NFP, DMI2 and CRE1) and four TFs (NSP1, NSP2, NF-YA and NF-YC). Transcripts showing ratios between 0.5 and 2 were considered as non-regulated at translational level and are presented in dark yellow or light orange. This category is represented by six transcripts encoding proteins with diverse functions (the RLK LYK3, the CCaMK DMI3 and its interacting protein IPD3, the ubiquitin E3 ligase LIN, and the TFs ERN1 and NIN) and by the highly structured RNA *ENOD40*. The third category includes only one transcript (encoding the ion channel DMI1), which exhibited a ratio < 0.5 and, therefore, it is considered as downregulated at translational level and presented in yellow. The arrow points to a growing infection thread (IT) represented in black in a curled root hair of a trichoblast.

bacterial infection. This group of genes constitutes an example of homo-directional transcriptional and translational upregulation during nodulation. Remarkably, *DMI1* was the only transcript among the analyzed genes that showed hetero-directional regulation upon bacterial infection (e.g., increased at the level of total RNA but decreased in their association to polysomes). This transcript might represent a category of mRNAs that upon rhizobial infection increase their rate of synthesis or stability, but once in the cytoplasm are stored in mRNPs complexes. Whether *DMI1* mRNA can associate more efficiently to polysomes at other stages of the symbiotic interaction remains to be elucidated.

The use of TRAP also allowed measuring the association of miRNAs to polysomes in the context of the symbiotic association.[8] All nine analyzed miRNAs were associated, although to different extent, with polysomes of *M. truncatula* roots; and three of them (miR166, miR396 and miR169) changed their levels of association in response to rhizobia. Remarkably, both miR166 and miR169 have been involved in nodule development in *M. truncatula*.[58,59] The percentage of association of miRNAs to polysomes might be influenced by the abundance and the degree of association to polysomes of their target mRNAs, as well as by the specific interaction with RNA binding proteins, but could also reflect the relative importance or contribution of the translational repression mode of action of each miRNA. The fact that miRNAs were found associated to actively translating polysomes might be indicative of a post-initiation repression mechanism of translation inhibition such as reduced elongation or ribosome drop-off. However, an inhibition of translational initiation or re-initiation might not be excluded, particularly for those miRNAs target transcripts that increase their levels of association to polysomes in response to rhizobial infection such as *NSP2* and *NF-YA/HAP2-1*. Further analysis of the distribution of miRNAs and their targets in polysomal fractions of different sedimentation coefficient, as well as the use of reporter genes fused to the recognition sites of individual miRNAs, would certainly help to address this controversy.

NF-YA/HAP2-1: A Gene with Multiple Tiers of Regulation

Genes of the *NF-Y/HAP* family encode the A, B and C subunits of the NF-Y heterotrimeric transcriptional complexes, which recognize with high sequence specificity the CCAAT box present in about 25–30% of eukaryotic promoters. Several genes encoding subunits of this transcriptional complex has been shown to play central roles in development. In plants, NF-Y subunits have been implicated in flowering time control, seed germination, ABA response as well as adaptation to drought stress and the unfolded protein response (for a recent review see ref[54]). The *M. truncatula NF-YA/HAP2-1* gene was initially identified in a large-scale transcriptome analysis as upregulated at steady-state mRNA abundance during nodule development. *NF-YA/HAP2-1* transcript levels significantly increased in nodules of 4 d and in roots at 3 and 6 d post-inoculation (dpi) with *Sinorhizobium meliloti* as compared with uninoculated roots.[60] Later on, Combier et al. demonstrated that

this gene was required for proper nodule development, probably controlling nodule meristem functions.[59] In situ hybridization of nodule sections revealed that *NF-YA/HAP2-1* mRNAs accumulated in the nodule meristematic zone. In addition, the analysis of *NF-YA/HAP2-1* mRNA sequence revealed that the 3' UTR region of this transcript contains two recognition sites for the miRNA miR169. Moreover, overexpression of miR169 resulted in a nodule phenotype similar to that observed by RNA interference of *NF-YA/HAP2-1*. Promoter-GUS fusion experiments have shown that expression of miR169 is limited to the infection zone of the nodule, particularly in the tissue adjacent to cells that accumulate *NF-YA/HAP2-1* mRNAs. These observations led to the proposal that in young nodules (at 10 dpi) expression of miR169 restricts accumulation of *NF-YA/HAP2-1* transcripts to the nodule meristematic zone by a mechanism of RNA cleavage. Experiments using the technique of rapid amplification of 5' complementary cDNA ends (5' RACE) evidenced that the cleavage of *NF-YA/HAP2-1* mRNA occurs predominantly at the first miR169 recognition site.[59] However, it must be noted that this technique is merely qualitative and is not indicative of the extent of the RNA cleavage. In our recent report, we have shown that *NF-YA/HAP2-1* was positively regulated at the translational level in response to *S. meliloti* at early stages of the interaction (2 dpi), and this was correlated with increased levels of the HAP2-1 protein in inoculated roots.[8] Concomitantly, we have observed that the association of miR169 with polysomes decreased upon infection with *S. meliloti*, leading to the suggestion that miR169 might contribute to the translational derepression of *NF-YA/HAP2-1* at early stages of the interaction. In a different report, Combier and colleagues revealed that expression of *NF-YA/HAP2-1* was also subject to trans-regulation by a small peptide encoded by an upstream open reading frame (uORF), which is produced by alternative splicing.[61] The alternative spliced (AS) form of *NF-YA/HAP2-1* mRNA, which accumulates at higher levels in non-inoculated roots, retains a long intron (865 bp) in the 5' leader sequence that contains three uORFs. This AS form of *NF-YA/HAP2-1* becomes predominant at later stages of nodule development (14–21 dpi), particularly in the infection zone. Accumulation of this AS form results in the expression of the peptide encoded by the first uORF (named uORF1p), which binds to the *NF-YA/HAP2-1* transcript and targets it for degradation, restringing expression of *NF-YA/HAP2-1* to the meristematic zone of the nodule.

All these evidence revealed that *NF-YA/HAP2-1* is regulated at multiple levels during root nodule symbiosis and led us to propose a regulatory mode of action for miR169 and uORF1p on the expression of *NF-YA/HAP2-1*, which is consistent with that previously proposed by Combier et al.[61] In uninoculated roots, a fraction of miR169 is associated with the *NF-YA/HAP2-1* mRNA present in the polysomal fraction of *M. truncatula* roots, limiting the rate of translation. At early time points after inoculation (2 dpi), when IT formation begins and cortical cell division is activated, miR169 partially dissociate from polysomes, allowing re-initiation of translation of *NF-YA/HAP2-1* mRNA, which is evidenced by the higher association of *NF-YA/HAP2-1* mRNA levels with polysomes and the increased levels of NF-YA/HAP2-1 protein observed upon inoculation. At subsequent stages of the interaction, when the nodule zone begins to differentiate, miR169 operates in the infection zone by an RNA cleavage mechanism that restricts accumulation of *NF-YA/HAP2-1* mRNA to the meristematic zone and leads to nodule growth. At later stages of nodule development (14–21 dpi), alternative splicing of *NF-YA/HAP2-1* mRNA results in the expression of the uORF1p in the infection zone, where its binds to *NF-YA/HAP2-1* mRNA transcripts and promotes its degradation, contributing to the persistence of the meristem in older nodules.

Perspectives

Forward genetic approaches have been very successful in identifying genes that are crucial for nodule development or bacterial infections, and allowed the dissection of the root nodule symbiotic pathway in model legumes. However, it is evident that other players need to be discovered to fulfill the pathway and genetic redundancy has been an obstacle toward this aim. On the other hand, selection of candidate genes for reverse genetic based on transcriptome analysis might exclude genes with important functions during nodule development that do not significantly change their mRNA abundance in the whole root in response to rhizobia (e.g., NSP1, DMI2, LYK3, DMI3). Some of these genes have been shown to be regulated at levels other than steady-state mRNA abundance. The previous section presented a nice example of the significant contribution of multiple tiers of post-transcriptional regulation of gene expression during a developmental process that also integrates signals derived from the microsymbiont and the environment (e.g., low nitrogen availability). It is clear that these tiers of regulation and its biological significance cannot be acceded simply by the analysis of whole organs transcriptomes. Another future challenge will be to access the regulation of gene expression in specific tissues or cell types of legume roots and nodules. A systematic analysis of gene expression at each level and in specific tissues needs the development (or adaptation) of new technologies that allow isolation of different mRNP complexes contained within root specific cell types (for a review on these technologies see ref[62]) and the subsequent large-scale analysis by RNA sequencing. These technologies will certainly help to better understand the mechanisms and significance of alternative splicing, selective mRNA translation, miRNA mediated translational repression, RNA cleavage, decapping and deadenylation during root nodule symbiosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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