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Computational investigation of small RNAs in the establishment of root nodules and arbuscular mycorrhiza in leguminous plants

Danfeng Jin[1,2], Xianwen Meng[1], Yue Wang[1], Jingjing Wang[1], Yuhua Zhao[1*] & Ming Chen[1*]

[1]*College of Life Sciences, Zhejiang University, Hangzhou 310058, China;*
[2]*Institute of Microbiology, Jiangxi Academy of Sciences, Nanchang 330096, China*

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Many small RNAs have been confirmed to play important roles in the development of root nodules and arbuscular mycorrhiza. In this study, we carried out the identification of certain small RNAs in leguminous plants (*Medicago truncatula*, soybean, peanut and common bean), such as miRNAs, tRFs and srRNAs, as well as the computational investigation of their regulations. Thirty miRNAs were predicted to be involved in establishing root nodules and mycorrhiza, and 12 of them were novel in common bean and peanut. The generation of tRFs in *M. truncatula* was not associated with tRNA gene frequencies and codon usage. Six tRFs exhibited different expressions in mycorrhiza and root nodules. Moreover, srRNA$^{5.8S}$ in *M. truncatula* was generated from the regions with relatively low conservation at the rRNA 3′ terminal. The protein-protein interactions between the proteins encoded by the target genes of miRNAs, tRFs and srRNAs were computed. The regulation of these three types of sRNAs in the symbiosis between leguminous plants and microorganisms is not a single regulation of certain signaling or metabolic pathways but a global regulation for the plants to own growth or specific events in symbiosis.

small RNA, arbuscular mycorrhiza, root nodule, leguminous plant, symbiosis

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INTRODUCTION

Many species of plants have the ability to form mutualistic symbiosis with a range of soil microorganisms. Based on these symbiotic associations, plants can obtain nutrients that are essential for their growth from microorganisms, and they simultaneously provide their partners with a carbon source (Oldroyd, 2013). There are two best known symbiotic interactions: one occurs between legumes and rhizobia (Gyaneshwar et al., 2011), and the other is between most land plants and arbuscular mycorrhizal (AM) fungi (Rajtor and Piotrowska-Seget, 2016). Leguminous plants, including peas, beans, alfalfa, peanuts, clovers and so on, are able to develop symbiotic relationships with both rhizobia and AM fungi to form root nodules and AM (Bazin et al., 2012).

Root nodules and AM stimulate the growth of both plants and microorganisms under the condition of nutritional deficiencies. Under nitrogen starvation, the plants exude flavonoids to encourage the growth of the bacterial population around the rhizosphere (Oldroyd and Downie, 2008). The bacteria receive the signals and produce nodulation factors (Nod factors) to activate the infection thread (Kant et al., 2016). The bacteria infect plant root cells through the infection thread and are released into membrane-bound compartments inside the cells (Deakin and Broughton, 2009; Maunoury et al., 2010). Finally, the parenchyma cells in the endodermis are stimulated by the secretions of rhizobia and produce large numbers of cortical cells, which enlarge the tissues and eventually form nodules. When forming AM,

*Corresponding authors (Ming Chen, email: mchen@zju.edu.cn; Yuhua Zhao, email: yhzhao225@zju.edu.cn)

strigolactones that are released by plants signal AM fungi around the root hair to stimulate spore germination, mycelia branching and appressoria formation (Besserer et al., 2006; Kosuta et al., 2003). The AM fungi produce mycorrhizal factors (Myc factors), leading to calcium oscillation, which also takes place in nodule formation (Gao et al., 2010; Kosuta et al., 2008; Maillet et al., 2011). The fungal hyphae enter the root epidermal cells by hyphopodia and get further into the inner cells to form arbuscules.

There are many aromatic compounds (e.g., flavonoids and strigolactones), saccharides (Nod factor, Myc factor and others) and proteins (e.g., some signal receptors and kinases) contributing to the plant-microbe symbiosis. The formation and development of root nodules and AM include a number of complex processes, so there are many regulators involved in these processes. MicroRNA (miRNA) is one of the numerous regulators. It regulates the gene expression level by recognizing the transcripts with perfect or nearly perfect base complementarity and eventually leads to target degradation or translational repression (Jin et al., 2013). It is reported that miRNAs, such as miR164, miR166 and miR169, influence nodule formation and development by regulating the expression of transcriptional activators, class III HD-zip transcription factors and CCAAT-binding transcription factors, respectively (Bazin et al., 2012; Mao et al., 2013). miR399 was suggested to play a role in the regulation of cellular responses during AM development for phosphate increases (Branscheid et al., 2010), and miR171h could regulate the Nod factor signaling and Myc factor signaling by binding the corresponding transcripts of GRAS family transcription factors (Branscheid et al., 2011).

Although numerous studies have been carried out on the function of miRNAs in the symbiosis between plants and soil microorganisms, no studies have shown the roles of other small RNAs (sRNAs) in symbiosis, such as tRFs (tRNA fragments) and srRNA (small rDNA-derived RNA). tRFs are a class of small RNAs derived from tRNAs (Garcia-Silva et al., 2012; Lee et al., 2009). They were reported as precisely generated functional sRNAs, evolutionarily conserved in all domains of life and associated with Argonaute (AGO) proteins (Kumar et al., 2014). The contribution of tRFs in the RNAi pathway during stress conditions such as drought (Hackenberg et al., 2015) and pathogen infection (Visser et al., 2014) was also reported in plants. srRNAs, originating from rRNAs, are reported to be produced as diRNAs (DSB-induced small RNAs). Wei et al. (Wei et al., 2013) found that srRNAs could combine with the AGO protein complex and were involved in metabolism regulation and other biological processes.

To systematically investigate the regulation of sRNAs during the establishment of root nodules and AM, we analyzed nine small RNA high-throughput sequencing datasets obtained from Gene Expression Omnibus (GEO) at NCBI. The characteristics of sequences and origins of tRFs and srRNAs in *Medicago truncatula* were investigated. Differentially expressed miRNAs and their targets in symbiosis were also detected. Our results provided a global view of these sRNAs and their potential roles in plant-microbe symbiosis.

RESULTS

High-throughput sequencing data with Illumina technology was collected to profile some sRNAs in root nodules of four legumes (*M. truncatula*, soybeans, common beans and peanuts) and that in AM of *M. truncatula*. After removing the adapters and filtering low-quality sequences, more than 92% of all reads were of the appropriate size (18–34 nt). Among these sRNAs, the 24-nt sRNAs were found to be the most abundant, followed by the 21-nt sRNAs (Figure 1). The GC content of the sRNAs in the length of 21 nt to 24 nt, as the main parts of sRNAs, was lower than others. The composition of 5′ terminal nucleotides in 20-nt to 25-nt sRNAs was also calculated. Most sRNAs in the length of 20-22 nt began with U, while the 23-nt to 25-nt sRNAs beginning with A had the highest proportion.

miRNA expression and regulation in root nodules and AM

In a recent release of miRBase (version 21), a total of 756, 639, 32 and 10 mature miRNAs were annotated in *M. truncatula*, soybean, peanut and common bean, respectively. For *M. truncatula*, 603, 626, 562 and 569 known miRNAs were detected in AM, nonmycorrhizal root (the control of AM), root nodules and uninoculated root (the control of root nodules), respectively. Thirty miRNAs were detected to be differentially expressed in *M. truncatula* AM or root nodules, while 18 of them were expressed differentially in soybean. Twelve homologous miRNAs were newly discovered miRNAs based on the sequences and annotated information of corresponding miRNAs in *M. truncatula* and soybean (Figure S1 in Supporting Information). The results showed that sequences of these miRNAs in four legumes were conservative. Thus, the function of newly discovered miRNAs could be predicted according to that of known homologous miRNAs in *M. truncatula* and other plants because miRNAs target the transcripts via base complementarity.

The expression levels of 30 miRNAs in each sample are shown in the heat map (Figure 2A and Table S1 in Supporting Information), and the interactions between miRNAs and their targets that were predicted are shown in Figure 2B and Table S2 in Supporting Information. The expressions of the miR169 family and miR171 family members, miR164,

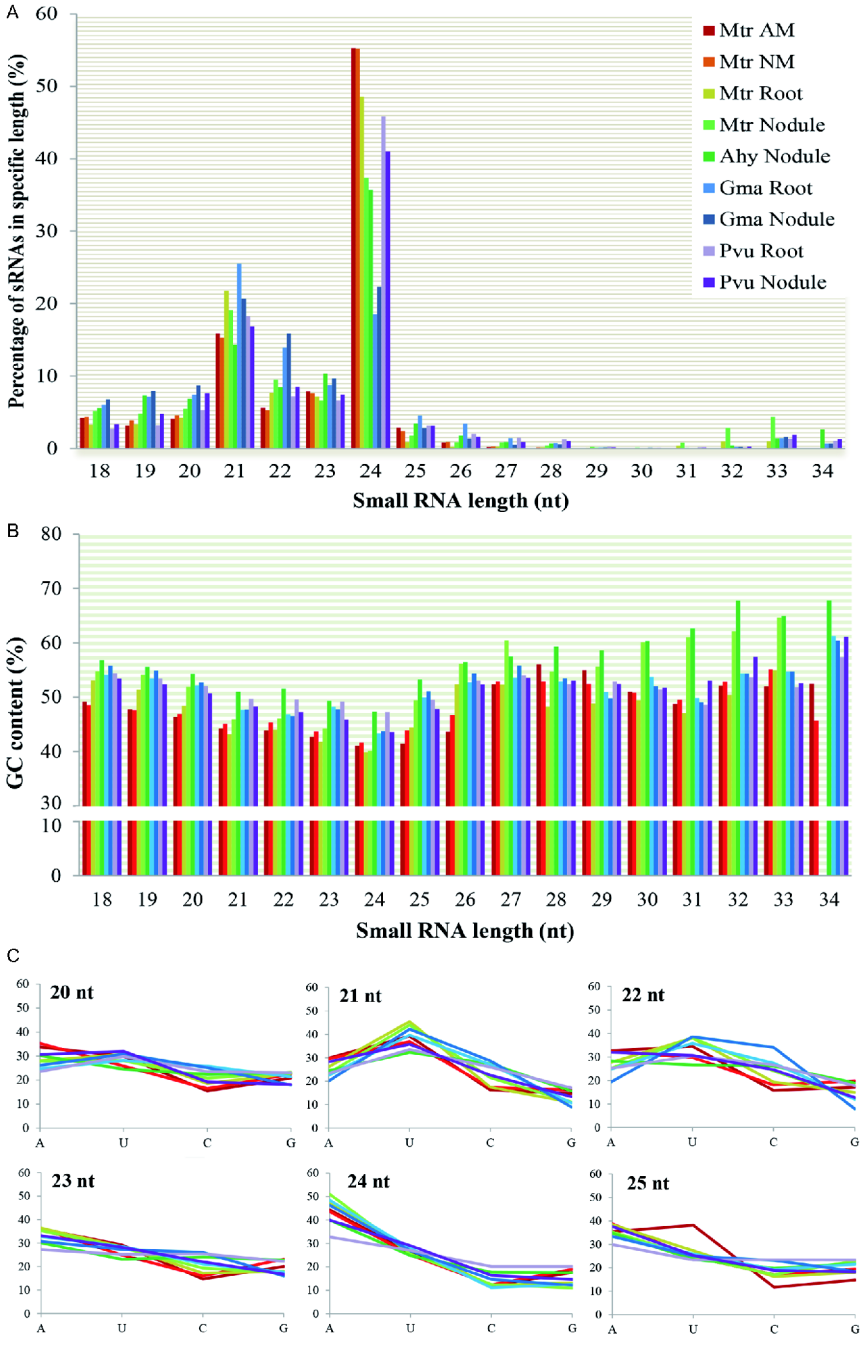

Figure 1 Sequence characteristics of small RNAs in leguminous plants. A, The length of small RNAs in each dataset. B, The GC contents of small RNAs in different lengths. C, The composition of 5′ terminal nucleotides of 20−25-nt sRNAs.

miR2119 and miR397, were down-regulated in root nodules compared with their controls, while miR160a/b, miR167a and miR482 had higher expression levels in root nodules. A special miRNA, i.e., miR399, was found to be expressed higher in soybean nodules but lower in common bean nodules than their controls. Moreover, its expression levels in root nodules and the control of *M. truncatula* were about the same. miR5204, miR5206, miR5559, miR5749, miR5751, miR5753, miR5755 and miR5758 were only found in *M. truncatula*. Three of them (miR5204, miR5206 and miR5559) were prominently down-regulated in root nodules, while the expressions of others were up-regulated. The comparison details are listed in Table S3 in Supporting Information.

miR160c, miR160f, miR169d, miR5204, miR5206 and miR5229 were found to be highly abundant in AM. In the miRNAs of interest, only miR164 and miR167a had the same expression trend in root nodules and AM. Five miRNAs had different expressional patterns in root nodules and AM, and there were also seven miRNAs that were only

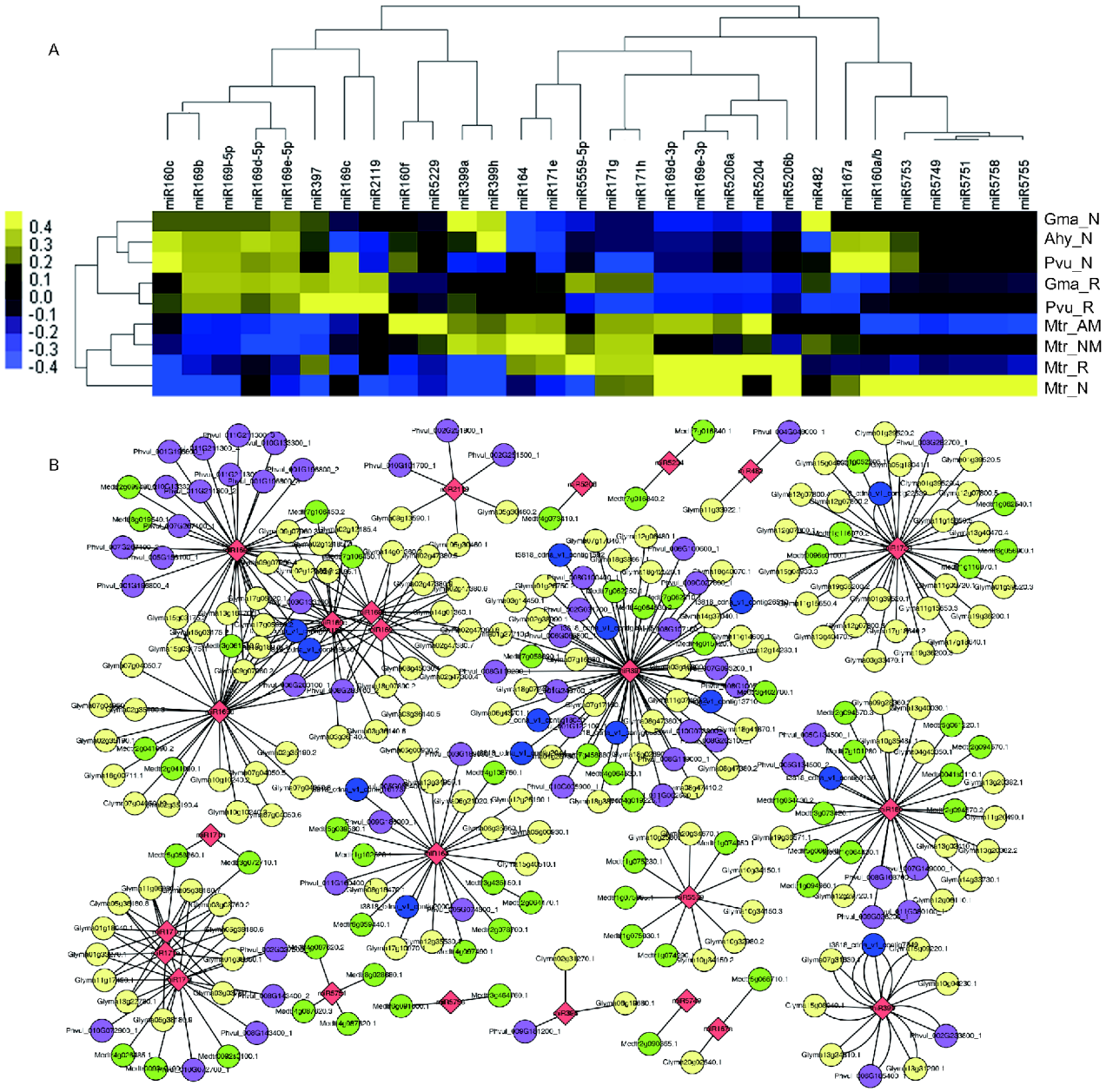

Figure 2 Clustering of differential expression miRNAs in symbiotic cells and regulation networks between miRNAs and their target genes in four leguminous plants. A, All miRNA expression data are normalized; yellow means high expression level and blue means low. The names for these samples are as follows: Ahy_N is root nodule of peanut; Gma_N is root nodule of soybean; Pvu_N is root nodule of common bean; Gma_R is uninoculated root of soybean (control of root nodule); Pvu_R is uninoculated root of common bean; Mtr_AM is arbuscular mycorrhiza of *M. truncatula*; Mtr_NM is non-mycorrhizal root of *M. truncatula*; Mtr_R is uninoculated root of *M. truncatula*; Mtr_N is root nodule of *M. truncatula*. B, miRNA-target regulatory networks for differential expression of miRNAs. Green, yellow, purple and blue cycles represent target genes in *M. truncatula*, soybean, common bean and peanut, respectively. Red diamonds represent miRNAs. Each line represents a matching site of miRNA in the corresponding target gene.

differentially expressed in one case.

Characteristics and distribution of tRFs in *M. truncatula*

The tRNA-derived sRNAs discovered from root nodules, AM and their controls accounted for 3.77%, 4.19%, 2.3% and 4.58%, respectively, of all small RNA reads in *M. truncatula*. According to our computational analysis, all tRNAs that carry 20 types of amino acids could be cleaved into tRFs, but the content of tRFs from different tRNAs were variable. As shown in Figure 3A, tRFs from $tRNA^{Ala}$, $tRNA^{Gly}$ and $tRNA^{Phe}$ had the most content, followed by $tRNA^{Arg}$ and $tRNA^{His}$. The expression trends of tRFs in four samples were consistent. For each tRF, its contents in dif-

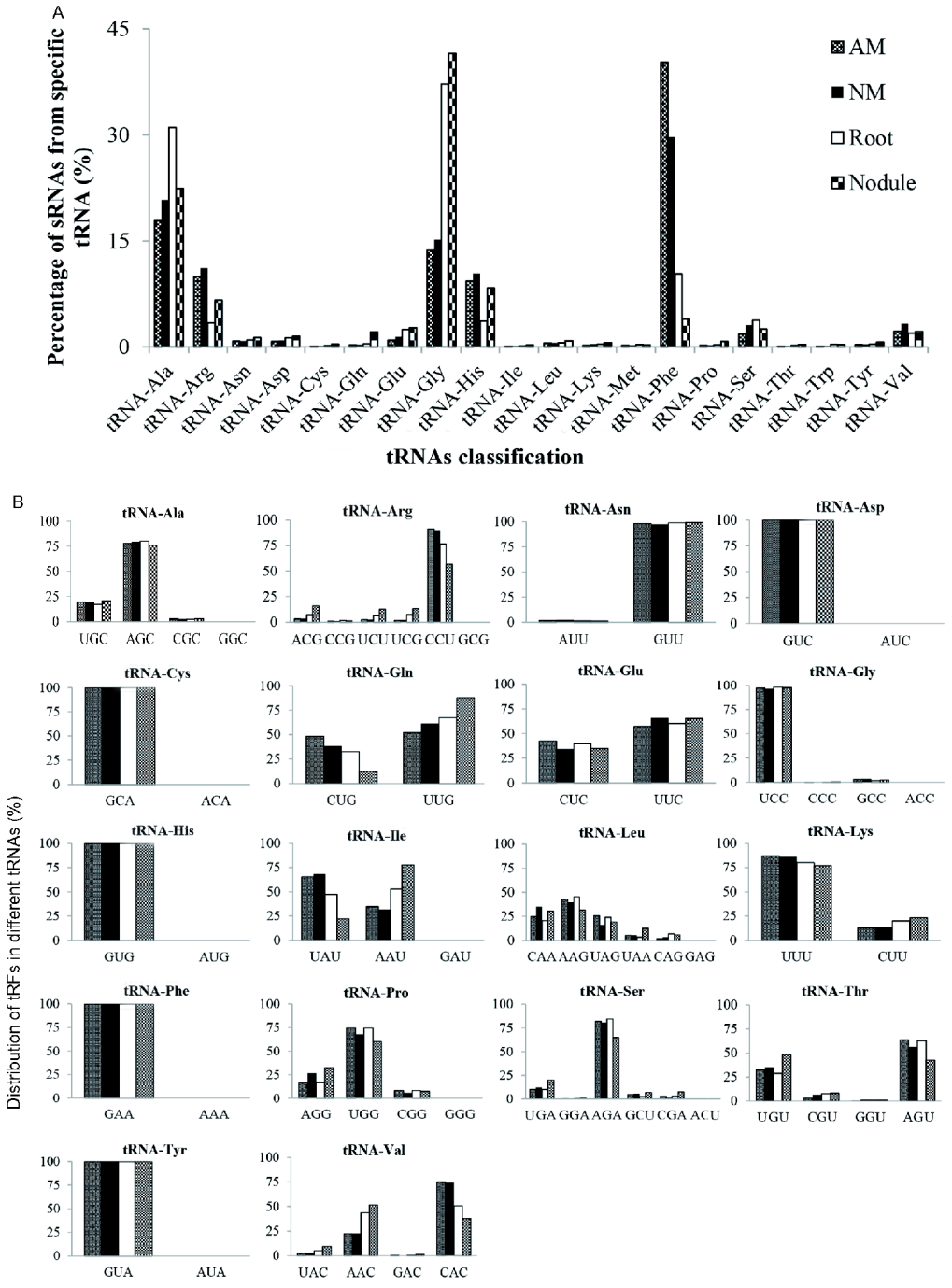

Figure 3 Characteristic and distribution of tRFs in *M. truncatula*. A, The contents of tRFs derived from different tRNAs. B, The distribution of tRFs from tRNAs carrying the same amino acid but with different anticodons. Four samples of *M. truncatula*are as follows: AM, arbuscular mycorrhiza; NM, nonmycorrhizal root; Nodule, root nodule; Root, uninoculated root as the control of root nodule.

ferent samples were extremely different. The content of tRFs from $tRNA^{Phe}$ in AM was much higher than that in root nodules, while the content of tRFs from $tRNA^{Gly}$ was lower. tRNAs carrying the same amino acids have different anticodons. The expression levels of tRFs from these tRNAs were very different in our results (Figure 3B and Table S1 in Supporting Information). Thirteen of 20 tRNAs with multiple anticodons could produce multiple tRFs. For each tRNA, the content of tRFs from one with a certain type of anticodon was much more than others. The remaining seven tRNAs each produced one class of tRF, in which the tRNAs transferring tryptophan and methionine (not shown) only have one anticodon, respectively. Pearson correlation coefficients between tRF concentrations and tRNA gene frequencies, codon usage in four samples were all below 0.3, and some were even close to 0 (Table S4 in Supporting Information).

The Pearson correlation analysis results suggested that there were no significant linear correlations between tRF production and tRNA gene frequencies as well as tRF production and codon usage. Besides, codon usage, tRNA gene frequency and the tRF concentration were found to be relatively independent in our research (Figure 4).

In our study, more than 50% of the tRFs were in the length range between 18 nt and 19 nt in the four samples. The 18-nt tRFs in AM were much more than that in other samples, while the 19-nt tRFs were fewer. This is to say, symbiosis can induce the cleavage of tRNAs in *M. truncatula* (Figure S2A in Supporting Information). Indeed, the sequence logos of 18-nt and 19-nt tRFs were similar to each other, except for the extra nucleotide of 19-nt tRFs (Figure S2B in Supporting Information). Nucleotides in some sites were predicted to be conservative. Guanine had the highest presenting frequency in most positions and followed by uracil. After mapping the tRFs to tRNAs, we found that more than 95% of the tRFs were from the 5′ and 3′ terminals of tRNAs. These tRFs were named as 5′ tRF and 3′ CCA tRF, respectively. The content of 5′ tRFs in root nodules was higher than that in the other three samples, while the content of 3′ CCA tRFs was lower (Figure S2C in Supporting Information). By comparing the sequences, cleavage of tRNAs was found to occur mainly at $G^{18}G^{19}$ in the D loop and about the 53rd conservative base in the TψC loop, leading to the formation of 5′ tRFs and 3′ CCA tRFs, respectively (Figure S2D in Supporting Information).

Prediction and function of tRF targets

Numerous studies have shown that tRFs have the ability to interact with some key elements in miRNA processing systems, such as Dicer and AGO family proteins (Loss-Morais et al., 2013). tRFs can also regulate the responses of organisms to environmental stimuli by inhibiting the translation of their target genes (Fu et al., 2009; Yamasaki et al., 2009). To investigate the biological roles of tRFs in *M. truncatula*, their target genes were predicted, and the GO annotation of these genes was performed. The results revealed that these target genes were involved in biological regulation, immune system processes, responses to stimuli and others (Figure S3 in Supporting Information). The differences in target GO annotations between AM and its control were focused on seven fields, such as nutrient reservoirs, translation regulators and reproduction. The genes enriched in the GO term of electron carrier and enzyme regulator in root nodules were nonexistent in controls.

According to the anticodons, tRNAs could be classified into 61 types, resulting in 61 types of tRFs. There were 52 tRFs found in *M. truncatula* in our study and only $tRF^{Ser(GGA)}$, $tRF^{Leu(UAA)}$, $tRF^{Arg(ACG)}$, $tRF^{Gln(UUG)}$, $tRF^{Thr(GGU)}$ and $tRF^{Pro(CCU)}$ were expressed differentially in AM and root nodules compared to their controls (Figure 5A). $tRF^{Ser(GGA)}$ and $tRF^{Leu(UAA)}$ were up-regulated in AM and root nodules, while $tRF^{Arg(ACG)}$, $tRF^{Thr(GGU)}$ and $tRF^{Pro(CCU)}$ were down-regulated. The expression of $tRF^{Gln(UUG)}$ was lower in AM but higher in root nodules. The interaction network between predicted tRFs and their targets are shown in Figure. 5B. Similar to miRNA, one tRF was predicted to target different genes, and different tRFs also had the same target genes. The targets of specifically expressed $tRF^{Gln(UUG)}$ were predicted to be the transcripts of natural resistance-associated proteins, phosphoglycerate dehydrogenase and epoxide hydrolase.

Characteristic, sources and functions of srRNAs in *M. truncatula*

The rDNA-derived sRNAs from the root nodule, AM and their controls were found to be 1%–2% of all small RNA reads in *M. truncatula*. srRNAs with a length of 18–21 nt had

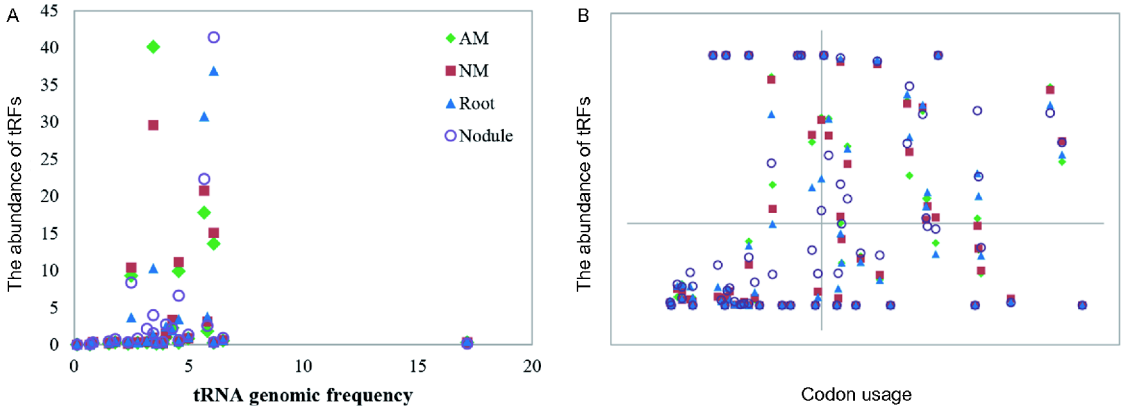

Figure 4 The analysis of linear correlations. A, Linear correlations between tRF concentration and tRNA gene frequency. B, Linear correlations between tRF concentration and codon usage. For scattered points, there are no linear correlations between tRFs concentration and tRNA gene frequency or codon usage, respectively.

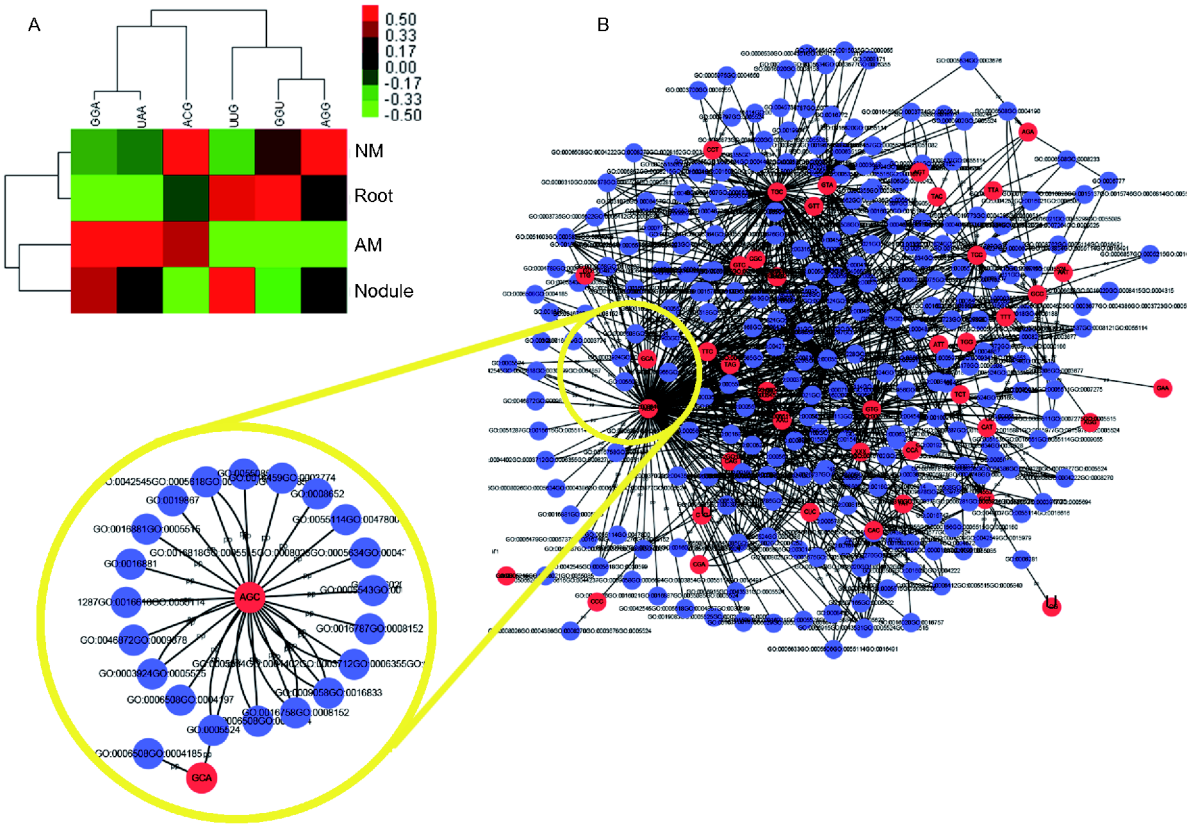

Figure 5 Clustering of differentially expressed tRFs in symbiotic cells and regulation network between tRFs and their target genes. A, Clustering of differentially expressed tRFs in four samples of *M. truncatula*. Red means high expression level and green means low. B, Regulatory relationship between tRFs and their target genes. Red cycles represent tRFs classified according to anticodons. Blue cycles represent the target genes.

higher concentrations in each sample, but there were also differences among the samples. The 19-nt srRNAs in AM and root nodules were fewer than that in the controls, but the content of 20-nt srRNAs in root nodules was higher (Figure S4A in Supporting Information). In these samples, more than 85% of the srRNAs originated from 18S rDNA, and the others were from 5.8S rDNA (<5%) and 25S rDNA (~10%) (Figure S4B in Supporting Information). The srRNAs were found to be produced in specific sites of 18S rDNA, 5.8S rDNA and 25S rDNA after mapping them to rDNAs (Figure S4C in Supporting Information).

Wei et al. (Wei et al., 2013) found that srRNAs are co-immunoprecipitated with AGO proteins in cells of *Arabidopsis*, *Drosophila* and humans. Lee et al. (Lee et al., 2009) also discovered that qiRNAs in the filamentous fungus *Neurospora crassa* could change the sensitivity to DNA damage by inhibiting protein translation, and qiRNAs originated mostly from the ribosomal DNA locus. In other words, srRNA can target mRNA and play regulatory roles similarly to miRNA. Therefore, the targets of srRNAs were predicted and listed in Table S5 in Supporting Information. To study the generation characteristics of srRNAs, we compared the gene sequences and structures of rRNAs in some leguminous plants. Taking 5.8S rRNA for instance, the gene sequences of 5.8S rRNAs in *Trifolium pretense*, *M. truncatula*, *M. sativa*, *Vicia sativa*, *Lathyrus cicera* and *Pisum sativum* were conservative, and srRNAs coming from these regions had relatively low conservation in the 3′ terminal (Figure S5 in Supporting Information). From our results, it was found that some miRNAs, tRFs and srRNAs could target the same genes. For example, the mRNA of ubiquitin ligase is a common target of miR5559, $tRF^{Val(AAC)}$ and $srRNA^{18S}$. Furthermore, there were protein-protein interactions between the proteins encoded by the target genes of miRNAs, tRFs and srRNAs (Figure S6A in Supporting Information). As shown in Figure S6B in Supporting Information, U-box domain ubiquitin ligase (GenBank: AET02051.1) can interact with stress-induced receptor-like kinase (GenBank: AES59896.2) and phragmoplast-associated kinesin-related protein (GenBank: AES71981.2). E3 ubiquitin ligase (GenBank: AES61911.1) can also interact with such proteins as ubiquitin-conjugating enzyme E2 (GenBank: AES82753.1), cysteine-rich receptor-like protein kinase (GenBank: AES89917.1) and the SAUR-like auxin-responsive family protein (GenBank: AES95106.1) (Figure S6C in Supporting Information).

DISCUSSION

The goal of this study was to find out the sRNAs that are related to the formation and development of root nodules and AM. In our results, the 24-nt sRNAs were found to be the most abundant and followed by the 21-nt sRNAs, which was in agreement with previous studies (Xie et al., 2004). Studies have shown that sRNAs with 30%–50% GC content were more effective than those with higher or lower GC content (Mishra et al., 2009). Thus, it can be observed that sRNAs with relatively low GC content in the length of 21–24 nt were the major contributors to regulation of gene expression, and there might be some correlation between GC content and RNA interference activity. It was known that Argonaute (AGO) proteins recruit small RNAs to form the core of RNA interference effector complexes, and different AGOs preferentially recruit small RNAs with different 5′ terminal nucleotides (Mi et al., 2008). The similar length and 5′ terminal nucleotide distribution in different leguminous samples found in this study indicated that sRNAs recruited by AGOs were highly conservative in leguminous plants.

It has been confirmed that nuclear factor-Y subunit genes (NF-Y), also called HAP in plants, are the targets of miR169, which play a key role in symbiotic nodule development and those processes that occur in response to adverse environmental conditions (Combier et al., 2006; Soyano et al., 2013). The down-regulation of miR169 in our results implied high expression of NF-Y and showed positive regulation in nodule development. According to the relevant research, targets of other miRNAs with a down-regulated expression, such as nodulation signaling pathway 1 (NSP1) and NSP2, NAC transcription factor, F-box family protein, laccase/diphenol oxidase family protein, auxin response factor (ARF) and nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins, are also necessary for nodule primordium formation and efficient nodule development (Hirsch and Oldroyd, 2009; Turner et al., 2013; Zhai et al., 2011). NSP2 is known for its participation in the Nod factor signaling pathway leading to root nodules formation, which is also indispensable for strigolactone biosynthesis that stimulates germination and growth of AM fungi (Akiyama et al., 2005; Liu et al., 2011). The reduction of miR171h in root nodules will result in a high content of NSP2 and stimulate nodule formation (Formey et al., 2014), while the increase of miR171h may decrease strigolactone content by down-regulating NSP2, thus controlling fungal colonization to keep balanced growth between plants and fungi. Manthey et al. (Manthey et al., 2004) proved that the target of miR5204 has enhanced expression in AM, but in our study, miR5204 was found to be highly abundant in AM. This was to say that the action of miR5204 to its target may not be a cleavage process and need further study to make clear. miR5229 was specifically detected in AM, but the pathway of miR5229 that regulates the development of AM is unclear, as the mechanism of mycorrhizal development is still indeterminate.

Studies have shown that $tRNA^{Gln}$ is the main source of tRF in *Haloferax volcanii* (Heyer et al., 2012), and most 19-nt tRFs in *Arabidopsis thaliana* were from $tRNA^{Asp}$ and $tRNA^{Gly}$ (Hsieh et al., 2009). In our results, tRFs from $tRNA^{Ala}$, $tRNA^{Gly}$ and $tRNA^{Phe}$ had the most content. These results showed that tRF production between species was very different. The differences of tRF contents in different samples indicated that external environment and some stimulation from abiotic factors could also induce tRNA cleavage into tRFs (Balaji and Smart, 2012; Hopper and Phizicky, 2003). Based on the existing research, the codon usage will be improved with an increase in tRNAs gene frequency, and high-usage codons require high concentrations of tRNAs to carry the corresponding amino acids (Novoa and Ribas, 2012). Assuming the probability of each tRNA generating a tRF is the same, tRNAs with high codon usage and a high gene frequency will produce more tRFs. However, codon usage, tRNA gene frequency and the tRF concentration were found to be relatively independent in our research. These results implied that the generation of tRFs was not random, and tRNAs had a specificity for tRF production, which was unrelated to the anticodon loop in *M. truncatula*. Maybe tRNA cleavage was related to the sequence of D and TψC loops and stems, because cleavage of tRNAs occurred mainly at $G^{18}G^{19}$ in the D loop and about the 53rd conservative base in the TψC loop (Cole et al., 2009).

By prediction, one tRF could target different genes, and different tRFs also had the same target genes. The genes regulated by multiple tRFs probably play important roles in biological processes (Sato and Yoshida, 2010). For example, the F-box protein can participate in the formation of root nodules (Mbengue et al., 2010), whose transcript can be targeted by five different tRFs in our prediction. Many protein encoding genes that play important roles in symbiosis or plant growth, such as the genes of the cell division cycle protein 48 homolog, NBS-LRR type resistance genes and receptor-like protein kinase (Radutoiu et al., 2003; Zhai et al., 2011), were shown to be regulated by different tRFs simultaneously. These results indicated that tRFs may play meaningful regulatory roles in plant-microbe interactions. Of course, it is necessary to further determine the accuracy of the targets.

At present, it was shown that the concentration of srRNAs from 28S rDNA was the highest in human liver and mouse neutrophils (Wei et al., 2013). This was very different from our results. Therefore, we speculated that the distance between species is a key factor causing srRNA differences. Since srRNAs are co-immunoprecipitated with AGO proteins in cells of *Arabidopsis*, *Drosophila* and humans (Wei et al., 2013), the targets of srRNAs were predicted and more or less involved in the process of symbiosis or nitrogen meta-

bolism. For example, srRNA^{18S} was predicted to have five targets, in which protein TAR1 can repress nitrogen metabolism by reducing the activity of laccase (Jiang et al., 2009); Kinesin-like protein is a type of calmodulin-binding protein that takes part in symbiotic processes by calcium oscillations in both AM and root nodules (Shi et al., 2013); the U-box domain-containing protein is an ubiquitin ligase, and its role has also been demonstrated to participate in the LYK3 signaling pathway in root nodule formation (Mbengue et al., 2010). Although our study only scratched the surface of srRNAs, and more experiments are needed to validate our results, it also demonstrated that srRNA was an important regulator in the process of plant development. The interactions among miRNAs, tRFs, srRNAs and their targets indicated that the regulation of three sRNA species in the symbiosis between leguminous plants and microorganisms is not a single regulation of certain signaling or metabolic pathways but a global regulation for the plants to own growth or specific events in symbiosis.

Small RNA regulatory network is a complex and diverse world. Many more approaches are required to understand the regulation of small RNAs in various life processes. Our studies could partially expand the current understanding of regulatory mechanisms in the symbiotic process between leguminous plants and microorganisms and the functions of plant sRNAs, thus inspiring further in-depth studies on plant non-coding RNAs.

MATERIALS AND METHODS

The small RNA sequencing data we used were acquired from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). The small RNAs were from the uninoculated root and the nodules of *M. truncatula*, *Arachis hypogaea*, *Glycine max* and *Phaseolus vulgaris* that were inoculated with *Sinorhizobium meliloti* strain 2011, *Bradyrhizobium japonicum* NC92, *B. japonicum* USDA110, and *Rhizobium leguminosarum* bv. viciae 3841, respectively (GEO accession: GSM769273, GSM769275, GSM769280, GSM769282, GSM769283, GSM769289, GSM769291) (Zhai et al., 2011). The small RNA sequencing data in *M. truncatula* roots colonized and not colonized by *Glomus intraradices* were also obtained as mycorrhizal and non-mycorrhizal samples (GEO accession: GSM643815, GSM643816) (Devers et al., 2011).

Annotated genome information

M. truncatula genomic, cDNA and annotation data for the release mt3.5 were downloaded from the *M. truncatula* project web page (http://mips.helmholtz-muenchen.de/plant/medi3/download/index.jsp) (Young, 2011). rRNA, tRNA and snoRNA sequences of four legumes were collected from the SILVA rRNA database (http://www.arb-silva.de/) (Quast et al., 2013), the Genomic tRNA Database (http://gtrnadb.ucsc.edu/) (Chan and Lowe, 2009), the Plant snoRNA Database (http://bioinf.scri.sari.ac.uk/cgi-bin/plant_snorna/home) (Brown, 2003), the PlantGDB Database (http://www.plantgdb.org) (Dong et al., 2004), and the Rfam database (http://rfam.sanger.ac.uk/) (Gardner et al., 2009). miRNA sequences (mature and precursor) were downloaded from miRBase (version 20) (Kozomara and Griffiths-Jones, 2014). The Gene Ontology (GO) of *M. truncatula* was extracted from agriGO (http://bioinfo.cau.edu.cn/agriGO/).

Computational analysis of small RNA datasets

For each dataset mentioned above, we filtered the low-quality reads and clipped the adaptor sequences and adaptor contaminants to generate unique reads using the FASTX-Toolkit. All reads were aligned to the reference genomes using Bowtie (Langmead et al., 2009) and some reads mapped to multiple positions were removed. The unique reads that were only mapped to one position in annotated genes of miRNA, tRNAs or rRNAs with no mismatches were saved for the following analyses. Given the incompleteness of the peanut and common bean genomes, we used these species' data for comparative analysis referenced to *M. truncatula* and soybean data. The count of each unique read was normalized to reads per million (RPM).

The miRExpress software (Wang et al., 2009) was used to detect the known miRNA expression profiles according to the sequences of mature miRNAs and precursors in miRBase 20. Homologous miRNAs in *A. hypogaea* and *P. vulgaris* were predicted based on known miRNAs in *M. truncatula* and miRNA properties like secondary structure, energy, alignment and conservation. One mismatch was permitted, and the maximal RNA fold energy (ΔG) was −21 kcal mol^{-1} (Artzi et al., 2008). To compare the miRNA expression levels in symbiotic and normal samples, all detected miRNAs were used to calculate fold changes by DEGSeq (Wang et al., 2010). The miRNAs with a $\log_2$|fold change| no less than 2 and a P value no more than 0.01 were considered to be differentially expressed. The online tool psRNATarget (Dai and Zhao, 2011) was employed to predict target genes of the small RNAs and WEGO was used for plotting GO annotation results (Ye et al., 2006). The results of each step were output filtering and reformatting by custom-written PERL scripts.

Calculation of the Pearson correlation coefficients

To analyze the interrelation between tRFs concentrations and

tRNAs gene frequencies and codon usage, the Pearson correlation coefficients, r, were calculated as follows:

where X_i and Y_i are the tRF concentrations and tRNAs gene frequencies (codon usage), respectively, in point i, and and are average values for all tRF concentrations and tRNA gene frequencies (codon usage), respectively.

Computational prediction of protein-protein interactions

Prediction of protein-protein interactions was performed based on the theory of evolutionary conservation of proteins (Gu et al., 2011). If 'protein A' and 'protein B' in *M. truncatula* are orthologous with 'protein A1' and 'protein B1' separately in *A. thaliana* and rice, and the interaction of 'protein A1' and 'protein B1' has been verified experimentally in the two species, 'protein A' and 'protein B' were predicted to interact with each other.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Figure S1 Nucleotide alignment of novel homologous miRNAs detected in common bean (pvu) and/or peanut (ahy). In each alignment, blue represents perfect match, and red represents one mismatch. Dots represent gaps.

Figure S2 General properties of tRFs. (a) The length of tRFs in each sample. (b) The sequence logo of tRFs in the length of 18 nt and 19 nt. The overall height of the stack indicates the sequence conservation at that position, while the height of symbols within the stack indicates the relative frequency of each nucleic acid at that position. (c) The relative position of tRFs in tRNAs. The ordinate represents the frequency of tRFs, while the abscissa represents locations on tRNAs from 5′ to 3′ terminal. (d) Secondary structure of tRNA. The red and blue arrows represent the splicing sites to produce 5′ tRF and 3′ CCA tRF, respectively.

Figure S3 GO annotations of the target genes of tRFs in (a) AM and its control as well as (b) root nodule and its control, respectively.

Figure S4 Characteristics of srRNAs in *M. truncatula*. (a) Lengths of srRNAs in different samples. (b) Contents of srRNAs from 18S, 5.8S and 25S rRNA. (c) A relative density estimation of srRNAs from each rRNA. Using the density of srRNAs from mycorrhizal rRNA with the highest content as 100%, the densities of other srRNAs were calculated according to their contents.

Figure S5 Multiple sequence alignment of 5.8S rRNA genes (partial, begin with 301st bases) in *Trifolium pretense*, *M. truncatula*, *M. sativa*, *Vicia sativa*, *Lathyrus cicera* and *Pisum sativum*. Sequence in red rectangular box was the origin of srRNA.

Figure S6 Protein-protein interactions between the proteins encoded by the target genes of miRNAs, tRFs and srRNAs. Blue, red and green cycles represent proteins encoded by the target genes of miRNA, tRF and srRNA, respectively. (b) and (c) illustrate the details of interactions in the yellow box in part A.

Table S1 The expression levels of miRNAs and tRFs in each sample

Table S2 The targets of miRNAs in *M. truncatula*, *G. max*, *P. vulgaris* and *A. hypogaea*

Table S3 miRNAs expression and their targets in root nodule and AM

Table S4 The Pearson correlation coefficients ‘r’ between the abundance of tRFs and tRNAs gene frequency and codon usage

Table S5 The targets of srRNAs predicted by psRNATarget

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