Article

Chinese Science Bulletin

Gene Engineering

October 2012 Vol.57 No.28-29: 3804–3810

doi: 10.1007/s11434-012-5197-9

MiR1511 co-regulates with miR1511* to cleave the *GmRPL4a* gene in soybean

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Received November 28, 2011; accepted April 1, 2012; published online May 23, 2012

MicroRNA1511 (miR1511) is a small RNA with unknown function identified in several plants by deep sequencing. In this study, we showed that this small RNA is an authentic miRNA by analyzing the structure of the precursor stem-loop containing the newly identified miR1511* sequence. We confirmed this result by Northern blotting analysis. We used 5′RACE to identify one of the target genes (*GmRPL4a*) cleaved by both miR1511 and miR1511*. The site cleaved by miR1511* was located in the first exon of *GmRPL4a*, and the site cleaved by miR1511 was located in the second exon. The expression level of miR1511/1511* was higher in leaves than in roots and stems. In contrast, the lowest level of *GmRPL4a* expression was in the leaves and the highest in the root. These results indicate that an miRNA can co-regulate with an miRNA* to cleave the same target gene in plants, and that the level of *GmRPL4a* mRNA is regulated by miR1511/1511*.

soybean, miR1511, miR1511*, *GmRPL4a*

Citation: Luo Z Q, Jin L G, Qiu L J. MiR1511 co-regulates with miR1511* to cleave the *GmRPL4a* gene in soybean. Chin Sci Bull, 2012, 57: 3804–3810, doi: 10.1007/s11434-012-5197-9

MicroRNAs (miRNAs) are widely found in animals and plants. They are expressed at high levels, and they have the characteristics of phylogenetic conservation and diversity [1–4]. Therefore, miRNAs have become a hotspot for biological research and many studies are now focusing on this topic. miRNAs are a family of single-stranded small RNAs that are 21–25 nucleotides (nt) in length. They can cleave or inhibit translation of a target mRNA via complementarity between the miRNA and its target mRNA [3,5,6]. The number of miRNAs in the miRBase database [7] has increased by approximately 100 times over the last decade, from 218 in release V1.0 (2002) to 21642 in release V18.0 (2011). At present, most studies on miRNAs focus on their identification and validation. A few studies have focused on how miRNAs regulate expression and other biological functions, using several conserved miRNAs in model organisms. For example, miR156 [8–10], miR159 [11], miR172 [12, 13], and miR396 [14,15] regulate the growth and development of leaves and flowers, while miR393 [16,17], miR398 [18,19], miR395 [20], and miR399 [21,22] play important roles in the responses of plants to environmental and nutrition stresses. However, the functions of numerous novel miRNAs have not yet been characterized. Soybean (*Glycine max*) is one of the most important oil, grain, and forage crops, but research on its miRNAs lags behind that on those in other model plants such as rice and *Arabidopsis*. To date, only a few soybean miRNAs have been analyzed in terms of their function [23–32].

miR1511 is a novel small RNA that was first identified in soybean [23], but has since been found in *Phaseolus vulgaris* [33], *Medicago truncatula* [34], and *Vitis vinifera* [35]. So far, there have been no experimental studies on miR1511. In this study, we verified the existence of miR1511 by sequencing and Northern blotting analyses. We found that miR1511 co-regulated with miR1511* to cleave and regulate the same gene, *GmRPL4a.* These results provide a theoretical foundation for the study of miR1511/1511* and their target genes.

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1 Materials and methods

1.1 Plant materials

We used the soybean (*G. max*) cultivar Zhongpin 95-5383, which was bred in our laboratory. Seeds were surface-sterilized in 8% Clorox for 4 min and then in 70% ethanol for 4 min. The seeds were rinsed three times with sterile deionized water [23]. Plants were grown in an illuminated incubator at 25°C, 65% relative humidity under a 16-h light/8-h dark photoperiod with a light intensity of 10000 lx. The leaves, stems, and roots of 20-d-old seedlings were collected, immediately frozen in liquid nitrogen, and stored at –80°C until use.

1.2 Collection of bioinformatics data and analytical tools

The soybean genome sequences were obtained from the Phytozome database (http://www.phytozome.net/). The analysis of the secondary structure of miRNA precursors was performed using the web-based software Mfold (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form). Target genes of miRNA were predicted by psRNATarget (http://plantgrn.noble.org/psRNATarget/) using data from DFCI Soybean Gene Index Release 15.0 (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=soybean) and soybean mRNA sequences obtained from the Phytozome database (ftp://ftp.jgi-psf.org/pub/JGI_data/phytozome/v6.0/Gmax/assembly/) according to Fahlgren's methods [36]. We used the Primer Premier Version 5.00 software package (Premier Biosoft International) to design primers [37] and Multalin (http://multalin.toulouse.inra.fr/multalin/multalin.html) for sequence alignments.

1.3 Small RNA extraction and enrichment

We extracted total RNA from each sample using the RNAiso Plus kit (TaKaRa Biotechnology, Dalian, China) following the manufacturer's instructions and Accerbi's methods [38]. The method for small RNA enrichment was as follows [39]: 400 μL (1–2 mg) total RNA was mixed with 50 μL each of 50% PEG8000 and 5 mol/L sodium chloride. The mixture was incubated on ice for 2 h and then centrifuged at 15000 × *g* for 10 min. A 1/10 volume of 3 mol/L sodium acetate (pH 5.2), two volumes of 95% ethanol, and 1 μL 20 mg/mL glycogen (MBI Fermentas, Vilnius, Lithuania) were added to the supernatant and the mixture incubated at –20°C for 2 h before sedimenting small RNAs at 15000 × *g*. The pellets were washed twice with 75% ethanol, dried briefly, and resuspended in 50% deionized formamide.

1.4 Northern blotting analysis

We conducted Northern blotting analysis as described elsewhere [40]. Briefly, 40 μg enriched small RNAs was separated on a 15% polyacrylamide gel containing 8 mol/L urea, then RNA was blotted onto positively charged nylon membranes (Amersham Life Science, Buckinghamshire, UK) using a semi-dry transfer cell (BioRad Laboratories, Richmond, CA, USA). After transfer, the membrane was crosslinked in an ultraviolet crosslinker (UVP, San Gabriel, CA, USA) in order of obverse, reverse, and obverse sides for 1 min each side. The membrane was baked at 80°C for at least 30 min and then prehybridized at 42°C in ULTRAhyb-Oligo buffer (Ambion, Austin, TX, USA). After 2 h, the oligonucleotide probes 5′ end-labeled with Opti-Kinase (USB Corp, Cleveland, OH, USA) were added and the membrane was left to hybridize overnight (14–24 h). The hybridized membrane was exposed to a storage phosphor screen (GE Healthcare, Milwaukee, WI, USA) and scanned using FX Pro Plus (BioRad Laboratories, Hercules, CA, USA).

1.5 Validation of target genes by 5′RACE

We carried out 5′RACE using the First-Choice RLM-RACE kit (Ambion Corp., Austin, TX, USA). The procedures were modified slightly, as follows [40]: the 5′RACE adapter was directly ligated to total RNA (1 μg) without calf intestinal phosphatase (CIP) and tobacco acid pyrophosphatase (TAP) treatment. We used random decamers for cDNA synthesis with M-MLV reverse transcriptase. Touch-down PCR was performed with the nested primer specific to the predicted miRNA target gene and the nested primer corresponding to the 5′RACE adapter. The amplification conditions were as follows: 94°C for 4 min; 12 cycles of 94°C for 30 s, 64–53°C for 30 s, 72°C for 30 s (–1°C/cycle) and 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s; with final extension at 72°C for 8 min. The two PCR amplifications were the same, but the template for the second PCR was 1/10 of the products of the first PCR. The PCR products from the second PCR amplification were electrophoresed on a 1.2% agarose gel. The expected fragments were isolated using a DNA gel extraction kit (Axygen Biotechnology, Hangzhou, China) and then ligated into the pMD18-T vector (TaKaRa Biotechnology) at 16°C for 1 h. *Escherichia coli* Top10 competent cells (Tiangen Biotech, Beijing, China) were transformed with the ligation products and spread onto LB agar plates containing 50 μg/mL ampicillin. The plates were incubated at 37°C for 12–16 h. Randomly selected colonies were cultured in liquid LB medium with 50 μg/mL ampicillin at 37°C on an oscillator for 6 h. Positive recombinant clones were screened by colony PCR. PCR products containing the expected inserts were sent to GENEWIZ (Beijing, China) for sequencing using M13 forward and reverse primers.

1.6 Real-time PCR analysis of target gene

cDNA was synthesized from 1 μg RNA using the Reverse

Transcription System (Promega Ltd., Southampton, UK) following the manufacturer's instructions. The transcript levels in various tissues were determined using the 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using FastStart Universal SYBR Green Master Mix (Roche Diagnostics GmbH, Mannheim, Germany). Each well contained 10 μL 2 × ROX, 0.5 μL forward primer (10 μmol L^{-1}), 0.5 μL reverse primer (10 μmol L^{-1}), 1 μL 1/10 diluted cDNA, and 8 μL water. The PCR program was as follows: 95°C for 10 min; followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. *ACT11* was used as the reference gene [41].

1.7 Probes and primers

The common probes and primers were synthesized by Sangon Biotechnology Ltd. (Beijing, China). The LNA-modified probes were purchased from Exqion Inc. (Vedbaek, Denmark), and included the miR1511 probe (Nor_1511), 5′-CCATGGTATCAGAGCCTGGTT-3′; the U6 snRNA probe (Nor_U6), 5′-GACCATTTCTCGATTTGTGCGTGTC-3′; and the miR1511* LNA probe, 5′-TG^{+}AAGC^{+}AGGA^{+}CCTG^{+}ATAC^{+}CAC-3′ (LNA_1511S) (the base before "+" was LNA-modified). Probes 5′ end-labeled with [γ-^{32}P] ATP (FuRui Biology Engineer Co. Ltd., Beijing, China) were used as hybridization probes. The adapters and primers for 5′RACE were as follows: 5′RACE adapter, 5′-GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA-3′; 5′RACE outer primer (5′OP), 5′-GCTGATGGCGATGAATGAACACTG-3′; 5′RACE inner primer (5′IP), 5′-GAACACTGCGTTTGCTGGCTTTGATG-3′; gene-specific primers Tg5580R1, 5′-GAATTTGCACTATAAAATCCGACAAAC-3′; Tg5580R2, 5′-CAAAGATATTTAGTTCTGATGGGCATAC-3′ (where R1 is the outer primer and R2 is the inner primer). The primers for real-time PCR were designed from the sequence of the cleavage site of the miRNA target gene: qRTmiR1511S-5580F, 5′-TGTGAAGAAGGCCACGCTGAAG-3′; qRTmiR1511S-5580R, 5′-CAGTCTTCCTCTTCTTGTCGAGC-3′ (primer pair was in the first exon across the cleavage site of miR1511*); and qRTmiR1511-5580F, 5′-GCTTCTGCCATCAAGTCTGCTG-3′; qRTmiR1511-5580R, 5′-ACTGTGAAACACCCAGCCACTTAG-3′ (primer pair was in the second exon across the cleavage site of miR1511). We used the following primer pair for amplification of the reference gene *ACT11*: ACT11F, 5′-ATCTTGACTGAGCGTGGTTATTCC-3′, ACT11R, 5′-GCTGGTCCCTGGCTGTCTCC-3′.

2 Results

2.1 Structure and chromosomal location of pre-miR1511

A total of 12 small RNA sequences located in the same region of chromosome 18 (21161219–21161347 bp, full length of 129 bp) were identified by high throughput sequencing of a small RNA library constructed from the soybean variety Zhongpin95-5383 (unpublished data). The predicted secondary structure of this sequence was a stem-loop structure. Among the 12 small RNAs, four were located on the 5′ arm of the stem-loop, six on the 3′ arm of the stem-loop, and two on the complementary strand. The sequence with the highest number of reads (371) was 21-nt long and was located on the 3′ arm of the stem-loop. This 21-nt sequence, which had only one more nucleotide (guanine) at the 5′ end than gma-miR1511, was located in the same region of the chromosome as gma-miR1511 obtained from miRBase (http://www.mirbase.org/). Therefore, this 21-nt sequence was designated as miR1511. Another 21-nt sequence (41 reads) located on the 5′ arm of the stem-loop was nearly complementary to miR1511, and was designated as miR1511*. The whole 129-bp sequence was located in an intergenic region of chromosome 18, and was designated as the miR1511 precursor pre-miR1511 (Figure 1) [2,42,43]. This sequence was located 10405 bp downstream of *Glyma18g19410.1* and 12789 bp upstream of *Glyma18g19420.1* (Figure 1(b)).

2.2 Target gene cleavage site analysis of miR1511/1511*

A total of 10 putative target genes of miR1511 were predicted, but 5′RACE analysis confirmed the cleavage site of only one gene, *Glyma10g05580*. The results of 5′RACE showed that three specific products were obtained by removing a single primer-binding PCR product (Figure 2(a)). The sequences of two bands were the binding sites of miR1511* and miR1511. Sequence alignment showed that the cleavage site of miR1511* was located in the first exon of *Glyma10g05580* and that of miR1511 was located in the second exon of *Glyma10g05580*, indicating that miR1511 and miR1511* both had a binding site on this target gene for co-cleavage (Figure 2(b)). Furthermore, another specific band may reflect cleavage of *Glyma10g05580* by an unknown miRNA; therefore, *Glyma10g05580* may be the target gene of more than one miRNA. *Glyma10g05580* belongs to the 60S ribosomal protein L4 family and is homologous to *RPL4a* in *Arabidopsis* (henceforth designated as *GmRPL4a*) (Figure 2(c)). There is another copy of *GmRPL4a* in the soybean genome, *Glyma13g19930*, which was designated as *GmRPL4d* because of its similarity to the *Arabidopsis RPL4d* gene. The predicted amino acid sequences of *GmRPL4a* and *GmRPL4d* had greater than 50% similarity to the 60S ribosomal protein L4 in human and mice and greater than 80% similarity to the *Arabidopsis* RPL4a and RPL4d proteins (Figure 2(d)).

2.3 Tissue specific expression analysis of miR1511/miR1511* and their target gene

To study the expression of miR1511/miR1511* in different

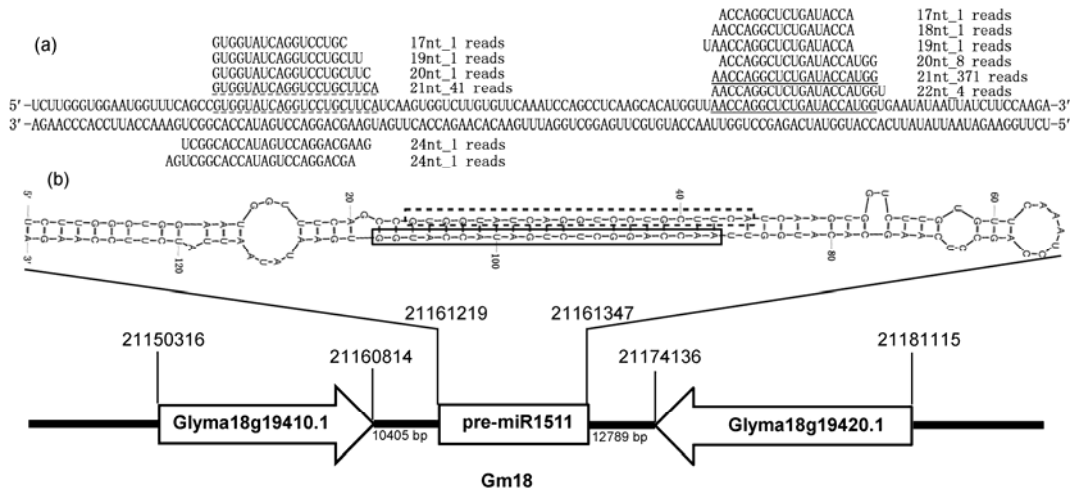

Figure 1 Sequence, stem-loop structure, and chromosome location of miR1511/miR1511*. (a) Length and read number distribution of pre-miRNA; (b) pre-miRNA stem-loop structure and genome location. Solid line indicates miR1511, dashed line indicates miR1511*.

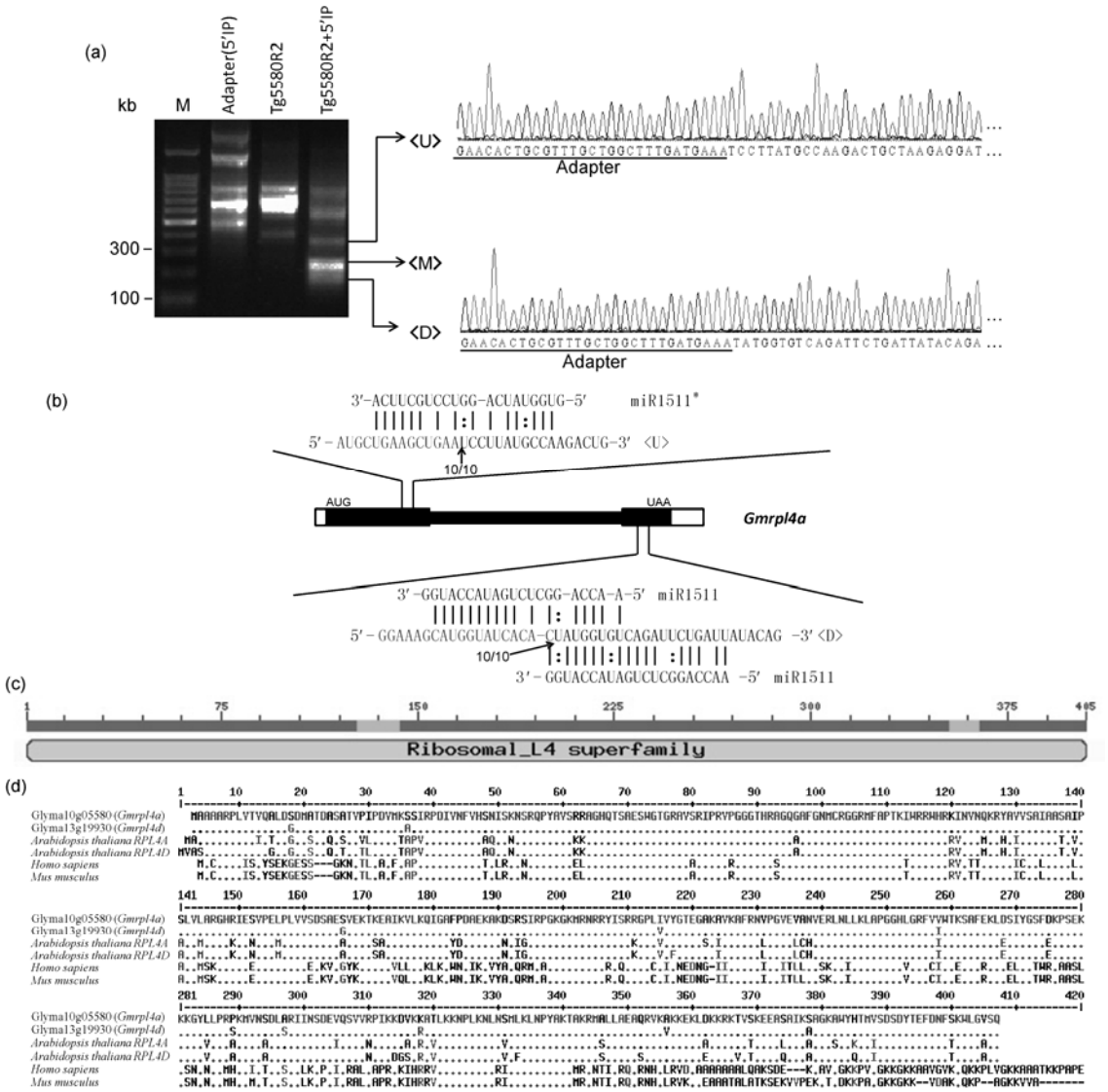

Figure 2 Target gene cleavage site analysis of miR1511/miR1511*. (a) 5′RACE and sequencing analysis; (b) schematic diagram of cleavage site; (c) predicted conserved region; (d) sequence alignment of RPL4 protein from soybean, *Arabidopsis*, human, and mice. M, 100 bp marker; ⟨U⟩, ⟨M⟩ and ⟨D⟩, band number; "." indicates the same amino acid.

tissues, we carried out Northern blotting analyses using RNA obtained from roots, leaves, and stems of Zhongpin95-5383 with miR1511/miR1511* specific probes and U6 snRNA as the internal reference. miR1511 was predominantly expressed in the leaves, with low to moderate expression in the roots and stems. The expression pattern of miR1511* was similar to that of miR1511 with weak signals in the leaves and stems. These results differ from those of a previous study in which miR1511 was expressed at its highest level in stems [28]. This difference might be because of differences in the variety and/or sampling period.

We designed two pairs of primers according to the sequence of the miR1511* and miR1511 cleavage sites in *GmRPL4a* mRNA. These primers were used to analyze the expression pattern of *GmRPL4a* in roots, stems, and leaves by real-time PCR. The relative expression patterns obtained using these two pairs of primers were similar. The highest expression level of *GmRPL4a* was in roots (more than twice that in the leaves) and it was expressed at moderate levels in stems (1.6–1.8 times that in leaves) (Figure 3(b) and (c)). Therefore, the expression pattern of *GmRPL4a* was negatively correlated with that of miR1511/1511*, suggesting that the expression of *GmRPL4a* decreased with increasing expression of miR1511/1511*.

3 Discussion

3.1 miR1511 is an authentic miRNA

Previously, high-throughput sequencing analyses identified miR1511 in soybean [23], *P. vulgaris* [33], *M. truncatula* [34], and grape [35], and its tissue-specific expression had been detected by Northern blotting [28]. However, the complementary strand of miR1511 (miR1511*) had never been identified. Identification of the complementary strand is a requirement for the classification of a small RNA as an miRNA [5,44]. In this study, we identified miR1511* by high-throughput sequencing and Northern blotting. The stem-loop structure analysis of the precursor indicated that miR1511 and miR1511* originate from two arms of the stem-loop structure with a 2-nt overhang at the 3′ end and a 3-bp mismatch in the complementary region. This structure was consistent with the standards for miRNAs [5], proving the authenticity of miR1511.

3.2 *GmRPL4a* was co-cleaved by miR1511 and miR1511*

We verified the cleavage sites in the target gene of miR1511, *GmRPL4a*, by 5′RACE. These results were consistent with soybean miRNA degradome sequencing data reported previously [30], but the cleavage site was slightly different. miR1511*, the complementary strand of miR1511, and its target gene were also identified by 5′RACE, indicating that miR1511/miR1511* could co-regulate the same target gene (Figure 2(b)). *GmRPL4a* and *GmRPL4d*, which show high sequence similarity to each other (90%), belong to the 60S ribosomal protein L4 family and have the same binding site for miR1511/1511* (Figure 2). High-throughput sequencing results showed that miR1511 also cleaved *GmRPL4d* [30], but this may not be accurate because of the short sequence read. The cleavage of *GmRPL4d* was not verified by 5′RACE in this study, which might be the result of the low expression level of *GmRPL4d* in the tissue examined, or some other unknown regulatory mechanism. The expression of *GmRPL4a* was negatively correlated with that of miR1511/1511* in roots, stems, and leaves, indicating that they may be controlled via the same regulatory mechanism. However, the expression level of *GmRPL4a* could not reflect regulation of miR1511 because of the possible influence of *GmRPL4d* and regulation of other miRNAs.

The basic function of ribosomal proteins, which are key components of the ribosomal subunit, is in ribosome assembly and protein synthesis. Many studies have shown that ribosomal proteins have various other ribosome-independent functions in plants and animals, playing roles in cell growth, differentiation, development, apoptosis, and regulation of stress responses [19–26]. Research on the mutants *rpl5a*, *rpl5b*, *rpl24b*, and *rpl28a* indicated that the entire ribosomal large subunit was involved in establishing abaxial-adaxial leaf polarity in *Arabidopsis*. The ribosome might be involved in the processing of one central network during the establishment of leaf polarity [25]. A study on mutants of 11 ribosomal proteins in *Arabidopsis* provided further evidence that the ribosome plays an important role in establishing leaf polarity. The *rpl4d* mutation did not affect cell proliferation but caused strong abaxialization of leaves in

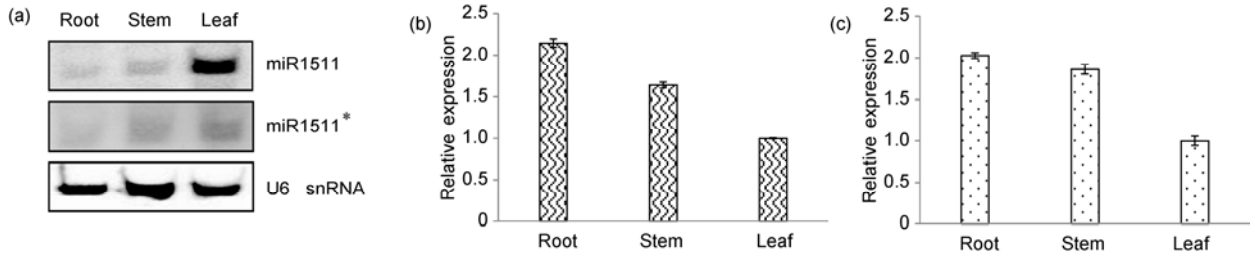

Figure 3 Expression of miR1511/miR1511* and target gene *GmRPL4a* in soybean roots, stems, and leaves. (a) Northern blotting of miR1511/miR1511* in different tissues; (b) and (c) real-time RT-PCR analysis of *GmRPL4a* in different tissues using primers designed from mR1511 cleavage site (b) and primers designed from mR1511* cleavage site (c).

the *as1* and *as2* backgrounds [26].

In this study, the deduced amino acid sequence of *GmRPL4a* showed high similarity (greater than 80%) to RPL4A and RPL4D proteins in *Arabidopsis* (Figure 2(d)). Also, the highest level of miR1511/1511* expression was in the leaves (Figure 3(a)). Therefore, based on the study of *RPL* genes in *Arabidopsis,* miR1511/1511* may play a role in regulating the development of soybean leaves. However, because the 5′RACE results on target cleavage suggested that *GmRPL4a* may be regulated by other miRNA(s) in soybean (Figure 2(a)), the contribution of miR1511/1511* to regulating soybean leaf development requires further study.

3.3 Functions of miRNA*

Generally, it is considered that miRNA*s integrate into the degradation pathway after the formation of mature miRNAs. However, in-depth studies of miRNAs [53] are providing more evidence that miRNA*s have other functions in plants and animals. In animals, miR199a* may play a role in inhibiting proliferation, movement, and invasiveness of cancer cells via negative regulation of mRNA and protein expression of its target gene *MET* (a proto-oncogene) and its downstream gene *ERK2* (extracellular signal-regulated kinase 2) [27]. In addition, miR199a* has a regulatory role in the formation of cartilage [55]. The expressions of miR-9 and miR-9* are significantly down-regulated in the cerebral cortex of patients with Huntington's disease, compared with that of healthy individuals. The target gene of miR-9 is *REST* and that of miR-9* is the *REST* co-repressor gene, *CoREST*, which form a double-negative feedback regulation loop [56].

In *Arabidopsis*, both miRNA* and miRNA could bind to different AGO proteins and form an RNA-induced silencing complex (RISC) because of the different 5′ terminal nucleotide. This indicates that miRNA*s have the essential requirements for functionality [57]. The levels of miR399* increased during low phosphorus stress, and it was transported from shoots to roots, like miR399 [58]. A previous study also revealed that the expression levels of some miRNA*s were the same as, or higher than, those of miRNAs in the apical meristem or mature leaves of soybean. In addition, *in situ* hybridization analysis indicated that miR166a/b and miR166a/b* showed different expression patterns.

Some miRNA*s are more abundant than miRNAs in the mycorrhizal symbiosis in *M. truncatula* [28]. According to data analysis of degradome sequencing, 44 target genes may be cleaved by different miRNA*s. The latest research on miR393* revealed that its overexpression in *Arabidopsis* resulted in a phenotype like that of the *memb12* mutant, the target gene mutant of miR393b*. miR393* and miR393 played the same role in plant resistance to exogenous bacterial invasion, but they combined with different AGO proteins to cleave a variety of target genes [60]. Although miRNA*s can regulate target genes both independently or with a corresponding miRNA, our study is the first report that a single target gene can be cleaved by an miRNA and its complementary strand miRNA*.

This work was supported by the National Natural Science Foundation of China (30871621 and 30490251), the National Major Special Project on New Varieties Cultivation for Transgenic Organisms (2009ZX08009-088B), and the National High Technology Research and Development Program of China (2006AA10110).

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