Published in final edited form as:
Planta. 2012 August ; 236(2): 437–445. doi:10.1007/s00425-012-1622-1.

Genome-wide analysis of microRNAs in rubber tree (*Hevea brasiliensis* L.) using high throughput sequencing

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Abstract

MicroRNAs (miRNAs) are short RNAs with essential roles in gene regulation in various organisms including higher plants. In contrast to the vast information on miRNAs from many economically important plants, almost nothing has been reported on the identification or analysis of miRNAs from rubber tree (*Hevea brasiliensis* L.), the most important natural rubber-producing crop. To identify miRNAs and their target genes in rubber tree, high throughput sequencing combined with a computational approach was performed. Four small RNA libraries were constructed for deep sequencing from mature and young leaves of two rubber tree clones, PB 260 and PB 217, which provide high and low latex yield, respectively. 115 miRNAs belonging to 56 known miRNA families were identified, and northern hybridization validated miRNA expression

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Note added in proof While our manuscript was under revision, a paper describing the identification of rubber tree miRNAs appeared in BMC Plant Biology (2012, 12:18).

Electronic supplementary material The online version of this article (doi:10.1007/s00425-012-1622-1) contains supplementary material, which is available to authorized users.

and revealed developmental stage-dependent and clone-specific expression for some miRNAs. We took advantage of the newly released rubber tree genome assembly and predicted 20 novel miRNAs. Further computational analysis uncovered potential targets of the known and novel miRNAs. Predicted target genes included not only transcription factors but also genes involved in various biological processes including stress responses, primary and secondary metabolism, and signal transduction. In particular, genes with roles in rubber biosynthesis are predicted targets of miRNAs. This study provides a basic catalog of miRNAs and their targets in rubber tree to facilitate future improvement and exploitation of rubber tree.

Keywords

miRNAs; Rubber tree; miR396; miR172; Rubber elongation factor; Hevamine A

Introduction

microRNAs (miRNAs) are a class of short, single-stranded, non-coding RNAs in plants and animals that are derived from primary miRNAs (pri-miRNAs) transcribed from specific *MIR* genes (see Chen 2009, Jones-Rhoades et al. 2006, and Voinnet 2009 for reviews on plant miRNAs). In plants, pri-miRNAs are processed by DICER-LIKE1 (DCL1) to produce 60–300 nucleotide (nt) hairpin RNAs known as pre-miRNAs, which are further processed into miRNA:miRNA* duplexes with 2 nt 3′ overhangs. A mature miRNA is derived from one strand of the duplex and bound by an argonaute protein, guiding the complex to target messages to effect posttranscriptional silencing. In plants, miRNAs guide the precise cleavage of their target RNAs or lead to their translational inhibition. Within the repertoire of miRNA species in any given organism, some miRNAs are conserved across large evolutionary distances while others are restricted to a particular species or lineage. In plants, roughly 20 miRNA families are conserved in angiosperms. Functional analyses of conserved miRNAs revealed their involvement in multiple developmental processes including leaf development and polarity, hormone signaling, meristem boundary formation and organ separation, lateral root formation, and floral organ identity specification (Chen 2009; Zhang et al. 2006). They also regulate plants' responses to environmental stimuli such as biotic and abiotic stresses (Shukla et al. 2008; Sunkar 2010; Sunkar et al. 2007). The evolutionary conservation of some miRNAs across diverse plant lineages enabled a powerful bioinformatics approach that predicts miRNAs from publicly available expressed sequence tag (EST) databases (Zhang et al. 2005). With this method, the number of known plant miRNAs has been dramatically increased in diverse plant species. However, this method is limited by the amount of genome sequence data available, especially for plants in the economically important Euphorbiaceae family.

Rubber tree (*Hevea brasiliensis* L.), belonging to the family Euphorbiaceae, is of major economic importance because its sap-like extract (known as latex) is the primary source of natural rubber. Natural rubber consists mainly of *cis*-polyisoprene, which is the most widely used natural plant polymer. Natural rubber has superior properties compared to synthetic rubber such as resistance to abrasion and impact, elasticity, efficient heat dispersal, and resilience and malleability at low temperature. These properties are lacking in synthetic rubber probably because they result from the unique secondary compounds (lipids, carbohydrates, and minerals) in rubber tree (Mooney 2009).

Identification of miRNAs and their target genes is essential for a full understanding of gene expression in plants during development and stress responses. Direct cloning followed by high throughput sequencing is considered the most effective way for miRNA discovery in plants (Lu et al. 2005), especially in plants without completely sequenced genomes. This

powerful technology enables the discovery of conserved miRNAs as well as species-specific miRNAs. Moreover, the read frequencies can reflect quantities such that the technology can be utilized for expression profiling.

Recently, Zeng et al. (2010) performed a genome-scale systematic study of miRNAs in Euphorbiaceae, by combining computational prediction and experimental analysis. They predicted 85 conserved miRNAs in castor bean (*Ricinus communis*), and experimentally verified and characterized 58 of the 85 miRNAs in at least 1 of 4 Euphorbiaceous plants: castor bean, cassava (*Manihot esculenta* Crantz), jatropha (*Jatropha curcas*), and rubber tree during seedling development.

To obtain a better knowledge of miRNAs and their target genes in rubber tree, we combined high throughput sequencing and bioinformatics analyses to identify miRNAs in rubber tree. In this study, we identify 115 known miRNAs that belong to 56 miRNA families and 20 novel miRNAs that do not match any known miRNAs in miRBase (Griffiths-Jones 2004; Griffiths-Jones et al. 2006, 2008). Targets of the known and novel miRNAs have been predicted, and genes acting in rubber biosynthesis are putative miRNA targets. Some miRNAs exhibit developmental stage-dependent or clone-specific accumulation patterns.

Materials and Methods

RNA extraction

Total RNA was extracted from young and mature leaves of the PB260 and PB217 clones of rubber tree using plant RNA isolation reagent (PRIR) (Invitrogen, USA). In brief, 1 g of tissue was ground in a frozen mortar until it became a fine powder. The frozen powder was transferred into a tube and resuspended in 5 ml of extraction buffer. The mixture was incubated for 5 min at room temperature and centrifuged for 10 min at 4 °C at 12,000*g* to precipitate insoluble material and the clarified supernatant was transferred to a clean tube. The supernatant was mixed with 2 ml of 5 M NaCl and 6 ml of chloroform by vortexing. The mixture was centrifuged for 10 min at 4 °C at 12,000*g* to separate the 2 phases; the top phase was then transferred to a new tube. The RNA was precipitated by adding 0.9 volumes of isopropanol followed by a 10 min incubation at room temperature. The mixture was centrifuged for 30 min at 4 °C at 12,000*g* and the pellet was washed with 5 ml of cold 75 % ethanol. The RNA pellets were dried and dissolved in RNase-free water. The total RNA was further assessed for quantity and quality using Nanodrop ND-1000 (Nanodrop technologies, USA) and denaturing gel electrophoresis and then stored at −80 °C.

Construction of small RNA libraries

500 μg of total RNA was precipitated with ethanol. The pellet was washed with 70 % ethanol, dried, and dissolved in RNase-free water. The quality and quantity of total RNA were subsequently determined by Nanodrop and denaturing agarose gel electrophoresis.

500 μg of RNA was resolved in a 15 % polyacrylamide/8 M urea/0.5x TBE gel. A gel slice corresponding to small RNAs between 18 and 30 nt was excised and the RNA was eluted and dissolved in 20-μl RNase-free water. Small RNAs were then ligated to a 3′ adaptor using T4 RNA ligase and the ligated species were recovered after size fractionation by denaturing polyacrylamide gel electrophoresis. The 5′ adaptor was then ligated and the ligated products were similarly recovered. The ligated small RNAs were reverse-transcribed to cDNA and amplified by PCR. The amplified products were purified by gel electrophoresis and sequenced in the Hiseq2000 (Illumina). In this study, libraries from young and mature leaves of PB217 and PB260 were separately constructed with 5′ adaptors containing different bar codes, mixed and sequenced together in one lane. The unique barcodes allowed the identification of reads from the different sources.

Identification of known and novel miRNAs in rubber tree

The raw sequences were filtered to remove low quality reads and the ones that passed the quality filter were trimmed to remove the adaptor sequences. Next, sequences of 20–24 nt that are represented by at least ten reads in all four libraries combined were selected as the raw small RNA sequences. The raw small RNA sequences were mapped to the miRNA database (http://www.mirbase.org/) (Griffiths-Jones 2004; Griffiths-Jones et al. 2006, 2008; Kozomara and Griffiths-Jones 2011) using the ssearch36 software in FASTA36 (Pearson 1991; Smith and Waterman 1981). Sequences containing not more than 2 nt mismatches were considered as known miRNAs in rubber tree.

The prediction of novel miRNAs was performed with a bioinformatics pipeline as described (Barrera-Figueroa et al. 2011). In brief, the raw small RNA sequences were mapped to the newly assembled rubber tree genome (http://www4a.biotec.or.th/rubber/Search) as well as to publicly available expressed sequence tags (ESTs) from rubber tree. The rubber tree sequences (ESTs or genomic sequences) with small RNA matches were interrogated in sliding windows for the potential to form secondary structures using UNAFold (Markham and Zuker 2008). The structures were further examined for free energy, mismatches between the miRNA and miRNA*, the number of asymmetric bulges in the stem region, dominance of the miRNA relative to other small RNAs in abundance, and precise cleavage as revealed by the presence of miRNA and miRNA* sequences, to satisfy the criteria defined for plant miRNA annotation (Meyers et al. 2008).

Target prediction

The potential targets of rubber tree miRNAs were predicted using the psRNATarget program (http://plantgrn.noble.org/psRNATarget/) (Dai and Zhao 2011) with default parameters. The program uses a scale of 0–5 to indicate the stringency of miRNA-target pairing with the smaller numbers representing higher stringency. A score of 3 or 3.5 was used in our target prediction. Since the EST database from rubber tree is much limited in size, the EST databases for leafy spurge (*Euphorbia esula* DFCI gene index release 1) and cassava (*Manihot esculenta* DFCI gene index release 1) were also used in the target searches.

Northern blot analysis

Total RNA isolation was performed using Plant RNA Isolation Reagent (Invitrogen) according to the manufacturer's instructions. For northern blot analysis, 10 μg of total RNA was resolved by electrophoresis in 15 % polyacrylamide/8 M urea/0.5x TBE gels. The RNA was transferred to Hybond N+ membrane and probed with labeled ^{32}P DNA oligonucleotides complementary to the miRNA sequence. Hybridizations were performed at 50 °C overnight. After hybridization, membranes were washed twice in 2x SSC/0.1 % SDS buffer and analyzed using a PhosphorImager (Amersham). Where indicated, membranes were stripped, re-exposed to a PhosphorImager screen to ensure complete signal removal and reused for a second hybridization. A DNA oligonucleotide complementary to 5S rRNA was used as a probe to detect 5S rRNA as an internal loading control. Sequences of the DNA oligonucleotide probes used for northern blotting are shown in Supplementary Table 6.

miRNA detection by stem–loop RT-PCR

miRNA detection by stem–loop RT-PCR was performed as described (Varkonyi-Gasic et al. 2007). DNase-treated total RNA from mature and young leaves from PB260 and PB217 clones were reverse transcribed to cDNAs using a stem–loop RT primer. The stem–loop RT, forward, and reverse primers were designed according to criteria previously described

(Varkonyi-Gasic et al. 2007) and their sequences are shown in Supplementary Table 6. To detect novel miRNAs, the PCR reactions were conducted with the following schemes: 94 °C for 2 min, followed by 28–35 cycles of 94 °C for 15 s and 60–64 °C for 1 min. The expected size of the PCR product obtained from this method should be ~60 bp.

Results

Construction and sequencing of rubber tree small RNA libraries

In general, two major approaches, computational and experimental, are used separately or in combination to identify miRNAs in plants. For rubber tree, pure computational approaches are limited by the lack of a completely sequenced and assembled genome and the small number of available ESTs such that the identification of all known conserved miRNA families or the prediction of novel miRNAs in rubber tree is not possible. Here we employed experimental cloning followed by high throughput sequencing to interrogate the small RNA repertoire of rubber tree. miRNAs were then identified from the small RNA reads based on their sequence similarity with known miRNAs. De novo miRNA prediction was also applied to the small RNA reads using the newly available, partial rubber tree genomic sequence assemblies (GSS) and the limited rubber tree EST database to identify novel miRNAs.

We constructed small RNA libraries using total RNAs extracted from young and mature leaves from two rubber tree clones, PB260 with a high latex yield and PB217 with a low latex yield. The four libraries were barcoded, mixed and sequenced on an Illumina HiSeq2000. A total of 203,588,835 sequences were recovered from the four libraries. The raw sequences were computationally analyzed to remove low quality sequences, adaptors, and reads shorter than 18 nt after adaptor removal to yield 69,205,862 small RNA sequences. As shown in Fig. 1, approximately 70 % of the small RNAs ranged 20–24 nt in length, with the 24 nt class being the highest in total abundance (21.5 %) followed by the 21 nt (18.6 %) and 18 nt classes (12.8 %). The size distribution of small RNAs from rubber tree mimics that of *Arabidopsis* (Kasschau et al. 2007).

Identification of known miRNAs in rubber tree

To identify miRNAs in rubber tree that are already known in other species, we searched the small RNA sequences of 20–24 nt in length for matches to mature miRNA sequences deposited in miRBase. We obtained a total of 115 potential miRNAs representing 56 miRNA families in rubber tree (Supplemental Table 1). The identified miRNA families are conserved in dicotyledonous plants only, both dicotyledonous and monocotyledonous plants, or in most land plants (Supplemental Table 1). Rubber tree miRNA families showed obvious differences in abundance between each other, as reflected by the read frequencies in the libraries (Supplemental Table 1; Fig. 2). For instance, reads for hbr-miR396, hbr-miR159, and hbr-miR167 were at 48,807, 43,127, and 18,330 transcripts per million (TPM), respectively; whereas the hbr-miR169 family was of relatively low abundance (113 TPM). The read frequencies indicated that miR396 (48,807 TPM) had the highest expression level in rubber tree leaves followed by miR159 (43,127 TPM), miR167 (18,330 TPM), miR166 (7,248 TPM), and miR858 (5,005 TPM) (Fig. 2).

The sensitivity of the high throughput sequencing technology also enabled the detection of miRNA*, the strand that pairs with the miRNA in the DCL1 product. 35 distinct miRNA* sequences representing 23 miRNA* families were found in the four libraries (Supplemental Table 2). The determination of which strand of the miRNA:miRNA* duplex is incorporated into the RNA-induced silencing complex (RISC), is largely based on the identity of the first nucleotide in plants. Plant miRNAs tend to begin with a U, a preferred 5′-nucleotide for AGO1, the major miRNA effector (Baumberger and Baulcombe 2005; Mi et al. 2008). The

miRNA* is usually not incorporated into RISC and is typically degraded and often not detectable in vivo. Most of the detected miRNA*s did not begin with a 5′ U, suggesting that they may not be bound by AGO1. Surprisingly, miR170*/171* (the two have the same sequence and are thus grouped together) was represented by more reads (1,748 TPM) than miR170 and miR171 (316 TPM as the sum of the two) in the four libraries combined. This raises the possibility that both strands of miR170/miR170* and miR171/miR171* have biological functions. Intriguingly, miR170*/171* reads were extremely enriched in young leaves of the rubber tree clone 260 (Supplemental Table 2), suggesting that the retention of star strands can occur in a developmental stage- and clone-specific manner.

Target prediction for known miRNAs in rubber tree

For most plant miRNAs, targets with nearly perfect complementarity can be identified with various algorithms. We employed psRNATarget (Dai and Zhao 2011) to predict the potential targets for conserved miRNAs in rubber tree. We used an expectation score of 3 or 3.5 (in a scale of 0–5 with the smaller numbers representing higher stringency) as the cutoff in the prediction. We first used the EST database of rubber tree (ESTTIK) for target finding because a complete genome sequence with annotated transcripts is not available. Due to the limited size of the EST database, potential targets were only found for 18 and 20 of the 56 miRNAs at the expectation scores of 3 and 3.5, respectively (Supplemental Table 3).

To better understand the targeting potential of the rubber tree miRNAs, we resorted to target prediction using the larger EST databases from two related plants in the same Euphorbiaceae family, *Manihot esculenta* (cassava) and *Euphorbia esula* (leafy spurge) with the assumption that the conserved miRNAs should have conserved targets in these related species. This effort identified potential target genes for 51 of the 56 miRNA families at the expectation score of 3.0 (Supplemental Table 3). When the expectation score was raised to 3.5, potential targets were also identified for the remaining five miRNA families (Supplemental Table 3). Of these predicted targets, a large category consists of transcription factor genes. Genes encoding Squamosa Promoter Binding protein, type III HD-Zip protein, Nuclear Transcription Factor Y subunit A (NFYA), PHAP2B and LIPLESS (AP2-domain containing proteins), a homeodomain protein, a Myb transcription factor, and AUX/IAA proteins were predicted as targets of miR156/157, miR165/166, miR169, miR172, miR396, miR858, and miR1510, respectively (Supplemental Table 3). A second large group consisted of genes related to plant defense and stress responses. For example, genes encoding glutathione-*S*-transferase, superoxide dismutase, and a hypoxia-responsive family protein were predicted targets of miR162, miR398, and miR535, respectively (Supplemental Table 3). Moreover, disease resistance genes are among the predicted targets of miR399, miR1507, and miR1510 (Supplemental Table 3). A third group of miRNA targets consisted of genes involved in signal transduction, especially in hormone signaling pathways. For instance, the auxin receptor *TIR1* was a predicted target of miR393. A gene annotated as “abscisic acid responsive element-binding protein 2” was a potential target of miR482. A gene encoding a mitogen-activated protein kinase kinase kinase was predicted to be a target of miR472. We also found some miRNAs with potential roles in nutrient assimilation: miR395, miR399, and miR1508 could target ATP-sulfurylase, phosphate transporter, and nitrate reductase genes, respectively. Interestingly, rubber elongation factor and Hevamine A, genes involved in rubber biosynthesis, were predicted targets of miR172 and miR160, respectively (Supplemental Table 3; Fig. 3).

We also attempted to predict targets for the detected miRNA* species in rubber tree small RNA libraries. Surprisingly, potential targets could be predicted for all 23 miRNA* families including miR170*/171* (Supplemental Table 4).

Discovery of novel rubber tree miRNAs

In the initial phase of our studies, our ability to predict miRNAs de novo was limited by the lack of a sequenced and assembled rubber tree genome. But we still attempted to predict novel miRNAs with the rubber tree EST database. Small RNA reads matching to the ESTs were interrogated with a bioinformatics pipeline (Barrera-Figueroa et al. 2011) that follows the updated plant miRNA annotation criteria (Meyers et al. 2008) for miRNA prediction. In total, 8,15,937 reads representing 5,471 unique sequences matched to the EST database and only 2 emerged from the pipeline as potential miRNAs. One was miR166 and the other did not match to any known miRNAs in miRBase, and is therefore a potentially novel miRNA (hbr-candidate 1; Table 1; Supplemental Figure 1). We did not find any targets for this miRNA from the rubber tree ESTs, but two potential targets were predicted from the leafy spurge and cassava ESTs (Supplemental Table 5; Fig. 3).

A partially sequenced and assembled rubber tree genome became available recently. We took advantage of this genomic resource to predict novel miRNAs from the high-throughput sequencing data. 11,708,729 reads representing 63,513 unique reads were mapped to the partial rubber tree genome. 37 potential miRNAs were predicted from these reads, among which 16 were known miRNAs. Among the 21 that did not match any known miRNAs in miRbase, 2 were removed due to suboptimal precursor structures to result in 19 candidate novel miRNAs (hbr-candidates 2–20; Table 1). Altogether, 20 novel miRNAs were predicted from rubber tree ESTs and genomic sequences (Table 1). The predicted targets of the 20 novel miRNAs included metabolic enzymes, transcription factors, and protein kinases (Supplemental Table 5; Fig. 3).

Accumulation of miRNAs in rubber tree

To confirm the in vivo accumulation and to examine the accumulation patterns of potential miRNAs, 19 miRNAs identified through sequence homology to known miRNAs, including 18 widely conserved miRNAs and a legume-specific miRNA (miR1511), were analyzed in leaves at different developmental stages and in the two rubber tree clones. Northern blot analysis was performed using antisense probe sequences from *Arabidopsis* miRNAs (Fig. 4). The expression was normalized to the abundance of 5S rRNA present in each RNA sample and the relative levels are shown in Supplemental Figure 2.

For all miRNAs examined, a signal was detected in at least one of the four samples, which confirmed their accumulation in vivo. Most of the miRNAs displayed clone-specific and/or developmental stage-specific expression patterns. For example, miR160, miR164, and miR319 were particularly enriched in young leaves of the clone PB260 (Fig. 4; Supplemental Figure 2). miR395 accumulated to higher levels in mature leaves than in young leaves in the clone PB217 (Fig. 4; Supplemental Figure 2).

We also examined the accumulation of four randomly chosen novel rubber tree miRNAs using a sensitive stem–loop RT-PCR approach (Varkonyi-Gasic et al. 2007). All four miRNAs accumulated in vivo (Fig. 5). Hbr-cand02, hbr-cand05 and hbr-cand12 were present in all four rubber tree samples, but hbr-cand13 was only present in the two mature leaf samples (Fig. 5).

Discussion

Using high-throughput sequencing, we generated millions of small RNA reads from young and mature leaves in two different rubber tree clones with differing latex yields. Of the millions of high-quality small RNA reads from the libraries, the 24 nt class exhibited the highest abundance followed by the 21 and 18 nt classes. Such size distribution is similar to that observed for *Arabidopsis thaliana* (Kasschau et al. 2007), *Oryza sativa* (Morin et al.

2008), *Medicago truncatula* (Szittya et al. 2008) and *Arachis hypogaea* (Zhao et al. 2010). In *Arabidopsis*, the 24 nt in class is composed almost exclusively of *DCL3*-dependent siRNAs (Kasschau et al. 2007; Xie et al. 2004). Conversely, 21 nt is the common length of miRNAs (Kasschau et al. 2007). We identified 115 conserved miRNAs representing 56 families and 20 novel miRNAs. miR396 was found to be the most abundant miRNA, accounting for close to 50 % of the total sequence reads. miR396 is conserved among dicot and monocot plants, and targets six growth-regulating factor (GRF) genes encoding putative transcription factors with roles in growth and cell proliferation in leaves in *Arabidopsis* (Wang et al. 2011). In *Arabidopsis*, miR396 is present at low levels in young leaves and its abundance gradually increases as leaves mature (Wang et al. 2011). This pattern of accumulation of miR396 was also seen in rubber tree, as reflected by the read frequencies in young and old leaves (Supplemental Table 1). Apart from the regulation of cell proliferation in plants, miR396 was also reported to be involved in responses to environmental stimuli such as drought stress (Gao et al. 2010). In addition to this miRNA, miR159/319, miR167, and miR166 families, which are believed to regulate MYB transcription factor, auxin responsive factor (ARF), and type III HD-Zip transcription factors, were also abundantly represented in our libraries. Several miRNA* species were found to accumulate to the levels comparable with their respective miRNAs, implicating a functional importance for these miRNA* species.

In this study, rubber elongation factor (*REF*) was predicted to be a target of miR172. REF is a major protein located on the surface of large rubber particles in latex and is involved in rubber biosynthesis (Priya et al. 2008). Hevamine A, a predicted target of miR160, is one of several genes encoding Hevamine, a chitinase/lysozyme activity found in the lutoid (vacuolar) fraction of rubber latex (Bokma et al. 2001, 1997; Subroto et al. 1996). Hevamine is important for plant defense against various bacterial and fungal pathogens. Further investigation into the regulatory relationship between the two miRNAs and their targets would potentially provide a means to enhance latex production in rubber tree.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Lijuan Ji and Theresa Dinh for technical advice. This work was partially supported by National Science and Technology Development Agency (grant No. BT-B-01-PG-14-5202), University of California, Riverside, and Mahidol University. Lertpanyasampatha M. is supported by the Royal Golden Jubilee Ph.D. program (no. PHD/0216/2550) and Fulbright-TRF Thai Junior Research Scholarship Program.

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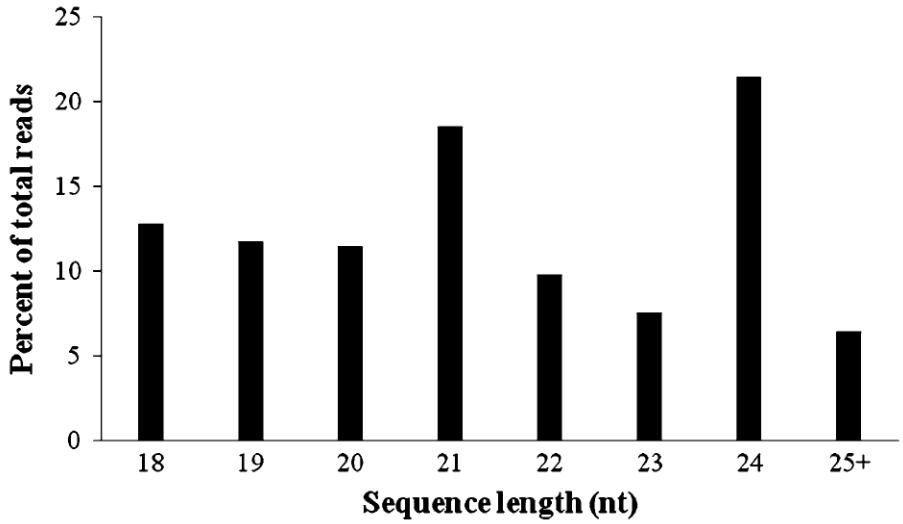

Fig. 1.

Size distribution of small RNAs in rubber tree leaves. The percentage of small RNAs of a particular length in the total small RNA population is shown

Fig. 2.

The read frequencies of conserved miRNAs in rubber tree leaves. The total number of reads corresponding to a particular miRNA family was normalized against the number of total small RNA reads and expressed in transcripts per million (TPM)

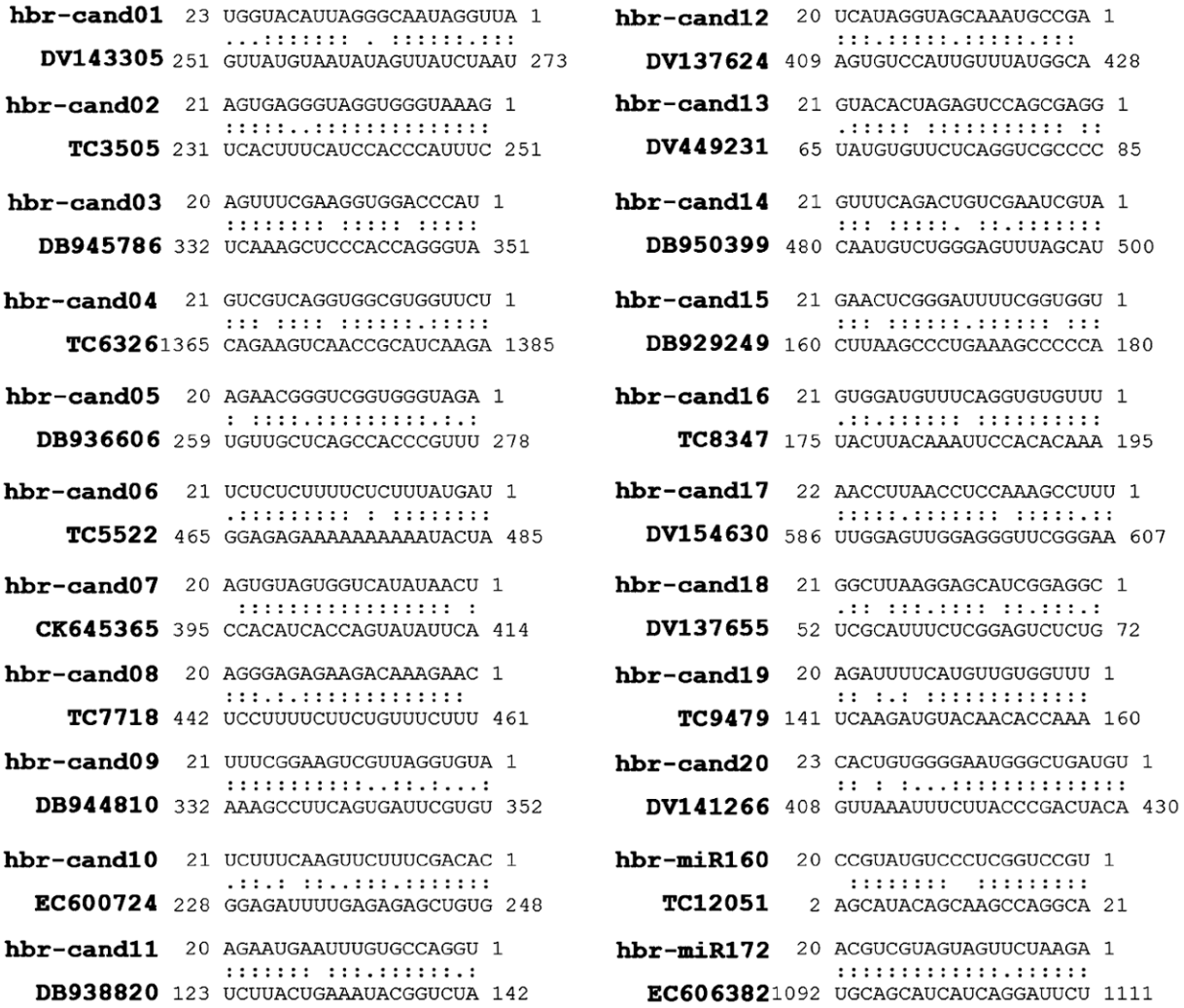

Fig. 3.

Diagrams of miRNA-target pairing between two conserved miRNAs (miR160 and miR172) and 20 novel miRNAs and their selected predicted targets. A–U and G–C hydrogen bonds are represented by *two dots* and the G–U hydrogen bonds are represented by a *single dot*. The *numbers* in the target genes represent the positions of the target sites within the indicated EST sequences. EC606382 is a GenBank accession number for a rubber tree EST. EC606382 encodes a protein identical to rubber elongation factor (GenBank accession P15252 from *Hevea brasiliensis*). The accession numbers for the other targets are from either the *Euphorbia esula* (leafy spurge) DFCI gene index release 1 or the *Manihot esculenta* (cassava) DFCI gene index release 1. TC12051 is a cassava EST that encodes a protein homologous to Hevamine A from rubber tree (GenBank accession P23472 from *Hevea brasiliensis*)

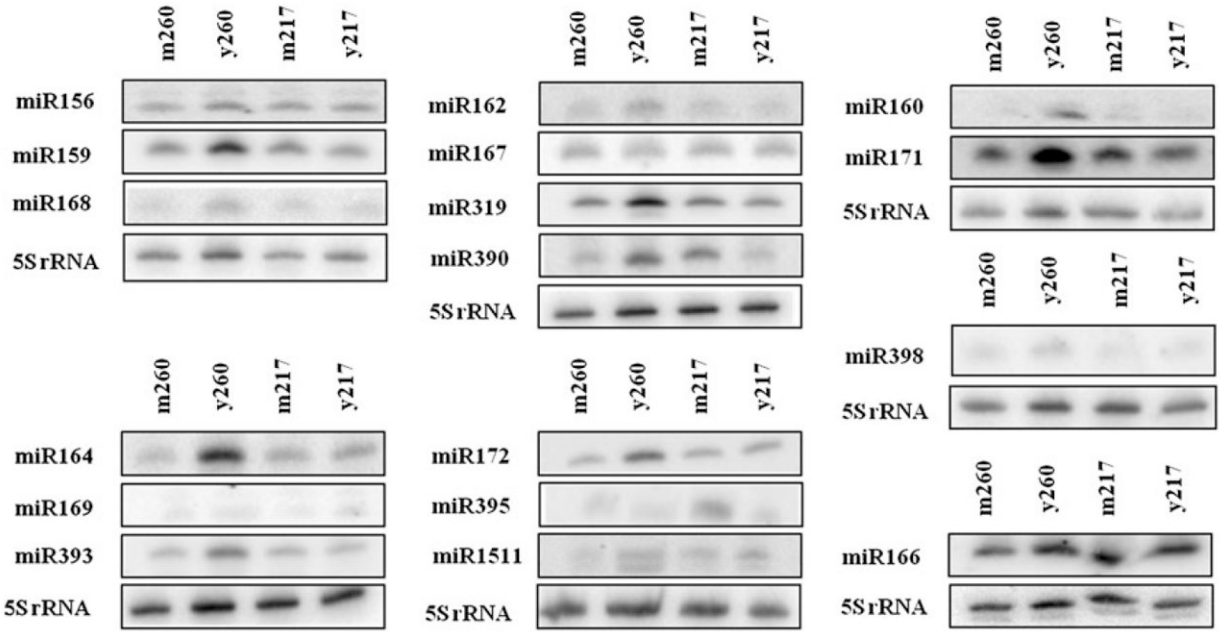

Fig. 4.

Northern blot analysis of rubber tree miRNA accumulation in leaves. Total RNA (10 μg) from mature (*m*) and young (*y*) leaves from two rubber tree clones PB260 (260) and PB217 (217) was resolved in 15 % polyacrylamide/8 M urea/0.5x TBE gels and subsequently transferred to membranes for northern blot analysis. Membranes were hybridized with an oligonucleotide antisense to each miRNA. 5S rRNA served as a loading control. In some cases, membranes were stripped and used for several hybridizations such that one 5S blot was the loading control for all the miRNAs shown above

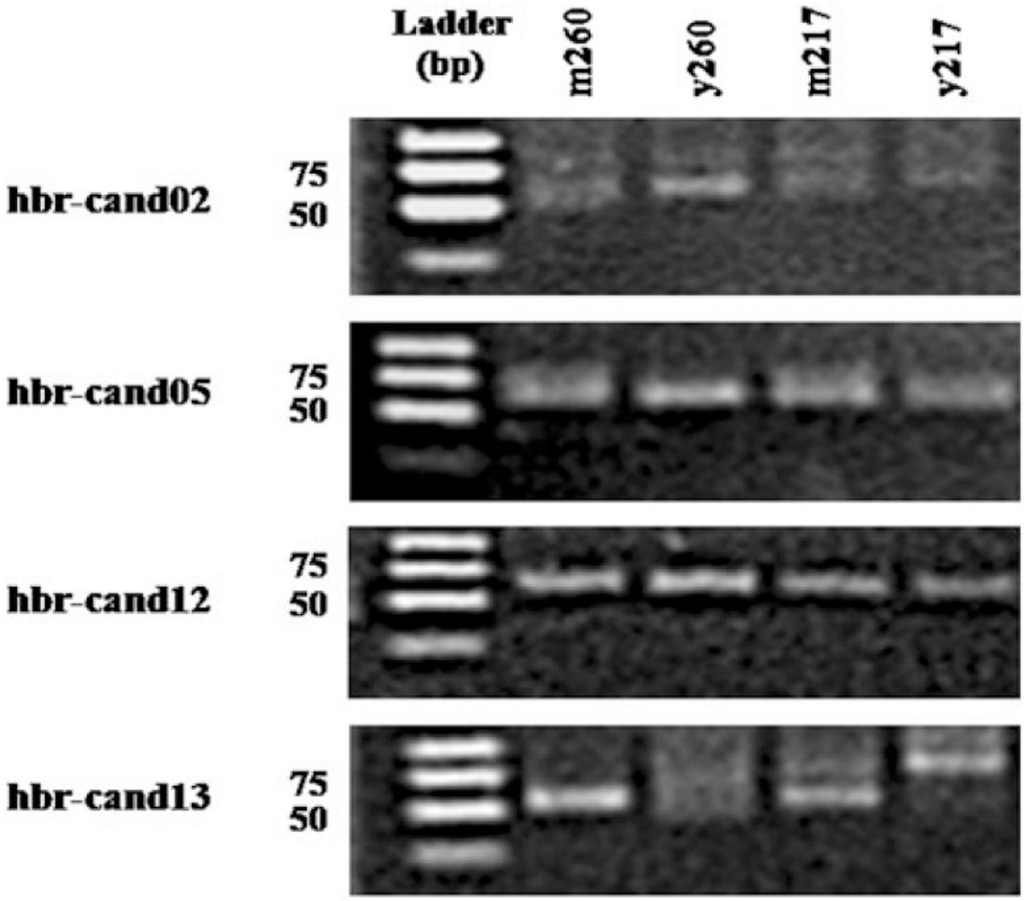

Fig. 5.
Stem–loop RT-PCR analysis of rubber tree miRNA accumulation in leaves. The expected size of the RT-PCR products is 60 bp. For hbr-cand02 and hbr-cand13, PCR were conducted for 35 cycles with annealing at 64 °C. For hbr-cand05 and hbr-cand12, PCR were conducted for 28 and 35 cycles, respectively, with annealing at 60 °C

Table 1

Predicted novel miRNAs from rubber tree

miRNA_ID	Mature miRNA	Length	Database[a]	Read frequency (in TPM)			
				217_m	217_y	260_m	260_y
Hbr-cand01	AUUGGAUAACGGGAUUACAUGGU	23	Rubber tree EST	0	0.2	0.3	0.3
Hbr-cand02	GAAAUGGGUGGAUGGGAGUGA	21	Rubber tree genome	0.3	1.3	0.2	45.6
Hbr-cand03	UACCCAGGUGGAAGCUUUGA	20	Rubber tree genome	0.2	0.2	0.3	0.1
Hbr-cand04	UCUUGGUGCGGUGGACUGCUG	21	Rubber tree genome	0	0.2	0.2	0.9
Hbr-cand05	AGAUGGGUGGCUGGGCAAGAAG	22	Rubber tree genome	108.2	4,322.5	108.3	4,590.1
Hbr-cand06	UAGUAUUUCUCUUUUCUCUCU	21	Rubber tree genome	0.9	0	1.1	4.7
Hbr-cand07	UCAAUAUACUGGUGAUGUGAU	21	Rubber tree genome	0.3	0	0.2	0.1
Hbr-cand08	CAAGAAACAGAAGAGAGGGAU	21	Rubber tree genome	0	0	0	0.9
Hbr-cand09	AUGUGGAUUGCUGAAGGCUUU	21	Rubber tree genome	0.1	0	0	0.5
Hbr-cand10	CACAGCUUUCUUGAACUUUCU	21	Rubber tree genome	8.7	9.5	7	20.8
Hbr-cand11	UGGACCGUGUUUAAGUAAGAA	21	Rubber tree genome	0.3	0	0.9	0.6
Hbr-cand12	AGCCGUAAACGAUGGAUACU	20	Rubber tree genome	21.6	21.4	10.2	0.8
Hbr-cand13	GGAGCGACCUGAGAUCACAUG	21	Rubber tree genome	6.1	4.2	3.3	1.8
Hbr-cand14	AUGCUAAGCUGUCAGACUUUG	21	Rubber tree genome	0.1	0.2	0.2	0.8
Hbr-cand15	UGGUGGCUUUUAGGGCUCAAG	21	Rubber tree genome	0.1	0	0	0.7
Hbr-cand16	UUUGUGUGGACUUUGUAGGUG	21	Rubber tree genome	0.4	0.4	0	3.4
Hbr-cand17	UUUCCGAAACCUCCAAUUCCAA	22	Rubber tree genome	14.8	9.4	32.3	22.1
Hbr-cand18	CGGAGGCUACGAGGAAUUCGG	21	Rubber tree genome	1.8	0.2	0.5	0
Hbr-cand19	UUUGGUGUUGUACUUUUAGAA	21	Rubber tree genome	48.7	44.9	85	622.7
Hbr-cand20	UGUAGUCGGGUAAGGGGUGUCACA	24	Rubber tree genome	0	0.5	0.2	1.8

TPM transcripts per million, *m* mature leaves, *y* young leaves, *217 and 260* two rubber tree clones

[a]The databases from which the miRNAs were predicted