

Identification of Conserved and Novel microRNAs from *Liriodendron chinense* Floral Tissues

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Abstract

Background: *Liriodendron chinense* (*L. chinense*) is an endangered basal angiosperm plant in China because of its low reproductive efficiency. Recently, miRNAs have obtained great attention because they can play important roles. Through high throughput sequencing technique, large amount of miRNAs were identified from different plant species. But there were few studies about the miRNAs in the basal angiosperms especially in the sexual reproduction process.

Results: Deep sequencing technology was applied to discover miRNAs in *L. chinense* flowers at different stages. After bioinformatic analysis, 496 putative conserved miRNAs representing 97 families and 2 novel miRNAs were found. Among them, one is previously regarded as gymnosperm specific. Their expressions were further validated by Real-time PCR for 13 selected miRNAs. Putative targeting genes were predicted and categorized with gene ontology (GO) analysis. About ten percents of the targets are involved in the reproduction process. Further expressional analysis showed that many of these miRNAs were highly related to the reproductive growth.

Conclusions: This is the first comprehensive identification of conserved and novel miRNAs in *L. chinense*. The data presented here might not only help to fill the gap of miRNA registered about basal angiosperm plants but also contribute to understanding the evolution of miRNAs. The differential expression of some of the miRNAs and the prediction of their target genes are also helpful in understanding the regulation of *L. chinense* sexual reproduction.

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Introduction

MicroRNAs are endogenous 21–24 nt small non-coding RNAs (sncRNAs) that have been found in a wide variety of organisms ranging from prokaryotes to eukaryotes [1,2]. They negatively regulate gene expression on transcriptional and post-transcriptional level [3], and play pivotal roles in many aspects of plant growth and development [4,5,6] including floral organ identity, female gamete formation and reproductive development [7,8]. In plant, most of the miRNA encoding genes are intergenic and rarely clustered in tandem. To broaden the knowledge of miRNAs, high throughput sequencing techniques have been applied and large amount of miRNAs from different plants were identified. Currently, 19 724 mature miRNAs belonging to 153 species are deposited in miRBase (release 17.0 version, April 2011) [9]. Among them, a total of 3362 are from 46 plant species. The majorities of these identified miRNAs are from monocots *Oryza sativa* and eudicots *Populus trichocarpa* and *Arabidopsis thaliana*. Other plants include algae *Chlamydomonas reinhardtii* [10] and *Porphyra yezoensis* [11], moss *Physcomitrella patens* [12], conifer *Pinus taeda* [13] and *Picea abies* [14], monocot *Brachypodium distachyon* [15], *Triticum aestivum* [16] and *Zea mays* [17], basal eudicot *Eschscholzia californica*

[18], core eudicot *Aquilegia coerulea* [19], *Arachis hypogaea* [20], *Populus euphratica* [21], *Medicago truncatula* [22], *Glycine max* [23] and Euphorbiaceous plants [24]. However there is little information on the miRNAs from basal angiosperm plants except a study on the miRNAs from *Selaginella moellendorffii* [25].

L. chinense is one of the two species in *Liriodendron* genus which belongs to basal angiosperm. It is the early branching angiosperm lineages (Fig. 1). Because it occupied pivotal positions in the phylogenetic tree, the plant of *Liriodendron* genus was always used to study the evolution of flowering plants. Although basal angiosperms only represent 3% of whole angiosperm species, most of the diversity in floral structure and organization was found among them [26]. In addition, *L. chinense* is ideal for landscaping because of its beautiful flowers and leaves. But it is endangering in China, which is mainly resulted by its low sexual reproductive efficiency. The molecular mechanism underlying its sexual reproduction barrier is totally unknown. Many internal factors including miRNAs have been found to play regulatory roles in the reproductive growth of plants. To profile the miRNAs in the flowers of *L. chinense* may help to explore the mechanisms that control the sexual reproductive process in this species, and hence to overcome its barrier in reproduction.

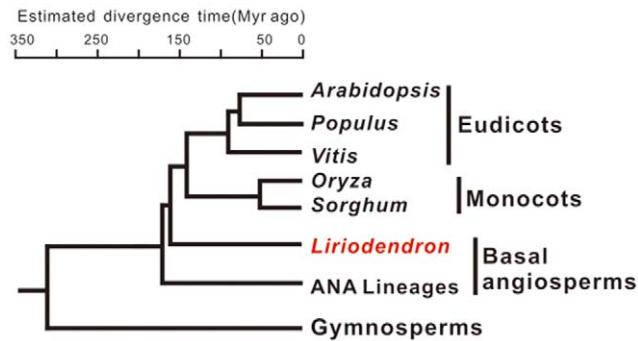


Figure 1. Phylogenetic position of *Liriodendron* genus (refer to literature [58]). ANA lineages stands for the genera Amborella, Nymphaea, Illicium, Trimenia and Austrobaileya.
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To date, there is not any study about the miRNAs in *L. chinense* and no miRNAs in the miRBase were identified from this species. Based on previous studies, it is well known that plants use both conserved and species-specific miRNAs to function in different biological processes [25,27,28,29]. A lot of miRNAs in Arabidopsis, nearly exactly match with those found in the gymnosperm Pinus genus [14,30]. However, there are still a large number of species or genus specific miRNAs in an individual plant [29]. So to profile the miRNAs in more basal plant species will help to understand not only the evolution of miRNAs but also the regulation of different biological processes at different evolutionary stages. In this study, a deep sequencing of miRNAs was conducted in the flower of *L. chinense*. Because of the absence of its genome information, only 496 conserved and 2 novel miRNAs were identified, most of the reads were assigned as unknown small RNAs. These data can not only help to comprehensively understand the miRNAs expression profile of *L. chinense* and their roles in reproductive growth regulation, but also expand the knowledge of plant miRNAs as a whole.

Results and Discussion

Complex Small RNA Population in *L. chinense*

To profile the miRNAs in *L. chinense* at reproductive growth stage, a small RNA (18–30 nt) library from the mixed flower tissues was first constructed. After sequencing with Solexa sequencing instrument, a total of 15,353,485 raw reads were acquired (Table 1). Among them, 1,111,113 (7.27%) low quality, adaptors and contamination sequences were discarded. The rest 14,165,979 (92.73%) of clean reads represented 5,185,931 unique sequences. Except for the RNAs with known function (recognized by Rfam), a large amount of small RNAs with unknown function (unannotated by Rfam) were found in *L. chinense* (Fig. 2). With the availability of more and more genomic information, their function might be discovered in the near future. The size of these small RNAs ranged from 18 to 35 nt (Fig. 3 left side). The 24 nt small RNAs were the most abundant. They accounted for more than 50% of the total clean reads; the second abundant population was the 21 nt small RNAs, and the 22 nt ones were at the third place.

Since the small RNAs might also include tRNA, rRNA, snRNA and snoRNA, further filtering by Blast with Rfam database was conducted to remove these small RNAs. After filtering, 696,864 miRNAs (5%) and 11,256,287 unannotated small RNAs (79%) were obtained. The ratio of tRNA, rRNA, snRNA and snoRNA was about 15% (Fig. 2). Actually, distribution pattern of the small RNAs' size did not change after removing the tRNAs, rRNAs,

Table 1. Statistics of small RNA sequencing.

Category	Sequences generated
Raw reads	15353485
High quality reads	15277092(100%)
Sequences <18 nt reads	1057858
Adaptors only reads	47580
N value contained reads	5675
Clean reads	14165979(92.73%)
Unique sequence	5185931

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snRNAs and snoRNAs (Fig. 3 right side), which implied that the small RNA library was with high quality.

The distribution pattern of the small RNA size in *L. chinense* was similar to that in other angiosperm species, such as Arabidopsis [31] and rice [30], which implied that *L. chinense* might possess similar processing components of small RNA biogenesis with rice and Arabidopsis. Whereas, the population of 24 nt RNAs was significantly low in conifer, which might be caused by the lack of DCL3 that mainly helps to produce 24 nt miRNAs in plants. It is reasonable in evolutionary history for their difference in the distribution pattern of small RNA. Because conifer is a gymnosperm plant, the other three are angiosperm plants. The comparison between basal angiosperm and gymnosperm species might help to understand the evolution of miRNAs biogenesis.

Identification of Conserved miRNAs in *L. chinense*

In *L. chinense*, there is no miRNA reported before. To identify the conserved miRNAs from our filtered data (the unique sequences except for the rRNA, snoRNAs, snRNAs and tRNAs), the sequences were aligned with all the miRNAs of Viridiplantae registered in miRBase 17. One or two mismatches were allowed during sequence alignment. Among the total 5,021,018 unique sequences input, 496 small RNAs matched to 97 known miRNAs families (Table S2) registered in miRBase. Among these 496 putative miRNAs, 102 had identical sequences and the rest had similar sequences with those miRNAs in the database (Table S2). These small RNAs were named as conserved miRNAs in *L. chinense*.

As we mentioned, this was the first comprehensive survey on miRNAs in basal angiosperm plants. To get a better understanding of the evolution of different miRNAs, the miRNA population in *L. chinense* was compared with those in other plants. Some representative genera including Pinus, Sorghum, Oryza, Vitis, Populus and Arabidopsis that stand for gymnosperms, monocots and eudicots plants were selected to conduct the comparison. Based on the statistic of miRBase and PMRD (<http://bioinformatics.cau.edu.cn/PMRD/>) and our results about *L. chinense*, we summarized the miRNA populations of 7 genera in figure 4. Twenty of the miRNAs exist in all the 6 angiosperm plant species; while only 10 of them also exist in pinus. As an important basal angiosperm species, *L. chinense* contains many valuable information to understand the evolution history of the miRNAs from gymnosperms to monocots and eudicots. For example, miR1310 is reported as a specific miRNA in gymnosperms plants [30], but it also existed in *Liriodendron* based on our results. This implied that miR1310 is not a gymnosperm specific miRNA. It might disappear during the evolution of the angiosperms. According

Composition of small RNAs

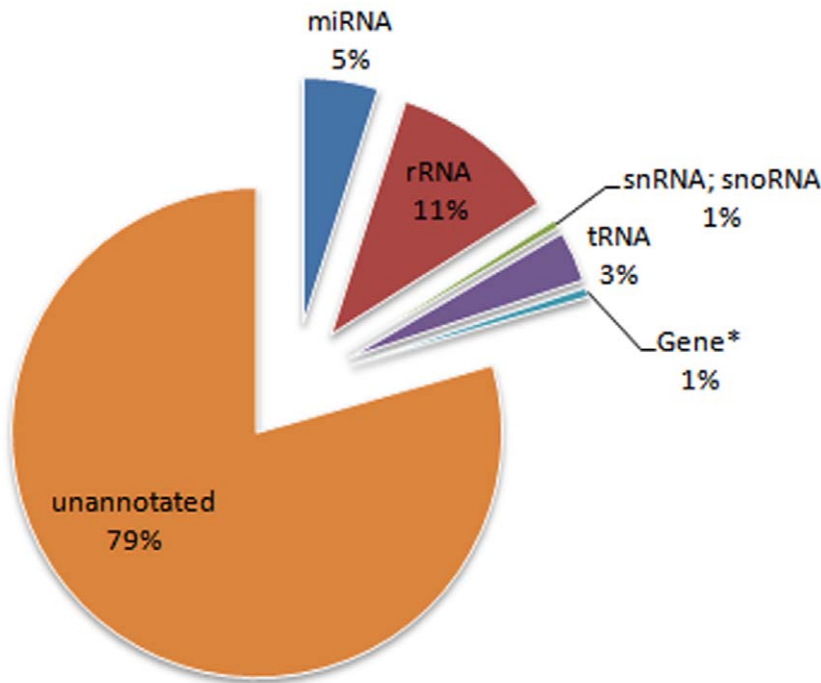


Figure 2. Composition of clean reads. The reads were annotated by Rfam.
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to the current data, some miRNAs might only exist in eudicots and *Liriodendron* like miR477, miR479, miR482, miR828 and miR846 and some miRNAs might only exist in monocots and *Liriodendron* like miR1863. It still needs more data to explain when these monocot or eudicot specific miRNAs diverged during the evolution.

Expression and Function Analysis of the Conserved miRNAs

The expression of plant miRNAs always appears to be spatio-temporal specific. To know their abundance in the flower of *Liriodendron*, the reads of 48 families of miRNAs that had more than 10 sequence counts were shown here (Fig. 5). The results

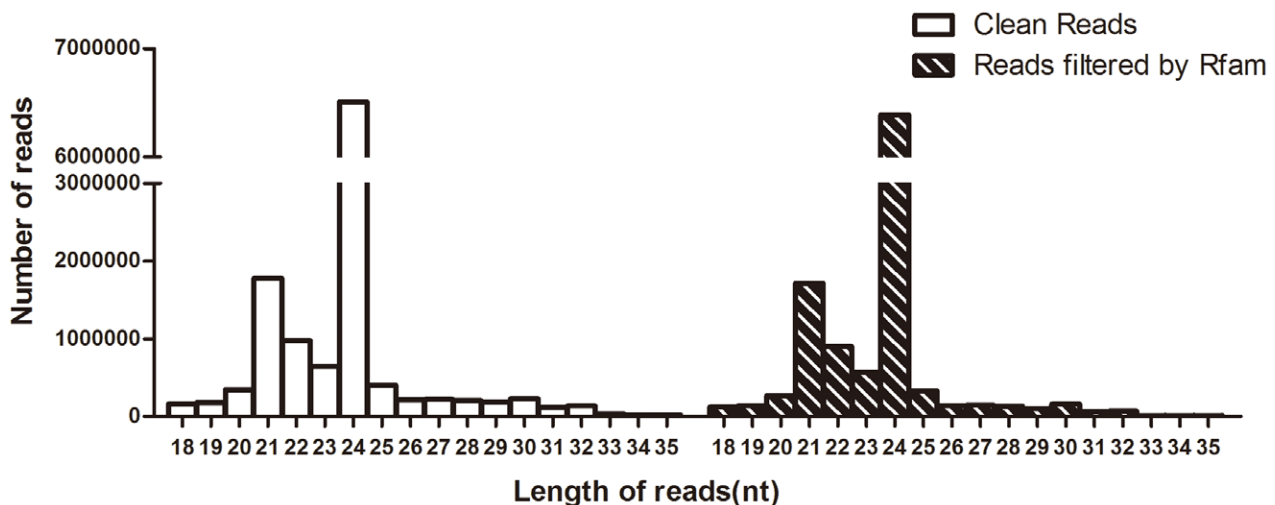


Figure 3. The length distribution of small RNAs. The open histogram at the left side shows the results from the total clean reads; the histogram with slash at the right side shows that the results after removing the reads for tRNA, rRNA, snRNA and snoRNA.
doi:10.1371/journal.pone.0044696.g003

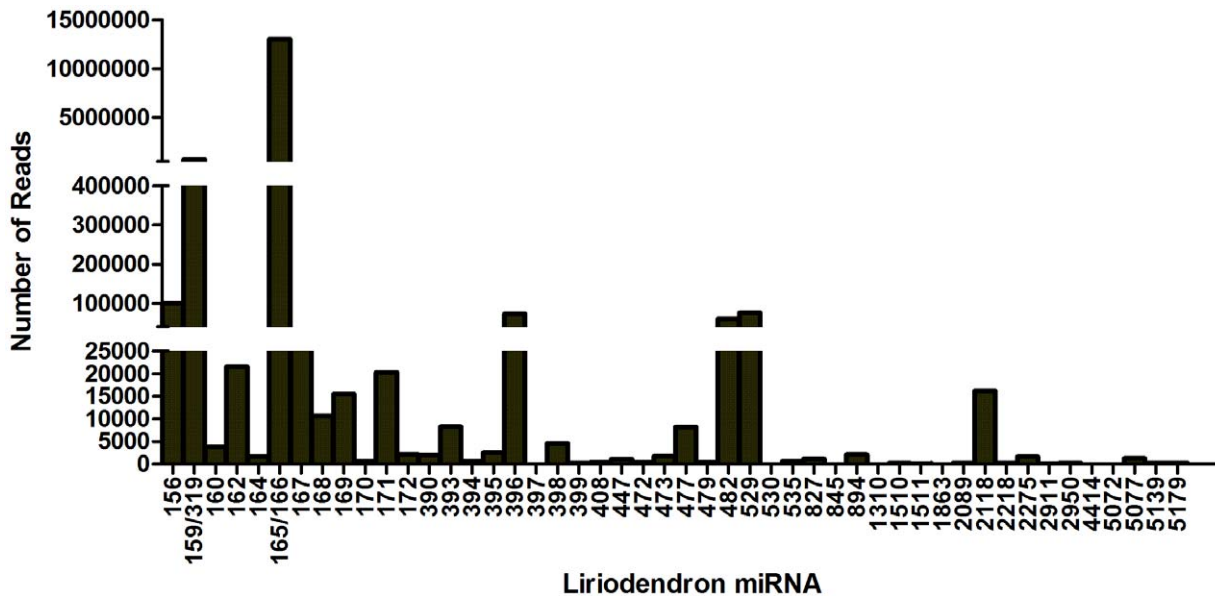


Figure 5. The abundance of some conserved miRNAs in flower of *L. chinense* based on sequencing data.
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MiR2275 was recently reported as a new class of miRNAs that specifically expressed in rice stamen and could produce phased small RNA. This class of miRNAs is possibly conserved in gramineous plants like rice and maize [42,43]. They could invoke a mass of siRNA (phased siRNAs) from dsRNA to regulate the inflorescence development of rice. Although the expression patterns of these miRNAs were similar in both basal and evolved angiosperm plants, it is still an open and interesting question to know if they all function similarly.

Interestingly, miR2118, which was reported to be similar with miR2275 specifically expressed in rice stamen, was specifically

expressed in the unpollinated pistils in *L. chinense*. It will be very interesting to know how its function evolved. Similarly expressed miRNAs included miR162 and miR169 (Fig. 7). To the contrary, the expression of miR159, miR535 and miR4376 were dramatically increased in pistil after pollination (Fig. 7). MiR159/319 family had been found to cleave the mRNAs of TCP and MYB transcription factors [41]. Over-expression of miR159 could cause male sterility in Arabidopsis [41], which is consistent with its low expression in anther (Fig. 7). It is very interesting to further study if the simultaneous highly expressed miR165/166 and miR159 in the flower of *L. chinense* had relation with its low setting percentage

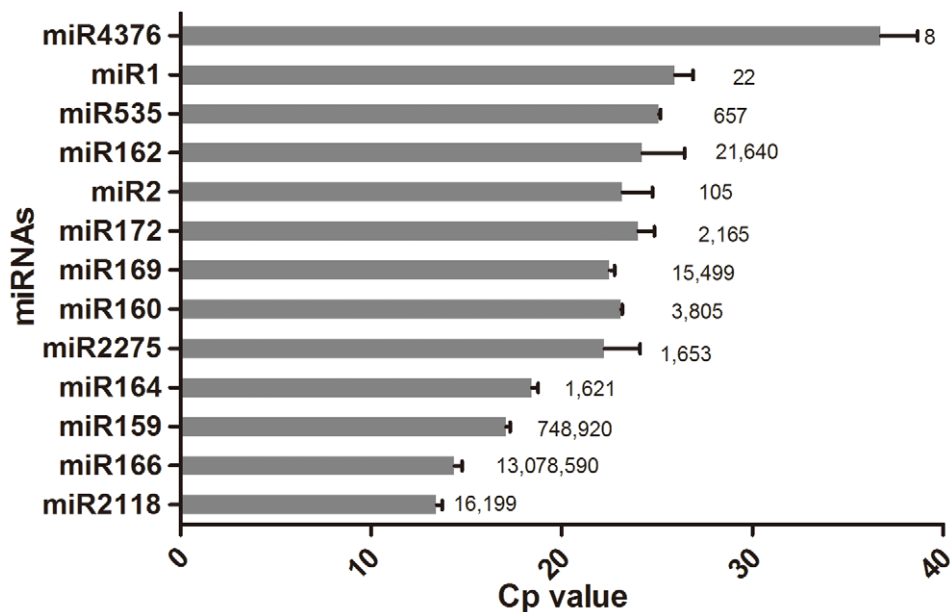


Figure 6. Real-time PCR validation of some miRNAs in *L. chinense*. the Cp value stands for the threshold cycle. Error bars indicate the standard deviation of three replicates. The corresponding sequencing reads number of individual miRNAs was on the right of columns and marked by rectangle.
doi:10.1371/journal.pone.0044696.g006

Table 2. Predicted targets of miRNAs that have high abundance in *L. chinense*.

miRNA	Reads counts	Target in <i>At and os</i> ¹	Putative target in <i>Liriodendron</i> ²
447	1114	2-PGK	Nucleotide binding protein
827	1165	ND	Phosphoribosyltransferase
5077	1302	ND	ND
164	1621	NAC	ND
2275	1653	ND	Prolyl oligopeptidase protein PPR protein
473	1754		ND
390	2091	ta-siRNA	Binding protein
894	2118		ND
172	2165	AP2	Kinesin Ribosomal protein
395	2513	APS	Vacuolar sorting protein SO ₂ transporter Cell division protein Clp protease subunit
160	3805	ARF	Arginine serine-rich splicing protein
398	4590	CytC oxidase	SOD CSD Pollen protein Transcription factor lim1
477	8170	ND	Aldehyde dehydrogenase Clathrin heavy chain
393	8255	b-ZIP	Sphingolipid desaturase
		F-box	
168	10670	AGO	HSP interaction protein
169	15499	HAP2	Ring-finger protein 26S proteasome subunit Galactosyltransferase
2118	16199	ND	Methyltransferase Ribosomal protein Vacuolar ATP synthase
171/170	20383	SCL	Protein kinase Methyltransferase protein Calcium ion binding Porin
162	21640	Dicer	Dipeptidyl peptidase
167	39273	ARF	ARF Cytochrome p450 Vascular protein
482	61179	ND	Eukaryotic initiation factor Chalcone flavanone isomerase
396	73058	GRF	GRF Ubiquitin protease 2b Cyclin
529	75594	SBP	SBP Chloroplast chaperonin Serine protease
156	100279	SBP	SBP Cytochrome P450
159/319	748920	MYB	TCP

Table 2. Cont.

miRNA	Reads counts	Target in <i>At and os</i> ¹	Putative target in <i>Liriodendron</i> ²
		TCP	sRNA methyltransferase Ribosome protein Reverse transcriptase
165/166	13078590	HD-ZIPIII	HD-ZIP III AP3-complex subunit RNA polymerase subunit Topoisomerase

¹The miRNA targets of *Arabidopsis thaliana* and *Oryza sativa* mainly consults reviewer papers [19,41,59].

²The GI number of the putative targets were provided in the Additional file 4. Abbreviations: 2-PGK, 2-phosphoglycerate kinase; AP2, APETALA2; APS, ATP-sulfurylase; ARF, auxin response factors; CytC, cytochrome; CSD, copper superoxide dismutase; AGO, ARGONAUTE; SCL, scarecrow-like; GRF, growth regulating factor; SBP, SQUAMOSA-promoter binding protein; ta-siRNA, trans-acting short interfering RNA; SOD, Superoxide Dismutase; PPR: pentatricopeptide repeat; HSP, heat shock protein.
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[44]. These miRNAs either increased or decreased their expression after pollination, which highly indicated that they might be involved in the regulation of pollination in *L. chinense*. But how these miRNAs or their target genes affect the pollination is still unknown. It will be worthy both theoretically and practically to explore their functions on controlling successful pollination in plants.

Identification of Novel miRNAs in *L. chinense*

L. chinense is an unsequenced species without any genomic information in the database. But there is an ESTs library available for its relative *L. tulipifera* [7]. In this study, we performed screening against *Liriodendron tulipifera* ESTs to predict precursor miRNAs in order to identify novel miRNAs. Using softwares miRDeep-P and miRNAFinder, we predicted several putative pre-miRNAs that could fold into classical stem-loop structures. Some of them had their mature transcripts detected in our sequencing data. To obtain a reliable result, only those with more than 20 reads were accepted as the real novel miRNAs. Based on these criteria, only 2 novel miRNAs were selected and designated as lc-miR1 and lc-miR2 (Table 3). The putative precursors from ESTs of *L. tulipifera* could fold into perfect stem-loop structures (Fig. 8). Lacking of genomic information might be the main reason that leads to the identification of only two novel miRNAs. Northern blotting was performed to validate the existence of these miRNAs. The expression of lc-miR1 and lc-miR2 could be detected in small flower (SF) and middle flower (MF) (Fig. 9&S1&S3). Both lc-miR1 and lc-miR2 had slight lower expression in MF than in SF

Table 3. Novel miRNAs in *L. chinense*.

miRNAs Name	Mature Sequence	Length (nt)	Energy (kcal)	Reads count
lc-miR1	CAUGGCAGUAGAAGAGAUACCG	22	-30.23	22
lc-miR1*	UGAUCGCUUCUAUUGUUUUUC	22	-30.23	15
lc-miR2	UCAACACUGAGGUCAUGGGUU	21	-24.94	105

doi:10.1371/journal.pone.0044696.t003

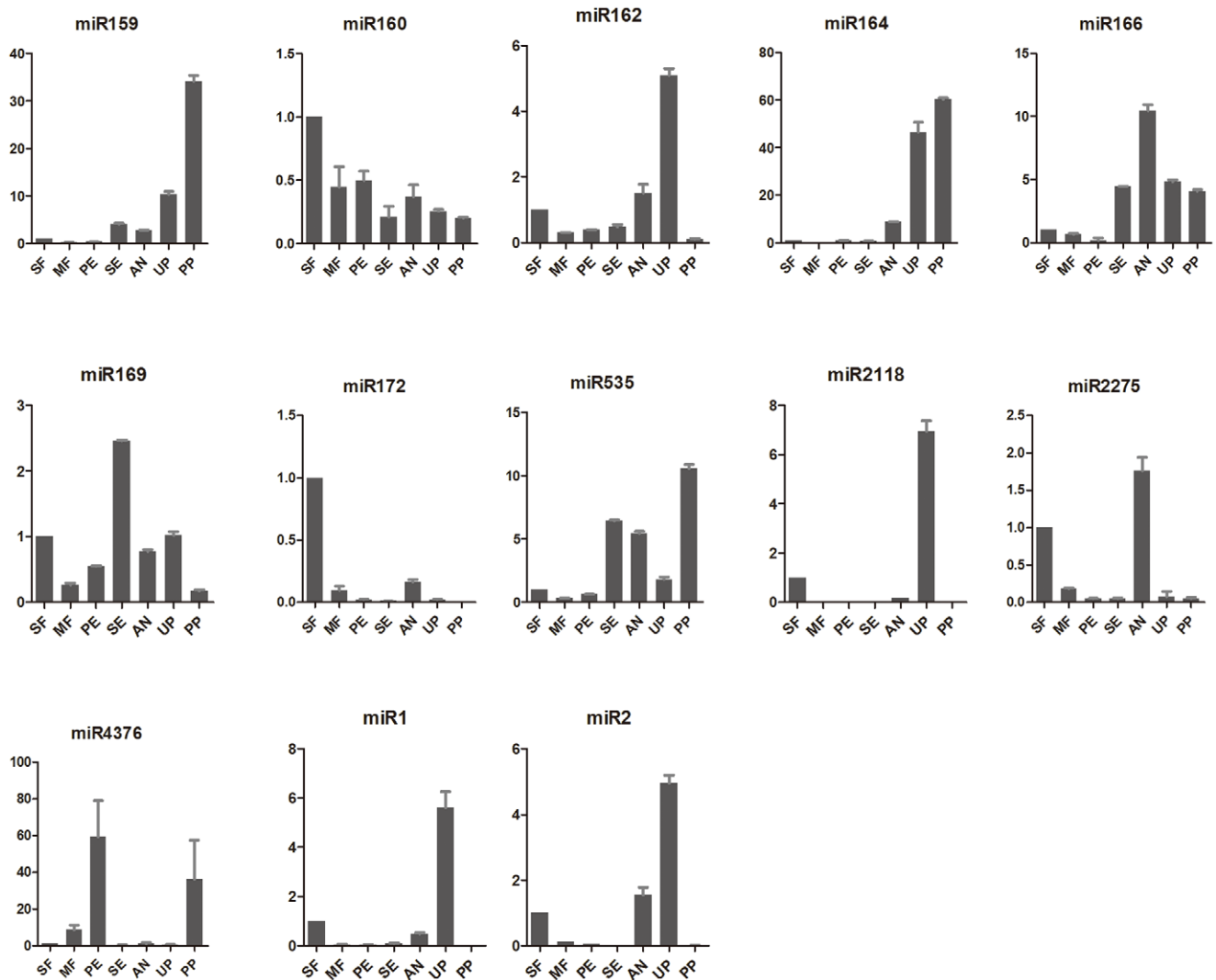


Figure 7. Relative expression level of some miRNAs in seven tissues. SF, small flower; MF, middle flower; PE, petal; SE, sepal; AN, anther; UP, unpollinated mature pistil; PP, pollinated pistil.
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(Fig. 9&S1&S3), which matched well with the real-time PCR results (Fig. 8). Furthermore, the expression of these two miRNAs and their putative target genes showed significant negative correlation (Fig. S1), which indirectly proved the existence of functional lc-miR1 and lc-miR2. Their mature sequences were aligned with all the miRNAs registered in the miRNA database miRBase (<http://www.mirbase.org/>) and PMRD (<http://bioinformatics.cau.edu.cn/PMRD/>), CSRDB (<http://sundarlab.ucdavis.edu/smrnas/>) and non-redundant sequences in Genebank. There was no homolog matching with them, which implied the two miRNAs found in our data are novel and might be *Liriodendron*-specific miRNAs. Both of them had high expression level in the un-pollinated mature pistil, whereas, both of their expression were sharply decreased after pollination (Fig. 7). This invoked us to analyze their target genes that might be important for the pollination. For miR1, one of the putative target genes encodes the large subunit of carbamoyl phosphate synthase (CPSase). The CPSase is a chloroplast protein responsible for the synthesis of carbamoylphosphate for both pyrimidine and arginine biosynthesis [45]. This is a highly controlled processes, and the transcription of CPSase encoding genes are sensitively

invoked by ornithine and feedback down-regulated by pyrimidine or arginine [46]. But how the regulation happens is still unknown. The results about lc-miR1 suggested that miRNAs might at least used to be involved in the regulation in *L. chinense*. It has been reported that accumulation of pyrimidine and purine in the style happened after the pollination in *Petunia* [47]. The sharply decrease of lc-miR1 might up-regulate CPSase and enhance the biosynthesis of pyrimidine for pollen growth. For lc-miR2, we further analyzed the putative 10 target genes. The alignment among them showed that 5 transcripts had high sequence similarity in some regions (Figure S2). In coincidence, the lc-miR2 target sites on them also located in this putative conserved region. This implied that the lc-miR2 might regulate a multi-gene family in *Liriodendron*. Blast analysis showed these transcripts had high similarity (61.15%) with Cysteine-rich receptor-like protein kinase (CRK) in *Medicago truncatula*. The CRK family in *Arabidopsis* was reported to be induced by pathogen infection, salicylic acid and reactive oxygen species [48,49]. Till now, no literature reported that the CRK family might be involved in the pollination process. But the study on self-incompatibility in Brassicaceae indicated that the cysteine-rich-motif containing

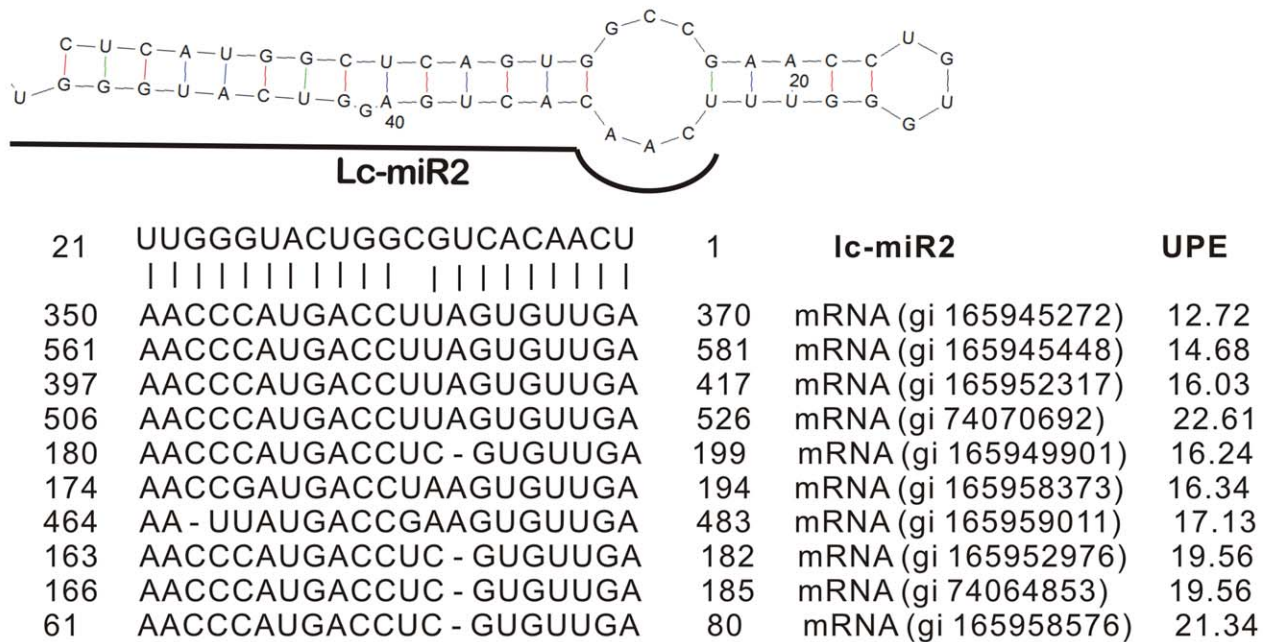
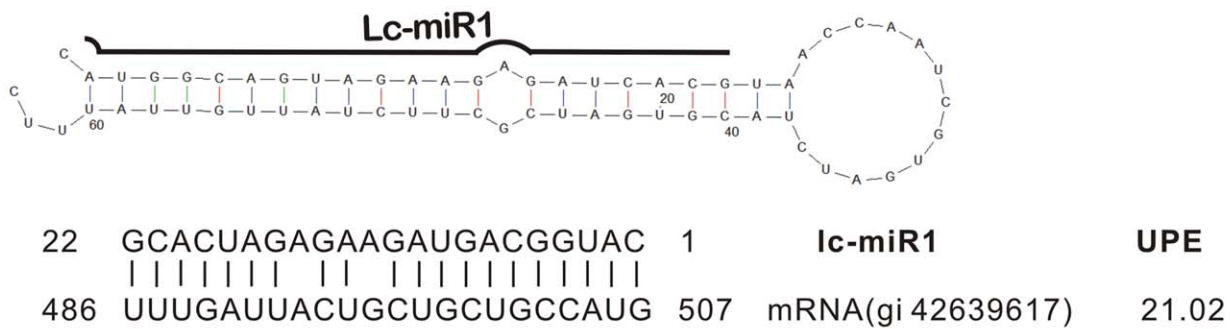


Figure 8. Putative stem-loop structures and target genes of novel miRNAs.
 doi:10.1371/journal.pone.0044696.g008

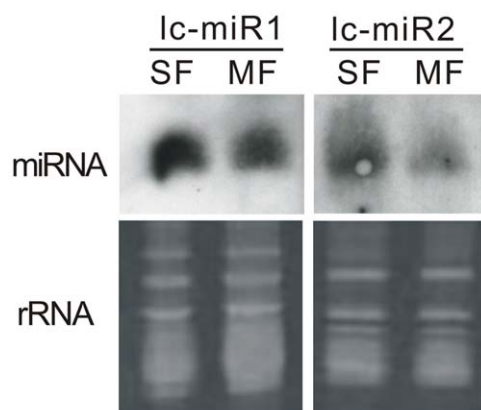


Figure 9. Northern blotting showing the expression of lc-miR1 and lc-miR2. rRNAs were used as loading control. SF, small flower; MF, middle flower.
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proteins like SRK and SCR were the important determinant factors in pollen recognition process [50]. Our findings in this paper indicate that the CRK proteins might be regulated by lc-miR2 during the pollination process in *L. chinense*. As mentioned above, *L. chinense* is endangering because of its low sexual reproductive efficiency. Up to now, it is still elusive about the molecular mechanism. The existence of these CRK like genes might provide a clue for us to further study the mechanism that leads to the low sexual reproductive efficiency in this species. The above analysis showed that lc-miR1 and lc-miR2 could be *Liriodendron*-specific miRNAs and play novel functions in this genus. It would be interesting that if this kind of regulation (through lc-miR1, lc-miR2 and their targets) also exists in other plants or not.

This is the first comprehensive analysis of miRNAs in *L. chinense*. A lot of conserved and novel miRNAs were identified. The data might be helpful not only in filling the gap of miRNA registered about basal angiosperm plants but also in understanding the evolution of miRNAs. Previous, Liang et al. have reported that many novel mRNAs were found in the *Liriodendron tulipifera* [51].

Our finding revealed that some novel miRNAs also existed in *L. chinense* and might be involved in the regulation of reproductive process. Exploring the regulatory mechanism of these miRNAs should be helpful to understand the sexual reproduction in these basal angiosperms. As an evolutionary important plant genus, *Liriodendron* might contain a certain amount of novel genes, including miRNA genes. With the availability of more genomic sequence information on *Liriodendron* genus, more miRNAs will be identified in this species. The cloning and identification of these genes and figuring out their regulation relationships would be very helpful for exploiting new genes and regulatory pathways and their evolution in plant.

Materials and Methods

Plant Material and Small RNAs Isolation

Young floral buds with the diameter of approximately 10.0–15.0 mm (small flower) and 15.0–20.0 mm (middle flower) in length and the petal (PE), sepal (SE), anther (AN), unpollinated pistil (UP) and pollinated pistil (PP) from open flowers of *L. chinense* were collected from wuhan botanical garden, wuhan city, China on April 15, 2011, and then kept at -80°C . Total RNA was extracted from the different organs of flower with Trizol (Invitrogen). After 15% polyacrylamide denaturing gel (8 M urea) electrophoresis, the small RNAs with size of 18–30 nt were excised from the gel and recovered by elution with 0.4 M NaCl overnight at 4°C . The elution solution was precipitated by adding three volume of absolute ethanol.

Small RNA Library Construction and Sequencing

The small RNAs were dephosphorylated by alkaline phosphatase for avoiding self-ligation and then sequentially ligated 3' and 5' RNA/DNA chimeric oligos adapters. After reverse transcription and PCR, the amplified products were sequenced by Solexa GAI (Majorbio, Shanghai, China).

Discovery and Analysis of Conserved Micro RNAs

The raw sequencing data were filtered with software Fastx-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) to delete the low quality reads, adapters and contamination. The clean reads were then annotated on Rfam 10.1 (<http://rfam.sanger.ac.uk>) to remove the rRNA, snRNA, snoRNA, tRNA. Then, the unique small RNAs filtered by Rfam were aligned with the data in miRBase 17 using Bowtie software (<http://bowtie-bio.sourceforge.net/index.shtml>) to search the conserved miRNAs in *L. chinense*. For the abundance analysis of miRNAs, the number of the read (read count) acquired by Solexa GAI was used.

Novel miRNA Prediction

The de novo prediction of novel miRNA was performed by miRDeep-P (<http://faculty.virginia.edu/lilab/miRDP/>) and miRNAfinder (<http://bioinfo3.noble.org/mirna/>). The putative novel miRNAs were screened in our sequencing data. The putative miRNAs that had more than 20 reads matching with unique sequences were taken as candidates. Then the candidates were validated further by realtime PCR. Their putative stem-loop structures were showed with RNAdraw software.

Prediction of miRNA Target

During the miRNA putative target analysis, psRNATarget (<http://plantgrn.noble.org/psRNATarget/>) [52] was used, the parameters were set as below: Maximum expectation: 3, Length for complementarity scoring: 20, Allowed maximum energy to unpair the target site (UPE): 25, Flanking length around target site

for target accessibility analysis: 17 bp, Range of central mismatch leading to translational inhibition: 9–11 nt. Currently there are no genome sequence of *L. chinense* provided in NCBI. So we used the total ESTs of genus *Liriodendron* as the pool for the putative miRNA targets prediction. The conserved and novel miRNAs identified in this study were used as baits. The blast and GO annotation of miRNA targets were using Blast2go software [53].

Northern Blotting for miRNAs

For Northern blot experiments, detailed procedures were referred to previous methods [54,55]. Briefly, 50 ug of total RNA was size-fractionated through electrophoresis on 17% (w/v) denatured PAGE gel with 7 M urea. RNA was blotted on to Hybond-XL membranes (GE Healthcare) by semi-dry transfer unit (Bio-Rad). Hybridization probes for lc-miR1 (CGTGATCTCTTCTACTGCCATG), lc-miR1* (GAAATAACAATAGAAGCGATCA) and lc-miR2 (AACCCATGACCTCAGTGTTGA) were synthesized from HuiRui Biotech (Shanghai, China).

Validation of miRNA Expression by Real-time PCR

The reverse transcription reaction and Real-time quantification was performed according to the protocol from chen [56]. The designed specific stem-loop primers and other primers for miRNAs are listed in Table S1. No RT primer and no RNA controls were performed during reverse transcription. Real-time PCR was carried out on Lightcycler 480 system (Roche) with SYBR Green I methods. The melting curves were analyzed to check the specificity of PCR products. All the reactions were run for three replicates. The values of the threshold cycle (Ct, it was named as Cp in Roche Lightcycler 480 system) were calculated using Lightcycler 480 software. Because the different primer pairs showed different amplification efficiency, for comparison of gene expression in different tissues, the efficiency-calibrated method was used [57]. The 18S rRNA was used as internal reference and the expression of other tissues were relative to that of small flower (SF).

Supporting Information

Figure S1 The novel miRNAs and their targets expression in different tissues by qRT-PCR.

(TIF)

Figure S2 The alignment of 10 putative targets of lc-miR2.

The high similarity region between the target genes of lc-miR2 was showed as red box and the putative target region by lc-miR2 was showed as blue box.

(PDF)

Figure S3 The expression of novel miRNAs in different tissues by Northern Blotting.

rRNAs were used as loading control. SF, small flower; MF, middle flower; PE, petal; SE, sepal; AN, anther; UP, unpollinated pistil.

(TIF)

Table S1 The primers used in this study.

(DOC)

Table S2 The 496 conserved miRNAs represented 97 miRNA families in *L. chinense*.

(XLS)

Table S3 The miRNAs targets predicted by psRNATarget and their GO analysis.

(XLS)

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Conceived and designed the experiments: PY KW. Performed the experiments: KW ML FG. Analyzed the data: KW. Contributed reagents/materials/analysis tools: KW FG ML. Wrote the paper: KW PY. Provided crucial suggestions on the experiment: YZ SL.

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