Plant Science 235 (2015) 46–57

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

High throughput sequencing of small RNA component of leaves and inflorescence revealed conserved and novel miRNAs as well as phasiRNA loci in chickpea☆

Sangeeta Srivastava[a,1], Yun Zheng[b,1], Himabindu Kudapa[c], Guru Jagadeeswaran[a], Vandana Hivrale[a], Rajeev K. Varshney[c,d,*], Ramanjulu Sunkar[a,**]

[a] Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK 74078 USA
[b] Faculty of Life Science and Technology, Kunming University of Science and Technology, 727, South Jingming Road, Kunming, Yunnan 650500, China
[c] International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad 502324, India
[d] School of Plant Biology and Institute of Agriculture, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia

ARTICLE INFO

Article history:
Received 4 February 2015
Received in revised form 27 February 2015
Accepted 3 March 2015
Available online 9 March 2015

Keywords:
Chickpea
MicroRNAs
PhasiRNAs
Posttranscriptional gene regulation

ABSTRACT

Among legumes, chickpea (*Cicer arietinum* L.) is the second most important crop after soybean. MicroRNAs (miRNAs) play important roles by regulating target gene expression important for plant development and tolerance to stress conditions. Additionally, recently discovered phased siRNAs (phasiRNAs), a new class of small RNAs, are abundantly produced in legumes. Nevertheless, little is known about these regulatory molecules in chickpea. The small RNA population was sequenced from leaves and flowers of chickpea to identify conserved and novel miRNAs as well as phasiRNAs/phasiRNA loci. Bioinformatics analysis revealed 157 miRNA loci for the 96 highly conserved and known miRNA homologs belonging to 38 miRNA families in chickpea. Furthermore, 20 novel miRNAs belonging to 17 miRNA families were identified. Sequence analysis revealed approximately 60 phasiRNA loci. Potential target genes likely to be regulated by these miRNAs were predicted and some were confirmed by modified 5′ RACE assay. Predicted targets are mostly transcription factors that might be important for developmental processes, and others include superoxide dismutases, plantacyanin, laccases and F-box proteins that could participate in stress responses and protein degradation. Overall, this study provides an inventory of miRNA–target gene interactions for chickpea, useful for the comparative analysis of small RNAs among legumes.

1. Introduction

Leguminous plants are the second most important crops that supply the human and animal diet. Small RNA analysis of *Medicago truncatula* and *Glycine max* (soybean) was an important step in identifying miRNAs in legumes [1–3]. Among legumes, chickpea (*Cicer arietinum* L.) is the second most important crop after soybean and is largely cultivated as a rainfed crop in dry and semi-dry regions. Given the nutritional and economic importance of this global crop, the chickpea genome has recently been sequenced for deeper insight into the gene content and organization [4,5].

Plant small RNA population comprises microRNAs (miRNAs), transacting siRNAs (derived from non-coding transcripts targeted by miRNAs and regulate the expression of mRNA targets), phased small interfering RNAs (phasiRNAs) and heterochromatic siRNAs and other endogenous small RNAs [6,7]. MicroRNAs are derived from longer primary miRNA transcripts that are capable of adopting hairpin-like structures. Such hairpin-like structures are recognized by DCL-1 and other proteins such as HYL1, SE and releases miRNA:miRNA*duplex (miRNA or the guide strand is functional molecule whereas miRNA* or passenger strand represent the opposite strand of the miRNA that is rapidly degraded). MicroRNA functions by loading into an Argonaute protein, the effector complex called as RNA-induced silencing complex [RISC] and guides the target mRNA cleavage or inhibits protein synthesis from the target mRNAs [6,7]. MicroRNAs in plants control seed, embryo, leaf, root and flower development as well as transition from juvenile, vegetative, and reproductive phases [6]. Plant miRNAs are also key players

☆ Chickpea small RNA dataset with accession ID GSE62216 have been deposited at GEO.

* Corresponding author at: ICRISAT, Patancheru, Hyderabad, India.
** Corresponding author. Tel.: +1 405 744 8496; fax: +1 405 744 7799.
E-mail addresses: R.K.Varshney@CGIAR.ORG (R.K. Varshney), ramanjulu.sunkar@okstate.edu (R. Sunkar).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.plantsci.2015.03.002

in acclimation to biotic and abiotic stress conditions [8,9]. Thus, identification of miRNAs can shed light on post-transcriptional gene regulation important for various aspects of chickpea development and acclimation to stress conditions.

Higher land plants are known to possess at least 22 families of highly conserved miRNAs and even more number of species-specific miRNAs. The availability of a complete genome is critical for identifying miRNAs, particularly novel miRNAs. Recently, a computational approach was used to predict conserved miRNA homologs in chickpea using the publicly available GSS and EST sequences of chickpea [10]. This study predicted 20 miRNA families in chickpea including miR5287, miR845, miR539, miR5021, miR1533 and miR414. While our manuscript was under preparation, Kohli et al. [11] reported cloning of miRNAs from chickpea but neither Kohli et al. nor our study found homologs of these above mentioned miRNAs in chickpea. In addition to the miRNAs, legumes also produce abundant quantities of phased siRNAs (phasiRNAs), a recently discovered class of small RNAs that are processed from non-coding and protein-coding loci [3,12–14]. The production of phasiRNAs is triggered by 22-nt miRNAs targeting transcripts. Some protein-coding genes such as nucleotide binding site-leucine-rich repeat (NBS-LRR), MYB, transport inhibitor response 1 (TIR1), and Ca^{2+}-ATPases generate phasiRNAs [14]. NBS-LRR genes have received much attention because of the vital roles they play in pathogen recognition and resistance. These NBS-LRR phasiRNAs may be more ubiquitous in legumes than non-legumes [3,12–14]. Identification of novel species-specific miRNAs and phasiRNAs requires a deep sequencing approach.

Our analysis of small RNA populations from leaves and flowers of chickpea (*C. arietinum* L.) was able to identify 60 miRNA families (38 conserved miRNA families represented by 96 miRNA homologs, and 17 novel miRNA families represented by 20 novel miRNAs). Additionally, we identified 60 phasiRNA loci in chickpea. Potential targets for the miRNAs were predicted by bioinformatics analysis. 5′ RACE assays were used for validation of some of the miRNA target genes in chickpea.

2. Materials and methods

2.1. Generation and sequencing of small RNA libraries

The chickpea (*C. arietinum* L.) genotype IC4958 was grown under greenhouse conditions at the International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India and the leaves and flowers from 8 to 9 week-old plants were sampled for analysis. Two different tissue sources will increase the ability to identify as many miRNAs as possible including tissue-specific ones. Other reason is that the small RNA component is more diverse (particularly 24-nt small RNA population is more abundant) in flowers of other plant species (for example, *Arabidopsis* and rice), and we wish to explore whether or not this is the case in chickpea. Total RNA was extracted from leaves and inflorescence by use of Trizol reagent according to the manufacturer's instructions, and small RNA libraries were constructed as described [15]. In brief, total RNA was size-fractionated on denaturing PAGE gel for 1.5 h, and small RNAs in the size range of 21–24 nucleotides were isolated. The isolated small RNAs were sequentially ligated with 3′ and 5′ RNA adaptors using a T4 RNA ligase. The ligated products were then converted into cDNA by use of superscript II reverse transcriptase. A PCR reaction was carried out on the cDNAs and the resulting PCR product with an expected size (93–96 bp) was isolated, purified and sequenced using Illumina GAII analyzer.

2.2. Sequence analysis

Small RNAs were extracted after trimming the adapter sequences from the raw reads. Then the read counts were established for unique small RNAs after removing redundant reads. These unique small RNAs were mapped to ribosomal RNAs, transfer, small nuclear, small nucleolar, RNAs were discarded by mapping to Rfam database (https://www.sanger.ac.uk/resources/databases/rfam.html) and those reads that were mapped to these different RNA categories were discarded. Similarly, reads mapped to messenger RNAs [by mapping to the annotated chickpea mRNAs (http://www.icrisat.org/gt-bt/ICGGC/GenomeSequencing.htm)] were also discarded. The filtered small RNA sequences were searched against the miRBase (www.mirbase.org) in order to identify homologs of the conserved miRNAs in chickpea. The remaining unique reads that could not be mapped to the above-mentioned RNA categories but were mapped to the chickpea genome (http://www.icrisat.org/gt-bt/ICGGC/GenomeSequencing.htm) were used to identify novel miRNAs on the basis of sequencing of miRNA* coupled with a predicted fold-back structure for their precursor sequence extracted from the chickpea genome.

2.3. Small RNA blot analysis

Small RNA blot analysis was performed as described previously [16]. Total RNA was resolved on 12% denaturing polyacrylamide gel and the RNA was transferred to a Hybond-N^+ membrane (GE). The membranes were probed with a p^{32}-labeled antisense oligonucleotide corresponding to the miRNA sequence. After hybridization, the membranes were exposed to phosphorscreen and scanned using Typhoon laser scanner.

2.4. miRNA target prediction and validation using 5′ RACE assay

To predict target mRNAs, the transcripts were searched for miRNA recognition sites by allowing for $\leq$3.5 number of mismatches [17]. In case of conserved miRNAs, the number of mismatches were relaxed slightly. Some of the predicted targets were validated using RLM-RACE assays [16]. Briefly, RNA adapter was ligated to the cleaved mRNAs that possess 5′ phosphate and the ligated product was amplified using a reverse-transcription polymerase chain reaction. The resulting PCR products were cloned and at least 10 independent clones were sequenced for each of the target transcript to determine the cleavage at the predicted site.

2.5. PhasiRNAs/phasiRNA loci identification

The unique small RNA reads were mapped to the genome and cDNA sequences of chickpea using SOAP2 [18]. A recently developed pipeline [19] was used to identify phasiRNA loci in chickpea genome.

3. Results and discussion

3.1. Overview of small RNA population in leaves and flowers of chickpea

The small RNA libraries from two different tissues (leaves and inflorescence/flowers) of chickpea were generated and sequenced by using the Illumina sequencer. These efforts yielded 13,491,628 and 25,259,578 reads from flowers and leaves, respectively. The reads were processed to extract small RNAs ranging from 18 to 29 nt, which resulted in 5,513,070 non-redundant reads (2,294,606 and 3,956,819 from flowers and leaves, respectively) from both the libraries (Table 1). The total small RNA read abundance versus the size distribution analysis in leaves and flowers revealed two peaks corresponding to the 21- and 24-nt sizes (Fig. 1). The peak for unique read abundance was greater for the 24- than 21-nt size, which suggests that the class comprises highly diverse

Table 1
Summary of small RNA libraries analysis in chickpea.

Reads mapped to	Flowers		Leaves	
	Total reads	Unique reads	Total reads	Unique reads
Non-coding RNAs	2,263,549	48,537	4,079,559	63,285
Conserved primary miRNA transcripts	690,461	7611	1,277,790	12,405
Messenger RNAs	857,407	94,565	1,500,531	153,333
Repeat elements	2,482,374	54,085	4,476,089	65,371
Chromosomes	8,495,149	1,471,107	15,377,289	2,394,242
Total	13,491,628	2,294,606	25,259,578	3,956,819

sequences, whereas the 21-nt size class mostly contained redundant sequences of miRNA homologs (Fig. 1).

On mapping small RNAs to various classes of RNAs, approximately 16% of the genome-matching unique reads in both libraries appeared to be degradation products from non-coding RNAs such as rRNAs and tRNAs, which were discarded (Table 1). Furthermore, small RNAs that were mapped to repeat-rich regions (17.96%) were removed. The remaining unique reads were mapped to the miRBase to identify "highly conserved" (conserved across all angiosperms that have been analyzed so far) and "known miRNAs" (mostly lineage-specific miRNAs but also miRNAs known in some of the plant species but not in all angiosperms) in chickpea. This analysis led to the identification of 96 miRNA homologs that can be grouped into 38 miRNA families (Table 2). These 96 miRNA homologs are likely derived from 157 miRNA loci in chickpea. Further we identified 20 novel miRNAs forming 17 families that were supported by miRNA* sequences in small RNA libraries along with fold-back structure predictions for these genomic loci. Sequence analysis of phasiRNAs in our small RNA population led to the discovery of approximately 60 phasiRNA loci in chickpea.

3.2. MicroRNA loci and their expression in leaves and flowers of chickpea

The highly conserved miRNA families showed significant size variation in terms of number of loci in chickpea. Of the highly conserved miRNA families, 19 families were represented by multiple loci and the remaining four families (miR168, miR394, miR397 and miR408) had just one locus in the chickpea genome (Fig. 2). Of the conserved miRNAs with multiple loci, the miR156 family had the most number of loci (17 loci), followed by the miR169 family (15 loci) and miR399 (13 loci), and both miR171 and miR172 had 10 loci each (Fig. 2). By contrast with the conserved miRNA loci, only two

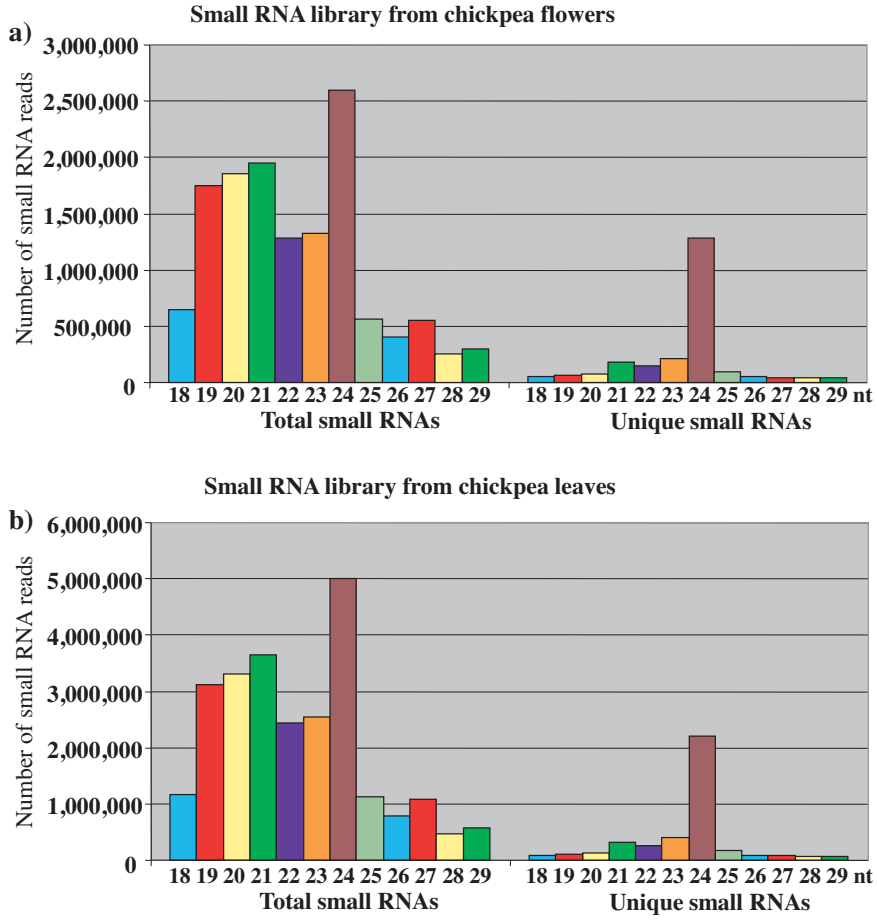

Fig. 1. Length distribution and abundance of small RNAs in chickpea leaves and flowers. Abundance of each size class of small RNAs based on nucleotide (nt) length in flowers (a) and leaves (b) plotted using total small RNA reads and unique reads.

Table 2
Conserved and known miRNAs and their read counts (total and normalized) in leaves and flowers of chickpea. A few miRNAs whose miRNA* are greater in abundances than their corresponding miRNAs are highlighted. (Normalized abundance is denoted as RPTM: reads per 10 million small RNAs.).

miRNA	Sequence	Flowers	Normalized (RPTM)	Leaves	Normalized (RPTM)
miR156a(4 loci)	UGACAGAAGAGAGUGAGCAC	73,481	54,464	130,194	51,542
miR156b(7 loci)	UUGACAGAAGAUAGAGAGCAC	40,758	30,210	70,032	27,725
miR156c	UUGACAGAAGAGAAUGAGCAC	3584	2656	6543	2590
miR156d	UUGACAGAAGAGAGAGAGCAC	1517	1124	2506	992
miR156e	UUGACAGAAGAUAGAGUGCAC	38	28	57	23
miR156f	UGACAGAAUAGAAUGAGCAC	4	3	3	1
miR156h	UGACAGAUAGAAAGUGAGCA	1	1	0	0
miR156i	UGACAGAAGAGAGAGAGCAC	124	92	197	78
miR159a(2 loci)	UUUGGAUUGAAGGGAGCUCUA	2736	2028	5340	2114
miR159c	UUGGACUGAAGGGAGCUC	10	7	26	10
miR159d	UGGACUGAAGGGGAGCUCCUUC	7	5	13	5
miR159e	UGGACUGAAGGGAGCUCCUUC	17	13	17	7
miR159f-2	UUGGACUGAAGGGGCCUCUU	67	50	123	49
miR160a	UGCCUGGCUCCCUGAAUGCCA	14	10	12	5
miR160b(3 loci)	UGCCUGGCUCCCUGUAUGCCA	494	366	825	327
miR162a	UCGAUAAACCUCUGCAUCCGG	73	54	136	54
miR162b	UCGAUAAACCUCUGCAUCCAG	42	31	64	25
miR164a	UGGAAAAGUGGAGCACGUGCA	40	30	57	23
miR164b	UGGAGAAGCAGGGCACAUGCU	8	6	8	3
miR164c (3 loci)	UGGAGAAGCAGGGCACGUGCA	33,978	25,185	59,602	23,596
miR164d	UGGAGAAGCAGGACACGUGCA	304	225	480	190
miR166a (5 loci)	UCGGACCAGGCUUCAUUCCCC	80,211	59,452	151,125	59,829
miR166d (4 loci)	UCGGACCAGGCUUCAUUCCUC	6130	4544	11,463	4538
miR166e	UCGGACCAGGCUUCAUUCCCG	8071	5982	15,510	6140
miR166f	UCGGACCAGACUUCAUUCCCC	38	28	63	25
miR167a	UGAAGCUGCCAGCAUGAUCU	249	185	475	188
miR167b	UGAAGCUGCCAGCAUGAUCUA	1347	998	2779	1100
miR167c	UGAAGCUGCCAGCAUGAUCUAA	2658	1970	5060	2003
miR167d (2 loci)	UGAAGCUGCCAGCAUGAUCUGA	4159	3083	7636	3023
miR167e	UGAAGCUGCCAGCAUGAUCUUA	1056	783	1907	755
miR167f	UGAAACAGCCGCAUGAUCUAA	0	0	3	1
miR167g	UGAAGUUGCCGCAUGAUCUAU	2	1	3	1
miR168	UCGCUUGGUGCAGGUCGGGAA	5343	3960	10,375	4107
miR169a (5 loci)	CAGCCAAGGAUGACUUGCCGG	977	724	1723	682
miR169b	UAAGCCAAGGAUGACUUGCCUA	2	1	11	4
miR169d	UAGCCAAGGAUGACUUGCCUA	7	5	16	6
miR169e (4 loci)	CAGCCAAGGUGAUUUGCCGG	0	0	2	1
miR169f	UGAGCCAGGGAUGACUUGCCG	6	4	5	2
miR169g	UGAGCCAGGAUGACUUGCCGG	15	11	19	8
miR169h	AAGCCAAAGAUGACUUGCCGA	0	0	3	1
miR169i	AGCCAAGGAUGACUUGCCGG	53	39	105	42
miR171a (5 loci)	UGAUUGAGCCGUGCCAAUAUC	362	268	653	259
miR171b	UUGAGCCGCGUCAAUAUCUCG	565	419	1082	428
miR171c	UUGAGCCGCGUCAAUAUCUUG	44	33	104	41
miR171d	UUGAGCCGUGCCAAUAUCAC	23	17	27	11
miR171e	UUGAGCCGCGCCAAUAUCACU	4	3	7	3
miR171f	UGAUUGAGUCACGCCAAUAUC	6	4	2	1
miR172a (2 loci)	AGAAUCUUGAUGAUGCUGCA	626	464	1152	456
miR172b (5 loci)	AGAAUCUUGAUGAUGCUGCAU	103,280	76,551	184,900	73,200
miR172c	CGAAUCCUGAUGAUGCUGCAG	13	10	42	17
miR172d	UGAAUCUUGAUGAUGCUGCA	21	16	43	17
miR172e	UGAAUCUUGAUGAUGCUACAU	1	1	2	1
miR319a	AGAGCUUUCUUCGGUCCACUC	263	195	459	182
miR319b (2 loci)	UUGGACUGAAGGGAGCUCCCU	58	43	112	44
miR319c	UUGGACUGAAGGGAGCUCCC	63	47	130	51
miR390a (2 loci)	AAGCUCAGGAGGGAUAGCGCC	139	103	257	102
miR390b	AAGCUCGGGAGGGAUAGCGCC	355	263	733	290
miR393a (2 loci)	UCCAAAGGGAUCGCAUUGAUCC	21	16	32	13
miR393b	UCCAAAAGGAUAGCAUGAUCC	2	1	0	0
miR394a (4 loci)	UUGGCAUUCUGUCCACCUCC	272	202	559	221
miR395a (3 loci)	UGAAGUGUUUGGGGGAACUCU	2	1	4	2
miR395b (3 loci)	UGAAGUGUUUGGGGGAACUC	0	0	2	1
miR395c	UGAAGUGUUUGGGGGAACAC	0	0	1	0
miR396a	UUCCACAGCUUUCUUGAACUU	1437	1065	2610	1033
miR396a*	**GCUCAAGAAAGCUGUGGGAGA**	**5750**	**4262**	**10,992**	**4352**
miR396b	UUCCACAGCUUUCUUGAACUG	716	531	1216	481
miR397	UCAUUGAGUGCAGCGUUGAUG	110	82	185	73
miR398a	UUGUGUUCUCAGGUCACCCCU	15	11	22	9
miR398a*	**GAGUGAAUCUUAGAACACAAGA**	**51**	**38**	**102**	**40**
miR398b (2 loci)	UGUGUUCUCAGGUCGCCCCUG	15	11	23	9
miR399a	UGCCAAAGGAGAGUUGUCCUG	0	0	3	1
miR399b	UGCCAAAGGAGAUUUGCCCUG	2	1	1	0
miR399c (2 loci)	UGCCAAAGGAGAGCUGCCCUG	4	3	4	2
miR399d (2 loci)	UGCCAAAGGAGAUUUGCCCAG	20	15	26	10
miR399e	UGCCAAAGGAGAUUUGCUCAG	2	1	3	1

Table 2 (*Continued*)

miRNA	Sequence	Flowers	Normalized (RPTM)	Leaves	Normalized (RPTM)
miR399h (4 loci)	UGCCAAAGGAGAGUUGCCCUG	7	5	13	5
miR399i	UGCCAAAGAAGAUUUGCCCCG	18	13	38	15
miR399j	UGCCAAAGGAGAGCUGCUCUU	0	0	2	1
miR408	AUGCACUGCCUCUUCCCUGGC	303	225	464	184
miR408*	**CAGGGAACAGGCUGAGCAUGG**	**1081**	**801**	**1783**	**706**
miR482	UUACCAAUUCCGCCCAUUCCUA	34	25	63	25
miR530	AGGUGCAGAUGUAUAUGCAGG	33	24	46	18
miR828	UCUUGCUCAAAUGAGUAUUCCA	0	0	10	4
miR1507	UUUCAUUCCAUACAUCGUCUAA	174	129	303	120
miR1509	UUAAUCAGGGAAAUCACAGUUG	903	669	1454	576
miR1511	AACCAGGCUCUGAUACCAUGA	1500	1112	2767	1095
miR1514	UUCAUUUUUAAAAUAGGCAUUG	330	245	580	230
miR2111a	GUCCUCGGAAUGCAGAUUAUC	72	53	124	49
miR2111b (5 loci)	UAAUCUGCAUCCUGAGGUUUA	61	45	89	35
miR2111c	UAAUCUGCAUCCUGAGGUGUA	3	2	1	0
miR2118	UUACCGAUUCCACCCAUUCCUA	1829	1356	3133	1240
miR2199	UGAUACACUAGCACGGAUCAC	903	669	1653	654
miR4376	UACGCAGGAGAGAUGAUGCCA	116	86	223	88
miR4416	UACAUGUCGCUCUCACCUGGA	366	271	591	234
miR5213-5p	UACGGGUGUCUUCACCUCUGA	817	606	1557	616
miR5234a (3 loci)	UUGUUGUGGAUGGCAGAAGAU	2	1	4	2
miR5234b (2 loci)	UGGUUUUGUUGUGGAUGGCAG	17	13	41	16
miR5770	UUAGAACUAUGGUUUGGACAA	15	11	38	15
miR4414a-3p	AACCAACGAUGCAGGAGCUGC	265	196	437	173
TAS3a.D8(+)	UUCUUGACCUUGUAAGACCUU	50	37	80	32
TAS3a.D7(+)	UUCUUGACCUUGUAAGACCUC	255	189	432	171
TAS3a_D5(+)	UUUUUGCUUUUGUGGAAGACA	1520	1127	2720	1077

miRNA families (miR2111 and miR5234) that belong to the "known miRNAs" had multiple loci (Table 3).

Because small RNAs were sequenced independently from leaves and flowers, the differences in miRNA abundance between these two tissues can be gauged. However, the normalized abundance of miR156, miR166, miR159, miR160, miR164, miR166, miR167, miR172 and miR396 revealed only minor differences between leaves and flowers (Table 2). These results were further confirmed using small RNA blot analysis of most of the conserved miRNA families (Fig. 3). Interestingly, the most abundantly expressed miRNA families had similar levels between leaves and flowers (i.e., most abundantly expressed family in leaves was also the most abundantly expressed miRNA family in flowers and vice versa). The top most abundantly expressed miRNA families included miR156, miR172, miR166 and miR164 in both tissues. Of note, miR6300 (annotated in miRBase) is represented by two isoforms (miR6300a and miR6300b) in chickpea. In flowers and leaves, miR6300 was the most abundantly expressed family with frequencies of 151,282 and 150,857 (reads per ten million-RPTM) in flowers and leaves, respectively for miR6300a, and 15,992 and 15,781 (RPTM), respectively, for miR6300b. However, the annotated miR6300 (from soybean) is ambiguous and needs experimental confirmation. Thus, we did not include these sequences in our list of miRNAs in chickpea. Similarly we found the miR405 homolog of *Arabidopsis* in chickpea libraries, but the annotation in *Arabidopsis* is still questionable, so we disregarded the annotation of this sequence in chickpea.

The abundance of several "highly conserved miRNA" families such as miR393, miR395, miR398, miR399, and "known miRNAs"

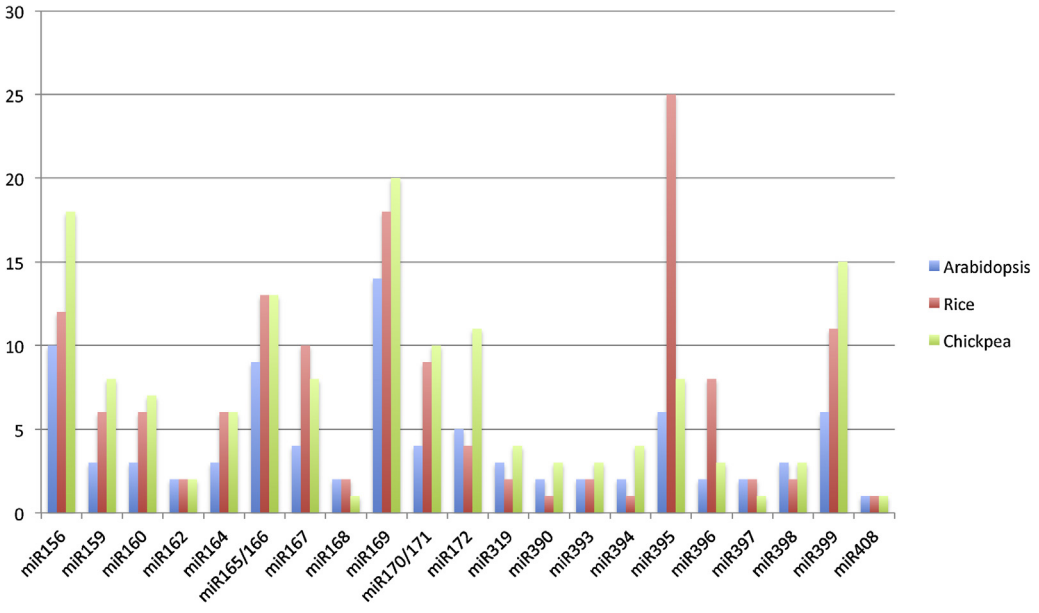

Fig. 2. Conserved miRNA family size in chickpea.

Table 3
Identified novel miRNAs and their normalized abundances in leaves and flowers of chickpea.

Novel Family	miRNA#id	miRNA sequence	Flowers	Normalized (RPTM)	Leaf	Normalized (RPTM)	miRNA* sequence
CauN#1	CauN#1	UUGUAUUUAUAUUACACAUGC	26	19	35	14	AUGUGUAAUAUAGAUGCAAAA
CauN#2	CauN#2	ACGACUGUUACAUCAUACAAC	29	21	73	29	UGUAUGGUGCAACAGUCGCAG
CauN#3	CauN#3	UAAAAUGGAAACAGCAGGAAA	19	14	14	6	UUCUGCUGUUUCCAUUUUAGC
CauN#4	CauN#4	UAACUAUUGCAGAAGAUCUAA	39	29	56	22	UAGAUCUUCUGCAAUAGUCAU
CauN#5	CauN#5	UAGCGACACGGAACGUCCAAC	5	4	11	4	UGGACGCUCCGUGCCACUAUC
CauN#6	CauN#6	UGAGGGAAGGUAUGGUUCCAC	29	21	35	14	GUAACCAUACCUUCUCUUAU
CauN#7	CauN#7	UGUUCCUCUUAUUUUAGCUGA	9	7	18	7	ACUAAAAUAAAGGGAUCAAA
CauN#8	CauN#8	UCAACCGGCAUCACAGCCAAC	333	247	589	233	UGGCUGUGAUGCCGGUUGAGG
CauN#9	CauN#9	UCCACUGAGGAAGAAGAAGCC	1446	1072	2734	1082	CUUCUUCUUCUUCAGUGGAUG
CauN#10	CauN#10	CUGUAGCAUCACUAUAGCCGC	21	16	37	15	GGCUAUAGUGGCGCUAUAGCG
CauN#11	CauN#11	GAAAUUGUAGUACUUUGUGAGGGC	22	16	41	16	CCUCACAAAAGACUACAAUUUCAG
CauN#12	CauN#12	UUUGUGAGUAGACCAAACCAAA	216	160	461	183	UUGGCUUGGUCUAUUGCAAAC
CauN#13	CauN#13a, b (2 loci)	UUCAGGUUUGUGAGAAAGGCU	15	11	33	13	CUUUCUCACAAACCUGAACAC
CauN#14	CauN#14a	CAAGAUUAUUAUAUAUAUCUUG	56	42	101	40	AGAUAUAUAUGAUUGUCUUAAG
	CauN#14b	CAAGAUUAUUGUAUAUAUCUUG	101	75	203	80	AGAUAUAUAUGAUUGUCUUAAG
CauN#15	CauN#15	UAGAAUUAGACAUAAAAGCAGAU	19	14	47	19	UGCUUGUGUCUGAUUCUAAA
CauN#16	CauN#16a, b (2 loci)	UUUUUGUAGUGUAUAUCCCAAA	2697	1999	5453	2159	UGGUUGUAUACUACAAAAAUA
CauN#17	CauN#17	AAGGGUCUGUUUGAGAGAAGUGGU	5996	4444	10,870	4303	CACUUUUUUCAAACAGUCCCCGAG

such as miR5234, miR5770 and miR828 was low both in flowers and leaves. miR395 and miR399 are induced during sulphate- and phosphate-deprived conditions, respectively in diverse plant species [2,20–22]. Similarly, miR393 and miR398 expression is also low in several plant species.

3.3. Differential expression of conserved miRNA isoforms or variants in chickpea

Most conserved miRNA families originate from multiple loci, and often their sequences slightly differ by 1 or 2 nt within the

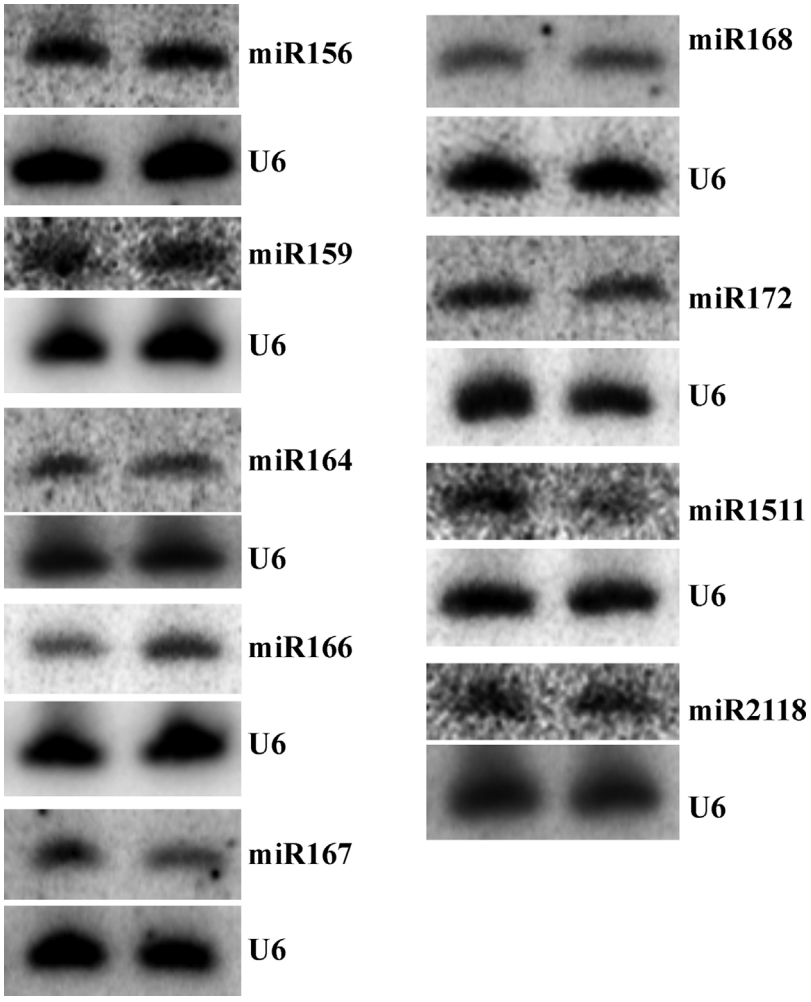

Fig. 3. Small RNA blot analysis of conserved miRNAs in flowers and leaves of chickpea.

canonical 21-nt sequence generating miRNA isoforms. As well, miRNA variants or isoforms occur from processing differences at their 5′ or 3′ ends that result in the addition or deletion of nucleotides. Investigating such incidences revealed the abundance of individual miRNAs within a family and within a tissue can be similar as well as distinct. For instance, the miR172e is barely detected in leaves and flowers whereas miR172b normalized frequencies ranged between 73,200 and 76,551 RPTM, respectively in flowers and leaves (Table 2). Similarly, the miR156 family is represented by eight miRNA isoforms, all of which showed distinct expression in chickpea (Table 2); miR156a is the most highly expressed, with approximately similar abundance in flowers and leaves (54,464 and 51,542 RPTM, respectively), followed by miR156b (30,210 and 27,725 RPTM, respectively); miR156c and miR156d have moderate abundance (2500–1000 RPTM), but miR156f and miR156h were barely expressed (Table 2). Similarly, of the eight miR169 isoforms, miR169a had greatest abundance, whereas the levels of the remaining seven isoforms (miR169b, d, e, f, g, h and i) was very low both in leaves and flowers. miR172 has five isoforms and only miR172b (with five loci in the genome) is highly expressed, followed by miR172a. miR172 targets AP2 factors that play an important role in flower development in *Arabidopsis* [23]. A high abundance of miR172 in chickpea flowers suggests a similar role as in *Arabidopsis*. Surprisingly, miR172 abundances in leaves are as high as in flowers (Table 2 and Fig. 3) suggesting that miR172 may be critical for normal flower development and other unidentified processes in leaves. The differential expression of family members likely fine-tunes targeted mRNA expression in a tissue- or cell-specific manner.miR828 homologs have been reported from various dicot species and a few gymnosperms (spruce and pine) (miRBase). In chickpea, miR828 levels were extremely low (4 RPTM) in flowers and could not be detected in leaves (Table 2). The 22-nt miR828 in *Arabidopsis* guides TAS4 transcript cleavage and triggers the production of trans-acting small interfering RNAs (tasiRNAs) [24–26]. A few other miRNA families also represented by 21-nt and 22-nt isoforms. For instance, three of the miR167 family (miR167c, d and e) are 22 nt and their expression was greater than the 21-nt isoforms (Table 2). Similarly, one of the miR169 isoforms (miR169b) is also 22 nt. The central feature of these 22-nt long miRNAs is that they trigger secondary siRNA biogenesis at the target locus [21,22]. Determining whether these 22-nt miRNAs can generate secondary siRNAs in chickpea needs further study.

Hu et al. [10] predicted miR5287, miR845, miR539, miR5021, miR1533 and miR414 as miRNAs in chickpea. However, the present study and an earlier study [11] did not find homologs of these miRNAs in the sequenced small RNA libraries of chickpea suggesting that these may not be miRNAs.

3.4. Greater abundance of miRNA* species relative to their miRNAs in chickpea

miRNA biogenesis produces equal abundance of the 5′and 3′ fragments (one being miRNA that mostly begins with U and has weak base-pairing at its 5′ end and the other being the miRNA* strand) from the miRNA precursor transcripts. Of these miRNA:miRNA*duplexes, miRNA* sequences are less stable and non-functional as compared with the miRNA that is stabilized when loaded into the RISC complex and regulates the expression of RNA targets. Remarkably, some miRNA* species accumulate to detectable levels and even silence the target mRNAs [27–29]. For instance, in *Arabidopsis*, a *SNARE* gene is targeted by miR393* [30] and a *Su (VAR)-3-9 homolog8* [31] is targeted by miR171*. Likewise, miR169* targets *BCP1* transcripts in *M. truncatula* [32]. We found that miRNA* species corresponding to miR396, miR398 and miR408 were at least threefold more abundant than their miRNA counterparts (Table 2 and Fig. S2). Intriguingly, a recent study also found greater expression of miR408* than miR408 in sacred lotus [29]. Further studies will reveal whether these miRNA* molecules have gene-regulatory functions in chickpea.

Supplementary Fig. S2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.03.002.

3.5. Novel miRNAs in chickpea

Besides the conserved miRNAs, plant species generate novel miRNAs that are species- or lineage-specific. Sequencing miRNA* sequences along with the predicted hairpin-like structure for primary miRNA transcripts is essential for annotating novel miRNAs in plants [33]. By strictly adhering to these criteria, we identified 20 novel miRNAs representing 17 novel miRNA families in chickpea (Table 3; Fig. S1). Of the three novel miRNA families (CauN#13, CauN#14 and CauN#16) with two loci each, only Cau#14 is represented by two isoforms (CauN#14 a and CauN#14b) that differed at position 11 from the 5′ end of the miRNA (Table 3). Most of the novel miRNAs had low expression, but some of the novel miRNA levels were comparable to that for conserved miRNAs that are expressed at moderate levels. For instance, CauN#9 had 1082 and 1072 RPTM in leaves and flowers, respectively, and CauN#16 had 2159 and 1999 RPTM, respectively (Table 3). The very high level expression of some of the novel miRNAs implies that they may have critical roles in chickpea development or other physiological processes. Conserved miRNAs are thought to be critically important for various developmental processes, whereas the recently evolved or evolving novel miRNAs could be important for species-specific gene regulatory functions [34,35].

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.03.002.

3.6. MicroRNA diversity in chickpea versus other legumes

Leguminous plants appear to have evolved lineage-specific miRNAs. Nevertheless, earlier studies revealed that not all of those miRNAs are present in all legumes investigated so far. Scrutiny of miRNAs in chickpea revealed that some of the miRNAs present in other legumes are absent in chickpea or vice versa. For instance, miR828 was reported only from soybean (miRBase) but not from any other legume. We found miR828 in chickpea (Table 2). Most importantly, miRNA homologs such as miR1507, 1508, 1509, 1510, 1512, 1514, 1520, 1521, 2086, 2109, 2119, 2199, 4414, 5213, 5232, 5234 and 5770 were mostly found in legumes such as *M. truncatula*, *G. max*, *G. soja*, *Lotus japonicus*, *Phaseolus vulgaris*, *Vigna unguiculata*, and *Acacia auriculiformis* (miRBase) (Table 4). Of these miRNAs, 1507, 1509, 1514, 2199, 4414, 5213, 5232, 5234 and 5770 were found in chickpea (Table 2). A recent study [11] have found miR2111, miR2118, miR5213 and miR5232 in chickpea and the authors report them as legume-specific miRNAs. We found all of these four miRNAs, except miR5232 in our small RNA libraries. However it is of note that miR2111 and miR2118 were not only found in legumes but also in plant species other than the legumes. For instance, miR2111 in *Arabidopsis thaliana*, *A. lyrata*, *Brassica napus*, *Vitis vinifera*, *Populus trichocarpa*, *Cucumis melo*, *Malus domestica* and *Manihot esculenta*, whereas miR2118 in rice, maize, sorghum, apple, *Brachypodium* and *Aegilops tauschii* were reported (www.miRBase.com) suggesting that miR2111 and miR2118 are not specific to legumes. Our small RNA analysis is not comprehensive and was limited to two tissue sources; therefore, analysis of small RNA populations in other tissues and under biotic and abiotic stress conditions could reveal whether other miRNAs mostly found in legumes are also expressed in chickpea.

Table 4
Distribution of potential legume-specific miRNAs in chickpea.

miRNA id#	*Mtr*	*Gma*	*Cau*	Other legumes
1507	■	■	■	*Lja, Vun*
1508		■		*Gso*
1509	■	■	■	*Gso*
1510	■	■		*Gso*
1512		■		
1514		■	■	*Pvu*
1520		■		
1521		■		
2086	■			*Acacia Sps*
2109		■		*Gso*
2199	■		■	
4414	■	■	■	
5213	■		■	
5232	■			
5234	■		■	
5770		■	■	

Cau, *Cicer aurietinum*; *Gma*, *Glycine max*; *Gso*, *Glycine sojae*; *Lja*, *Lotus japonicus*; *Mtr*, *Medicago truncatula*; *Pvu*, *Phaseolus vulgaris*; *Vun*, *Vigna unguiculata*.

3.7. Trans-acting small interfering RNAs in chickpea

TasiRNAs are derived from TAS transcripts as a result of miRNA-guided cleavage on the target transcripts. TasiRNAs can determine leaf patterning and adaxial–abaxial polarity in *Arabidopsis* [36,37]. The most conserved TAS gene in plants is *TAS3*-tasiRNA, which is targeted by miR390. Two variants or isoforms of miR390 are present in chickpea; miR390a (two loci) and miR390b. TAS3-siRNA targeting auxin response factors (ARFs) were recovered in both tissue libraries of chickpea (Fig. 4 and Table 5). TAS3-derived tasiRNA targets ARF2, 3 and 4, the transcription factors involved in auxin response, and hence influences many developmental traits.

3.8. phasiRNAs

The 22-nt miRNAs (miR1507, 1508, 1509, 1510, 1514, 2109, 2119, 2199 and miR472/miR482/miR2118) function in triggering the 21-nt phasiRNA biogenesis at the target loci once the target transcript is cleaved [3,12,13]. Some of these phasiRNAs in turn could regulate specific targets, like tasiRNAs [12]. Remarkably, several of these 22-nt miRNAs (miR1507, miR1509, miR1514, miR482 and miR2118) have been found and were expressed at

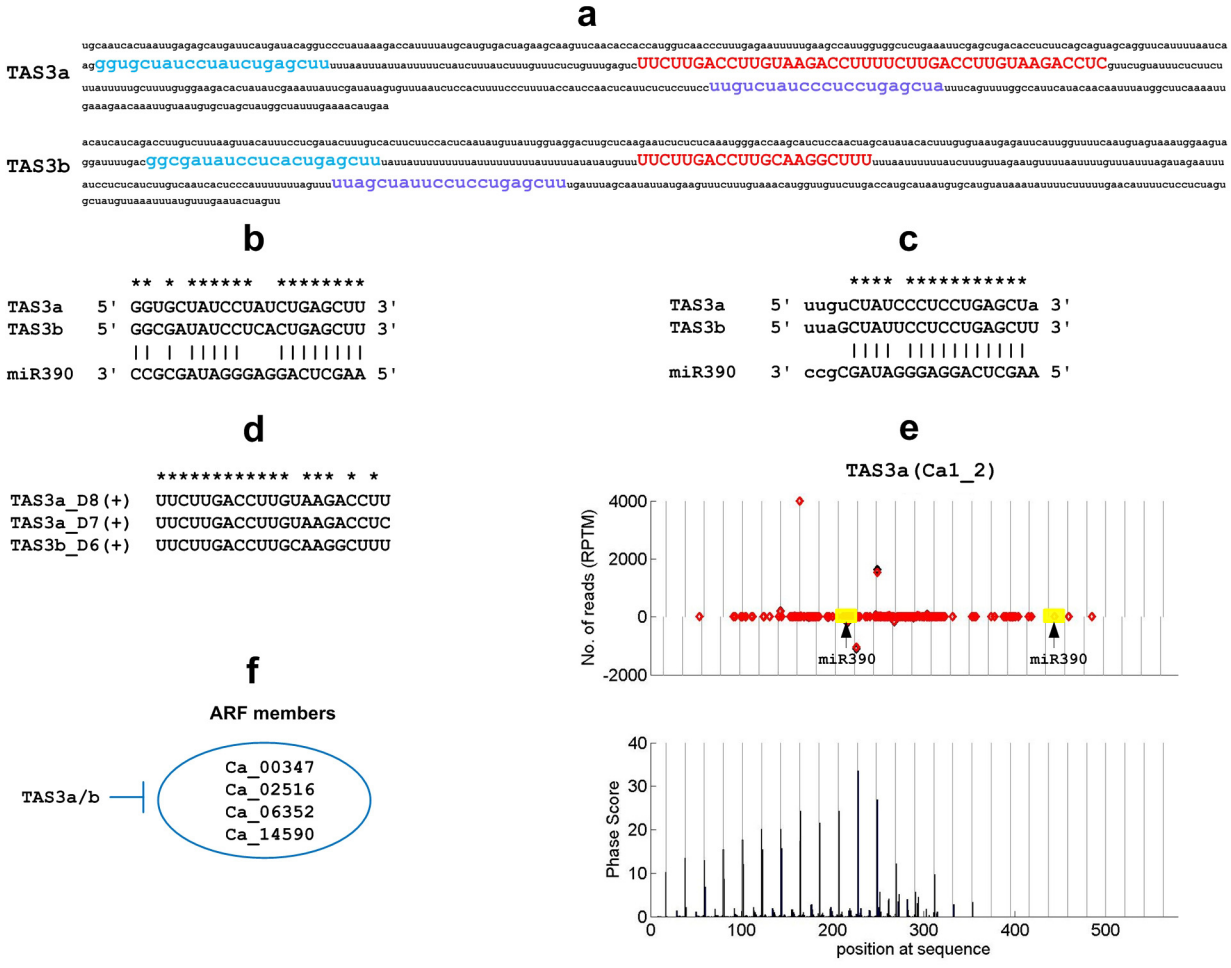

Fig. 4. TAS3 loci and predicted targets of tasiRNAs in chickpea. (a) The nucleotide sequences of the two TAS3 loci in chickpea. The blue and purple regions are 5′ and 3′ miR390 complementary sites, respectively. The regions of upper case nucleotides are mature tasiRNAs that target ARF transcripts (b) The 5′ miR390 binding sites on TAS3 transcripts. (c) The 3′ miR390 binding sites on TAS3 transcripts. (d) The mature tasiRNAs that target ARF family members derived from TAS3a/b loci. (e) The reads distribution and phase score of TAS3a. The black and red diamonds represent the number of 21 nt long reads, vertical axis, that appeared at the position of the phasi loci and horizontal axis, in the flower and leaf small RNA libraries, respectively. The vertical gray lines with distances of 21 nt are the phased positions from the position with highest phase scores of the PHAS loci. The yellow boxes in the read distribution panel represent the miR390 complementary sites. (f) The predicted targets of tasiRNAs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 5
Summary of predicted miRNA targets in chickpea.

miRNA family	Target gene family	Number of genes
miR156/157	Squamosa promoter-binding proteins	10
miR159/319	MYB transcription factors	2
miR159/319	TCP transcription factors	3
miR160	Auxin Response factors	3
miR162	Dicer Like protein	1
miR164	NAC domain protein	4
miR165/166	Homeo domain-Zip transcription factors	4
miR167	Auxin response factors	3 (4)
miR168	Argonautes	2
miR169	HAP2/NFY transcription factors	3
miR170/171	Scarecrow-like transcription factors	2
miR172	AP2 domain transcription factors	2
miR390/391	TAS3-primary transcripts	2
miR393	F-Box protein	1
miR394	F-Box protein	1 (4)
miR395	ATP sulfurylases	2
miR395	sulfate transporters	2
miR396	Growth regulating factors	4
miR397	Laccases	9
miR398	Cu/Zn superoxide dismutases (CSD)	3
miR398	Copper chaperone for the CSD	1
miR399	Phosphate transporter	1 (4.5)
miR399	Ubiquitin-conjugating enzymes (E2 ligase)	2
miR408	Plantacyanins	2
miR1507	NBS-LRR disease resistance proteins	8 (2 of them with 4 mismatches)
miR1511	Ca_19634-unannotated protein	1
miR1514	bZIP transcription factor, PPR protein, neuroblastoma-related protein, exocyst complex protein	1 each
miR2111	JmjC-domain containing protein, Kelch-like repeat containing protein, neuroblastoma-related protein, and receptor-like protein kinase and a protein phosphatase	1 each
miR2118	NBS-LRR disease resistance proteins	2
miR5213-5p	Methyl transferase-like protein, mitochondrial Rho GTPase, TIR-NBS-LRR disease resistance protein	1 each
miR5234b	Lin-like protein	1
miR5770	Amine oxidase and ATP-dependent RNA helicase	1 each
TAS3-siRNA	Auxin response factors	7

Number in parenthesis indicates if mismatches in target complementary sites exceeds 3.5.

moderate levels in the tissues analyzed in chickpea. Some (miR1507 and miR2118 have been predicted to target 7 and 2 NBS-LRR genes, respectively) of these were predicted to target NBS-LRR genes (Table 4). The normalized abundance of miR2118 in chickpea was greater than that for other miRNA families of this class (Table 2).

The phylogenetic distribution of miR2118/miR482/miR472 miRNAs is highly variable among different plant species. miR2118 is present in *Gingko* and Norway spruce (gymnosperms), which suggests an early evolution and ancient regulatory mechanism of these phasiRNAs in plants [3]. Both miR2118 and miR482, but not miR472, have been found in chickpea (Table 2). The identification of the 482/2118 family and their target sites on NBS-LRR transcripts in chickpea suggests that their interaction could generate phasiRNAs from their RNA targets. At least one such Phas-NBS-LRR locus (Phas_ca-6-1; Tables S1 and S2) targeted by miR2118 was found in chickpea. There may be additional NBS-LRR phasiRNA loci but because the chickpea genome annotation is not comprehensive, we were unable to perform this analysis extensively.

Supplementary Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.03.002.

Supplementary Table S2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.03.002.

PhasiRNA-generating loci are named phasi genes. We found several phasi genes targeted by the typical 22-nt miRNAs such as miR2118 and miR828 (two miR2118 target sites on Phas_Ca6_1 [the target locus encodes NBS-LRR gene], miR828 site on Phas_Ca4_4; Tables S1 and S2). As well, in a few other instances, 21-nt canonical miRNAs such as miR156, miR393, miR5021 could trigger the biogenesis of phasiRNAs (Table S1). Secondary siRNA biogenesis at these loci is known in plants [3,38].

Besides targeting *NBS-LRR* genes, some of these miRNAs regulate the expression of other protein-coding genes. For instance, in *M. truncatula*, *DCL2* genes are cleaved by the 22-nt miRNA miR1507 and produce secondary phasiRNAs [3]. In soybean, miR2118 targets *GmSGS3a* transcripts, one of the main enzymes involved in TAS3-derived tasiRNA biogenesis [3]. Whether miR1507 and miR2118 in chickpea also target these genes needs further analysis.

3.9. Prediction of putative targets for the chickpea miRNAs and their confirmation

miRNA functions can be deduced by identifying their targets that function in specific biological pathways or processes. Plant miRNA:target predictions are based on the near-perfect complementarily of miRNA sequences with their target mRNAs [17,35]. To predict mRNA targets for miRNAs in chickpea, we performed BLAST searches of annotated transcripts using miRNA sequences and allowing $\leq$3.5 mismatches [17]. Targets were predicted for most of the conserved miRNAs in chickpea and included transcription factors such as squamosa promoter binding (SBP) transcription factors, MYB transcription factors, TCP factors (teosinte branched 1, cycloidea, PCF [TCP]-domain protein family), NAC (NAM, ATAF, and CUC) domain-containing transcription factors, ARFs, Nuclear transcription factor Y (NF-Y) transcription factors, scarecrow-like (SCL) transcription factors, apetala2 (AP2)-like transcription factors and growth regulating factors (GRFs) [17] (Table 5). F-box proteins involved in protein degradation are predicted targets for miR393 and miR394 in chickpea (Table 4). Argonaute 1-like for miR168, plantacyanin and laccase for miR408, and ubiquitin-conjugating

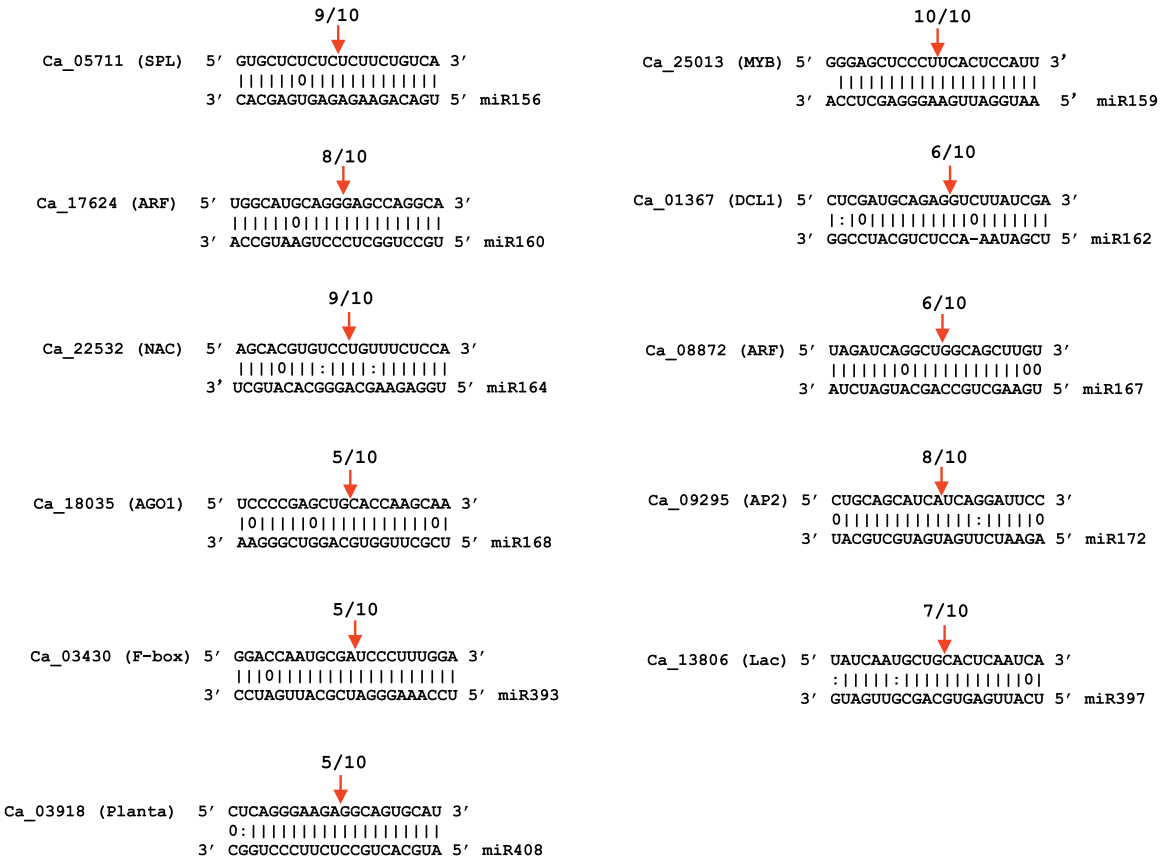

Fig. 5. Validation of conserved miRNA targets in chickpea using modified RACE assays. The alignments include messenger RNA and miRNA. The perfectly complementary matches are indicated with straight lines, mismatches with colons, and G-U wobbles with a circle. The fraction shown in parenthesis is number of cloned products that terminated at the predicted cleavage site.

enzyme for miR399 are among the predicted targets [18] (Table 5). We validated several conserved miRNA targets in chickpea by 5′ RACE assay (Fig. 5). Most of the miRNA-directed cleavages on the target transcripts were found at the predicted cleavage sites (between nucleotides 10 and 11 from the 5′ end of the miRNA) [2]. These targets include SPB factor (targeted by miR156), MYB transcription factor (targeted by miR159), ARFs (targeted by miR160), DCL1 (targeted by miR162), HD-Zip (targeted by miR164), Argonaute 1 (targeted by miR168), AP2 (target of miR172), F-box protein (target of miR393) and plantacyanin (targeted by miR408) (Fig. 5).

Interactions of plant miRNA families and target gene families are often characterized as one miRNA family targeting one target gene family, with only a few exceptions such as miR395 targeting sulfate assimilation genes (*APS*) and a sulfate transporter (*AST68*) or miR399 targeting phosphate transporter as well as UBC24 or miR398 that targets *Cu/Zn SODs* and a *CCS1* gene [9]. One common theme in all these cases is that despite one miRNA targeting two distinct target gene families, both target gene families function in the same biochemical pathway [34]. In contrast, six distinct miRNA families – miR1507, miR1509, miR1510, miR1515, miR2109, and miR2118 – target only one target gene family (i.e., *NBS-LRR* genes in legumes [2,3,12,13]) and several of these miRNAs have been identified in chickpea.

Several of the predicted targets for the conserved miRNAs are thought or shown to function in regulating diverse developmental processes. For instance, SPL transcription factors (miR156 targets) regulate phase transition in plants, especially from the juvenile to adult phase [39]. Surprisingly, miR156 levels were abundantly expressed in flowers of chickpea. Such observations were noted earlier in switchgrass and sorghum [20,22]. The conserved expression of miR156 in flowers or inflorescence suggests additional but yet-unidentified roles for this miRNA in plants. By targeting *TCP* factors, miR319 controls leaf morphogenesis in *Arabidopsis* [40]. Auxin, a major plant hormone, regulates plant growth and development, and a few components such as TIR1 (auxin receptor) and ARFs that are part of the auxin signaling pathway are targets for different miRNA families. miR393 targets mRNAs encoding TIR1 and other closely related F-box proteins [17], and the transcripts of ARFs are targeted by miR160 and miR167 [6].

3.10. Potential functions of chickpea small RNAs in nodulation

miRNAs in legumes function in nodulation as well [32,40–42]. miR169 regulates the meristem maintenance and bacterial release in nodules by controlling the spatial distribution of the HAP2-1 transcription factor [43]. miR166 controls *HD-ZIPIII* expression, which regulates meristem activity and vascular differentiation in nodules [44,45]. *Bradyrhizobium japonicum* inoculation induced the accumulation of miR393 in soybean roots, which suggests an important role for this miRNA in nodulation [40]. Besides the conserved miRNAs, other less-conserved miRNAs identified in chickpea appear to play important regulatory roles in nodulation in other legumes. For instance, in roots inoculated with the symbiotic bacterium *B. japonicum*, miR1511 targets transcripts encoding a phosphatase 2C and is related to nodule development [42]. miR482 might play a role in nodulation by regulating *NBS-LRR* genes in soybean, besides being involved in plant–pathogen resistance

[42]. Similarly, miR4416 in soybean is predicted to be associated with nodulation because it is highly expressed in nodules [46]. Likewise, miR1507 is nodulation-responsive [46]. Because most *PHAS* loci targeted by miR1507 and miR2118 encode NBS-LRR resistance proteins, their role in disease resistance as well as nodular symbiotic interactions in legumes is expected. Taken together, several miRNAs known to be involved in nodulation are found in chickpea. Identification of several miRNAs in chickpea that play a role in nodulation in other legumes suggests that the identified miRNAs might play similar roles in chickpea.

4. Conclusions

In summary, we identified 96 known miRNA homologs (38 miRNA families) in chickpea. Most importantly, we uncovered the differential abundance of miRNA variants or isoforms and differences in terms of the presence or absence of some of the miRNAs that target NBS-LRR genes in chickpea. Additionally, we uncovered 20 novel miRNAs in 17 novel miRNA families in chickpea as well as approximately 60 phasiRNA loci. Target genes for the conserved miRNAs have been predicted and some validated. Thus, we provide a better understanding of miRNA-guided posttranscriptional regulation in chickpea and a good resource for the comparative analysis of miRNA and phasiRNAs among legumes.

Acknowledgements

This research was supported by the Oklahoma Agricultural Experiment Station to RS and by the National Natural Science Foundation of China (grant no. 31460295) and a start-up grant (no. 14078285) of Kunming University of Science and Technology, China, to YZ. SS and VH acknowledge a DBT-overseas postdoctoral fellowship, Government of India, and a UGC postdoctoral fellowship, Government of India, respectively. HK acknowledges a DST-INSPIRE fellowship, Government of India.

References

[1] G. Szittya, S. Moxon, D.M. Santos, R. Jing, M.P. Fevereiro, et al., High-throughput sequencing of *Medicago truncatula* short RNAs identifies eight new miRNA families, BMC Genomics 9 (2008) 593.
[2] G. Jagadeeswaran, Y. Zheng, Y. Li, L. Shukla, J. Matts, et al., Sequencing of a small RNA library from *Medicago truncatula* revealed four families of novel legume-specific and candidate microRNAs, New Phytol. 184 (2009) 85–98.
[3] J. Zhai, D.H. Jeong, E. De Paoli, et al., MicroRNAs as master regulators of the plant NB-LRR defense gene family via the production of phased, trans-acting siRNAs, Genes Dev. 25 (2011) 2540–2553.
[4] R.K. Varshney, C. Song, R.K. Saxena, et al., Draft genome sequence of chickpea *Cicer arietinum* provides a resource for trait improvement, Nat. Biotechnol. 31 (2013) 240–246.
[5] M. Jain, G. Misra, R.K. Patel, P. Priya, S. Jhanwar, et al., A draft genome sequence of the pulse crop chickpea (*Cicer arietinum* L.), Plant J. 74 (2013) 715–729.
[6] M.W. Jones-Rhoades, D.P. Bartel, B. Bartel, MicroRNAs and their regulatory roles in plants, Annu. Rev. Plant Biol. 57 (2006) 19–53.
[7] R. Sunkar, J.K. Zhu, Micro RNAs and short-interfering RNAs in plants, J. Integr. Plant Biol. 49 (2007) 817–826.
[8] L. Shukla, V. Chinnusamy, R. Sunkar, The role of microRNAs and other endogenous small RNAs in plant stress responses, Biochem. Biophys. Acta Gene Regul. Mech. 1779 (2008) 743–748.
[9] R. Sunkar, Y. Li, G. Jagadeeswaran, Functions of microRNAs in plant stress responses, Trends Plant Sci. 17 (2012) 196–203.
[10] J. Hu, L. Sun, Y. Ding, Identification of conserved microRNAs and their targets in chickpea (*Cicer arietinum* L.), Plant Signal. Behav. 8 (4) (2013) e23604.
[11] D. Kohli, G. Joshi, A.A. Deokar, A.R. Bhardwaj, M. Agarwal, et al., Identification and characterization of wilt and salt stress-responsive microRNAs in chickpea through high-throughput sequencing, PLOS ONE 9 (2014) e108851.
[12] P.V. Shivaprasad, H.M. Chen, K. Patel, D.M. Bond, B.A. Santos, et al., A microRNA superfamily regulates nucleotide binding site-leucine-rich repeats and other mRNAs, Plant Cell 24 (2012) 859–874.
[13] F. Li, D. Pignatta, C. Bendix, J.O. Brunkard, M.M. Cohn, et al., MicroRNA regulation of plant innate immune receptors, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 1790–1795.
[14] Q. Fei, R. Xia, B.C. Meyers, Phased, secondary, small interfering RNAs in post-transcriptional regulatory networks, Plant Cell 25 (2013) 2400–2415.
[15] J.L. Reyes, C. Arenas-Huertero, R. Sunkar, Cloning of stress-responsive microRNAs and other small RNAs from plants, Methods Mol. Biol. 639 (2010) 239–251.
[16] R. Sunkar, A. Kapoor, J.K. Zhu, Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by down-regulation of miR398 and important for oxidative stress tolerance, Plant Cell 18 (2006) 2051–2065.
[17] M.W. Jones-Rhoades, D.P. Bartel, Computational identification of plant microRNAs and their targets, including a stress-induced miRNA, Mol. Cell 14 (2004) 787–799.
[18] R. Li, C. Yu, Y. Li, T.-W. Lam, S.-M. Yiu, et al., SOAP2: an improved ultrafast tool for short read alignment, Bioinformatics 25 (15) (2009) 1966–1967.
[19] Y. Zheng, S. Wang, R. Sunkar, Genome-wide discovery and analysis of phased small interfering RNAs in Chinese sacred lotus, PLOS ONE 9 (12) (2014) e113790.
[20] Z. Li, Y. Zheng, G. Jagadeeswaran, Y. Li, K. Gowdu, et al., Identification and temporal expression analysis of conserved and novel miRNAs in *Sorghum*, Genomics 98 (2011) 460–468.
[21] G. Jagadeeswaran, Y.F. Li, R. Sunkar, Redox signaling mediates the expression of a sulfate-deprivation-inducible miR395 in *Arabidopsis*, Plant J. 77 (2014) 85–96.
[22] J. Matts, B.A. Roe, R. Sunkar, Identification of microRNAs and their targets in switchgrass, a model cellulosic biofuel plant species, J. Plant Physiol. 167 (11) (2010) 896–904.
[23] X. Chen, A microRNA as a translational repressor of APETALA2 in *Arabidopsis* flower development, Science 303 (2004) 2022–2025.
[24] L. Wu, L. Mao, Y. Qi, Roles of DICER-LIKE and ARGPNAUTE proteins in TAS-derived small interfering RNA-triggered DNA methylation, Plant Physiol. 160 (2012) 990–999.
[25] J.T. Cuperus, A. Carbonell, N. Fahlgren, H. Garcia-Ruiz, R.T. Burke, et al., Unique functionality of 22-nt miRNAs in triggering RDR6-dependent siRNA biogenesis from target transcripts in *Arabidopsis*, Nat. Struct. Mol. Biol. 17 (2010) 997–1003.
[26] H.M. Chen, L.T. Chen, K. Patel, Y.H. Li, D.C. Baulcombe, et al., 22-Nucleotide RNAs trigger secondary siRNA biogenesis in plants, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 15269–15274.
[27] L.C. Hsieh, S.I. Lin, A.C. Shih, J.W. Chen, W.Y. Lin, et al., Uncovering small RNA-mediated responses to phosphate deficiency in *Arabidopsis* by deep sequencing, Plant Physiol. 151 (4) (2009) 2120–2132.
[28] L. Guo, Z. Lu, The fate of miRNA* strand through evolutionary analysis: implication for degradation as merely carrier strand or potential regulatory molecule? PLoS ONE 5 (6) (2010) e11387.
[29] Y. Zheng, G. Jagadeeswaran, K. Gowdu, S. Wang, S. Li, et al., Genome-wide analysis of microRNAs in sacred lotus, *Nelumbo nucifera* (Gaertn), Trop. Plant Biol. 6 (2013) 117–130.
[30] X. Zhang, H. Zhao, S. Gao, W.C. Wang, S. Katiyar-Agarwal, et al., *Arabidopsis argonaute* 2 regulates innate immunity via miRNA393(*)-mediated silencing of a golgi-localized SNARE gene, MEMB12, Mol. Cell 6 (2011) 356–366.
[31] P.A. Manavella, D. Koenig, I. Rubio-Somoza, H.A. Burbano, C. Becker, D. Weigel, Tissue-specific silencing of *Arabidopsis* SU(VAR)3-9 HOMOLOG8 by miR171a*, Plant Physiol. 161 (2013) 805–812.
[32] E.A. Devers, A. Branscheid, P. May, F. Krajinski, Stars and symbiosis: microRNA- and microRNA*-mediated transcript cleavage involved in arbuscular mycorrhizal symbiosis, Plant Physiol. 156 (2011) 1990–2010.
[33] B.C. Meyers, M.J. Axtell, B. Bartel, D.P. Bartel, D. Baulcombe, et al., Criteria for annotation of plant microRNAs, Plant Cell 20 (2008) 3186–3190.
[34] E. Allen, Z. Xie, A.M. Gustafson, J.C. Carrington, MicroRNA-directed phasing during trans-acting siRNA biogenesis in plants, Cell 121 (2005) 207–221.
[35] R. Sunkar, X. Zhou, Y. Zheng, W. Zhang, J.K. Zhu, Identification of novel and candidate miRNAs in rice by high throughput sequencing, BMC Plant Biol. 8 (1) (2008) 25.
[36] X. Adenot, T. Elmayan, D. Lauressergues, S. Boutet, N. Bouché, V. Gasciolli, H. Vaucheret, DRB4-dependent TAS3 trans-acting siRNAs control leaf morphology through AGO7, Curr. Biol. 16 (9) (2006) 927–932.
[37] N. Fahlgren, T.A. Montgomery, M.D. Howell, E. Allen, S.K. Dvorak, A.L. Alexander, J.C. Carrington, Regulation of AUXIN RESPONSE FACTOR3 by TAS3 ta-siRNA affects developmental timing and patterning in *Arabidopsis*, Curr. Biol. 16 (9) (2006) 939–944.
[38] H.M. Chen, Y.H. Li, S.H. Wu, Bioinformatic prediction and experimental validation of a microRNA-directed tandem *trans*-acting siRNA cascade in *Arabidopsis*, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 3318–3323.
[39] R.S. Poethig, Small RNAs and developmental timing in plants, Curr. Opin. Genet. Dev. 19 (2009) 374–378.
[40] S. Subramanian, Y. Fu, R. Sunkar, W.B. Barbazuk, J.K. Zhu, O. Yu, Novel and nodulation-regulated microRNAs in soybean roots, BMC Genomics 9 (2008) 160.
[41] Y. Wang, P. Li, X. Cao, X. Wang, A. Zhang, X. Li, Identification and expression analysis of miRNAs from nitrogen-fixing soybean nodules, Biochem. Biophys. Res. Commun. 378 (2009) 799–803.
[42] H. Li, Y. Deng, T. Wu, S. Subramanian, O. Yu, Misexpression of miR482, miR1512, and miR1515 increases soybean nodulation, Plant Physiol. 153 (2010) 1759–1770.
[43] J.P. Combier, F. Frugier, F. de Billy, A. Boualem, F. El-Yahyaoui, S. Moreau, et al., MtHAP 2-1 is a key transcritional regulator of symbiotic nodule development

regulated by microRNA169 in *Medicago truncatula*, Genes Dev. 20 (2006) 3084–3088.
[44] A. Boualem, P. Laporte, M. Jovanovic, C. Laffont, J. Plet, J.P. Combier, et al., MicroRNA166 controls root and nodule development in *Medicago truncatula*, Plant J. 54 (2008) 876–887.
[45] P. Bustos-Sanmamed, J. Bazin, C. Hartmann, M. Crespi, C. Lelandais-Brière, Small RNA pathways and diversity in model legumes: lessons from genomics, Front. Plant Sci. 4 (2013) 236–274.
[46] M. Turner, O. Yu, S. Subramanian, Genome organization and characteristics of soybean microRNAs, BMC Genomics 13 (2012) 169.