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Genome-wide Characterization of MicroRNAs from Mungbean (*Vigna radiata* L.)

Sujay Paul[1*] and Amita Pal[1]

[1]Division of Plant Biology, Bose Institute, P 1/12, CIT Scheme VII M, Kolkata - 700054, India.

Authors' contributions

This work was carried out in collaboration between both authors. Author SP designed the study, performed the experiments and wrote the first draft of the manuscript. Author AP helped to write the manuscript. Both authors read and approved the final manuscript.

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Short Communication

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ABSTRACT

Aims: MicroRNAs (miRNAs) are endogenous, short (~21-nucleotide), non-coding RNA molecules that play important roles in post-transcriptional gene silencing by directing target mRNA cleavage or translational inhibition. The main aim of this study is to identify and characterize miRNAs from economically important and high stress tolerant crop mungbean (*Vigna radiata* L.).
Study Design: Conserved miRNAs and their targets were characterized from mungbean using computational and RT-PCR approach.
Place and Duration of Study: Division of Plant Biology, Bose Institute, P 1/12 CIT Scheme VII M, Kolkata- 700054, India between January 2011- November 2015.
Methodology: Conserved miRNAs and their targets from mungbean were identified in this study using homology based strict filtering approach. Software tools such as mfold and psRNATarget were used during this study. Predicted mungbean miRNAs were validated by RT-PCR technique.
Results: In this study using recently published draft genome sequence of mungbean (*Vigna radiata* L.) and applying genome-wide computational-based approaches a total of 56 potentially conserved microRNAs belonging to 28 families were identified. 3 putative mungbean miRNAs (vra-miR160a, vra-miR162b and vra-miR398b) were successfully validated by RT-PCR. Using psRNATarget tool a

**Corresponding author: E-mail: sujay.p07@gmail.com;*

total of 88 potential miRNA target transcripts were also recognized for the identified mungbean miRNAs which include a number of transcription factors.
Conclusion: For the first time 56 conserved microRNAs and 88 potential target sequences were identified in mungbean. Predicted target transcripts were found to be involved in development, metabolism and stress responses.

Keywords: Vigna radiata (mungbean); MicroRNA (miRNA); Minimum Folding Free Energy (MFE); Minimum Folding Free Energy Index (MFEI); miRNA targets.

ABBREVIATIONS

miRNA : MicroRNA
MFE : Maximum Folding Free Energy
MFEI : Maximum Folding Free Energy Index
psRNATArget : Plant Small RNA Target Analysis Server

1. INTRODUCTION

Mungbean (*Vigna radiata* (L.) R. Wilczek var. Radiata), a high-stress tolerant grain legume has been considered as one of the most important staple food of India. This grain legume cultivated throughout Asia where almost 90% of global production currently occurs and also in several dry regions of southern Europe and the southern United states. MicroRNAs are endogenous, non-coding, small RNAs ranging in length from 20-24 nucleotides. Post-transcriptional gene regulation mediated by endogenous miRNAs play a pivotal role in various aspects of plant development as well as adaptation to biotic and abiotic stresses [1,2]. In plants, mature miRNAs are generated from longer stem-loop RNA precursors (pre-miRNAs) with the aid of ribonuclease III-like dicer (DCL1) enzyme. Despite the fact that microRNAs have a great role in stress responses, till date no scientific initiative has been taken to study mungbean miRNAs and their targets in detail. With the recent draft genome sequence available [3], it is important to exploit this information for better understanding the physiological processes in mungbean.

2. MATERIALS AND METHODS

2.1 *In silico* Prediction of Mungbean miRNAs

For the computational identification of potential conserved miRNAs in mungbean, a reference set of plant miRNAs (downloaded from miRbase 21) was searched against whole genome sequence of mungbean. The set consisted of a total 1832 known mature miRNA sequences including *Arabidopsis thaliana* (427) *Glycine max* (639), *Medicago truncatula* (756) and *Phaseolus vulgaris* (10). The detail methodology, described previously by Paul et al. [4] was illustrated graphically in Fig. 1 with minor modifications. In brief, the aforesaid set of known miRNA sequences were BLASTn search against mungbean genome and sequences with ≤1 mismatches were chosen manually. The possible precursor (pre-miRNA) sequences of approximately 400-nt (200 nt upstream and 200 nt downstream to the BLAST hit region) were extracted and sequences coded for proteins were removed. Stable secondary structures of the remaining precursor sequences were predicted using mfold web server (http://unafold.RNA.albany.edu/?q=mfold/mfold-references) following previously described filtering criteria [5] such as: (i) the secondary structure of the precursor sequences should have the stem-loop structure that contains a mature miRNA sequence within one arm and no loop or break in the mature miRNA sequences; (ii) the potential miRNA sequence should not be located on the terminal loop of the hairpin structure; (iii) mature miRNAs should have fewer than nine mismatches with the opposite miRNA* sequence [6]; and (iv) the predicted stem-loop candidates should have higher MFEIs and negative minimum folding free energies. The formula for calculating MFEI is as follows:

$$\text{MFEI} = \frac{(\text{MFE}/\text{length of RNA sequence}) \times 100}{\%\text{GC content}}$$

2.2 MicroRNA Expression Analysis

For the experimental validation of predicted mungbean miRNAs by RT-PCR (reverse transcription), small RNA was first isolated from mungbean leaves using mir Premier microRNA Isolation Kit (Sigma-Aldrich). 1 µg of aforesaid mungbean small RNA was polyadenylated (using modified oligo dT primer) and reverse transcribed at 37ºC for 1 h in 10 µl reaction mixture using Mir-X miRNA First-Strand Synthesis kit (Clontech). The obtained cDNA was then amplified by GeneAmp PCR system 2400 (Perkin Elmer) using entire predicted miRNA sequence as sense primer and adapter specific mRQ 3' primer provided with Mir-X miRNA qRT-

PCR SYBR kit (Clontech) as antisense primer. 100 ng cDNA was used as template for the PCR. The PCR was programmed as follows: initial denaturation at 95℃ for 3 min, followed by 35 cycles of denaturation at 94℃ for 30 s, annealing at 60℃ for 30 s, extension at 72℃ for 25 s and a final elongation step at 72℃ for 7 min.The resulting PCR products (~ 70 bp) were checked in 2% agarose gel with EtBr staining.

2.3 Target Prediction of Mungbean miRNAs

The Plant Small RNA Target Analysis Server (psRNATarget) was used in this study to predict mungbean miRNA targets (http://plantgrn.noble.org/psRNATarget/). Due to non-availability of mungbean protein database in psRNATarget server target transcript search was performed against protein database of *Glycine max*. The following parameters were employed in prediction of miRNA targets in mungbean: a) Maximum exception of 3.0, length of complementarity score: 20.b) Target accessibility - allowed maximum energy to unpair the target site (UPE): 25. c) Flanking length around the target accessibility analysis: 17 bp upstream and 13 bp in downstream. d) Range of central mismatch leading to translation inhibition: 9–11 nt.

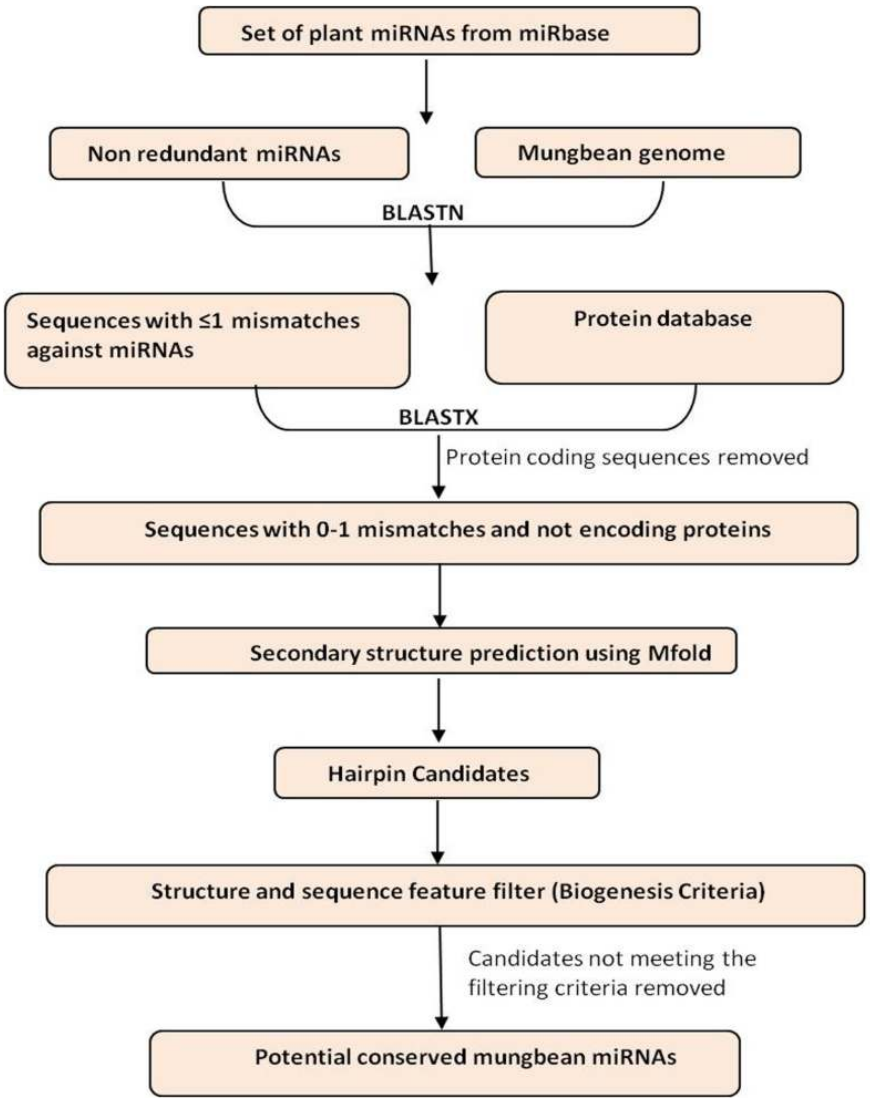

Fig. 1. Schematic representation of mungbean (*Vigna radiata* L.) miRNA search procedure

3. RESULTS AND DISCUSSION

3.1 Characterization of Mugbean miRNAs

With high stringent filtering approach, a total of 56 potential conserved miRNAs belonging to 28 families were identified in mungbean (Table 1). Among them, 29 miRNAs (~52%) were located in 5' arm of the precursor while 27 (~48%) located in 3' arm suggesting that mungbean miRNAs are located in both the arms of the precursor void any preference. Precursors of mungbean also showed great variability in their size ranging from 65 to 183 with an average of 106 ± 31 (Table 1) which represent good agreement to those reported for other plant species such as soybean, cotton and maize [5,7,8]. Vra miR2111b showed the shortest precursor length of 65 nt while vra miR166h-5p showed the longest one of 183 nt. The MFEI is a useful criterion for distinguishing miRNAs from other types of coding or non-coding RNAs. In this study, the identified precursors have high MFEI values (0.70–1.51) with an average of 0.99 ± 18.0 which is much higher than that of tRNAs (0.64), rRNAs (0.59), or mRNAs (0.62–0.66) respectively [9]. The secondary structure of the precursors with higher MFEI values is presented in Fig. 2 (Top 20).

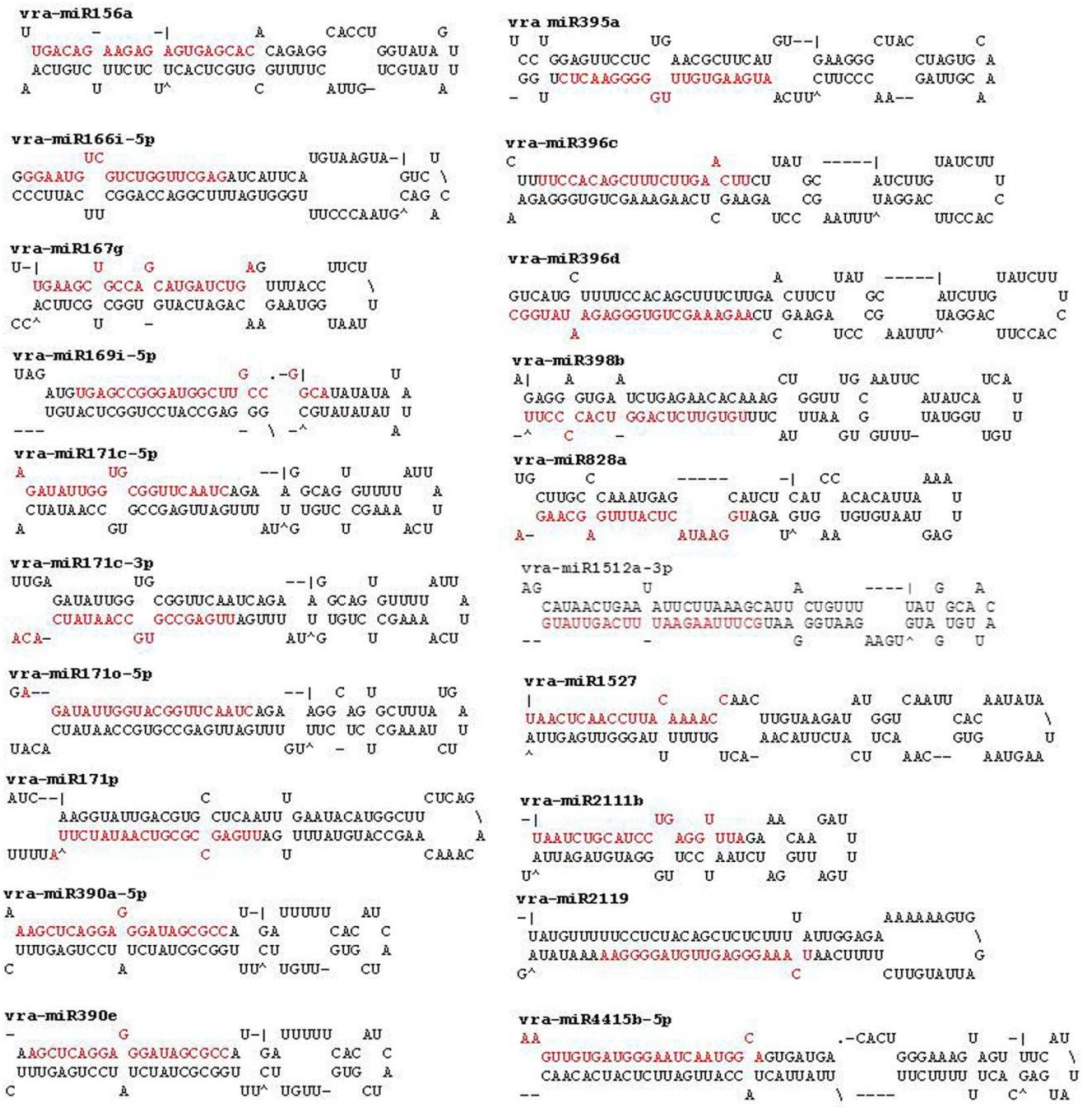

Fig. 2. Secondary structure (stem loop) of the mungbean miRNA precursors with higher MFEI values (Top 20). microRNAs are represented with red font

Table 1. Potential conserved miRNAs in *Vigna radiata*

Identified miRNAs	LM (nt)[a]	miRNA sequences	Accessions	Strand	Location	NM (nt)[b]	LP (nt)[c]	MFEs (ΔG)	MFEIs
vra-miR156a	20	UGACAGAAGAGAGUGAGCAC	gi|949042834	+/+	5’	0	83	-41.70	1.16
vra-miR160a	21	UGCCUGGCUCCCUGUAUGCCA	gi|948541905	+/+	5’	0	81	-37.40	0.91
vra-miR162a	20	UCGAUAAACCUCUGCAUCCA	gi|949042873	+/+	3’	0	96	-39.30	0.94
vra-miR162b	21	UCGAUAAACCUCUGCAUCCAG	gi|949042873	+/+	3’	0	89	-35.00	0.87
vra-miR164a	21	ACCUCUUCGUCCCGUGCACGU	gi|949042843	+/-	3’	0	151	-58.20	0.84
vra-miR164c	21	UGGAGAAGCAGGGCACGUGCG	gi|949042843	+/-	3’	1	161	-60.40	0.82
vra-miR166d	21	UCGGACCAGGCUUCAUUCCCC	gi|949042884	+/+	3’	0	150	-55.60	0.89
vra-miR166h-3p	21	UCUCGGACCAGGCUUCAUUCC	gi|949042884	+/+	3’	0	151	-54.80	0.86
vra-miR166s	20	UCGGACCAGGCUUCAUUCCC	gi|949042884	+/+	3’	0	157	-62.60	0.92
vra-miR166h-5p	21	GGAAUGUUGUUUGGCUCGAGG	gi|949042777	+/+	5’	0	183	-78.50	1.02
vra-miR166i-5p	20	GGAAUGUCGUCUGGUUCGAG	gi|949042801	+/+	5’	0	84	-43.40	1.06
vra-miR166u	20	UCUCGGACCAGGCUUCAUUC	gi|949042884	+/+	3’	0	142	-50.30	0.84
vra-miR167a	21	UGAAGCUGCCAGCAUGAUCUA	gi|949042861	+/+	5’	0	85	-38.80	0.90
vra-miR167g	22	UGAAGCUGCCAGCAUGAUCUGA	gi|949042801	+/+	5’	0	69	-36.30	1.20
vra-miR168a	21	UCGCUUGGUGCAGGUCGGGAA	gi|949042755	+/+	5’	0	91	-39.90	0.75
vra-miR169a	21	CAGCCAAGGAUGACUUGCCGG	gi|949042812	+/+	5’	0	181	-66.80	0.87
vra-miR169f	21	CAGCCAAGGAUGACUUGCCGG	gi|949042812	+/+	5’	0	97	-36.80	0.86
vra-miR169i-5p	23	UGAGCCGGGAUGGCUUGCCGGCA	gi|949042812	+/+	5’	0	101	-52.50	1.14
vra-miR169u	21	CAGCCAAGGAUGACUUGCCGU	gi|949042843	+/+	5’	0	106	-36.70	0.80
vra-miR171b-3p	21	CGAGCCGAAUCAAUAUCACUC	gi|949042777	+/+	3’	0	93	-33.80	0.89
vra-miR171c-5p	21	AGAUAUUGGUGCGGUUCAAUC	gi|949042812	+/+	5’	0	82	-44.10	1.42
vra-miR171c-3p	21	UUGAGCCGUGCCAAUAUCACA	gi|949042812	+/+	3’	0	87	-44.10	1.34
vra-miR171e	21	UGAUUGAGCCGUGCCAAUAUC	gi|949042812	+/+	3’	0	121	-45.60	0.88
vra-miR171j-5p	21	UAUUGGCCUGGUUCACUCAGA	gi|949042812	+/+	5’	0	115	-42.90	0.88
vra-miR171j-3p	21	UUGAGCCGUGCCAAUAUCACG	gi|949042873	+/+	3’	0	96	-41.50	0.97
vra-miR171k-5p	21	CGAUGUUGGUGAGGUUCAAUC	gi|949042812	+/+	5’	0	91	-38.20	0.95
vra-miR171k-3p	21	UUGAGCCGCGCCAAUAUCACU	gi|949042812	+/+	3’	0	91	-38.50	0.99
vra-miR171o-5p	21	AGAUAUUGGUACGGUUCAAUC	gi|949042777	+/+	5’	0	85	-44.90	1.32
vra-miR171p	21	UUGAGCCGCGUCAAUAUCUUA	gi|949042834	+/+	3’	0	91	-45.80	1.35
vra-miR172h-5p	21	GCAGCAGCAUCAAGAUUCACA	gi|949042801	+/+	5’	0	81	-35.50	0.96
vra-miR319b	20	UUGGACUGAAGGGAGCUCCC	gi|949042812	+/+	3’	0	177	-73.00	0.99
vra-miR390a-5p	21	AAGCUCAGGAGGGAUAGCGCC	gi|948541341	+/+	5’	0	74	-38.80	1.17

Identified miRNAs	LM (nt)[a]	miRNA sequences	Accessions	Strand	Location	NM (nt)[b]	LP (nt)[c]	MFEs (ΔG)	MFEIs
vra-miR390e	20	AGCUCAGGAGGGAUAGCGCC	gi|948541341	+/+	5’	0	73	-38.50	1.17
vra-miR393a	22	UUCCAAAGGGAUCGCAUUGAUC	gi|949042884	+/+	5’	0	108	-48.50	0.99
vra-miR393b	21	UUUGGGAUCAUGCUAUCCCUU	gi|949042821	+/+	3’	0	90	-34.40	0.92
vra-miR393c-3p	21	AUCAUGCUAUCCCUUUGGAUU	gi|949042861	+/+	3’	0	86	-34.10	0.92
vra-miR394a-5p	20	UUGGCAUUCUGUCCACCUCC	gi|949042834	+/+	5’	0	96	-30.40	0.72
vra-miR395a	21	AUGAAGUGUUUGGGGGAACUC	gi|949042755	+/+	3’	0	91	-47.10	1.07
vra-miR396b-3p	21	GCUCAAGAAAGCUGUGGGAGA	gi|949042812	+/+	3’	0	101	-40.30	1.00
vra-miR396c	21	UUCCACAGCUUUCUUGAACUU	gi|949042812	+/+	5’	0	93	-40.30	1.06
vra-miR396d	24	AAGAAAGCUGUGGGAGAAUAUGGC	gi|949042812	+/+	3’	0	105	-46.40	1.05
vra-miR396g	21	UUCUUGAACUUCUUAUGCAUC	gi|949042812	+/+	5’	0	70	-18.20	0.70
vra-miR397a	21	UCAUUGAGUGCAGCGUUGAUG	gi|949042812	+/+	5’	0	85	32.60	0.96
vra-miR398b	21	UGUGUUCUCAGGUCACCCCUU	gi|948539988	+/+	3’	0	97	-42.60	1.15
vra-miR399a	21	UGCCAAAGGAGAGUUGCCCUG	gi|949042801	+/+	3’	0	83	-39.80	1.02
vra-miR399e	21	UGCCAAAGGAGAUUUGCCCAG	gi|948541893	+/+	3’	0	95	-43.00	0.98
vra-miR482-5p	22	GGAAUGGGCUGAUUGGGAAGCA	gi|949042843	+/-	3’	1	91	-30.50	0.85
vra-miR530a	20	UGCAUUUGCACCUGCACUUU	gi|949042873	+/+	5’	0	161	-56.80	0.85
vra-miR530c	21	UGCAUUUGCACCUGCACUUUA	gi|949042873	+/+	5’	0	162	-57.40	0.84
vra-miR828a	22	UCUUGCUCAAAUGAGUAUUCCA	gi|949042873	+/-	3’	0	81	-31.20	1.04
vra-miR1512a-3p	21	GCUUUAAGAAUUUCAGUUAUG	gi|949042843	+/+	3’	0	87	-34.00	1.26
vra-miR1527	20	UAACUCAACCUUACAAAACC	gi|949042873	+/+	5’	0	100	-31.10	1.04
vra-miR2111b	21	UAAUCUGCAUCCUGAGGUUUA	gi|948541628	+/+	5’	0	65	-33.30	1.51
vra-miR2119	21	UCAAAGGGAGUUGUAGGGGAA	gi|948539988	+/+	3’	0	90	-41.20	1.42
vra-miR4415b-5p	24	AAGUUGUGAUGGGAAUCAAUGGCA	gi|949042843	+/+	5’	0	158	-57.00	1.10
vra-miR5770a	21	UUAGGACUAUGGUUUGGACGA	gi|948542439	+/+	5’	0	153	-52.20	0.97

[a]LM = Length of mature miRNAs; [b]NM= Number of mismatch; [c]LP = Length of precursor

3.2 Experimental Validation of Putative Mungbean miRNAs

The efficiency of the computational strategy was further verified by RT-PCR based experimental procedure. The randomly selected three miRNAs vra-miR160a, vra-miR162b and vra-miR398b from mungbean were subjected for validation studies. All these mungbean miRNAs showed confirmation through experimental validation (Fig. 3).

3.3 Potential Targets of Putative Mungbean miRNAs

A total of 88 potential targets were identified and most of them were functionally categorized as transcription factors. Important transcription

Table 2. Potential targets of identified *Vigna radiata* miRNAs

miRNA	Targeted protein (Number)
miR156	SBP - Medicago truncatula (5)
miR162	60S ribosomal protein (1)
miR164	NAM protein (1)
	NAC domain protein (7)
miR166	Class III HD-Zip (8)
	Disease resistance protein-like protein (1)
	Nucleolar GTPase (1)
miR169	CCAAT-box transcription factor complex WHAP12 (5)
	Nuclear transcription factor Y (1)
miR171	Tubulin beta chain (2)
	Cysteine-rich repeat secretory protein 9 precursor (1)
	Leaf senescence protein-like (1)
	Leucine-rich repeat (1)
miR172	Ubiquitin carrier protein (5)
	Ethylene-responsive transcription factor (1)
miR390	Protein kinase Pti1 (2)
miR393	Auxin-responsive factor TIR1-like protein (2)
	Transport inhibitor response 1 (4)
	Chlorophyll a-b binding protein (1)
	Early nodulin (1)
miR394	Somatic embryogenesis receptor kinase (1)
miR395	ATP sulfurylase (4)
miR396	Malic enzyme (1)
miR397	Diphenol oxidase (3)
	Laccase (2)
	Acetyl-CoA synthetase (1)
miR398	Superoxide dismutase [Cu-Zn] (2)
miR399	Phosphate transporter (1)
miR482	Peroxisomal copper-containing amine oxidase (2)
miR530	Probable UDP-N-acetylglucosamine (1)
miR828	MYB protein (1)
	Adenylosuccinate-AMP lyase (1)
	Syringolide-induced protein (1)
miR1512	Ser/Thr protein kinase (1)
	Uridylate kinase (1)
	Phosphatidic acid phosphatase alpha (1)
	Glucose-1-phosphate adenylyltransferase (1)
	Chalcone--flavonone isomerase (1)
miR1527	Cyclin (1)
miR 2111	Nodulin-like protein (1)
miR 2119	Alcohol dehydrogenase (3)
miR4415	L-ascorbate oxidase precursor (1)
	LATE BLOOMER (1)
miR5770	Diamine oxidase (4)

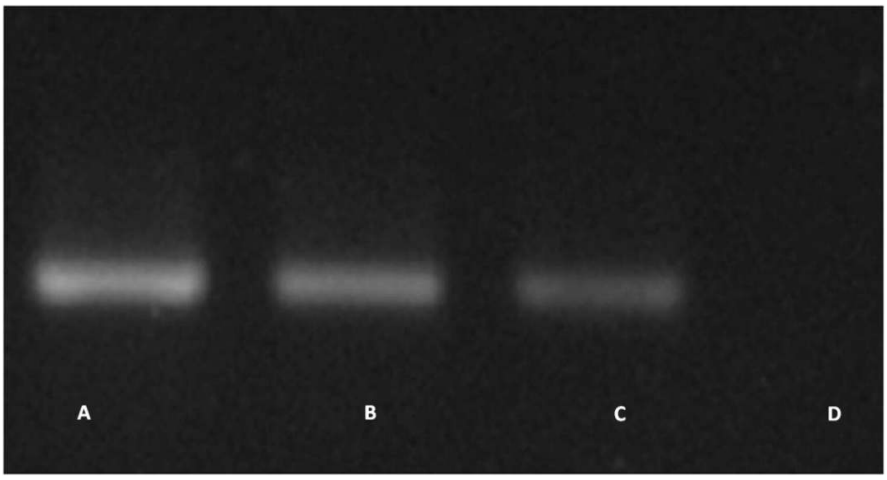

Fig. 3. Validation of some mungbean miRNAs by RT-PCR. The resulting PCR products are checked in 2% agarose gel with EtBr staining. A) vra-miR160a, B) vra-miR162b, C) vra-miR 398b and D) Negative control

factors targeted by mungbean miRNAs include Squamosa promoter-binding protein/SPB (miR156), NAC domain protein (miR164), Class III HD-Zip (miR166), CCAAT-box transcription factor complex (miR169), Nuclear transcription factor Y (miR169) Auxin-responsive factor TIR1-like protein (miR393), Superoxide dismutase [Cu-Zn] (miR398), MYB protein (miR828) (Table 2). These transcription factors are known to play role in metabolic processes and stress response signaling in plants.

Abiotic and biotic stress factors are the major constrains for crop production. MicroRNA represents a class of small regulatory biomolecules that are employed by the host as a counter measure to resist biotic and abiotic stresses. However, the molecular mechanism behind the microRNA-mediated stress responses in plants are still not very clear and hence stress tolerant crop like mungbean is currently in a great demand to study the role of stress-responsive miRNAs. Nevertheless, identification of miRNAs and their targets is the key step to initiate a miRNA-related study in a crop plant. Our results may apparently basic but we believe that identification of 56 potentially conserved mungbean miRNAs, their precursors, and 88 potential targets will be of immensely helpful for future research on miRNA-mediated gene regulation and stress tolerance in economically important crops.

4. CONCLUSION

In this study a total of 56 conserved miRNAs belonging to 28 families were first time identified from mungbean. To validate the expression of potential miRNAs in mungbean, a RT-PCR experiment was performed and 3 miRNA families were detected. Moreover, a total of 88 potential targets were predicted and they were found to be involved in development, metabolism and stress responses.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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