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Identification and profiling of high temperature responsive miRNAs in French bean (*Phaseolus vulgaris L*)

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ABSTRACT

MicroRNAs (miRNAs) are group of small, non-coding RNAs that play important roles in plant growth, development and stress response. There have been an increasing number of investigations aimed at discovering miRNAs and analyzing their functions in model plants. In this study, we constructed high temperature stress induced small RNA libraries and characterized 26 potential miRNAs belonging to 21 families in French bean. A total of 140 annotated potential targets were found, of which majority were transcription factors (MYB, *bHLH*, GRF1, bZIP, NAC etc.,) which may play an important role in stress resistance. RT-qPCR and Northern blot analysis revealed differential expressions of candidate miRNAs and their target genes. The observed induction of miRNA expression is correlated with the down regulation of their targets. Investigation of gene ontology linked with targets of miRNAs forecasted their involvement in various biological functions. We anticipate the further studies may offer new avenues in developing stress tolerant variety of French bean.

1. INTRODUCTION

The modern globalization and extensive industrialization has resulted in reduced agricultural production. The exposure to various stressful environmental conditions and their sessile nature made plants to evolve advanced adaptive strategies to cope and sustain. These adaptations exhibited as physiological responses which were fine-tuned through induction of cascades of molecular mechanisms operating at transcriptional levels [1]. Advancement in the genomic technologies emphasized the role of non-coding RNAs as king-tuners of genomes and un-wrapped their involvement in genome dynamics, evolution and regulation. miRNAs are 21-24 nucleotide long, single stranded RNA molecules coded by their own transcripts known to exhibit gene regulation through target mRNA cleavage or translational repression mediated via RISC complex. Several roles of gene regulation have been attributed to these molecules which include development, signalling, defence and stress response. A number of miRNAs were demonstrated to function in biotic and abiotic stress responses in plants [2-4]. The role of miRNAs in plants infected by pathogenic bacteria, viruses, nematodes, and fungi has been widely reported [5, 6]. Various functions of miRNAs during abiotic stress has been established, which include cold [2, 7], drought [8] and oxidative stress induced by heavy metals, salinity, and nutrient deficiency [9]. Diverse set of miRNAs were also identified with heat stress response in wheat [10], Brassica rapa [11], barley [12]. French bean is one of the most important legume crop grown worldwide.

The major cues affecting the crop yield includes high temperature, nutrient deficiency, drought and high salinity. Constant rise in ambient temperature has been considered as the detrimental factor and affect the life processes which are multifarious, often lethal bringing alterations in plant growth, development, physiological processes and yield. Majority of the studies have concentrated on regulation of genes which are responsible for synthesis of osmo-protectants, detoxifying enzymes, transporter and other regulatory proteins [1].

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Recently Arenas-Huertero *et al.* [13] identified conserved and novel miRNAs which responded to drought and salinity. In addition Valdes-Lopez *et al.* [14], used macroarray-hybridization approach to identify miRNAs responded to nutrient deficiency and manganese toxicity and demonstrated the involvement of miR399 in the PHR1 signaling pathway. Recently, Pelaez *et al.* [15] used high throughput sequencing for the identification and characterization of *P. vulgaris* miRNAs under salt stress. Previously we have reported the altered expression of miRNAs render plant tolerant towards salt, drought and nutrition depletion [16, 17]. In this study, we aimed to decipher a set of conserved and novel miRNAs responding to high temperature stress. The stress modulated expression of miRNAs was validated through stem-loop quantitative PCR and Northern blot to establish the negative correlation of expression of the candidate miRNAs and their targets. The gene ontology studies revealed many of the targets identified with the candidate miRNAs were involved in maintaining cellular homeostasis.

2. MATERIALS AND METHODS

2.1 Plant materials and stress treatment

Seeds of French bean (*Phaseolus vulgaris* Selection - 9) were surface sterilized and grown under controlled conditions at 28 °C day/25 °C night with 12 h light/12 h dark photo period. After 6 day of germination, seedlings were exposed to high temperature stress [42°C for 1 h (induction); 45 °C for 1 h and 48 °C for 6 h respectively]. Tissues were harvested immediately and stored at -80 °C for further analysis.

2.2. Small RNA extraction and cloning

Total RNA was isolated from tissue using TRizol (Invitrogen) according to the manufacturer's instructions and treated with RNAase-free DNAase I (Promega). Small RNAs (200 nt) were separated on a denaturing 15 % polyacrylamide gel. Molecules ranging from 18 to 26 nt were excised and recovered. The sRNAs were then 3' (5'-ACTGTAGGCACCATCAAT-3' underlined *BanI* site) and 5' (5'AAACCATGGTACTAATACGACTCACTAAA-3' underlined *RsaI* site) adapter-ligated by T4 RNA ligase (Fermentas). At each step, their lengths were verified and purified by Urea-PAGE. The adapter-ligated sRNAs were transcribed into cDNA by Super-Script II Reverse Transcriptase (Invitrogen). Reverse transcription was performed using the adapter primers and the resulting cDNAs were PCR amplified with family specific primers at 95 ºC for 20s; 56-58 ºC for 30s; 72 ºC for 30s cycled for 30 and final extension at 72 ºC for 5 min in thermo cycler (Kyratec). The amplicon were purified and ligated into *pGEMT* Easy (Promega). The colonies were screened for the ligated products using gene specific primers. The plasmid DNA from recombinant colonies showing the correct product size were subjected to *RsaI* and *BanI* digestion and rechecked for the insert size. This procedure screened out the colonies carrying adapter self-ligation and possibly the degraded mRNA products carrying the restriction sites. The colonies carrying the correct size fragments were further screened using the adapter primers in combinations with vector primers. The PCR positive clones were sequenced and processed for BLAST analysis against the NCBI genomic data sets.This strategy also revealed that in some colonies two miRNAs were ligated together.

2.3 Data analysis

The sequences were filtered for tRNA/rRNA contamination using Rfam database (http://www.sanger.ac.uk/Software/Rfam). Putative origins for the remaining sequences were identified by BLASTN search against French bean genome sequence. The sequences with perfect (0-3) matches with small RNA sequences were used for fold back secondary structure prediction with MFOLD [18]. A segment was considered a valid miRNA candidate if its secondary structure met the criteria according to Meyers *et al.* [19]

2.4 Prediction of Potential Target mRNAs

Target prediction for the miRNAs was based on the principle of nearly perfect complementation between the miRNA and target mRNAs. The identified conserved and putative novel miRNAs were all submitted for target gene prediction using psRNATarget (http://plantgrn.noble.org/psRNATarget/) and psRobot (http://omicslab.genetics.ac.cn/psRobot/). French bean transcript sequences downloaded from Phytozome version 9 (www.phytozome.net) were used to predict the putative targets with default parameters. Sequences with a score of less than 4 were regarded as miRNA target genes. On the basis of their functions putative targets were classified using Gene Ontology (GO) annotations from agriGO (http://bioinfo.cau.edu.cn/agriGO).

2.5 Semi-quantitative RT-PCR validation of miRNA Expression

To validate the expression of the high temperature stress miRNAs in French bean, stem loop RT-qPCR was performed using SYBR Green PCR Master mix (Takara) on Light cycler 96® (Roche). Each PCR reaction (20 µl) included 2 µl cDNA, 10µl SYBR Green Master mix, 1 µl sequence specific forward primer (10 µM), 1 µl Universal reverse primer (10 µM) and 6 µl sterile water. The miRNA expression was normalized against U6 gene. The reactions were performed at 95 ºC for 10 min followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min with a final dissociation 72 ºC for 30s, with two biological replicates; the data was analysed based on efficiencies and fold changes. For target validation each reverse transcriptase reaction contain 2.5 µg of total DNA free RNA, 1 µl oligo dT and 1 µl dNTPs mix (10µM). The reaction was incubated for 5 min at 65 ºC and snap cooled subsequently, 5X First strand buffer, DTT, Ribolock and Superscript II enzyme (Invitrogen) were added. This reaction was incubated for 30 min at 42 ºC followed by 70 ºC using forward and reverse gene specific primers. The reactions were carried as described earlier and signals were quantified relative to GAPDH mRNA using the $2^{-\Delta\Delta CT}$ method.

2.6 Northern Blot analysis

In order to validate the miRNA expression, northern blot hybridization was conducted using high sensitive miRNA Northern blot assay kit (Signosis, USA). 30 µg total RNA of each sample was electrophoresed on 15% polyacrylamide gel and transferred to membrane. Antisense RNA biotin labeled in the 5' end (Invitrogen) was used as hybridization probes. The SYBR Green® II stained (Biotech) 5S rRNA was used as loading control. All the primers used in the study are mentioned in Suppl. 1.

3.RESULTS AND DISCUSSION

3.1 Identification of miRNAs and their targets

To explore the role of miRNAs under high temperature, small RNA libraries were constructed from stress exposed seedlings. 3,000 clones were screened for the sequences representing candidate miRNAs. 80% of the sequences were 18-26 nt in length and the remaining sequences had either shorter fragments or self -ligated adapters. Further, the sequences were mapped to French bean nuclear genome (http://www.phytozome.net), 97% of the sequences exhibited at least one match with the genome. Several sequences were mapped to chloroplast/mitochondrial genomes and few represent possible regulatory products/orgenellar RNAs (Figure 1). The tRNA/rRNA contamination was clarified by Blastn against Rfam database.

Based on initial survey of secondary structure and homology of mature miRNA sequences with miRBase v20 (www.miRBase.org), we were able to identify 26 conserved miRNAs corresponding to 21 miRNA families and two miR* sequences with two predominant size classes of 20 and 21 nt (Figure 2).

The detection of miRNAs is a strong clue, albeit not infallible, about the existence of precursor hairpin structures with negative folding-free energies ranging from -19.99 to -82.28 kJ and their MFEI was <-0.85 adds credence to the authenticity of the predicted candidates (Table 1). Majority of the sequences are starting with 5' Uridine, which is in accordance with defined structure of miRNAs. The miRNA sequence that remained after exclusion of conserved and their variants were considered as novel. These sequences exhibited ≥4 mismatch with conserved miRNAs and has been named as *Pvu Nx* (for *Phaseolus vulgaris* Novel, x being the number). We were able to identify 8 new members belonging to four miRNA families (Suppl. 2, Suppl. 3, and Suppl. 4).

To clarify the biological functions of the newly identified miRNAs, we examined putative target genes using the *psRNATarget* and *psRobot* programs with default parameters. We observed the candidate miRNA targeting many genes involved in different cellular functions suggests that these miRNAs may play multiple roles.

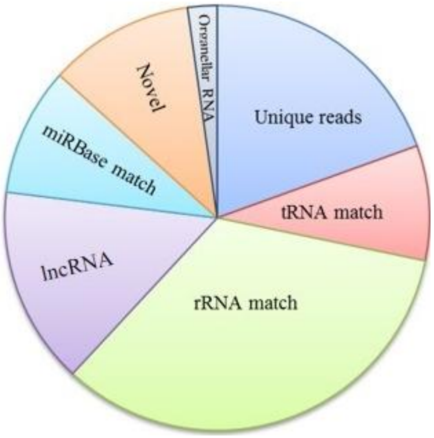


Fig. 1: Familial classification of small RNA raw sequences obtained from Sanger sequencing. The unique reads represent the small RNA reads filtered from Rfam. Novel miRNAs represent small subset of unique reads which does not show homology with conserved miRNAs from miRBase.

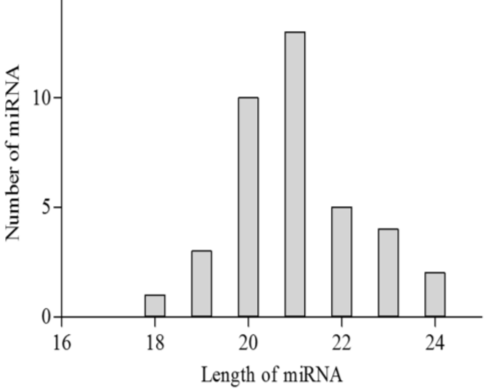


Fig. 2: Length distribution of the small RNA library in French bean.

Table 1: Conserved miRNAs identified with French bean expressed under high temperature stress.

miR	Sequence	Length	MFE	Family
Pvu seq 3	UGAAGUGUUUGGGGGAACUC	20	-80.3	miR395
Pvu seq 5	ACCAUCACCGAGGUGUACGA	20	-54.14	miR2111
Pvu seq 6	UAUGGCCGCUGCUGUGAUGAUGA	23	-13.85	miR397
Pvu seq 7	UAUGGCCGCUGCUGUGAUGAUGAUGA	24	-29.88	miR2653
Pvu seq 8	GUGGAGGUCGGUGCCGCG	20	-58.14	miR858
Pvu seq 9	GAUCAAAAUUUUAUCAUUUUUAC	23	-19.99	miR1438
Pvu seq 10	AUUCGUGUUCAGAAAGGAGA	20	-21.8	miR2674
Pvu seq 11	AGGACUCGGCAUGGCUCCUCCCAC	24	-61.45	miR160*
Pvu seq 13	UGGGAGGAGCCAACCCUAGUG	21	-42.8	miR396
Pvu seq 14	AAGGCACAGUCAAUUCCAGGUU	21	-37.23	miR2905
Pvu seq 16	UUAUGUGUAAAUGAAUGAAAU	21	-18.4	miR319
Pvu seq 17	UUGGACUGAAGGGACCUCCUU	21	-18.4	miR319
Pvu seq 18	UGAAGCUGCCAGCAUGAUCU	20	-64.8	miR167
Pvu seq 19	AAGCUGCCAGCAUGAUCUGA	20	-53.02	miR167
Pvu seq 20	UUGGGCAAAUCUCCUUUGGCA	21	-41.55	miR399
Pvu seq 21	UUGCCGAUUCCACCCAUUCCUAU	23	-56.00	miR2118
Pvu seq 22	UUAAUUGAGCCGCGUCAAUAUC	22	-64.5	miR171
Pvu seq 23	ACGAUGAUGAUGAGGAUGA	19	-51.76	miR414
Pvu seq 26	ACAGAUCAUGUGGCUGCUUCA	21	33.9	miR167
Pvu seq 27	UAUUAAUCUUGAUGAUGCUGCA	22	-40.3	miR172
Pvu seq 28	AGAAUCUUGAUGAUGCUGCA	20	-40.3	miR172
Pvu seq 29	GGAAUAUUGAUGAUGCUGAU	20	-37.4	miR172
Pvu seq 32	AAGACAUCGCCAAGGAGACU	20	-45.7	miR169
Pvu seq 33	CUAUUAAUCUUGAUGAUC	18	-44.0	miR172
Pvu seq 34	UUAAUCUUGAUGAUGCUGC	19	-29.9	miR172
Pvu seq 35	UGGUGCACUUGAUGAUGCUG	20	-31.5	miR172
Pvu seq 36	UGUGUUCUCAGGUCACCCCUU	20	-37.6	miR398

Suppl. 1: List of primers used in the study

A) Primers used for small RNA library construction

miRNA	5’- 3’ Sequence
miR162	ATCGTAGGCACCTGAAA
miR159	AGCTGCTGACTCGTTGGTTC
miR167	CGTAGGGGAGAAGATGGGGACGAT
miR157	GCGTGATTCAACTTGGCCTTGTCG
miR397	TACAAGCACACCACAATCATCACCA
miR395	GGTAATCTGCATCCTGAGGTTTA
miR398	GTTGGAGGTTGCTTGTGGAAT
miR169	CAGCAAAAGGAAGTCGAGGA
miR396	TGAAGAAGATAGTCCCCTTAACACC
miR156	GCGCCACAGAAGAGAGTGAGCAC
miR402	GCGCCACGCCTATTAAACCTCTG
miR172	GCGCCACTTGATGATGCTGCAT
miR393	GCGCCACAAGGAGATTTGCCCTG
miR165	GCGCCACTACTTCGGACCAGGCT
miR168	GCGCCACGGTTGCAGGTCGGGAA
miR319	GCGCCACTGAAGGGAGCTCCCT
miR399	GCGCCACGGGATCGCATTGATCC
Universal Reverse Primer	GTGCAGGGTCCGAGGT

B) Stem – loop primers used for mature miRNA validation

miRNA	Primer sequence 5’-3’
Pv seq 3	SL:GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGAGTTC FP:GCCGGGTGAAGTTGTTTCC RP: CCAGTGCAGGGTCCGAGGTA
Pv seq 10	SL:GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCTCCT FP:GCCGGGATTCGTGTTCAG RP: CCAGTGCAGGGTCCGAGGTA
Pv seq 12	SL: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC FP:GATGCGACTCGGCATGGC RP: CCAGTGCAGGGTCCGAGGTA
Pv seq 13	SL:GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCACTAG FP: GATGCGTGGGAGGAGCCA RP: CCAGTGCAGGGTCCGAGGTA
Pv seq 17	SL:GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGGAG FP:GAGCTGGTTGGACTGAAGG RP: CCAGTGCAGGGTCCGAGGTA

Pv seq 20	SL:GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCCAA FP:GCCGGGTTGGGCAAATCT RP: CCAGTGCAGGGTCCGAGGTA
Pv seq 25	SL:GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATACTC FP:GAGCTGGATATTGGGACGG RP: CCAGTGCAGGGTCCGAGGTA
Pv seq 27	SL:GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCAGC FP:GCGATGGCCTATTAATCTTGA RP: CCAGTGCAGGGTCCGAGGTA
Pv seq 30	SL:GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC FP:GAGCTGGCAATTTGGGTGC RP: CCAGTGCAGGGTCCGAGGTA
Pv seq 36	SL:GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGGGG FP:GGACGGTGTGTTCTCAGG RP: CCAGTGCAGGGTCCGAGGTA

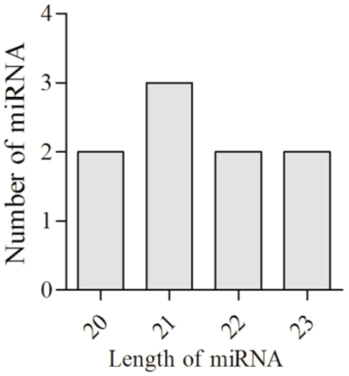
C) Primers used for expression analysis Target genes using qPCR

miRNA	Target gene	Expression	Primer sequence 5’-3’
Pvu seq3	ATP sulpharylase	Down regulated	FP: GTAAGCTTGTTCCTCGGCCT RP: GCATCAGCTTTGCATCTGCA
Pvu seq10	*MYB*- domain protein	Up regulated	FP: CTTCCTCTAGCTTTCTCTCGC RP: AAATCGTAGTGGCGGTCTGA
Pvu seq13	Serine carboxy peptidase	Down regulated	FP: AGTGAGCGAGTGAGTAGGGT RP: TCGGTTTCAACAAGCTCTCGA
Pvu seq16	Lipoyl transferase	Down regulated	FP: TAAGAGCCCCTTGCAAGCTA RP: CGACCAAGGAAGTTCCACAT
Pvu seq20	Stress inducible protein	Up regulated	FP: TCAATGATGGTTTTGCCTCA RP: CATGAGCTTCTCGATGTCCA
Pvu seq21	LRR-NBS	Up regulated	FP: TCTTCATGCTCAACTTGGCA RP: CAGGTAGCTGCAGGTCTTGA
Pvu seq36	Heat shock transcription factor	Up regulated	FP: ACGAGATCGCTTCACACCTC RP: CAAAGGTACGCCGGGGATAA

Suppl. 2: Description of novel miRNAs characterized under high temperature stress.

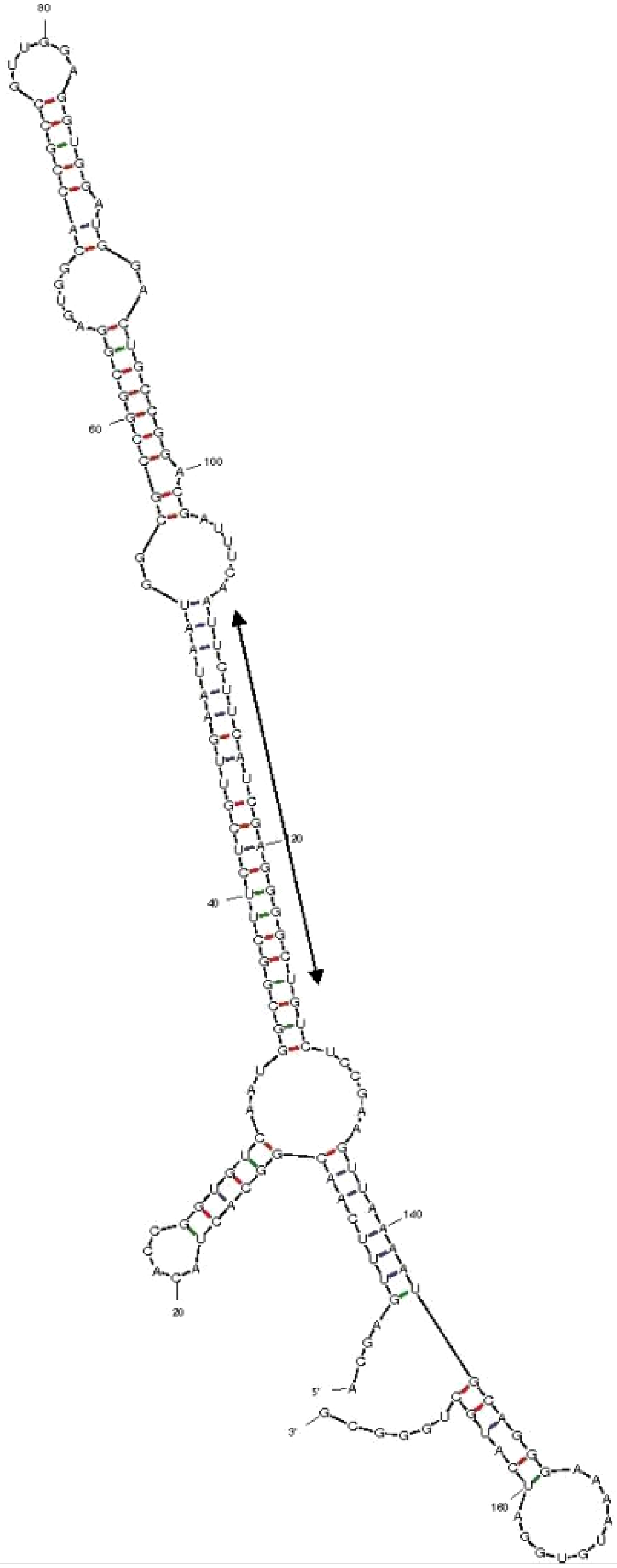
miRNA	Sequence 5’-3’	Length	MFEI	Family	Chromosome no.	Genomic location
Pvu N1	CUUCAUCGAGGGGCUGUCUCC	21	-0.94	miR1919	Chromosome 11	3’UTR
Pvu N2	UGCUGCCCCUCGGCGGUCUC	20	-0.98	Undefined	Chromosome 02	Intron
Pvu N3	UUACCGGCGUUACGUGGAGUCG	22	-0.89	Undefined	Chromosome 02	5’ UTR
Pvu N4	UUGCAGAACCUGGAAUUGACUGU	23	-0.81	miR2905	Chromosome 08	CDS
Pvu N5	AACAGGGCGGGGAACAGGUGGUG	23	-0.85	miR2030	Chromosome 01	3’ UTR
Pvu N6	AUAUUGGGACGGAGGGAGUAU	21	-0.84	miR6224	Chromosome 06 & 08	Intron
Pvu N7	CAAUUUGGGUGCCCCUGCUG	20	-0.86	Undefined	Chromosome 04	5’UTR
Pvu N8	UUGGGUGCCCCUGCUGUUUCUU	22	-0.91	Undefined	Chromosome 04	5’UTR

*MFEI = (MFE/ Length of pri-miRNA) *100 ÷ G+C %*

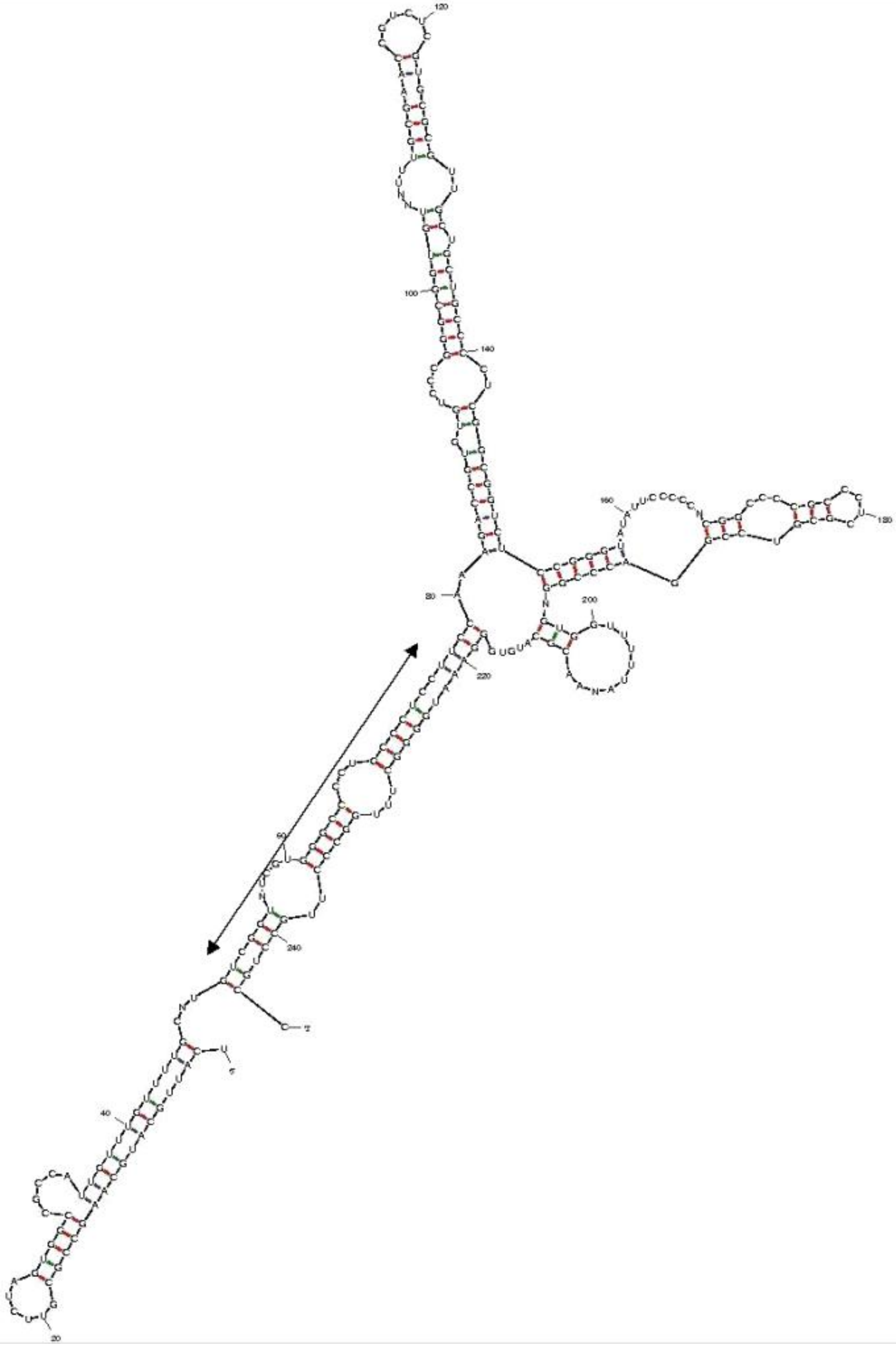


Suppl. 3: Length preference of novel miRNAs

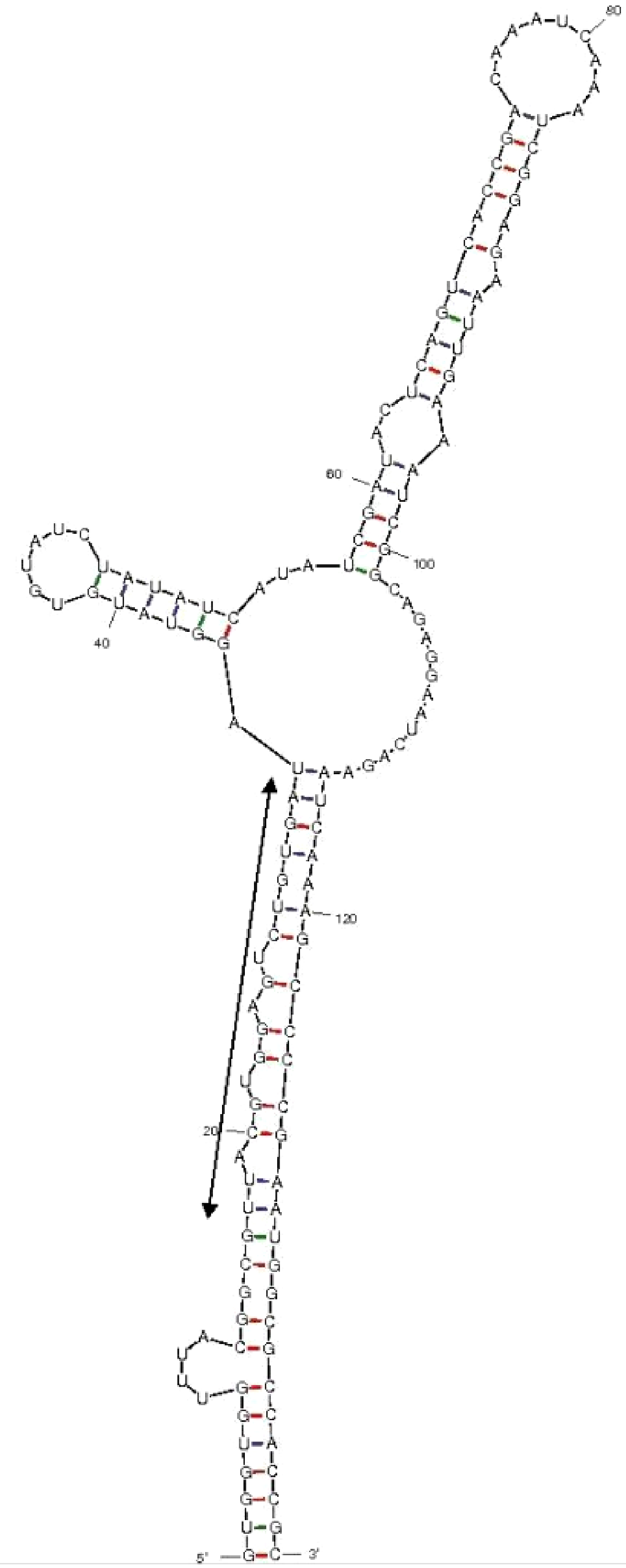
Suppl. 4: Structure of novel miRNAs



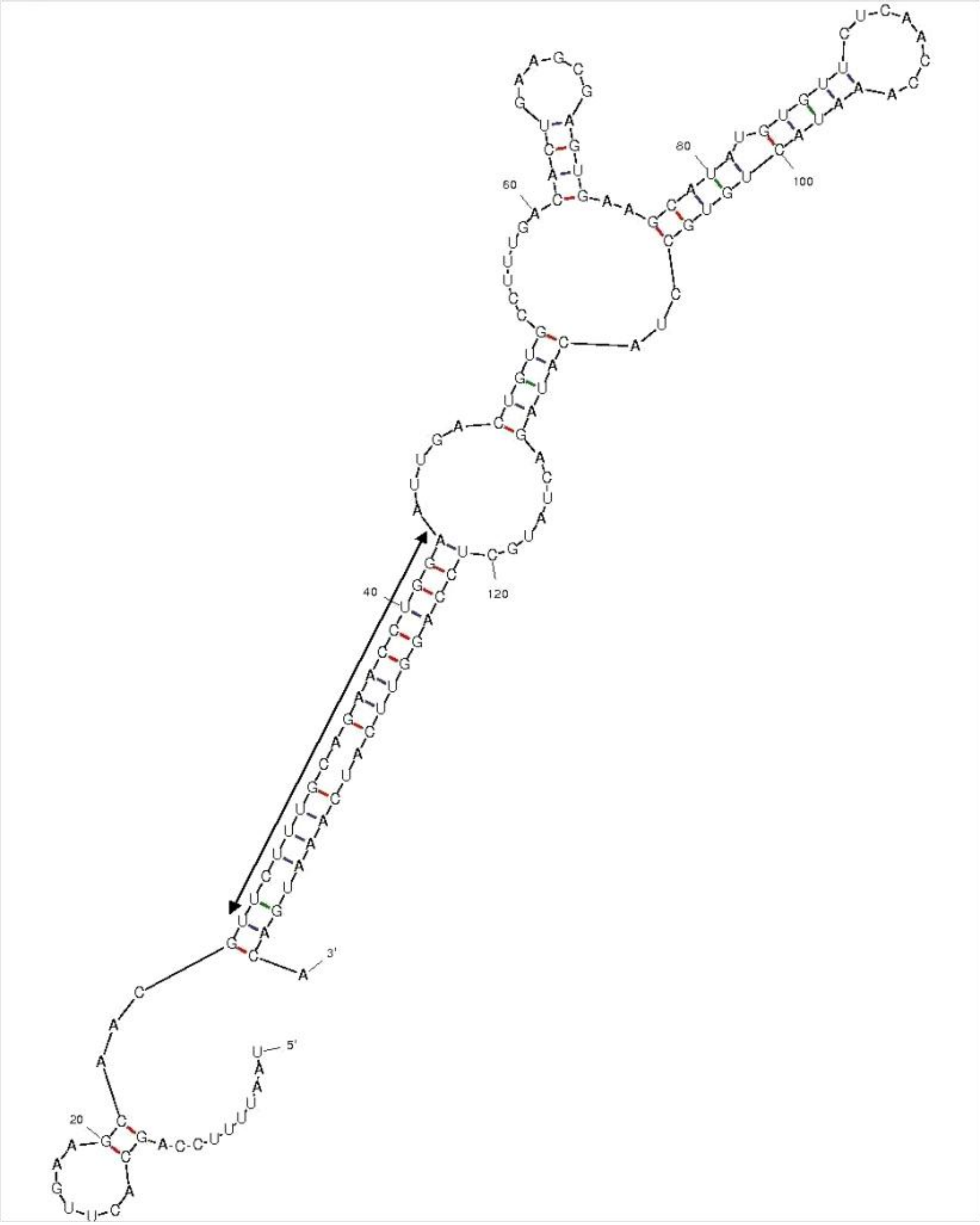
PvuN1



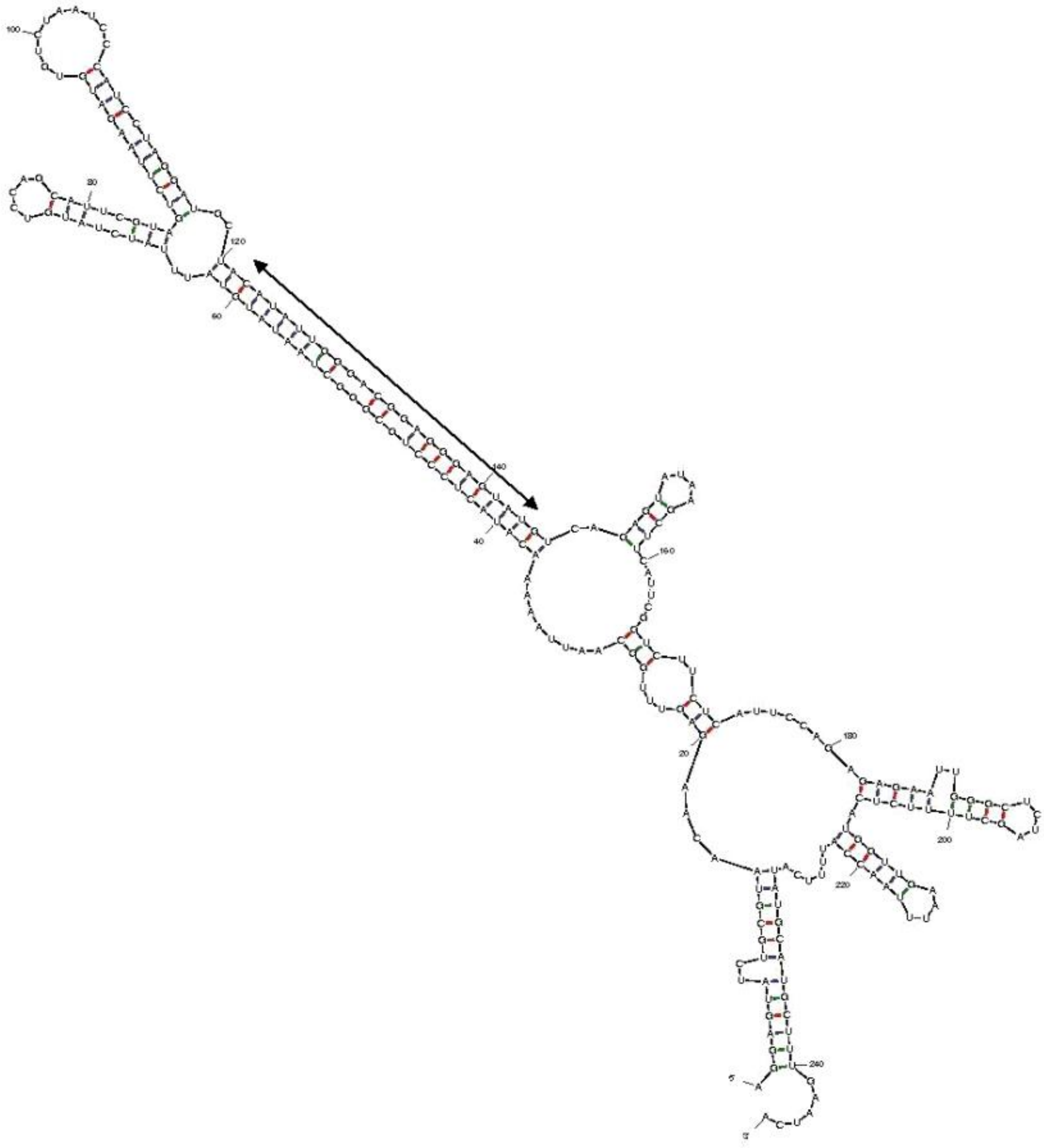
PvuN2



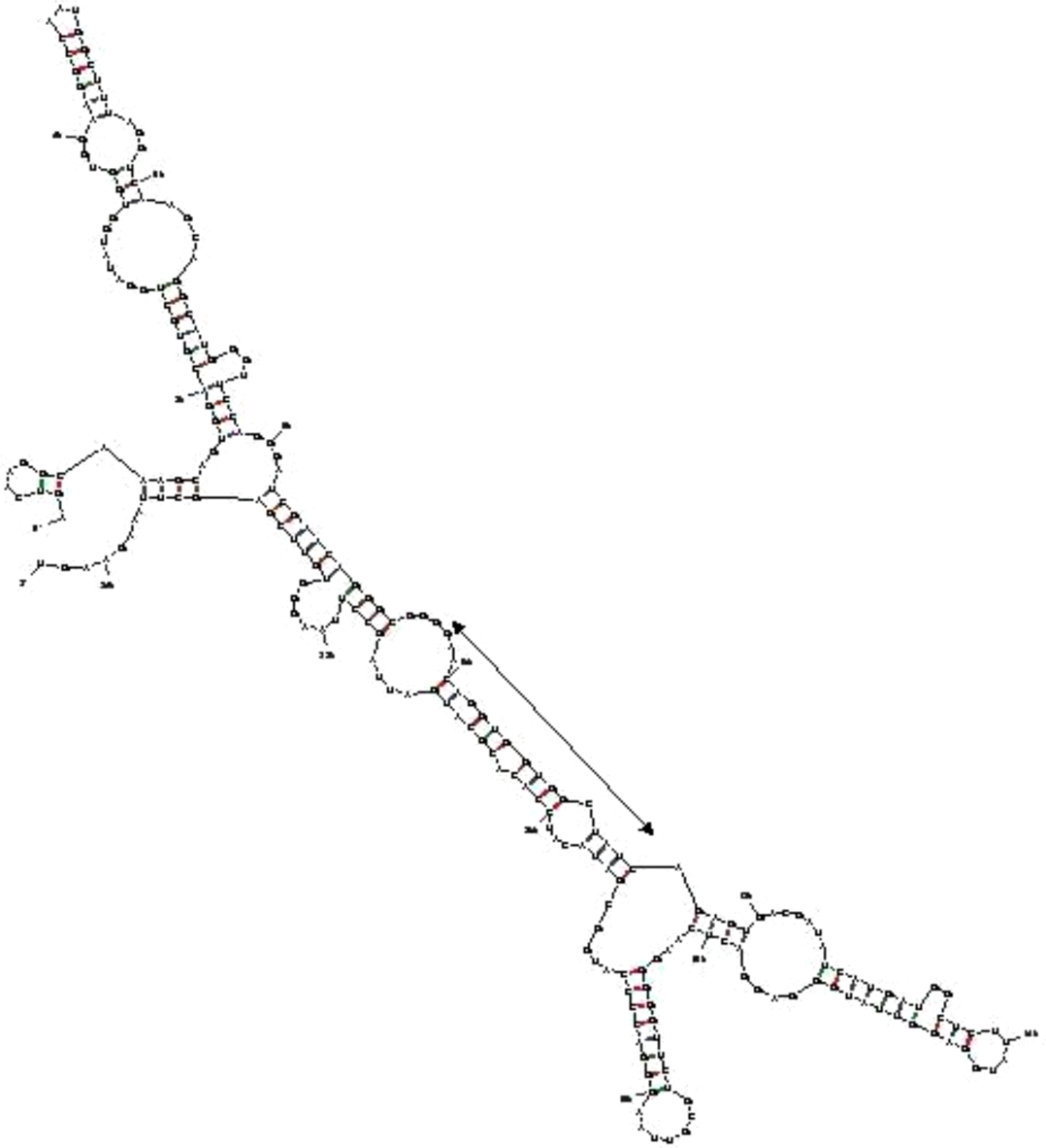
PvuN3



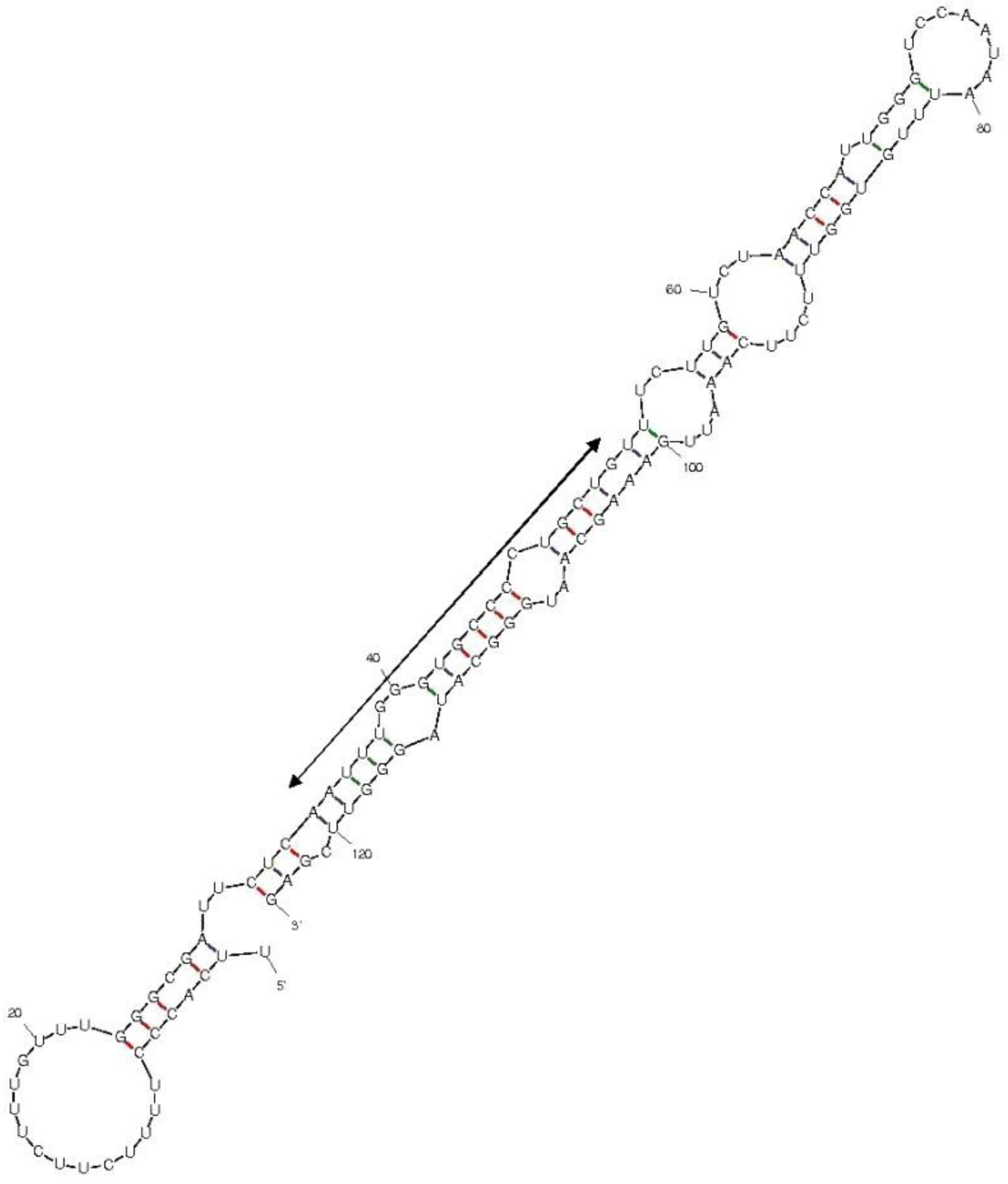
PvuN4



PvuN5



PvuN6



PvuN7

A total of 250 genes were analyzed; 140 genes were annotated and most of the miRNAs exhibited multiple targets. Pvu seq23 has maximum hits with 25 targets while, Pvu seq7, Pvu seq9, Pvu seq16, Pvu seq24, Pvu seq30, and Pvu seq32 targeted single gene. Recognition of multiple targets emphasizes the role of miRNAs in intricate gene regulatory network. Most targets were transcription factors which include NBS- LRR, MYB, bHLH, NAC, F-box, GRF1, bZIP, RING-H2 finger, AP2, ARF etc., others represent metabolic enzymes, membrane proteins and signal transducers (Table 2; Suppl. 5). To determine the roles of identified putative target annotation was performed. The frequency of GO terms associated with predicted targets were enriched with biological function and linked to molecular components (Figure 3). Most of the targets were found to exhibit molecular function with binding activity and majority were transcription factors, followed by metabolic enzymes, membrane proteins and signaling molecules. The transcription factors targeted by the candidate miRNAs include, TPR like super family proteins, MYB, bHLH, GRF1, NAC, GATA, AP2, Homeo box-leucine zipper, NBS-LRR disease resistant protein. Stress specific expression of miRNAs belonging to miR156, miR162, miR169,miR172, miR393, miR396, miR395, miR398, miR408 family were studied by high-throughput sequencing at whole genome level in several plants such as, Arabidopsis [20, 21], Rice [22], Tomato [23], Wheat [10], Potato [24], Medicago trancatula [25, 26], and Cotton [27]. In this study, we established a set of putative conserved miRNAs in French bean responding to high temperature. Many studies reported miRNAs associated with high temperature stress in other plants including wheat [28], Arabidopsis [29], Brassica [12], Barley [13], Rice [30], Celery [31] etc., however, no reports are found with respect to miRNA expression under high temperature stress in French bean.

Table 2 Targets genes identified for conserved miRNAs in French bean

miRNA	GO number	Description
Pvu seq3	GO:0004781,GO:0000103	ATP sulfurylase 1
	GO:0003676,GO:0008270	Zinc finger protein 1
		α-crystallin domain 32.1
	GO:0008762,GO:0016491,GO:0050660,GO:0055114, GO:0003885,GO:0016020	D-arabinono-1,4-lactone oxidase family protein
		Tetratricopeptide repeat (TPR)-like superfamily protein
	GO:0008152	Aconitase/3-isopropylmalate dehydratase protein
	GO:0004455,GO:0009082,GO:0055114,GO:0008652, GO:0016491	ketol-acid reductoisomerase
Pvu seq5	GO:0003677,GO:0006355	AP2/B3-like transcriptional factor family protein
		Histone deacetylase 9
		Putative ATPase
Pvu seq6	GO:0004672,GO:0005524,GO:0006468,GO:0003824, GO:0016773,GO:0009103,GO:0016020	Protein kinase superfamily protein
	GO:0003779,GO:0007010,GO:0015629,GO:0030036	Profilin 5
Pvu seq7	GO:0005507,GO:0009055	Blue-copper-binding protein
Pvu seq8	GO:0004672,GO:0005524,GO:0006468	Protein kinase superfamily protein
	GO:0008270,GO:0047134,GO:0055114	Cysteine/Histidine-rich C1 domain family protein
		Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
	GO:0005506,GO:0009055,GO:0016705,GO:0020037, GO:0055114	Cytochrome P450, family 710, subfamily A, polypeptide 1
	GO:0003700,GO:0043565,GO:0006355	WRKY DNA-binding protein 32
Pvu seq9	GO:0016758,GO:0008152	UDP-glucosyl transferase 73C2
	GO:0005515	Transducin family protein / WD-40 repeat family protein
Pvu seq10	GO:0003677	Myb domain protein 20
		C2H2 and C2HC zinc fingers superfamily protein
	GO:0005515,GO:0005247,GO:0006821,GO:0055085, GO:0016020	Chloride channel C
	GO:0005515	Disease resistance family protein / LRR family protein
Pvu seq13	GO:0004185,GO:0006508	Serine carboxypeptidase-like 31
		ATP-dependent helicase family protein
	GO:0003676	RNA-binding (RRM/RBD/RNP motifs) family protein
Pvu seq14	GO:0045454	PDI-like 1-6
	GO:0005515,GO:0006886,GO:0016192,GO:0030131	Clathrin adaptor complexes medium subunit family protein
	GO:0003677,GO:0005524,GO:0003676,GO:0008026, GO:0004386,GO:0008270,GO:0016818	DNA/RNA helicase protein
Pvu seq15		NEP-interacting protein 2
		RING/U-box superfamily protein
	GO:0004672,GO:0005524,GO:0006468,GO:0005515	Leucine-rich repeat protein kinase family protein
Pvu seq16	GO:0016415,GO:0009107,GO:0005737	Lipoyltransferase 2
Pvu seq17	GO:0016779,GO:0009058,GO:0009435	Nicotinate/nicotinamide mononucleotide adenyltransferase
		ARM repeat superfamily protein

Pvu seq18	GO:0072488,GO:0016020,GO:0008519	Ammonium transporter 2
	GO:0016747	Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase
	GO:0004672,GO:0005524,GO:0006468,GO:0048544, GO:0005529	Protein kinase superfamily protein
Pvu seq19	GO:0006355,GO:0005634,GO:0003677,GO:0009725	Auxin response factor 8
	GO:0006355,GO:0005634,GO:0003677,GO:0009725	Auxin response factor 6
		Ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein
	GO:0016747	Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase
Pvu seq20	GO:0004672,GO:0005524,GO:0006468	Protein kinase family protein
	GO:0004553,GO:0005975	O-Glycosyl hydrolases family 17 protein
	GO:0005515	Stress-inducible protein, putative
Pvu seq21	GO:0043531,GO:0006915,GO:0005524	LRR and NB-ARC domains-containing disease resistance protein
	GO:0043531,GO:0005515,GO:0007165,GO:0005622, GO:0045087,GO:0031224,GO:0004888,GO:0006915, GO:0005524	Disease resistance protein (TIR-NBS-LRR class), putative
	GO:0005515,GO:0043531,GO:0007165,GO:0005622, GO:0005524,GO:0045087,GO:0031224,GO:0004888, GO:0006915	Transmembrane receptors;ATP binding
Pvu seq22		GRAS family transcription factor
Pvu seq23	GO:0008152	Pyridoxal phosphate (PLP)-dependent transferases superfamily protein
	GO:0043531,GO:0005515,GO:0007165,GO:0005622, GO:0045087,GO:0031224,GO:0004888,GO:0006915, GO:0005524	Disease resistance protein (TIR-NBS-LRR class) family
	GO:0004672,GO:0005524,GO:0006468,GO:0005515	PEP1 receptor 1
	GO:0004672,GO:0005524,GO:0006468,GO:0005515	Leucine-rich repeat transmembrane protein kinase family protein
	GO:0003700,GO:0043565,GO:0046983,GO:0006355	bZIP transcription factor family protein
	GO:0003700,GO:0006355	Integrase-type DNA-binding superfamily protein
		RING/U-box superfamily protein
	GO:0004190,GO:0006508	Eukaryotic aspartyl protease family protein
	GO:0030599,GO:0042545,GO:0005618,GO:0004857	Plant invertase/pectin methylesterase inhibitor superfamily
	GO:0016020,GO:0009765	Chlorophyll A/B binding protein 1
	GO:0008168,GO:0008152	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
		Oxidative stress 3
	GO:0003700,GO:0043565,GO:0006355,GO:0003677, GO:0005634	Homeobox-leucine zipper protein family
	GO:0003700,GO:0008270,GO:0043565,GO:0006355	GATA transcription factor 9
	GO:0003697	Primosome PriB/single-strand DNA-binding
		Pentatricopeptide repeat (PPR) superfamily protein
	GO:0003700,GO:0043565,GO:0006355	WRKY family transcription factor
		Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
	GO:0016788,GO:0006629	GDSL-like Lipase/Acylhydrolase superfamily protein
	GO:0003676	RNA-binding (RRM/RBD/RNP motifs) family protein
	GO:0005524,GO:0016887,GO:0016020	ABC-2 type transporter family protein
	GO:0004129,GO:0005507,GO:0016020,GO:0009055, GO:0022900,GO:0016021	Cytochrome oxidase 2
	GO:0003700,GO:0043565,GO:0006355	WRKY DNA-binding protein 75
	GO:0030528,GO:0006355	bHLH DNA-binding superfamily protein
	GO:0005506,GO:0009055,GO:0016705,GO:0020037, GO:0055114	Cytochrome P450, family 71, subfamily B, polypeptide 34
	GO:0000166,GO:0046872,GO:0003824,GO:0008152, GO:0016020,GO:0015662,GO:0006812,GO:0006754	ATPase E1-E2 type family protein / haloacid dehalogenase-like hydrolase family protein
	GO:0008138,GO:0006470	Mitogen-activated protein kinase phosphatase 1
	GO:0031072	DNAJ heat shock N-terminal domain-containing protein
	GO:0005515,GO:0005524,GO:0016818,GO:0006355, GO:0005634	Growth-regulating factor 1
	GO:0003700,GO:0043565,GO:0006355,GO:0005634	Heat shock transcription factor A2
Pvu seq26		Transmembrane protein-related
	GO:0003677,GO:0006306	Methyltransferase 1
	GO:0003677	Myb domain protein 21
Pvu seq27		Cyclin related
Pvu seq28	GO:0055085,GO:0016021	Nodulin-like / Major Facilitator Superfamily protein
Pvu seq29	GO:0003700,GO:0006355	related to AP2.7
	GO:0003700,GO:0006355	Integrase-type DNA-binding superfamily protein
	GO:0003700,GO:0043565,GO:0006355	Homeobox-leucine zipper family protein / lipid-binding START domain-containing protein
	GO:0004672,GO:0005524,GO:0006468	Casein kinase alpha 1

Pvu seq31	GO:0005515,GO:0007165,GO:0005622,GO:0045087, GO:0031224,GO:0004888	Disease resistance protein (TIR-NBS-LRR class) family
	GO:0003700,GO:0006355	Related to AP2.7
		Pentatricopeptide (PPR) repeat-containing protein
	GO:0016491,GO:0016706,GO:0055114	Gibberellin 3-oxidase 1
		HEAT/U-box domain-containing protein
	GO:0005515	Syntaxin of plants 71
	GO:0005215,GO:0006810,GO:0055085,GO:0016021	Sulfate transporter 91
Pvu seq32	GO:0022857,GO:0055085,GO:0016021	Major facilitator superfamily protein
	GO:0005524,GO:0016887,GO:0042626,GO:0006810, GO:0055085,GO:0016021	Multidrug resistance-associated protein 10
Pvu seq33	GO:0008236,GO:0006508	Alpha/beta-Hydrolases superfamily protein
	GO:0005524,GO:0044267,GO:0051082,GO:0006457	TCP-1/cpn60 chaperonin family protein
	GO:0005506,GO:0009055,GO:0016705,GO:0020037, GO:0055114	Cytochrome P450, family 704, subfamily B, polypeptide 1
	GO:0016020	Nodulin MtN21 /EamA-like transporter family protein
	GO:0043531,GO:0005524,GO:0006915	NB-ARC domain-containing disease resistance protein
	GO:0016742,GO:0009058	Formyltransferase, putative
	GO:0004672,GO:0005524,GO:0006468,GO:0005515	NSP-interacting kinase 1
	GO:0045454,GO:0016209,GO:0016491,GO:0055114, GO:0015035,GO:0009055,GO:0006662	Thioredoxin 2
	GO:0004672,GO:0005524,GO:0006468	NIMA-related kinase 2
Pvu seq34	GO:0003700,GO:0006355	Related to AP2.7
		Cleavage and polyadenylation specificity factor 30
		Late embryogenesis abundant protein (LEA) family protein
	GO:0005506,GO:0009055,GO:0016705,GO:0020037, GO:0055114	cytochrome P450, family 81, subfamily D, polypeptide 8
	GO:0043531,GO:0005515,GO:0007165,GO:0005622, GO:0045087,GO:0031224,GO:0004888,GO:0006915, GO:0005524	Disease resistance protein (TIR-NBS-LRR class) family
	GO:0005515	Cystathionine beta-synthase (CBS) family protein
	GO:0030528,GO:0006355	basic helix-loop-helix (bHLH) DNA-binding superfamily protein
Pvu seq36		NB-ARC domain-containing disease resistance protein
	GO:0004672,GO:0005524,GO:0006468,GO:0005515	Leucine-rich receptor-like protein kinase family protein
	GO:0003700,GO:0043565,GO:0046983,GO:0006355	ABA-responsive element binding protein 3
	GO:0003676,GO:0005524,GO:0008026,GO:0004386	DEA(D/H)-box RNA helicase family protein
	GO:0016998	Peptidoglycan-binding LysM domain-containing protein
	GO:0009055,GO:0016491,GO:0055114,GO:0050660, GO:0008033	FAD-dependent oxidoreductase family protein
	GO:0003700,GO:0043565,GO:0006355,GO:0005634	Heat shock transcription factor B4
	GO:0005215,GO:0006810,GO:0055085,GO:0016020	Xanthine/uracil permease family protein
	GO:0008168	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
Pvu seq 37	GO:0008270	GATA type zinc finger transcription factor family protein

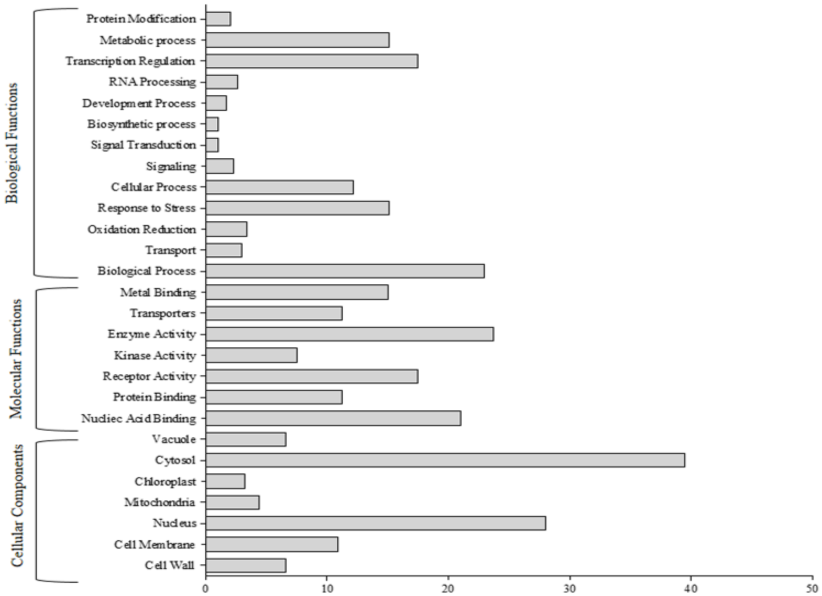


Fig. 3: Gene ontology of the predicted targets of miRNAs identified in French bean. Categorization of miRNA-target genes were performed according to the cellular component, molecular function and biological process.

3.2 Expression patterns of conserved and newly identified miRNAs in French bean

To validate the expression of high temperature responsive miRNAs, we randomly selected eight conserved miRNAs (Pvu seq 3, Pvu seq 10, Pvu seq 13, Pvu seq 16, Pvu seq 20, Pvu seq 21, Pvu seq 27 and Pvu seq 36). Temporal expression of miRNAs were compared with the mock, 10 miRNAs showed differential expressions and could be categorized into three groups: ten fold up regulation was found with Pvu seq 3 while and Pvu seq 16 were up-regulated by 5 fold. Pvu seq 36 and Pvu seq 21 were down regulated by 5 fold. The fold changes were interpreted as logarithm ratio of expression under stress library to the control library. However, Pvu seq 20 and Pvu seq 27 were not affected (Figure 4).

The expression patterns were further verified by Northern blotting and the analogous results were observed. The expression profiles strongly indicate that different miRNA regulation might completely or partially contribute to stress tolerance and differential expression of miRNA is expected to have opposite effect on its target gene. The expression profiles of novel miRNAs are shown in Suppl. 6. The expression patterns of conserved miRNAs were consistent with earlier reports [29, 11]. Through expression abundance of miRNA repertoires many miRNAs were found to have a wide range of expression levels, may be due to miRNA processing and/or stress associated regulation. The up-regulation of the Pvu seq13 and Pvu seq16 appears to play negative role in cell proliferation due to reduced cell division by repressing the target genes GRF and TCP transcription factors respectively. The over expression of miR396 resulted in reduced cell number at the site of cell proliferation and established the interaction of miRNA and GRFs during leaf growth [32]. The qPCR results revealed the expression of target genes is negatively correlated with the expression of their respective candidate miRNAs. Repressions of the targets were found with the up-regulation of their miRNAs and vice versa. We observed the significant fold changes in the expression of miRNAs and their targets. ATP sulpharylase, Serine carboxy peptidase and Lipoyl transferase, were repressed by 2.5, 1.0 and 0.5 fold, while Stress inducible protein, LRR-NBS factor, MYB domain protein, and Heat shock transcription factor B4, were up regulated by 0.2, 2.0, 3.0, and 3.5 fold respectively. It is observed that, despite of the expression trends of miRNAs, the fold changes were different. However, the target genes exhibited marginal changes in their expression. This may be due to the involvement of transcription regulatory factors other than miRNAs whose expression may not alter due to stress induction (Figure 5). Pvu seq10, Pvu seq21 and Pvu seq36 were observed to be repressed upon high temperature stress by 5 folds. Meanwhile, we also noted the up-regulation of their targets MYB, LRR-NBS and Heat Shock Transcription factor B4, however, the fold change with respect to LRR-NBS was found to be statistically not significant. MYB is a major transcription factor in plants, which play vital roles viz., development, metabolism, hormone signal transduction, disease resistance and environmental cues [33]. OsMYB48 was expressed under PEG, abscisic acid, H2O2 and dehydration treatments [34]. It is also demonstrated the coordination of 6 MYB factors with SLIM during sulphate assimilation [35]. Mir398 forms the major miRNA studied under high temperature stress. It is shown that down regulation of miR398 in response to oxidative stress would induce the accumulation of its targets genes CSD1, CSD2 and CCS [36]. Guan *et al.* [29] demonstrated the repression of miR398 in heat exposed Arabidopsis and expression of genes encoding heat stress transcription factors (HSF genes) and heat shock proteins (HSP genes) is reduced in heat-sensitive transgenic plants expressing miR398-resistant forms of CSD1, CSD2 or CCS but is enhanced in the heat-tolerant csd1, csd2 and ccs plants.

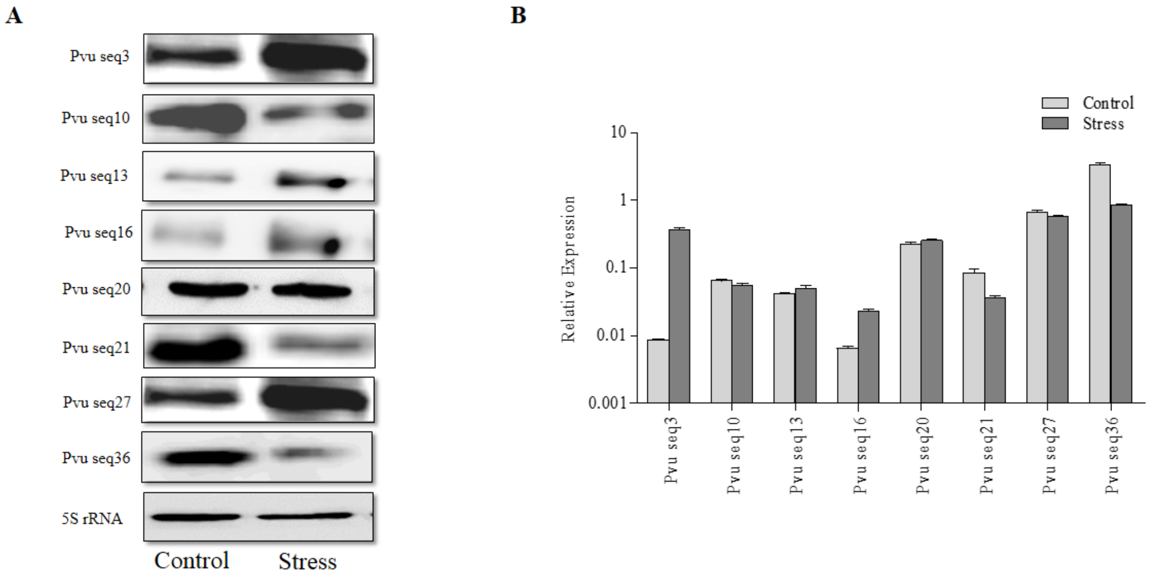


Fig. 4: Expression of miRNAs in French bean. Differentially expressed miRNAs were detected by stem loop qRT-PCR and validated by Northern analysis. Six day-old seedlings (two true leaves) were exposed to high temperature stress at 48 ºC for 6 h, as described in methods. Total RNA from each treatment was extracted, pooled, and determined by RNA gel blotting. **A)** Northern blot of candidate miRNAs **B)** qRT-PCR analysis of conserved mature miRNAs. The standard deviations of the data were obtained from the three independent experiments with student's t-test ($p \leq 0.05$) using statistics software Graphpad Prism v.5

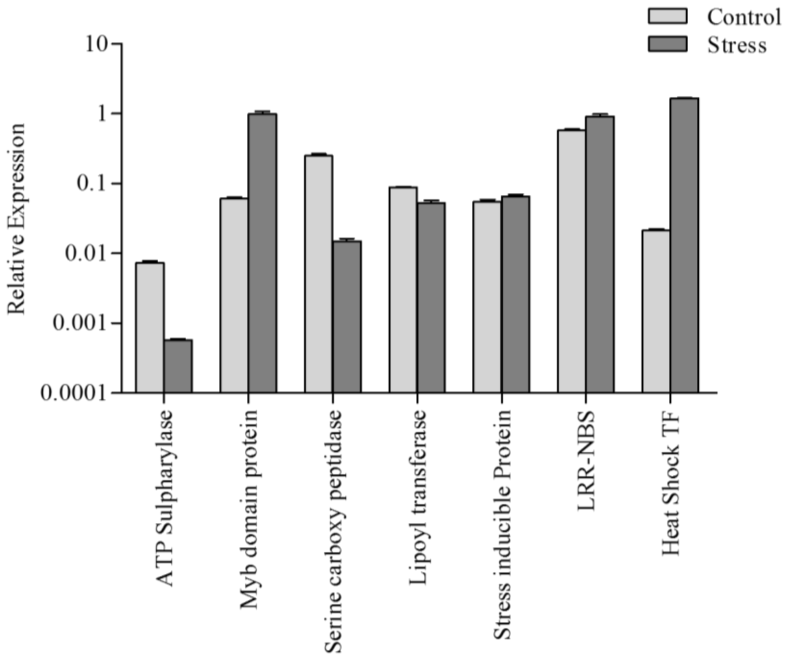


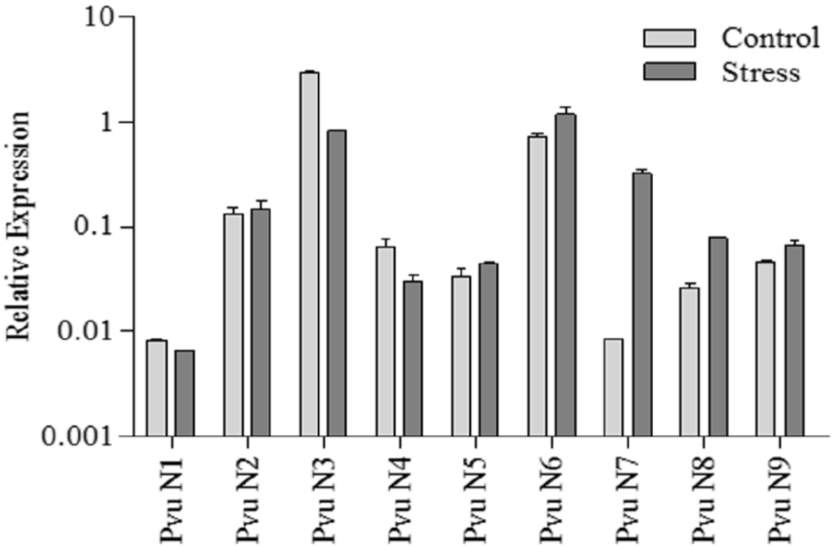
Fig. 5: Validation of predicted targets of miRNAs identified in French bean. Differential expression of miRNA targets were confirmed through qRT-PCR. The standard deviations of the data were obtained from the three independent experiments with student's t-test ($p \leq 0.05$) using statistics software Graphpad Prism v.5

Suppl. 5: Description of genes targeted by high temperature responsive miRNAs.

miRNA ID.	Target_Acc.	Target Annotation
Pvseq3	Phvul.007G062900.1|PACid:27161269	ATP sulfurylase 1
Pvseq3	Phvul.007G118500.1|PACid:27161275	zinc finger protein 1
Pvseq3	Phvul.008G264700.1|PACid:27153124	alpha-crystallin domain 32.1
Pvseq3	Phvul.001G074300.1|PACid:27163090	D-arabinono-1,4-lactone oxidase family protein
Pvseq3	Phvul.001G095200.1|PACid:27163727	Tetratricopeptide repeat (TPR)-like superfamily protein
Pvseq5	Phvul.001G010200.1|PACid:27162704	Unannotated
Pvseq6	Phvul.002G086900.1|PACid:27169951	blue-copper-binding protein
Pvseq6	Phvul.002G132600.1|PACid:27170126	Unannotated
Pvseq6	Phvul.007G249400.1|PACid:27159595	Protein kinase superfamily protein
Pvseq6	Phvul.007G249400.2|PACid:27159596	Protein kinase superfamily protein
Pvseq6	Phvul.010G005700.1|PACid:27140434	Protein of unknown function (DUF3527)
Pvseq6	Phvul.010G005700.2|PACid:27140436	Protein of unknown function (DUF3527)
Pvseq7	Phvul.002G086900.1|PACid:27169951	blue-copper-binding protein
Pvseq9	Phvul.001G182500.1|PACid:27162031	UDP-glucosyl transferase 73C2
Pvseq9	Phvul.006G059700.1|PACid:27166626	Domain of unknown function (DUF2431)
Pvseq9	Phvul.004G028400.1|PACid:27156596	Protein of unknown function (DUF1666)
Pvseq9	Phvul.011G010400.1|PACid:27152977	transducin family protein / WD-40 repeat family protein
Pvseq9	Phvul.006G192000.1|PACid:27165602	FBD, F-box and Leucine Rich Repeat domains containing protein
Pvseq10	Phvul.008G236500.1|PACid:27155183	myb domain protein 20
Pvseq10	Phvul.009G089000.1|PACid:27146943	C2H2 and C2HC zinc fingers superfamily protein
Pvseq13	Phvul.006G189100.1|PACid:27166985	serine carboxypeptidase-like 31
Pvseq13	Phvul.008G253400.1|PACid:27153972	Eukaryotic aspartyl protease family protein
Pvseq13	Phvul.005G133500.1|PACid:27149640	ATP-dependent helicase family protein
Pvseq13	Phvul.005G133500.2|PACid:27149641	ATP-dependent helicase family protein
Pvseq13	Phvul.006G150400.1|PACid:27165494	RNA-binding (RRM/RBD/RNP motifs) family protein
Pvseq14	Phvul.011G179700.2|PACid:27152902	Ca(2)-dependent phospholipid-binding protein (Copine) family
Pvseq14	Phvul.011G179700.1|PACid:27152903	Ca(2)-dependent phospholipid-binding protein (Copine) family
Pvseq14	Phvul.007G203200.1|PACid:27160187	Clathrin adaptor complexes medium subunit family protein
Pvseq14	Phvul.010G049600.2|PACid:27141248	glycine-tRNA ligases
Pvseq14	Phvul.010G049600.1|PACid:27141247	glycine-tRNA ligases
Pvseq14	Phvul.005G168200.1|PACid:27148705	PDI-like 1-6
Pvseq14	Phvul.005G128000.1|PACid:27149260	DNA/RNA helicase protein
Pvseq15	Phvul.008G252000.1|PACid:27154830	Unannotated
Pvseq15	Phvul.007G276100.1|PACid:27159665	Unannotated

Pvseq15	Phvul.007G276100.2|PACid:27159664	Unannotated
Pvseq15	Phvul.007G275900.1|PACid:27161179	Unannotated
Pvseq15	Phvul.003G267100.2|PACid:27142387	NEP-interacting protein 2
Pvseq15	Phvul.003G267100.1|PACid:27142386	NEP-interacting protein 2
Pvseq15	Phvul.007G276700.1|PACid:27159477	Unannotated
Pvseq15	Phvul.009G133400.1|PACid:27147127	Unannotated
Pvseq15	Phvul.007G277100.1|PACid:27159851	Unannotated
Pvseq15	Phvul.007G276200.1|PACid:27161431	Unannotated
Pvseq15	Phvul.002G105100.1|PACid:27169086	RING/U-box superfamily protein
Pvseq15	Phvul.007G277000.1|PACid:27160958	Unannotated
Pvseq15	Phvul.007G276500.1|PACid:27160750	Unannotated
Pvseq15	Phvul.007G276400.2|PACid:27161554	Unannotated
Pvseq15	Phvul.007G276400.1|PACid:27161553	Unannotated
Pvseq15	Phvul.007G276000.1|PACid:27158898	Unannotated
Pvseq15	Phvul.002G060200.1|PACid:27167520	Leucine-rich repeat protein kinase family protein
Pvseq17	Phvul.008G279200.1|PACid:27153060	nicotinate/nicotinamide mononucleotide adenyltransferase
Pvseq17	Phvul.004G026300.2|PACid:27157421	ARM repeat superfamily protein
Pvseq17	Phvul.004G026300.1|PACid:27157420	ARM repeat superfamily protein
Pvseq17	Phvul.004G026300.3|PACid:27157419	ARM repeat superfamily protein
Pvseq18	Phvul.003G267400.1|PACid:27142383	ammonium transporter 2
Pvseq18	Phvul.005G183200.1|PACid:27149455	hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase
Pvseq18	Phvul.007G077300.1|PACid:27161606	Protein kinase superfamily protein
Pvseq19	Phvul.008G242400.1|PACid:27154534	auxin response factor 8
Pvseq19	Phvul.006G084200.2|PACid:27166910	auxin response factor 8
Pvseq19	Phvul.006G084200.1|PACid:27166909	auxin response factor 8
Pvseq19	Phvul.002G233600.1|PACid:27167802	auxin response factor 8
Pvseq19	Phvul.002G177600.1|PACid:27168787	auxin response factor 8
Pvseq19	Phvul.008G197600.1|PACid:27156308	auxin response factor 6
Pvseq19	Phvul.005G141300.1|PACid:27149358	Ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein
Pvseq19	Phvul.005G183200.1|PACid:27149455	hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase
Pvseq19	Phvul.007G077300.1|PACid:27161606	Protein kinase superfamily protein
Pvseq19	Phvul.003G124900.1|PACid:27142544	Alkaline-phosphatase-like family protein
Pvseq19	Phvul.003G122400.1|PACid:27143589	P-glycoprotein 11
Pvseq20	Phvul.009G125200.1|PACid:27146257	Plant protein of unknown function (DUF869)
Pvseq20	Phvul.009G125200.2|PACid:27146258	Plant protein of unknown function (DUF869)
Pvseq20	Phvul.008G137900.1|PACid:27156262	
Pvseq20	Phvul.003G256300.1|PACid:27142161	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
Pvseq20	Phvul.003G256100.1|PACid:27142677	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
Pvseq20	Phvul.004G127100.2|PACid:27157223	Microtubule associated protein (MAP65/ASE1) family protein
Pvseq20	Phvul.004G127100.1|PACid:27157222	Microtubule associated protein (MAP65/ASE1) family protein
Pvseq20	Phvul.002G060800.2|PACid:27170426	stress-inducible protein, putative
Pvseq20	Phvul.002G060800.1|PACid:27170425	stress-inducible protein, putative
Pvseq21	Phvul.011G201000.1|PACid:27152390	LRR and NB-ARC domains-containing disease resistance protein
Pvseq21	Phvul.011G203000.1|PACid:27151641	NB-ARC domain-containing disease resistance protein
Pvseq21	Phvul.010G004700.1|PACid:27140917	LRR and NB-ARC domains-containing disease resistance protein
Pvseq21	Phvul.011G181500.1|PACid:27152520	NB-ARC domain-containing disease resistance protein
Pvseq21	Phvul.004G036300.1|PACid:27158025	NB-ARC domain-containing disease resistance protein
Pvseq21	Phvul.004G036300.2|PACid:27158024	NB-ARC domain-containing disease resistance protein
Pvseq21	Phvul.010G136700.1|PACid:27140167	disease resistance protein (TIR-NBS-LRR class), putative
Pvseq21	Phvul.010G025000.1|PACid:27140443	Disease resistance protein (TIR-NBS-LRR class) family
Pvseq21	Phvul.002G129200.1|PACid:27168586	LRR and NB-ARC domains-containing disease resistance protein
Pvseq21	Phvul.002G131100.1|PACid:27169705	LRR and NB-ARC domains-containing disease resistance protein
Pvseq21	Phvul.002G131200.2|PACid:27171328	LRR and NB-ARC domains-containing disease resistance protein
Pvseq21	Phvul.002G131200.1|PACid:27171329	LRR and NB-ARC domains-containing disease resistance protein
Pvseq22	Phvul.010G072700.1|PACid:27140442	GRAS family transcription factor
Pvseq22	Phvul.010G072900.1|PACid:27141042	GRAS family transcription factor
Pvseq22	Phvul.008G143400.1|PACid:27153384	GRAS family transcription factor
Pvseq22	Phvul.008G143400.2|PACid:27153383	GRAS family transcription factor
Pvseq26	Phvul.010G100800.1|PACid:27140279	transmembrane protein-related
Pvseq27	Phvul.010G096500.1|PACid:27141429	Arabidopsis thaliana protein of unknown function (DUF821)
Pvseq27	Phvul.007G045300.1|PACid:27160020	cyclin-related
Pvseq28	Phvul.002G087600.1|PACid:27168324	Nodulin-like / Major Facilitator Superfamily protein

Pvseq29	Phvul.005G138300.2|PACid:27148711	related to AP2.7
Pvseq29	Phvul.011G071100.2|PACid:27152560	related to AP2.7
Pvseq29	Phvul.005G138300.1|PACid:27148710	related to AP2.7
Pvseq29	Phvul.011G071100.1|PACid:27152559	related to AP2.7
Pvseq29	Phvul.001G174400.1|PACid:27163589	related to AP2.7
Pvseq29	Phvul.002G016900.1|PACid:27167533	Integrase-type DNA-binding superfamily protein
Pvseq29	Phvul.003G241900.1|PACid:27141828	Integrase-type DNA-binding superfamily protein
Pvseq29	Phvul.007G240200.1|PACid:27161577	related to AP2.7
Pvseq29	Phvul.001G233200.2|PACid:27162634	Unannotated
Pvseq29	Phvul.001G233200.1|PACid:27162633	Unannotated
Pvseq29	Phvul.011G079800.1|PACid:27152785	Homeobox-leucine zipper family protein / lipid-binding START domain-containing protein
Pvseq29	Phvul.001G033700.2|PACid:27162998	AT3G20430.1
Pvseq29	Phvul.001G033700.1|PACid:27162997	AT3G20430.1
Pvseq29	Phvul.009G117500.1|PACid:27145893	like heterochromatin protein (LHP1)
Pvseq29	Phvul.005G030900.1|PACid:27149167	S-locus lectin protein kinase family protein
Pvseq29	Phvul.002G012700.1|PACid:27167926	casein kinase alpha 1
Pvseq29	Phvul.004G132000.1|PACid:27157216	Peroxidase superfamily protein
Pvseq31	Phvul.010G152200.1|PACid:27141178	gibberellin 3-oxidase 1
Pvseq31	Phvul.002G197200.1|PACid:27170332	O-acetyltransferase family protein
Pvseq31	Phvul.002G004400.1|PACid:27167872	pentatricopeptide (PPR) repeat-containing protein
Pvseq31	Phvul.008G250900.1|PACid:27153923	HEAT/U-box domain-containing protein
Pvseq31	Phvul.007G221500.1|PACid:27161668	syntaxin of plants 71
Pvseq31	Phvul.008G153500.1|PACid:27156331	transporter associated with antigen processing protein 1
Pvseq31	Phvul.010G132000.1|PACid:27139847	Disease resistance protein (TIR-NBS-LRR class) family
Pvseq31	Phvul.009G249300.1|PACid:27145337	Tetratricopeptide repeat (TPR)-like superfamily protein
Pvseq31	Phvul.002G095200.1|PACid:27169074	sulfate transporter 91
Pvseq31	Phvul.010G084500.2|PACid:27140589	RNA 2\'-phosphotransferase, Tpt1 / KptA family
Pvseq31	Phvul.010G084500.1|PACid:27140590	RNA 2\'-phosphotransferase, Tpt1 / KptA family
Pvseq33	Phvul.003G252500.1|PACid:27143606	Major facilitator superfamily protein
Pvseq33	Phvul.009G165200.1|PACid:27146040	Unannotated
Pvseq36	Phvul.011G201200.1|PACid:27152203	retinoblastoma-related 1
Pvseq36	Phvul.011G201200.2|PACid:27152204	retinoblastoma-related 1
Pvseq36	Phvul.008G267500.1|PACid:27154019	DEA(D/H)-box RNA helicase family protein
Pvseq36	Phvul.002G146700.1|PACid:27170419	Xanthine/uracil permease family protein
Pvseq36	Phvul.008G244000.1|PACid:27155417	RNA-binding KH domain-containing protein
Pvseq36	Phvul.009G138500.1|PACid:27147122	Leucine-rich receptor-like protein kinase family protein
Pvseq36	Phvul.006G016600.1|PACid:27165672	AT2G24960.2
Pvseq36	Phvul.006G016600.2|PACid:27165673	AT2G24960.2
Pvseq36	Phvul.004G095400.1|PACid:27157244	Duplicated homeodomain-like superfamily protein
Pvseq36	Phvul.009G227600.1|PACid:27146507	O-fucosyltransferase family protein
Pvseq36	Phvul.008G057600.1|PACid:27153747	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
Pvseq37	Phvul.009G181200.1|PACid:27146418	GATA type zinc finger transcription factor family protein



Suppl. 6: Expression assay of novel miRNAs. Expressions were analyzed by RT-qPCR, and normalized against U6 gene. Analysis was carried out in three biological replicates and the student's *t* test was performed with $p \leq 0.05$

4. CONCLUSION

Cloning of small RNAs is a starting point to understand their number, diversity and possible roles in different organisms. In this study, we have identified a sum of 36 miRNAs representing 21 families modulated by high temperature and potential targets suggest that miRNAs are directly or indirectly involved in stress response. Validation of these miRNAs emphasis the negative correlation with their target genes. The present work provides important and fairly extensive knowledge about miRNAs and their functions in French bean. Additionally, this knowledge may be useful in studies concerned with methods to increase the yield whilst improving their ability towards stress adaptations.

5. CONTRIBUTIONS

Ms. Jyothi has contributed in bringing the data of the following; Construction of cDNA library, characterization of miRNAs and qPCR analysis. Ms. Usha, Ms. Suchithra B and Dr. Sharadamma were involved in computational analysis of miR targets and Northern blot analysis. Dr. Devaraj supported technically for preparing research manuscript. Prof. Rai and Dr. Nagesh Babu are research supervisors/corresponding authors. All the authors have contributed for bringing of this manuscript.

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