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Identification and Characterization of High Temperature Stress Responsive Novel miRNAs in French Bean (*Phaseolus vulgaris*)

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Abstract MicroRNAs are important gene regulators controlling almost all biological and metabolic functions. They elicit their regulatory response through modulation of their target gene expression. In this study, we identified eight novel microRNAs (miRNAs) belonging to four miRNA families and one miR* sequence from the French bean genome which responded to high temperature. The precursor miRNAs varied in length and showed conserved signatures of RNA polymerase II transcripts in their upstream regions. Promoter region analysis indicated the prevalence of MYB and WRKY binding sites emphasizing auto-inhibition of miRNA biogenesis. The genomic organization study revealed the presence of 150 putative regulatory motifs of which 41 are unique. Common motifs shared by miRNAs involved in more than one abiotic stresses were also identified. Further, the miRNA validation was carried out by stem-loop real-time PCR, and the results emphasize that the differential expression of miRNAs confers stress tolerance. Functional analysis revealed that most of the targets represent transcription factors. The results obtained would provide new insights to the complex regulatory mechanism employing small non-coding regulatory RNAs toward stress adaptation.

Keywords Differential expression · Heat stress · MYB · Transcription start site · Promoters · Regulatory motifs · WRKY

Abbreviations

GO	Gene Ontology
miRNA	MicroRNA

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TF	Transcription factor
TSS	Transcription start site
TFB	Transcription factor binding site
UTR	Untranslated region
LEA	Late embryogenic abundant proteins

Introduction

Climatological extremes including high temperature were predicted to have a general negative effect on plant growth and development, leading to catastrophic loss of crop productivity and resulting in widespread famine. Future agricultural production and thus global food security will encounter additional challenges from human population growth. The growing food demand and the threat of heavy crop loss due to global climate change impose the urgent development of strategies to substantially improve food availability. Higher plants exposed to temperature at least 5 °C above their optimal growing conditions induce dramatic resetting of physiological and molecular mechanisms in order to facilitate continued homeostasis and survival by regulated gene expression. To date, a large number of genes that respond to high temperature stress at transcriptional level have been identified [1]. The products of target genes play an important role not only in protecting cells from high temperature but also in regulating genes for signal transduction in stress response. However, the detailed regulations of these genes are poorly understood.

Recently, small non-coding RNAs specifically microRNAs (miRNAs) are gaining importance as stress adaptive molecular switches that fine-tune the gene expression at post-transcriptional level. They have been reported to be involved in various biological functions including organ development [2, 3], hormone signaling [4], defense against pathogens [5], and abiotic stress responses [6]. Many plants have been explored for the involvement of miRNAs in response to salinity [7], cold [8], heavy metal [9], drought [10, 11], nutrition depletion [12–15], and different biotic stresses [16]. Stress-induced miRNAs downregulate their target genes, which may be negative functional proteins involved in stress response. Conversely, other miRNAs are downregulated, leading to the accumulation of their target messenger RNAs (mRNAs) that contribute positively to stress adaption. It is essential to note that most of these miRNAs target genes encoding transcription factors, which place miRNAs at the center of gene regulatory networks [13]. Diverse stresses are known to trigger widespread changes in miRNA levels in part through altered *MIR* transcription [17, 18]. As like of the protein coding genes, the transcription of miRNA genes is regulated by a set of biogenic factors. A network of transcription factors associated with RNA binding proteins interacts with distinct regions on pri-miRNA during processing to recruit DCLI, HYL, and SE proteins to form stable plant microprocessor unit. Transcription factor binding sites (TFBs) that are located in the upstream region of the genes regulate gene expression. The architecture of the TFBs may be conserved to a particular class of genes or genes co-expressed to a particular stress. The phenomenon may also operate even in the regulation of miRNA genes. The distribution and accumulation of TFBs vary between different miRNAs and even with the members of the same family and confers characteristic regulation of miRNA generation in a tissue/stage-specific manner [19]. This high specificity relays over the mechanism of miRNA function.

The navigation toward exploring the structure of miRNA genes provides insights toward the complexity of eukaryotic gene regulation which involves feedback regulation and auto-

inhibition. It is evident that miRNAs even self-regulate the expression of their genes. miR162 in Arabidopsis targets *DCL I* gene, the key enzyme of miRNA maturation. The target site of miR162 resides at the transcription factor binding motif present around the promoter sequence of pre-miRNA [20]. Although some independent studies were reported in identifying the known transcription regulatory elements in miRNA genes, no report is found toward analyzing the genomic organization and genetic structure owing to the differential expression of miRNAs under high temperature in French bean.

Materials and Methods

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 a 12-h light/12-h dark photo period. After 6 days 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). Tissues were harvested immediately and stored at −80 °C for further analysis.

Small RNA Extraction and Cloning

Total RNA was isolated from tissues using TRizol (Invitrogen) according to the manufacturer's instructions and then treated with RNAase-free DNAase I (Promega). Small RNAs (200 nt) were separated on a denaturing 15 % urea polyacrylamide gel. Molecules ranging in size from 18 to 26 nt were excised and recovered using 30 μl RNAase-free water. The soluble RNAs (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 complementary DNA (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 (Supplementary File 1) at 95 °C for 20 s, 56–58 °C for 30 s, 72 °C for 30 s cycled for 30, and final extension at 72 °C for 5 min in a thermo-cycler (Kyratec, Australia). The amplicons were purified and ligated into *pGEMT* Easy (Promega, USA). The colonies were screened for the ligated products using gene-specific primers. The plasmid DNA from recombinant colonies showing the correct PCR size was then 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.

Data Analysis

All sequences were used to search for the Rfam database (http://www.sanger.ac.uk/Software/Rfam) [21, 22] to filter most ribosomal RNA sequences. Putative origins for the remaining

sequences were identified by BLASTN against French bean ESTs. The sequences with perfect (0-3) matches with small RNA sequences were used for fold back secondary structure prediction with MFOLD (http://mfold.rna.albany.edu) [23]. A segment was considered a valid miRNA candidate if its secondary structure met the criteria according to Meyers et al. [24].

Prediction of miRNA Targets

Target prediction for the miRNAs was based on the principle of nearly perfect complementation between the miRNA and target mRNAs. The newly identified miRNAs were analyzed for targets using psRNATarget (http://plantgrn.noble.org/psRNATarget/) [25] and psRobot (http://omicslab.genetics.ac.cn/psRobot/) [26]. French bean transcript sequences downloaded from Phytozome version 9 (www.phytozome.net) [27] 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) [28].

Characterization of miR Genes

Retrieval of Promoter Sequences

The miRNAs were assumed as independent transcription units to have uniformity. Upstream sequences of 1 kb were retrieved at the beginning of the pre-miRNA for the prediction of transcription start site (TSS) for all the types of miRNA (genic and intergenic). The TSS and TATA-box predictions were made using TSSP web tool (http://linux1.softberry.com) [29]. Putative promoter sequences from −1000 to −1 from the TSS were retrieved for all classes of miRNA and used for motif search and identification of strong motifs.

Scanning for Transcription Factor Binding Motifs

The candidate miRNA genes were scanned for putative TFBs using (1) plant *cis*-acting regulatory DNA elements (PLACE) signal scan search, to identify the known cis-regulatory elements (http://www.dna.affrc.go.jp/PLACE) [30]; (2) NSITE (http://linux1.softberry.com/) [31]; and (3) MELINA-II (http://melina2.hgc.jp/public/index.html) [32]. We have used four algorithms to predict the motifs (1) Consensus, (2) Gibbs Sampler, (3) MD SCAN (with default parameters), and (4) MEME (with a cutoff E-value of 1 with *anr* (any number of repetitions) mode). The motifs identified by at least two programs were considered as strong motifs. The motifs which were not detected in PLACE database were considered as unique (novel) motifs. To interpret the genomic locations of miRNA genes, the genomic coordinates of pre-miRNAs were overlapped at the transcript genomic region.

Semi-quantitative RT-PCR Validation of miRNA Expression

To validate and quantitate the expression levels of the high temperature stress miRNAs, RT-qPCR was performed using SYBR Green PCR Master mix (Takara) on Light cycler 96 Real-time PCR (Roche). Each PCR reaction (20 μl) included 2 μl cDNA, 10× 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 (Supplementary File 2). The reactions were performed at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min with a final dissociation curve analysis. All reactions were run with two biological replicates. The data was analyzed using $2^{-\Delta\Delta CT}$ method.

Results

Identification of High Temperature Responsive miRNAs

Small RNAs were documented not only to modulate a series of complex developmental events but also to regulate defense under abiotic stress conditions. To explore the small RNA pool under high temperature, we constructed small RNA libraries from high temperature treated French bean seedlings. Small RNA ranging from 18 to 26 nt were isolated by size fractionation, ligated to 5′ and 3′ adapters, cloned, and sequenced. A total of 3000 clones were sequenced (approximately one third of the library), of which 2000 cDNA sequences were between 18 and 26 nt; the remaining were shorter fragments or self-ligated adapters. BLASTN revealed that these sequences had at least one match in the French bean nuclear genome. Several clones were mapped to chloroplast/mitochondrial genomes and may represent degradation or possible regulatory products/organellar RNAs. The largest class of sequences represent fragments of abundant non-coding RNAs (rRNA, tRNA, small nuclear/nucleolar RNA), as determined by Rfam database. miRNAs were distinguished from endogenous siRNA on the basis of the ability of miRNA surrounding sequences to adopt hairpin structure (Supplementary File 3). Based on initial survey of folded structure and homology of mature miRNA with miRBase, the miRNA sequence that remained after exclusion of conserved and their variants with ≥4 mismatches was considered as novel and has been named as PvuNx (for *P. vulgaris* Novel, x being the number). With this, we were able to identify eight novel miRNAs belonging to four miRNA families with MEFI of −0.81 to -0.98 (Table 1).

Table 1 Novel miRNAs expressed under high-temperature stress in French bean found to regulate cellular homeostasis

miRNA	Sequence 5′-3′	Length	MFEI	Family
PvuN1	CUUCAUCGAGGGGCUGUCUCC	21	−0.94	miR1919
PvuN2	UGCUGCCCCUCGGCGGUCUC	20	−0.98	Undefined
PvuN3	UUACCGGCGUUACGUGGAGUCG	22	−0.89	Undefined
PvuN4	ACUCGGCAUGGCUCCUCCCAC	21	−0.90	miR160*
PvuN5	UUGCAGAACCUGGAAUUGACUGU	23	−0.81	miR2905
PvuN6	AACAGGGCGGGGAACAGGUGGUG	23	−0.85	miR2030
PvuN7	AUAUUGGGACGGAGGGAGUAU	21	−0.84	miR6224
PvuN8	CAAUUUGGGUGCCCCUGCUG	20	−0.86	Undefined
PvuN9	UUGGGUGCCCCUGCUGUUUCUU	22	−0.91	Undefined

Target Prediction and Functional Analysis of Stress-Responsive miRNAs

A single miRNA is known to regulate the expression of many genes or members of a gene family. To find the putative targets of novel miRNAs, we used psRNATarget and psRobot. The detailed analysis revealed that the majority of the miRNAs have more than one potential regulatory target. To investigate the biological significance of the target genes, it is important to discuss the gene ontology (GO) descriptions. The GO analysis shows that predicted targets were involved in a wide range of regulatory functions and specific biological processes (Fig. 1). The transcripts representing the genes with known functions were categorized according to the ontological definitions of GO terms (Table 2). Most of the miRNA target genes assigned to the binding category were involved in nucleic acid, protein, and ion binding. Since these sequences are encoded for transcription factors, this is in accordance with previous reports [33].

Validation of Novel miRNAs by Stem-Loop RT-PCR

Stress-specific expressions and the validation of the newly identified miRNAs were studied by stem-loop real-time PCR. Although high temperature affected the expression of all the miRNAs, the expression levels determine the extent of their involvement. Of the eight miRNAs, PvuN1, PvuN3, and PvuN5 were downregulated with fold change of 0.5, 1.5, and 1.0, respectively. PvuN7 was upregulated by threefold while PvuN6, PvuN8, and PvuN9 showed marginal upregulation; however, PvuN2 remains unaffected (Fig. 2). These differential expression patterns prompt the modulation of miRNA expression by high temperature.

Characterization of miRNA Genes

To decipher the structural features of miRNA genes expressed under high temperature, genomic locations of the newly identified miRNAs were surveyed. PvuN2, PvuN3, PvuN4, PvuN5, PvuN7, PvuN8, and PvuN9 exhibited complete genotyping with the existence of TSS, regulatory motifs, and promoter sites. Most of the miRNAs showed the presence of TSS in the region of −300. However, TSS was not found in PvuN1 and PvuN6. Meanwhile, PvuN4, PvuN7, and PvuN8 possessed multiple TSS, their positions were determined around −100 to −600 bp (from the pri-miR start site) (Fig. 3), and TATA box was predicted in the region of −35. Although PvuN8 and PvuN9 originated from the same gene loci, their expression preference and the TSS/promoter occupancy make them different and represented as two different miRNAs. Chromosome-wise categorization of miRNAs was carried out to identify the common chromosomal regions of miRNAs involved under high temperature. The upstream and downstream regions of miRNA were analyzed for their positions and nearest genes. Chromosomes 4 and 6 were found to harbor more miRNAs that are involved in abiotic stress. The distribution of miRNA genes was found to be multi-positional (chromosomal). The loci of *PvuN7* was positioned on Chr06 and Chr08 while *PvuN5* exhibited multi-loci over Chr08. The rest of the *miR* genes were positioned over a single chromosome. The generic positions depict that most of the miRNAs were located in intron regions which emphasize the role of introns as non-protein coding genes and origins of endogenous non-coding RNAs specifically miRNAs (Table 3).

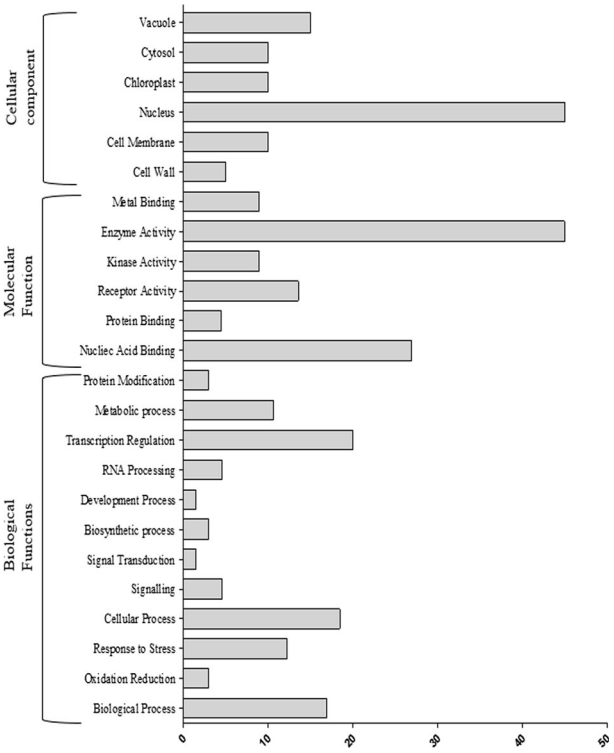

Fig. 1 Ontological definitions of the genes targeted by novel miRNAs expressed under high temperature in French bean

To elucidate the features of transcription regulation of miRNA, sequences were further analyzed for the presence of transcription factor binding sites (TFBs), using NSITE, PLACE, and MELINAII. A total of 145 conserved and 41 unique regulatory motifs (Fig. 4) were identified. We retrieved 150 significant non-redundant TFBs including MYB, WRKY, bZIP, CCAAT, GC-rich, and TC-rich motifs, and positional preferences showed that TFBs were concentrated at −400 to −600 positions (Fig. 5 and Table 4). MYB and WRKY/W-Box were determined to be located around −300. These results suggest that the upstream region up to 1 kb might play an important role in the transcription of miRNA genes. The identified TFBMs were known to be involved in regulation of stress-responsive genes, which indicates that the differential expression of miRNAs may be modulated by employing the same elicitor moieties engaged in regulation of protein-coding genes.

Discussion

In the long course of evolution, plants have evolved the complex intricate gene regulatory mechanism involving the stress-specific expression of genes controlled by transcription

Table 2 Targets genes identified for novel miRNAs in French bean

miRNA	GO number	Target description
PvuN1	GO:0004842,GO:0016567,GO:0000151	ARM repeat superfamily protein
	GO:0000166,GO:0046872,GO:0003824,GO:0008152, GO:0016020,GO:0015662,GO:0006812, GO:0006754	ATPase E1-E2 type family protein/ sshaloaciddehalogenase-like hydrolase family protein
	GO:0019538	Double Clp-N motif-containing P-loop nu cleoside triphosphate hydrolases super family protein
PvuN2	GO:0045454,GO:0005515	Tetratricopetide-repeat thioredoxin-like 1
	GO:0004672,GO:0005524,GO:0006468,GO:0005515	ERECTA-like 1
	GO:0003723,GO:0016779,GO:0006396	Polynucleotide adenylyltransferase family protein
	GO:0004252,GO:0006508,GO:0042802,GO:0043086	Subtilase family protein
		Nucleolar protein gar2-related
		C2H2 and C2HC zinc fingers superfamily protein
	GO:0005089	*rho*guanyl-nucleotide exchange factor 1
PvuN4	GO:0016415,GO:0009107,GO:0005737	Lipoyltransferase 2
PvuN5		Glutamine dumper 3
PvuN6		Transmembrane protein-related
PvuN7		Kinase-related protein
	GO:0005506,GO:0009055,GO:0016491,GO:0050660, GO:0016021,GO:0055114,GO:0004601, GO:0050664	NADPH/respiratory burst oxidase protein D
PvuN8	GO:0003824,GO:0008152	Long-chain acyl-CoA synthetase 2

factors. Recent research emphasizes the role of miRNAs as key gene regulators under various abiotic stress including salinity, drought, metal toxicity, and nutrition depletion [34–41]. Off the small non-coding RNAs studied, miRNAs gain significant attraction owing to their endogenous nature and their function as *cis*-regulatory molecules. Most of the studies focused on the expression trends of candidate miRNAs and established the stress/tissue specificity. However, the information about their transcriptional regulation is very limited. In this study, we aimed to decipher the differential expression of novel miRNAs and their genomic organization under high temperature in French bean.

Sequences obtained from the cDNA libraries were analyzed for small non-coding RNAs and mapped to French bean genome. Those sequences which satisfied the miRNA criteria of Meyers et al. [19] but did not show homology with the existing members were considered as candidate miRNAs expressed specifically under high temperature. One predominant feature of miRNA is that the mature miRNA expresses predominantly than its precursor and, hence, the expression analysis of mature sequence validates the stress-specific expression of miRNAs. Since real-time PCR assay is considered as the gold standard approach to validate the RNA expression, we employed the stem-loop RT-qPCR which is distinct between the isoforms and specificity of miRNAs. It is apparent that the differential expression of miRNA was exhibited

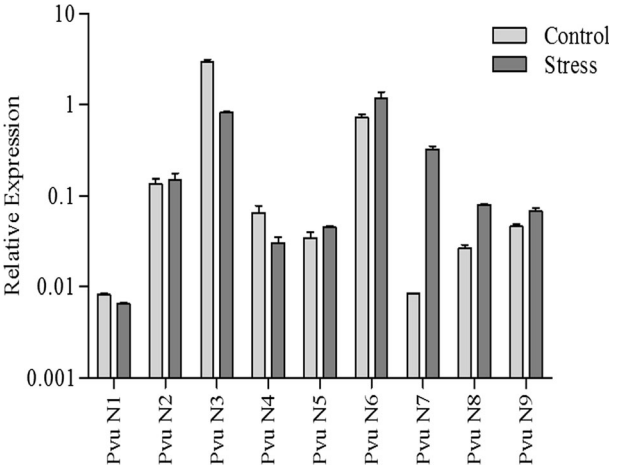

Fig. 2 Expression of novel miRNAs was analyzed using stem-loop RT-qPCR. The fold changes were calculated using $2^{-\Delta\Delta CT}$ method and normalized against U6 gene

by the candidates, and their fold changes resemble their involvement as gene regulators. We speculate that the expression modulation of miRNAs would induce plants to reprogram their cellular composition to sustain the induced environmental cues. However, the miRNA forms the inner layer of intricate gene regulatory network; the small changes in their expression would have a significant effect on the abundance of their targets. The functional analysis of induced miRNAs was determined by identifying their targets. The identified targets were major transcription factors, membrane proteins, and metabolic enzymes. Our GO studies emphasized that miRNA exerts gene regulation through the repression or expression of these transcription factors and other stress-induced metabolic enzymes. Under the GO category of catalytic activity, isomerase, transferase, and oxido-reductase activities were enriched in

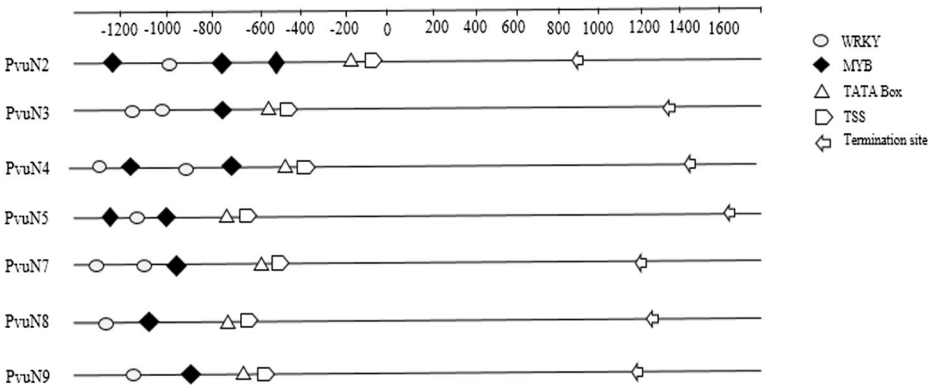

Fig. 3 Positional preference of TSS and TATA box. The pri-miRNA start site is defined as 0. Positions of TSS, TATA box, TFBs, and termination end sites are depicted relative to the pri-miRNA start site

Table 3 Genomic location of novel miRNAs responsive to high temperature in French bean

Sl No.	miRNA	Chromosome no.	Genomic location	TSS	TATA box	Nearest gene
1	PvuN1	Chromosome 11	3′UTR	–	–	Phvul.007G119400 (Unannotated)
2	PvuN2	Chromosome 02	Intron	−122	−160	Terpene synthase
3	PvuN3	Chromosome 02	5′UTR	−480	−515	Nucleotide-sugar transporter
4	PvuN4	Chromosome 06	5′UTR	−384	−419	RNA binding family protein
5	PvuN5	Chromosome 08	CDS Intron CDS Intron	−645	−679	Undefined protein ARM repeat super family protein LEA protein RING 1 A
6	PvuN6	Chromosome 01	3′UTR	–	–	UDP-glucosyl transferase
7	PvuN7	Chromosome 06 Chromosome 08	Intron Intron	−695	−565	Ribose -5-phosphate Isomerase type A Serene-threonine protein phosphatase
8	PvuN8	Chromosome 04	5′UTR	−644	−670	Ypt/Rab-GAP domain superfamily protein
9	PvuN9	Chromosome 04	5′UTR	−544	−600	Ypt/Rab-GAP domain superfamily protein

response to high temperature and genes associated with oxidative stress due to excess accumulation of ROS and/or due to insufficient antioxidant defenses [42, 43]. Tetratricopeptide repeat domain protein was found to be one of the targets of PvuN2, which in association with FKPB-propyl isomerase acts as chaperone and is involved in cell cycle, transcription, and protein transport complexes [44].

To further elucidate the inducibility of miRNA-mediated gene regulation, in silico analysis of *cis*-acting elements in plant promoters has been used with success in studies of miRNA expression profiles. Existence of promoters, TSS, regulatory motifs, and polyA tail

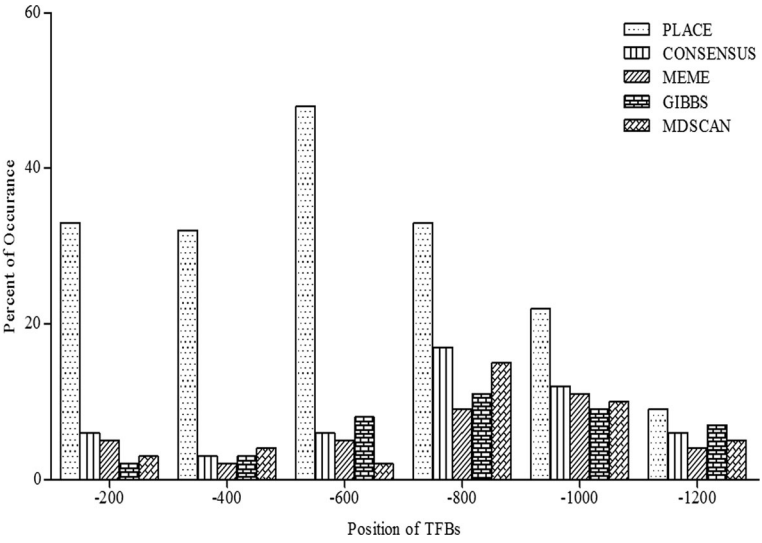

Fig. 4 Comparison of *cis*-regulatory elements identified with various algorithms. The number of elements identified at a given position varies with algorithm. The non-redundant motifs were considered and their functional definitions were obtained from PLACE

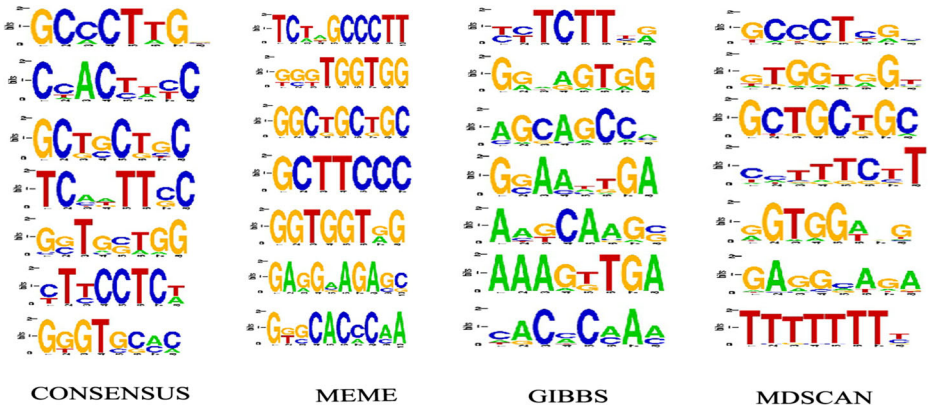

Fig. 5 Sequence logo of the unique motifs identified in French bean novel miRNA genes

Table 4 Known *cis*-regulatory elements of French bean novel miR genes

Sl no	Motif logo	Motif sequence	Algorithm	Function
1	NODCON2GM	CTCTT	PLACE	Involved in regulation of genes that are responsive to water stress
2	ACGTABOX	TACGTA	PLACE	Required for etiolation-induced expression of erd1
3	ACGTTBOX	AACGTT	PLACE	Binding sequence of AtERFs
4	ANAERO2CONSENSUS	AGCAGC	PLACE	ARR1 is a response regulator
5	ARR1AT	NGATT	PLACE	HBP-1 binding site of wheat histone H3 gene
6	BOXLCOREDCPAL	ACCWWCC	PLACE	DCMYB1 binding
7	CACTFTPPCA1	YACT	PLACE	Key component of Mem1 (mesophyll expression module 1) found in the cis-regulatory element in the distal region of the phosphoenolpyruvate
8	CCAATBOX1	CCAAT	PLACE	"CCAAT box" found in the promoter of heat shock protein genes
9	CGCGBOXAT	VCGCGB	PLACE	Ca++/calmodulin binding
10	CURECORECR	GTAC	PLACE	TAC is the core of a CuRE (copper-response element) found in Cyc6 and Cpx1 genes
11	DPBFCOREDCDC3	ACACNNG	PLACE	bZIP transcription factors
12	GAREAT	TAACAAR	PLACE	ERE (ethylene responsive element)" of tomato
13	GATABOX	GATA	PLACE	ARE (GA-responsive element)
14	MYB2CONSENSUSAT	YAACKG	PLACE	MYB recognition site found in the promoters of the dehydration-responsive gene
15	POLLEN1LELAT52	AGAAA	PLACE	MYC recognition site found in the promoters of the dehydration-responsive gene rd22
14	WBBOXPCWRKY1	TTTGACY	PLACE	WRKY proteins bind specifically to the DNA sequence motif (T)(T)TGAC(C/T)
15	MYBPLANT	MACCWAMC	PLACE	Plant MYB binding site
16	P1BS	GNATATNC	PLACE	PHR1-binding sequence of Phosphate starvation genes

characterizes the eukaryotic gene. In order to define these structural features, 1-kbp upstream and downstream sequences were fetched from the mapped sequences, and genomic distributions were predicted. Chromosome 6, 8, and 11 host majority of the stress-specific miRNA genes in introns. PvuN5 exhibited multiple positions on chromosome 8 of which two were introns and two were embedded in the LEA gene. This suggests that miRNA may act as both mirtron and/or intragenic and exhibit the transcriptional regulation. Many reports suggests that miRNA genes are inverted repeats and are found randomly in plant genome [42–44]. Some of these belong to non- autonomous DNA transposons or miniature inverted-repeat transposable elements (MITEs) [45]; however, we could find the loci of the identified miRNAs distributed over introns and exons. The intronic miRNAs targeted their neighboring protein-coding genes, while the exonic miRNAs upon generation were found functional at inter-genic locations. It has been reported that sometimes, the genes in which the miRNAs were embedded are also their putative targets of regulation, and in such cases, the expression of miRNAs and the putative targets are tightly co-regulated [46].

The dynamic network of gene transcriptional regulation is modulated by the function of *cis*-acting regulatory elements. Stress-specific differential expression of miRNAs fascinates the further elucidation of regulatory motifs in promoter regions. The rapid processing of pri-miRNAs challenges the traditional global TSS mapping strategies, and therefore only a limited number of miRNA TSSs were identified to date [46, 47]. Conserved motifs flanking the TSS revealed the prevalence of adenine at TSS similar to that observed in Arabidopsis and flax. In most of the identified novel miRNAs, TATA box was found at −35 position relative to TSS, suggesting that French bean miRNA genes are transcribed by RNA pol II and have same promoters as the protein coding genes. The non-TATA box containing miRNA genes (*PvuN1* and *PvuN6*) might fall into the class termed TATA-less (promoters) generally reported for housekeeping and developmental genes; similar observations were reported in Arabidopsis and rice [48]. A total of 145 distinct transcription factor binding sites (Supplementary File 4) were observed of which 41 found to be unique. MYB and WRKY binding motifs occupies the majority of the regulatory motifs, suggesting their key role in stress responses and transcription of miRNA genes as well. MYB is known to take part in complicated signaling network through which plants respond to changes in the surrounding environment. The high occurrence of MYB may represent cross talk between disease and drought response mechanism, accounting for characteristic high inducibility of miRNAs in poplar [49]. The fact that the MYBs are widely spread in plants suggests that these transcription factors play a wide role in stress adaptive mechanism. Some MYBs are involved in the regulation of cell proliferation, differentiation, and apoptosis and determine the fate of plant cells. MYB sites are identified in all miRNA genes implying that they are positively regulated by the transcription factors. The expression of these targets is modulated by a negative feedback loop that buffers small changes in the level of their mRNAs.

WRKY transcription factor binding sites (W-Box) form another abundant TFBs, global regulators of plant responses to biotic and abiotic cues. Pandey et al. (2009) [50] reported the WRKY-small RNA interactome in plant defence mechanism. Pathogen attack triggers the expression of WRKY genes that regulate cellular miRNA populations; meanwhile, several differentially regulated miRNAs modulate WRKY TF levels by targeting their transcripts. The involvement of WRKY TFs in modulating the expression of several miRNAs while their

transcription is partly under miRNA surveillance adds another dimension to the regulatory complexity that must be sorted out.

Our study showed that most of the miRNAs use their own transcription initiation regions with few members appearing to share with their host genes, suggesting that the alternative post-transcriptional processing plays a key role in determining the fate of each primary transcript. The observations revealed the existence and positional preferences of intronic miRNA TSSs which are significantly far from their host TSS and are evolutionarily conserved. Further investigations on upstream regulatory motifs, transcription factors, and validation of the targets will allow us to construct a detailed miRNA-mediated gene regulatory network which is critical for complete understanding of plant stress response.

Conclusion

In this study, we identified 8 novel miRNAs responding differentially under high temperature. The functional characterization revealed that most of the targets represent transcription factors. The navigation toward genomic organization of miRNA genes revealed that the majority of the miRNAs were intronic and possess their own transcription start sites and promoter sequences. Forty-one unique and 150 significant regulatory motifs were identified of which MYB and WRKY were abundant. The identification of TSS and TFBs in the upstream region of the miRNA strongly supports that the miRNA gene expression is independent irrespective of their genomic location. Our observations on the promoters and motifs will contribute to the characterization of miRNA promoters, thereby helping in understanding the regulation of miRNA gene transcription.

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Conflict of Interest The authors declare that they have no conflict of interest.

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