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Boron toxicity induces altered expression of miRNAs in French bean (*Phaseolus vulgaris L.*)

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ABSTRACT

French bean (*Phaseolus vulgaris* L.), a legume grown all over the world, is an important pulse crop of India whose yield is affected by various biotic and abiotic stresses. Micro RNAs (miRNAs) have been shown to play an important role in the regulation of plant responses to several stresses. Boron toxicity is a significant limitation to cereal crop production. In this study, RT-qPCR confirmed seven miRNAs responsive to high concentrations of boron, exhibited differential expression trends compared with the control. Target prediction and their functional analysis showed that most of the miRNA targets represent transcription factors regulating expression of stress-related genes. GO results supported our hypothesis that miRNAs were involved in diverse cellular processes, including plant circadian cycle, vegetative development, transcription, and cross adaptation. Our research characterized a subset of miRNAs that would facilitate understanding the regulatory mechanisms of small non-coding regulatory RNAs involved in stress tolerance.

1. INTRODUCTION

Boron toxicity is an important agronomic problem that limits crop productivity worldwide. Higher concentrations of boron might occur naturally (in the soil or in groundwater), or acquired by the soil due to activities such as mining, use of fertilizers, or through irrigation water. The permissible levels of Boron in irrigation water range from 0.3 mg per litre to 4 mg per litre [1]. The symptoms of boron toxicity are visible chlorotic/necrotic patches on leaves. Most of the studies have mainly concentrated on the toxic effects of heavy metals and macronutrients such as cadmium [2], lead [3,4], mercury [4], copper [5,6], arsenic [7] etc., However, limited research has concerned about the toxic effects of trace elements such as boron. Research on heavy metal toxicity regulations has been done extensively. The regulatory mechanism involves the intracellular metal chelation by a series of Cys-rich peptides [8]. Loss of function within the synergistic network employing metal transporters, chelators and sequesters is the basal cause of plant hypersensitivity. Transcriptional and post-transcriptional gene regulation is an important step in response to metal toxicity or metal deficiency and there exists large lacunae in understanding plant metal homeostasis. With the advent of high throughput sequencing, small RNA mediated gene regulations has gained prominence in unravelling the critics of plant gene regulatory mechanism. MicroRNAs, a class of small non-coding RNAs that regulate mRNA level by either vitiating target mRNA or attenuating its translation. Several investigations escalated the number of miRNAs identified and demonstrated their crucial role in various plant metabolic functions. Many studies reported the involvement of miRNAs in various biological processes such as cell cycle, cell death, floral development and several physiological responses [9]. Increasing evidences also emphasized the influence of various biotic and abiotic stress factors on expression of miRNAs [10]. Through microarray, *Ding et al.* reported the altered expression of miR168, miR528, and miR162 under cadmium stress and defined their targeted genes were involved in biogenesis of miRNAs [11]. They also determined the miRNA genes possessed MREs (metal responsive cis-regulatory elements) in their upstream sequences which control the expression of these miRNAs. The overexpression of miR192 caused due to increased cadmium concentrations retarded the seed germination and seedling growth in rice [12]. *Ozhuner et al*. demonstrated the elevated levels of boron induced over expression of miR156d, miR171a, miR397, and miR444a in leaves which were not detected in root while, miR172, miR399, miR2021, miR5053 and miR5066 were expressed in both leaf and root [13]. This exemplifies the tissue-specific expression of miRNAs under stress.

French bean is an important legume crop cultivated for its seeds and pods. The crop yield is found to be affected by drought, high salt,

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temperature and availability of essential nutrients. Many groups involved in identification and characterization miRNAs under various abiotic stress conditions. *Arenas-Huertaro et al.* proposed the differential expression of miR2118, miR159a, miR1514a, miR482, miR2119, miR166a, miR319c, and miR399a would render plant tolerant towards drought and salt stress [14]. *Valdes-Lopez et al.* through next-generation sequencing confirmed the miRNAs respond stress specifically and demonstrated miR399 is crucial in phosphate uptake and acts as a signaling molecule during phosphate starvation [15]. *Pelaez et al.* exploited high throughput sequencing to characterize the complete set of miRNAs responded to salt stress [16]. *Naya et al*. through expression analysis functionally characterized the role of miR398b in copper homeostasis. miR398b targets Cu/Zn superoxide dismutase, which was also determined to target stress up-regulated Nodulin 19 in root, nodules, and leaves of French bean [6]. Although extensive research substantiates the key role of miRNAs in various stress responses, resource against the boron stress-responsive miRNAs in legumes specifically French bean is scanty.

Our study aims to characterize putative miRNAs from boron exposed French bean seedlings. To understand the impact of boron toxicity on the expression of conserved stress-responsive miRNAs, RT-qPCR studies were carried out. Further, B responsive miRNAs were cloned and sequenced. Functional analysis and Gene Ontology (GO) study revealed that miRNAs affect plant circadian cycle and vegetative development. We anticipate our effort would provide new insights into the metal stress regulatory pathways acting in plant metal homeostasis.

2. MATERIALS AND METHODS

2.1. Plant Materials and Stress Treatment

Surface sterilized French bean seeds were grown under controlled conditions at 28°C day/25°C night with 12 h photoperiod. 6 days old seedlings were hydroponically treated with 5 ppm of boric acid, in half strength Hoagland medium for 48 h and a group of seedlings was maintained as control [17]. Post-stress treatment, both test, and control tissues were stored at −80°C until further analysis.

2.2. RT-qPCR Validation of miRNA Expression

To evaluate the stress-induced expression of miRNAs and validate the effect of boron toxicity, RT-qPCR was performed using stress responsive miRNA family specific primers designed based on the available literature and SYBR Green PCR Master mix (Takara) on Light cycler 96® (Roche). Total RNA was isolated from the stressed and control samples and cDNA was synthesized using Universal Reverse primer and MMLV Reverse transcriptase (Invitrogen, USA). Each PCR reaction (20 µl) included 2 µl cDNA, 10 µl SYBR Green Master mix, 1 µl sequence-specific forward primer (10 µM), 1 µl Universal reverse primer (10 µM) and 6 µl sterile water (Supplementary file 1). The reactions were performed for 10 min at 95ºC followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min with a final extension 72ºC for 30 s, with two biological replicates; the data were analyzed based on efficiencies and fold changes. The reactions were carried as described earlier and signals were normalized against U6 snoRNA and the relative expressions were calculated using the $2^{-\Delta\Delta CT}$ method and the fold changes were determined by *Fold change* = log_2 *ratio of normalized expression of miRNAs under stress to that of control samples*. Statistics software GraphPad Prism v.5 was used to obtain standard deviations of the data obtained from the three independent experiments with student's t-test ($p < 0.05$).

2.3. Characterization of Boron Responsive miRNAs

To define the miRNA sequence of boron stress-responsive miRNAs, RT-qPCR products were subsequently cloned and sequenced. 50 µl of RT-qPCR product was purified by Nucleospin DNA Cleanup kit (Takara, Japan) according to manufacturer's instructions. The PCR products were subjected to gel purification and products were ligated to pGEMT Easy vector system I (Promega). Cloning was performed with 5 µl of 2X Rapid Ligation Buffer, 1 µl of pGEM-T Easy Vector (50 ng), and 1 µl of T4 DNA ligase (3 units/µl) was added to the purified and quantified RT-qPCR products in a final volume of 10 µl at 4°C overnight and transformed into E. coli (DH5α). The colony PCR was carried out using gene-specific primers and the PCR positive clones were sequenced and processed for BLAST analysis.

2.4. Data Analysis

Rfam database (http://www.sanger.ac.uk/Software/Rfam) was used to check the sequences for tRNA/rRNA contamination. BLASTN search against French bean genome was performed to identify putative origins. 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].

2.5. 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) [20]. French bean transcript sequences downloaded from Phytozome version 9 (www.phytozome.net) [21] 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 Blast2Go [22].

2.6. Characterization of miR Genes

The determination of *MIR* regulatory motifs and TSS (Transcription Start Site) prediction was carried out as described previously [23]. Briefly, 2 kb upstream sequences were retrieved at the beginning of the pre-miRNA for the prediction of 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) Putative promoter sequences from −1,000 to −1 from the TSS were retrieved for all classes of miRNA and used for motif search and identification of strong motifs.

2.7. Scanning for Transcription Factor Binding Motifs (TFBs)

The candidate miRNA genes were scanned for putative TFBs using two modules (1) PLACE (Plant cis-acting regulatory DNA elements) signal scan search, to identify the known cis-regulatory elements (http://www.dna.affrc.go.jp/PLACE/signalscan.html) and (2) MELINA-II (http://melina2.hgc.jp/public/index.html) [24]. 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 cut-off 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.

1.8. 5'-RACE Validation of Target Genes

The cDNA was amplified with mirRacer 5' primer (5'-GGACACTGACATGGACTGAAGGAGTA-3') and mirRacer 3' primer (5'-ATTCTAGAGGCCGAGGCGGCCGACATG-3') to generate a pool of non-gene-specific product. These miRacer primers are complementary to the 5' and 3' adaptors, respectively. The conditions used for the amplification were carried out for 25 cycles at a final annealing temperature of 60°C. 5' miR-RACE reactions were performed with the mirRacer 5' primer and miRNA-gene-specific forward and reverse primers and a piece of Poly(T) and 5' adaptor. In each case, a unique gene-specific DNA fragment was amplified. After the amplification, the 5' RACE PCR products were separated in a 2.5% agarose gel with ethidium bromide staining. The gel slices containing DNA with a size of about 60 bp were excised and the DNA fragments were purified using an agarose gel DNA purification kit (Takara, Japan), according to the manufacturer's instructions. The DNA fragment was directly sub-cloned with the pGEMT Easy vector (Promega). Colony PCR was performed using the PCR specific primer pairs as above. The 5' RACE clones with PCR products were sequenced.

3. RESULTS

3.1. Validation of miRNA Expression Using Stem-Loop RT-qPCR

To determine the influence of boron toxicity, the RT-qPCR was carried out using family-specific primers for sixteen miRNA families reported as responsive to abiotic stress (Supplementary fig 1). We found most of the miRNAs are sensitive to the elevated boron concentration. However, we observed noticeable fold changes among seven miRNAs, miR396, miR398, miR812, miR845, miR2593, miR2608 and miR2905 (Figure 1A) with an average up-regulation by 2 folds. Higher boron availability down-regulated miR398 and miR2608 by 1.75, and 2.7 folds respectively. miR2593 was found to be highly induced by 3 folds and miR845 by 2.6 folds. miR396, miR812 and miR2905 were induced by 1.5, 1.7 fold and 1 fold respectively (Figure 1B).

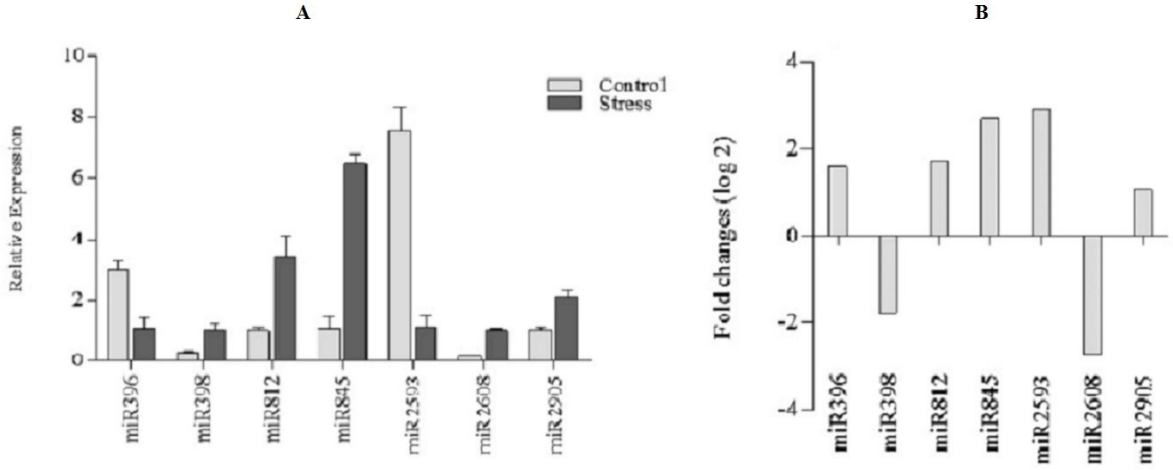

Figure 1: Expression analysis of boron stress-responsive miRNAs in French bean. A. Relative expression of miRNAs was studied using RT-qPCR. The expressions are normalized against U6. The study was carried in three independent experiments and Standard deviations and t-test were performed with $p < 0.05$. **B.** Fold change of respective miRNAs compared to control sample.

3.2. Determination of Boron Stress-Responsive miRNAs in French Bean

To determine the sequences of boron stress-responsive miRNAs, the RT-qPCR products were cloned and plasmids were sequenced. The filtered sequences were analysed further for putative miRNA and their precursors. The sequences of length 18-27 nt were mapped to French bean genome to obtain their true precursors forming stable secondary structure with MFEI values >0.85. The length of the miRNA sequences ranged from 19–24 nt and majority of the sequences starts with A, characteristic of plant miRNAs. The length of the precursor sequences varied from 230-330 with high MFE. The MFEI validates the identified pri-miRNA structure. Table 1 describes the genomic location and MFEI values of miRNAs found sensitive to boron toxicity.

3.3. Computational Prediction of Putative Targets and Their Annotations

To obtain a complete view of the mode of action of boron stress-responsive miRNAs, the target prediction was performed using psRNATarget with French bean transcriptome as background database with default parameters. The target hits were annotated to define their functions using Blast2Go (Supplementary file 2). Our results showed most of the targets were involved in biological process maintaining cellular homeostasis. Majority of them were transcription factors which include GRF, MYB, GATA, BTB-TAZ and NEP Interacting Protein 2 and RNA binding RINT1/TIP. Genes involved in biological function include aspartyl amino peptidase, hydrolase, lipoyltransferase, glucouronosyl transferase, etc. (Table 2). Gene ontology studies also reported the miRNA targets were exclusively involved in plant growth and development as most of the targets exhibited enzyme regulation during reproduction and developmental stages, 8 of which exhibited nucleotide binding, 10 were involved in metabolic functions and 13 engaged in cellular processes (Figure 2).

3.4. Functional Characterization of miRNA Genes

To gain further insights towards the genomic organization of boron stress-responsive miRNAs, computational search for putative TSS, TATABOX, and TFB motifs and polyA hangs were carried out. For the analysis 2 kb, upstream and downstream sequences from the precursor start site was obtained and processed. The identified miRNA genes exhibited the TSS around −600 to −300 from the pri-miRNA

start site and their respective TATA box was found −35 to their TSS. However, we could not define the TSS and TATA box for miR396 and can be considered under the category of TATA less genes. Majority of the miRNA genes exhibited polyA strings at +900 from pri-miRNA endpoint. Further, to determine the TFB motifs surrounding the TSS and regulating the miRNA gene expression we employed MELINA II and NSITE algorithms and description of the motifs were defined with PlantCare and PLACE database. The motifs which were found in all the five algorithms was considered as putative TFB of the given miRNA gene. And we noticed MYC/MYB, WRKY and MRE binding sites were commonly found in all miRNA genes. Other motifs include ABRE, DREB, HD-ZIP, bHLH domains (Table 3). The identified TFB motifs were known to involve in tissue and organ development, stress responses including abiotic cues such as light, metal uptake, nutrient absorption and phytohormone signalling. These results suggest the upstream regions up to 2 kb were crucial in miRNA gene expression.

Table 1: Characteristics of miRNAs responded to Boron toxicity French bean.

Sl. No	miR Family	Sequence 5'-3'	miRNA length	Precursor length	MFEI	Chromosomal loci	Expression
1	miR396	AGGAGCCAACCAUAGCCAU	19	275	−1.17	Chr9	Up regulated
2	miR398	UGUGUUCUCAGGUCGCCCCUG	21	322	−0.86	Chr2	Down regulated
3	miR812	GUGGGAGGAGCCAUGCCGAGU	21	234	−0.96	Chr6	Up regulated
4	miR845	AUUCGUGUUCAGAAAGGAGA	20	320	−1.03	Chr4	Up regulated
5	miR2593	UUGCAGAACCUGGAAUUGACUGU	23	323	−0.88	Chr7	Up regulated
6	miR2608	AGGACUCGACAUGGCUCCUCC	21	234	−0.96	Chr5	Down regulated
7	miR2905	AAGGCACAGUCAAUUCCAGGUU	22	322	−0.88	Chr7	Up regulated

Table 2: Description of genes targeted by boron stress induced conserved miRNAs in French bean.

Sl. No	miRNA Id	Gene ID	Target Function
1	miR396	Phvul.009G047000.1|PACid:27147196	Growth-regulating factor 7
2	miR398	Phvul.009G181200.1|PACid:27146418	GATA type zinc finger transcription factor family protein
3	miR845	Phvul.008G236500.1|PACid:27155183	MYB domain protein 20/ Protein odorant1
		Phvul.009G089000.1|PACid:27146943	C2H2 and C2HC zinc fingers superfamily protein
4	miR812	Phvul.006G150400.1|PACid:27165494	RNA-binding (RRM/RBD/RNP motifs) family protein
		Phvul.007G103100.1|PACid:27158970	Aspartyl aminopeptidase
		Phvul.010G073600.1|PACid:27140612	Bag family molecular chaperone regulator 4
		Phvul.002G011700.1|PACid:27170559	BTB and TAZ domain protein 4
5	miR2593	Phvul.007G045700.1|PACid:27160487	Lipoyltransferase 2
		Phvul.008G090500.1|PACid:27156134	UDP-glycosyltransferase 73B4
6	miR2608	Phvul.007G032300.1|PACid:27160981	Alpha/beta hydrolase
7	miR2905	Phvul.003G267100.2|PACid:27142387	NEP-interacting protein 2
		Phvul.003G267100.1|PACid:27142386	NEP-interacting protein 2
		Phvul.002G105100.1|PACid:27169086	NEP-interacting protein 1
		Phvul.002G060200.1|PACid:27167520	Protein NSP-interacting kinase 2
		Phvul.003G220400.1|PACid:27142206	Glucuronosyltransferase pgsip8-like
		Phvul.006G020300.2|PACid:27166277	RINT-1/TIP-1 family
		Phvul.006G020300.1|PACid:27166276	RINT-1/TIP-1 family

3.5. Validation of Target Genes

High complementarity of the miRNA with their target genes results in their degradation. To validate the targets in order to achieve reduce false negatives among the computationally predicted targets, the RLM-RACE was performed. We found most of the perfect complementary pairs produced target cleavage at position 10, with the cleavage site preceded by nucleotide sequence of XXA/CCXX (where X represents any nucleotide) (Figure 3). Perfect binding with 'seed sequence' and the complementary target sequence obeys the principle of 5' seed sequence alignment which further establishes the guanine targets with specific cleavage sites.

4. DISCUSSION

Recently, microRNAs were established as key gene regulators and many studies evidenced role in various plant physiological processes including plant stress responses. Studies revealed the altered climatic

conditions influence the expression patterns of miRNAs thereby encouraging the modulated expression of genes to acquaint stress tolerance [25]. In this view, we aimed to define the influence of boron toxicity on French bean in terms of altered expression of conserved stress-responsive miRNAs. We studied the expression of sixteen miRNAs under B toxic conditions out of which seven miRNAs found to be crucial. However, of the seven miRNAs responded only two (miR396 and miR398) were conserved candidates and rest (miR812, miR845, miR2593, miR2608, miR2905) forms a group of non-conserved stress-responsive miRNAs. The qPCR studies clearly demonstrated the average fold change as 70% up-regulation with respect to control.

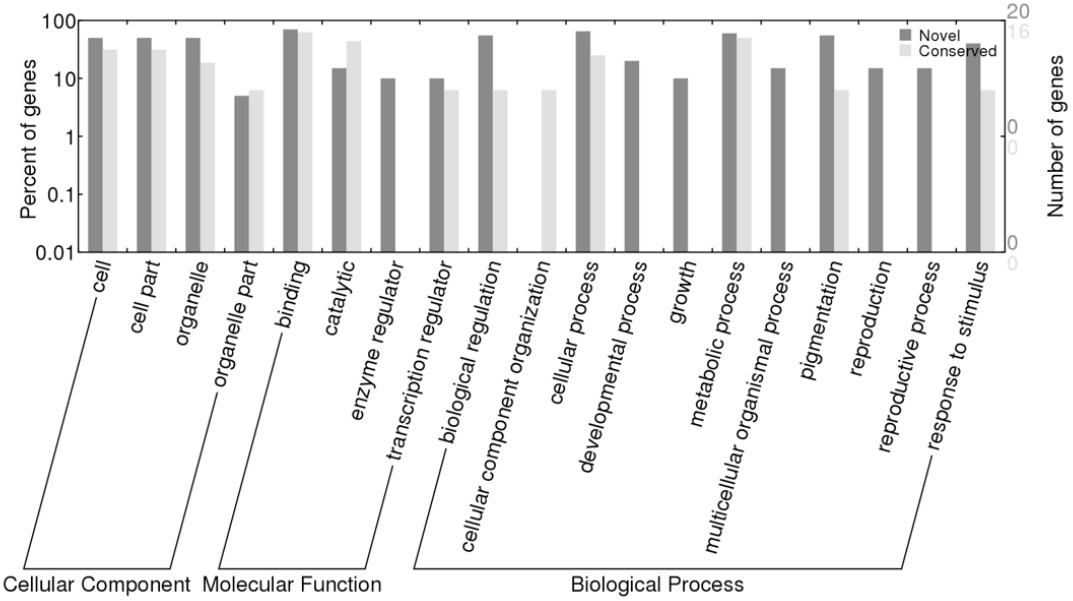

Figure 2: Functional analysis of Boron stress-responsive miRNAs. miRNA function was predicted by defining the biological function of its target gene. The GO analysis of targets was performed using Blast2Go and BGI WEGO. The graph represented is obtained from BGI-WEGO.

Table 3: Genomic features of novel miRNAs expressed under boron stress in French bean.

Sl. No	miRNA ID	Chromosome number	Chromosomal loci	TSS position	TATA box position	TFBM position	TFBM Description	Poly A
1.	miR396	Chr9	Intron	Not defined		1896 1116 1008	WRKY W-Box MYB2	3110
2.	miR398	Chr2	Intron	1513	1478	1085 1074 946	BPC1 WRKY EREBP	3052
3.	miR812	Chr6	Intron	1513	1478	1851	CT Element	3049
4.	miR845	Chr4	Intron	225	193	1976 435	AT Rich HH AP3+P1 heterodimer	3550
5.	miR2593	Chr7	Intron	1787	1753	1895 1894	GAGA element AREB2/ABF4	3120
6.	miR2608	Chr5	Intron	540	503	1851 1378	CT Element AACA Motif	Not identified
7.	miR2905	Chr7	Intron	782	753	1795 1515 1379	OXII ZFHD1 C-Rich R	3129

Further the functional annotation of miRNAs responded to boron toxicity can be performed by defining the role of their target genes. The computational prediction of target genes was carried out using psRNA Target and the functional annotation was performed using Blast2GO. Our results revealed most of the targets are transcription factors as targets of conserved miRNAs and metabolic enzymes form the targets of non-conserved miRNAs. We observed down-regulation of miR398 and miR2608 the miRNAs targeting GATA transcription factors and hydrolases. GATA family transcription factors forms large group of zinc finger proteins which bind to GATA domain and involved in development, nitrogen metabolism [26], plant circadian regulation and light-regulated photomorphogenesis [27]. In plants GATA TFs are demonstrated to regulate light-sensitive genes and involve in plant circadian cycle [28,29]. Overexpression of GmGATA44 in *Arabidopsis* affected Chlorophyll biosynthesis under low nitrate conditions [30]. *Luo et al*. demonstrated the GATA TFs forms key

signaling molecules in light integrated brassinosteroid pathways [31]. In *Arabidopsis*, miR398 was found to target two closely related Cu/Zn superoxide dismutase coding genes, cytosolic CSD1 and chloroplastic CSD2, and a reduced level of miR398 led to improved tolerance of transgenic lines compared with the wild-type plants under oxidative stress conditions [32]. It is evidenced as miR398 is significantly involved in maintaining plant metal homeostasis. The excess copper resulted in down-regulation of miR398 resulting in accumulation of its targets CSD1 and CSD2 and aid to scavenge the ROS thus generated [33]. Conversely, under copper deficiency, miR398 is induced down-regulating the CSDs, ROS scavenging is taken are by FSD1 which acts in concert with miR156. Suppression of miR156 under copper deficiency results in accumulation of SPL proteins. SPL proteins binds to GTAC motif of FSD1 and CSD2 simultaneously inducing and suppressing the expression of genes respectively [34]. Tissue-specific expression trend was observed with miR398 in excess zinc exposed *Arabidopsis*. It is noticed that, the transcripts of miR398b/c are reduced in leaves but no response in roots [8]. Similarly, in the French bean, leaves exhibited lowered miR398b transcripts while it is accumulated in roots and nodules during manganese toxicity [14]. This implies down-regulation of miRNAs would render plant to activate ROS scavengers as excess production of ROS is a consequence of boron toxicity and disturbs photosynthetic activity [35].

Figure 3: Validation of miRNA target genes. The target sequences of the miRNAs were validated for the specific cleavage sites by RLM-RACE and found most of the miRNA mediated target cleavage at position 10.

The up-regulated miRNAs constitute to target TF genes crucial in plant growth and cellular proliferation. Induction of miR396 resulted in degradation of GRFs, the plant-specific TFs essential for cell proliferation and growth [9]. It is shown that miR396 expression is induced in majority of stress conditions such as drought, salinity, nutrient depletion, and metal toxicity. Drought influences up-regulation of miR396 in *Arabidopsis*, Rice, Medicago, and Cotton [36]. Similarly, salt stress and severe cold conditions also induce miR396 expression [37] while hypoxic conditions were shown to repress miR396 [38]. It is imperative that the miRNA is responsive to various environmental stimuli and modulates plant stress responses and proposed key causes of plant retarded growth and impaired development. The other development specific TF targeted by boron responsive miRNA includes MYB (miR845) and BTB & TAZ domain proteins (miR812). Further, it emphasis the MYB would play major role in stress response. Some MYBs are involved in the regulation of cell proliferation, differentiation, and apoptosis and determine the fate of plant cells. Studies report MYB as a conserved target of miR159. Differential expression of miR159 is observed in rice exposed to drought where it is repressed by two-fold, evidences the species-specific nature of miRNA expression [39]. The miR159-MYB101 network was established to be essential for modulation of vegetative growth whilst controlling the salt stress-induced premature transition to reproductive phase in potato [40]. miR159 forms the key enzyme involved in hormonal signaling. The expression of miRNA is altered during ABA treatment where it accumulates in germinating seeds leading to desensitization due to reduced MYB33 and MYB101 levels, the TFs essential for hormonal signaling [41].

BTB and TAZ domain proteins are cleaved by miR812 under elevated

boron levels. BTB-TAZ forms member of Calmodulin-binding proteins and are engaged in plant signaling pathways [42]. Recent evidence implies their role in gametophyte format ion in *Arabidopsis*, light signals, nutrient status and hormonal responses [43]. The TFs are induced under no light conditions and involve in sugar-mediated inhibition of germination. Overexpression of BT2, a BTB-TAZ protein, potentiates auxin responses in postgermination and vegetative development stages [44].

The other genes identified as putative targets of boron responsive miRNAs include aminopeptidases, hydrolases, lipoyl transferase, glucosyl transferases etc., and are actively involved in cellular metabolism. Thus, we proclaim, boron toxicity symptoms such as chlorosis, retarded growth, reduced biomass etc., are due to impaired expression of metabolic enzymes that are crucial for maintaining sugar/lipid homeostasis. This signifies the determination of non-conserved miRNAs in the boron stressed small RNA library as they regulate the metabolic process to achieve cellular homeostasis at a faster rate and forms the front line of defense. Thus we infer, the altered expression of conserved stress-responsive miRNAs may engage in regulating stress responses at basal level and confers long-lasting tolerance mechanisms to evolve; while, the differential expression of non-conserved miRNAs are crucial in stress recovery pathways by altering the metabolome of the plant thus offering an immediate response to environmental stimuli.

The dynamics of miRNA expression can be thoroughly understood by gaining insights to genomic organization of miRNA genes. Featuring of miR genes in terms of TSS, TATA position and regulatory motifs would aid in defining regulation of MIR transcription and it is modulated by the function of cis-acting regulatory elements. Differential expression analysis of miRNAs facilitates the elucidation of promoter motifs. The traditional global TSS mapping and their delineation of pri-miRNAs challenges have led to a limited number of miRNAs TSSs identified till date [45].

Conserved motifs −35 positions adjoining to TSS suggest French bean miRNAs were transcribed by RNA pol II and same promoter coding sequence, in addition, similar kind of results were obtained in *Arabidopsis* and flax. miR396 termed as TATA-less (promoters) is further considered as housekeeping gene. Forty-one distinct transcription factor binding sites were found to be unique among 110 that were identified. MYB and WRKY dwells the majority of the regulatory motifs and may play a vital role in transcribing miRNA involved in stress responses. Of which MYB represents cross-talk between disease and drought response mechanisms and modulated by a negative feedback loop that buffers small changes in the level of their mRNAs [46]. In this study, most of the miRNAs were annotated which use their own transcription initiation regions and a few members appear to be shared with host genes, suggests the alternative post-transcriptional events would determine the fate of each primary transcript. It also reveals the existence and positional preferences of intronic miRNA, TSSs, and evolutionary relationship. Further, functional characterization of transcription factors and their validation will decipher us to explore the detailed miRNA-mediated gene regulatory network during abiotic stress conditions.

5. CONCLUSIONS

The study investigates small RNAs in French bean associated with boron toxicity and is the first of its kind. The expression patterns of seven miRNAs were studied and their putative target genes were predicted and annotated by GO, to explore gene functions. The majority of the identified miRNAs were significantly responsive to boron stress. The functional analysis confirmed the putative miRNAs engaged in a network of growth and plant stress signaling. This study will provide useful information to deepen the understanding of the function and regulatory mechanisms of miRNAs in boron toxicity.

6. CONFLICT OF INTEREST

Authors declare no conflict of interest.

7. ACKNOWLEDGMENTS

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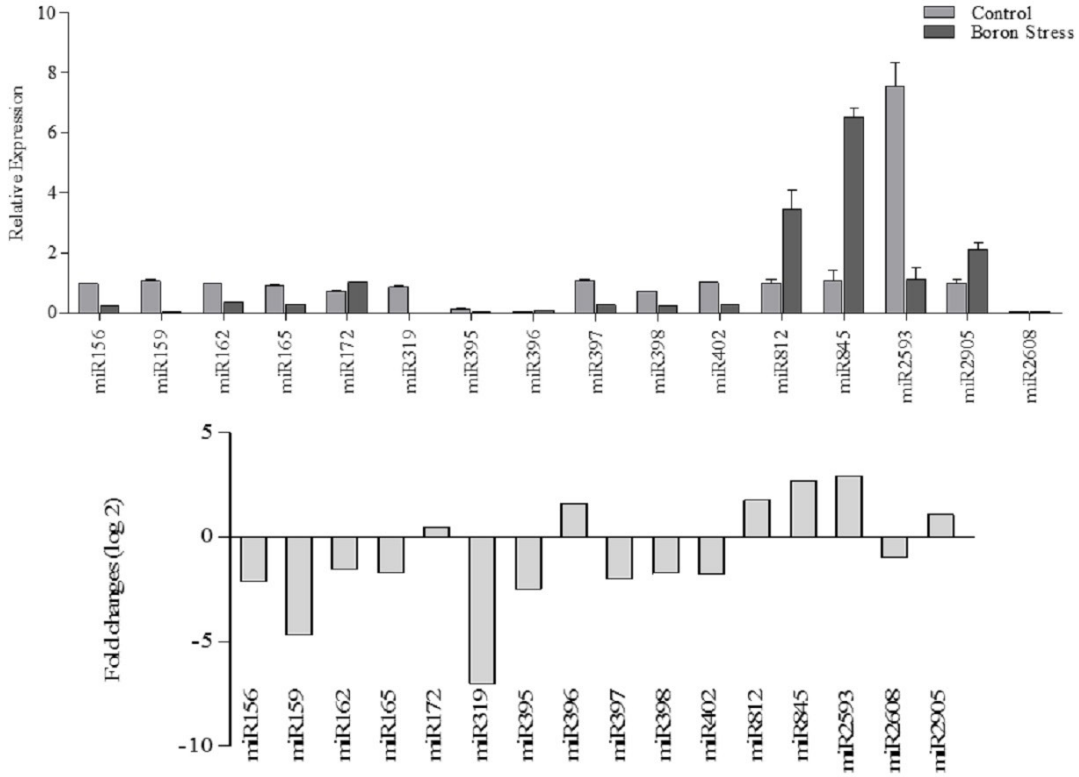
Supplementary File 1: List of Primers.

miRNA Family	Forward Primer sequence (5'-3')	Universal Reverse Primer (5'-3')
miR156	TGACAGAAGAGAGAGA	GTGCAGGGTCCGAGGT
miR159	CTTCCATATCTGGGGA	GTGCAGGGTCCGAGGT
miR162	TCGATAAACCTCTGCA	GTGCAGGGTCCGAGGT
miR165	GGAATGTTGTCTGGAT	GTGCAGGGTCCGAGGT
miR172	AGAATCTTGATGATGC	GTGCAGGGTCCGAGGT
miR319	TTGGACTGAAGGGAGC	GTGCAGGGTCCGAGGT
miR393	TCCAAAGGGATCGCAT	GTGCAGGGTCCGAGGT
miR395	ATGAAGTGTTTGGGGG	GTGCAGGGTCCGAGGT
miR396	GTTCCACAGCTTTCTTG	GTGCAGGGTCCGAGGT
miR397	GCGGCGGTCATTGAGT	GTGCAGGGTCCGAGGT
miR398	TGTGTTCTCAGGTCGCCCC	GTGCAGGGTCCGAGGT
miR399	GCGGCGGTGCCAAAGGA	GTGCAGGGTCCGAGGT
miR402	GCGGCGGTTCGAGGCCT	GTGCAGGGTCCGAGGT
miR812	GACGGACGGTTAAACGT	GTGCAGGGTCCGAGGT
miR845	CGGCTCTGATACCAAT	GTGCAGGGTCCGAGGT
miR2593	TTAAATGAATGAACCT	GTGCAGGGTCCGAGGT
miR2608	GTTGTACATATATCACT	GTGCAGGGTCCGAGGT
miR2905	TACATGTCAGTGACAA	GTGCAGGGTCCGAGGT
U6	GAGAAGATTAGCATGG	CACGAATTTGCGTGTCATCCTT

Supplementary File 2: Description of targets predicted by psRNATarget and their gene ontology descriptions.

miRNA_Acc.	Target_Acc.	Inhibition	Multiplicity	Target_Desc.
miR396	Phvul.009G047000.1|PACid:27147196	Cleavage	1	growth-regulating factor 7
miR398	Phvul.009G181200.1|PACid:27146418	Translation	1	GATA type zinc finger transcription factor family protein
miR812	Phvul.006G150400.1|PACid:27165494	Cleavage	1	RNA-binding (RRM/RBD/RNP motifs) family protein
miR812	Phvul.007G103100.1|PACid:27158970	Translation	1	Zn-dependent exopeptidases superfamily protein
miR812	Phvul.010G073600.1|PACid:27140612	Cleavage	1	BCL-2-associated athanogene 4
miR812	Phvul.002G011700.1|PACid:27170559	Cleavage	1	BTB and TAZ domain protein 4
miR845	Phvul.008G236500.1|PACid:27155183	Translation	1	myb domain protein 20
miR845	Phvul.009G089000.1|PACid:27146943	Cleavage	1	C2H2 and C2HC zinc fingers superfamily protein
miR2593	Phvul.007G045700.1|PACid:27160487	Translation	1	lipoyltransferase 2
miR2593	Phvul.008G090500.1|PACid:27156134	Cleavage	1	UDP-glycosyltransferase 73B4
miR2608	Phvul.007G032300.1|PACid:27160981	Translation	1	alpha/beta hydrolase
miR2905	Phvul.003G267100.2|PACid:27142387	Cleavage	1	NEP-interacting protein 2
miR2905	Phvul.003G267100.1|PACid:27142386	Cleavage	1	NEP-interacting protein 2
miR2905	Phvul.002G105100.1|PACid:27169086	Cleavage	1	RING/U-box superfamily protein
miR2905	Phvul.002G060200.1|PACid:27167520	Cleavage	1	Leucine-rich repeat protein kinase family protein
miR2905	Phvul.003G220400.1|PACid:27142206	Translation	1	Nucleotide-diphospho-sugar transferases superfamily protein



Supplementary Fig. 1: Expression analysis of conserved stress responsive miRNAs under Boron toxicity in French bean. To analyse the impact of boron toxicity on expression patterns of miRNAs, 16 stress responsive miRNAs was selected and their expression trends were studied by RT-qPCR. The data was normalized against U6 gene.