Journal of Stress Physiology & Biochemistry, Vol. 19, No. 4, 2023, pp. 120-131 ISSN 1997-0838

ORIGINAL ARTICLE

OPEN ACCESS

Differential Expression of miRNAs under Boron Toxicity Affects Circadian Cycle and Vegetative Development in French Bean

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Received June 26, 2023

French bean is (*Phaseolus vulgaris*) widely grown legume worldwide and is the most important pulse crop in the Indian subcontinent. The productivity is adversely affected by a large number of biotic and abiotic stresses. Micro RNAs (miRNAs) have been implicated in the regulation of plant responses to several biotic and abiotic stresses. Boron (B) toxicity is a significant limitation to cereal crop production worldwide. In this study, RT-qPCR confirmed seven miRNAs were responsive to boron toxicity. The known predicted targets and their functional analysis revealed that most of the targets represent transcription factors. GO results supported our hypothesis that miRNAs were found to be involved in diverse cellular processes, including plant circadian cycle, vegetative development, transcription, and cross adaptation. The results obtained would provide new insights to the complex regulatory mechanism employing small non-coding regulatory RNAs toward stress adaptation.

Key words: expression assay, metal toxicity, MRE, transcription factors

Boron toxicity is an important agronomic problem that limits crop productivity worldwide. High concentrations of B may occur naturally in the soil or in groundwater, or be added to the soil from mining, fertilizers, or irrigation water. The permissible concentrations of B in irrigation water range for 0.3 mg per litre to 4 mg per litre (Keren and Bingham, 1985). The symptoms of boron toxicity are visible chlorotic/necrotic patches in leaf tissue and phloem tissue. Most of the studies have mainly concentrated on the toxic effects of heavy metals and macro nutrients such as cadmium (Xu *et al.*, 2013), lead (He *et al.*, 2014; Wang *et al.*, 2015), mercury (Zhou *et al.*, 2012), copper (Naya *et al.*, 2014), arsenic (Srivastava *et al.*, 2013) etc., However, limited research has concerned about the toxic effects of trace elements such as Boron. Extensive research on metal toxicity regulations have been made at physiological, biochemical and molecular levels. The best described mechanism involves the intracellular metal chelation by a series of Cys-rich peptides such as glutathione (GSH), phytochelatins (PCs) and metallothioneins (MT) (Gielen *et al.*, 2012). 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 a key step in response to metal exposure or metal deficiency network, 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. Overwhelming 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 wide range of biological process such as cell cycle, apoptosis, floral development and several physiological responses (Jones-Rhoades *et al.*, 2006). Increasing evidences also emphasized the influence of various biotic and abiotic stress factors on expression of miRNAs (Khraiwesh *et al.*, 2012). Through microarray, Ding *et al.*,(2011) reported the altered expression of miR168, miR528, and miR162 under cadmium stress and defined their targeted genes were involved in biogenesis of miRNAs. They also determined the miRNA genes possessed MREs (metal responsive cis-regulatory factors) in their upstream sequences which control the expression of these miRNAs. The over-expression of miR192 caused due to increased cadmium concentrations retarded the seed germination and seedling growth in rice (Ding *et al.*, 2013). Ozhuner *et al.*, (2013) 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. This exemplifies the tissue specific expression of miRNAs under stress.

French bean is one the most important legume crop grown for its seeds and pods. The crop yield is found to affect by drought, high salt, temperature and availability of essential nutrients. Many groups involved in identification and characterization miRNAs under various abiotic stress conditions. Arenas-Huertaro *et al.*, (2009) proposed the differential expression of miR2118, miR159a, miR1514a, miR482, miR2119, miR166a, miR319c, and miR399a would render plant tolerant towards drought and salt stress. Valdes-Lopez *et al.*, (2010) through next generation sequencing confirmed the miRNAs respond stress specifically and demonstrated miR399 is crucial in phosphate uptake and acts as a signalling molecule during phosphate starvation. Palaez *et al.*, (2012) exploited high throughput sequencing to characterize complete set of miRNAs responded to salt stress. Naya *et al.*, (2014) 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. Although overwhelming 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.

In this study, we aimed to characterize set of putative miRNAs from boron exposed French bean seedlings. To understand the impact of B toxicity on expression of

conserved stress responsive miRNAs, RT-qPCR studies were carried out. Further, B responsive miRNAs were cloned and sequenced. Functional analysis and 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.

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 12 h light/12 h dark photo period. After 6 day of germination, the seedlings hydroponically treated with 5ppm of boric acid, in half strength Hoagland media for 48 h meanwhile a group of seedlings were maintained as control. Tissues were harvested immediately and stored at -80 °C for further analysis.

RT-qPCR validation of miRNA expression

In order to define the stress specific expression of miRNAs and validate the effect of boron toxicity their expression, 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, 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 (Supplementary file 1). 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 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 of ratio of normalized expression of miRNAs under stress to that of control samples*. The standard deviations of the data were obtained from the three independent experiments with student's t-test ($p < 0.05$) using statistics software Graphpad Prism v.5

Characterization of Boron responsive miRNAs

To define the miRNA sequence of boron stress responsive miRNAs evaluated by expression assay, the RT-qPCR products were subsequently cloned and sequenced. Briefly, 50 µl of RT-qPCR product was purified by Nucleospin DNA Cleanup kit (Takara, Japan) according to manufacturer's instructions. The purified DNA was analysed and quantified on 5% Agarose gel. The purified RT-qPCR 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) were added to the purified and quantified RT-qPCR products in a final volume of 10 µl at 4 °C overnight and transformed into *Escherichia coli* (DH5α). The colony PCR was carried using gene specific primers and the PCR positive clones were sequenced and processed for BLAST analysis against the NCBI genomic data sets.

Data analysis

The sequences were filtered for tRNA/rRNA contamination using Rfam database (http://www.sanger.ac.uk/Software/Rfam). Putative origins were identified by BLASTN search against French bean genome. The sequences with perfect (0-3) matches with small RNA sequences were used for fold back secondary structure prediction with MFOLD (Zuker, 2003). A segment was considered a valid miRNA candidate if its secondary structure met the criteria according to Meyers *et al.*, (2008)

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)(Dai *et al.*, 2011). French bean transcript sequences downloaded from Phytozome version 9 (www.phytozome.net) (Goodstein *et al.*, 2012) 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 (Conesa and Gotz, 2008)

Characterization of miR genes

The determination of *MIR* regulatory motifs and TSS prediction was carried as described (Jyothi *et al.* 2015). Briefly, 2 kb upstream sequences were retrieved at the beginning of the pre-miRNA for the prediction of TSS (Transcription Start Site) 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.

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) (Okumara *et al.*, 2007). 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.

RESULTS

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 (data not shown, 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 (Fig 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 (Fig 1B).

Determination of boron stress responsive miRNAs in French bean

To determine the exact sequences of boron stress responsive miRNAs, the RT-qPCR products were cloned and plasmids were sequenced. The filtered sequences considered and 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 (Supplementary file 2). Table I describes the genomic location and MFEI values of miRNAs found sensitive to boron toxicity.

Computational prediction of putative targets and their annotations

To obtain 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 3). Our results showed most of the targets were involved 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 II). 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. Majority exhibited nucleotide binding (8) and involved in metabolic functions (10) and engaged in cellular

processes (13) (Fig 2) (in parentheses number of gene sequences).

Functional characterization of miRNA genes

To gain further insights towards 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 2kb 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 our, miRNA genes exhibited polyA strings at +900 from pri- miRNA end point. 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 III). 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 phyto-hormone signalling. These results suggest the upstream regions upto 2 kb were crucial in miRNA gene expression.

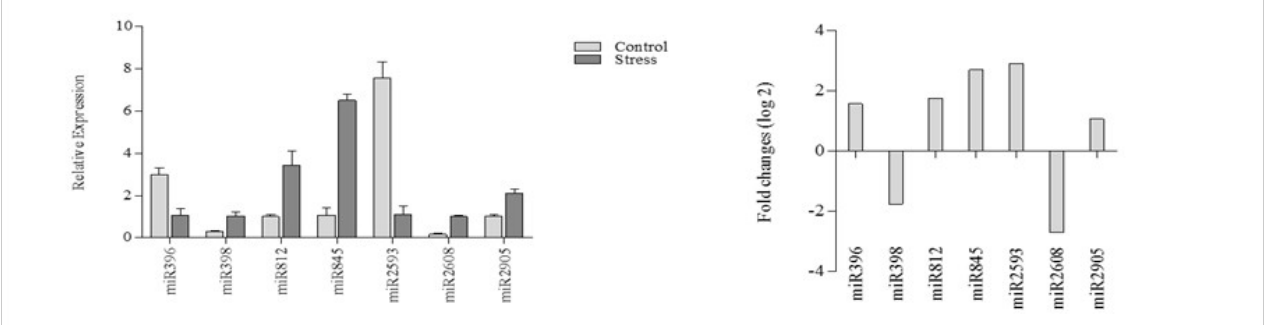

Figure 1: Expression analysis of boron stress responsive miRNAs in French bean.A.Relative expression of miRNAs was studied using RT-qPCR. The expressions are normalized against U6. The study was carried in three independent experiments and Standard deviations and t-test was performed with $p<0.05$. B.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

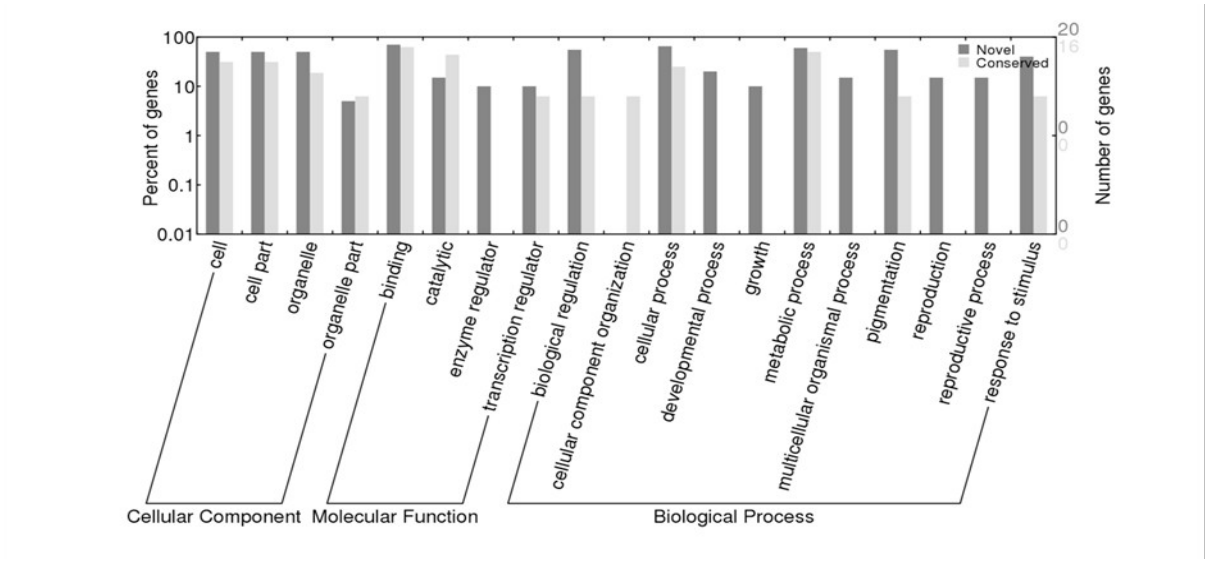

Figure 2: Functional analysis of Boron stress responsive miRNAs. miRNA function was predicted by defining the biological function of its target gene. The GO analysis of targets was performed using Blast2Go and BGI WEGO. The graph represented is obtained from BGI-WEGO.

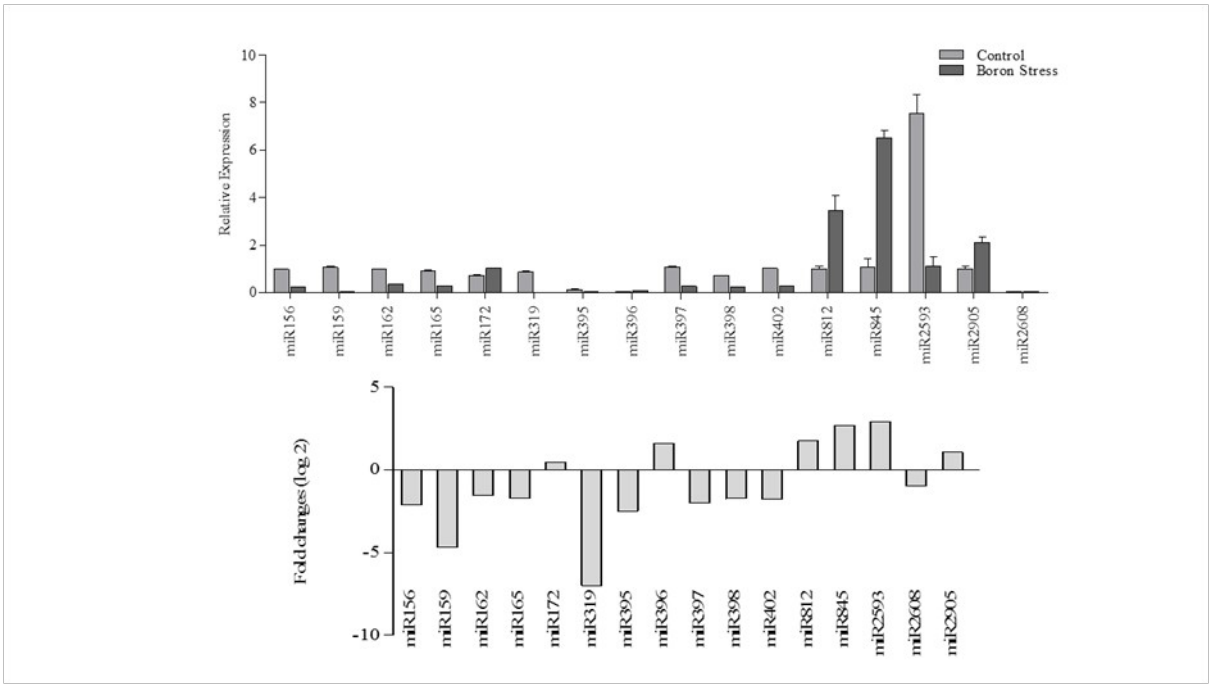

Figure 3: Supplementary Fig 1 Expression analysis of conserved stress responsive miRNAs under Boron toxicity in French bean. To analyse the impact of boron toxicity on expression patterns of miRNAs, 16 stress responsive miRNAs was selected and their expression trends were studied by RT-qPCR. The data was normalized against U6 gene.

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

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

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 modulated expression of genes to acquaint stress tolerance (Contreras-Cubas *et al.*, 2012). In this view, we aimed to define the influence of born

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 crucially. 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.

Further the functional annotation of miRNAs responded to B toxicity can be performed by defining the role of their target genes. The computational prediction of target genes was carried out using psRNATarget 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 forms 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 zinc finger proteins which bind to GATA domain and involved in development, nitrogen metabolism (Bi *et al.*, 2005), plant circardian regulation and light regulated photomorphogenesis (Arguello-Astorga and Herrera-Estrella, 1998). In plants GATA TFs are demonstrated to regulate light sensitive genes and involve in plant circardian cycle (Manfield *et al.*, 2007; Jeong and Shih, 2003). Over expression of GmGATA44 in Arabidopsis affected Chlorophyll biosynthesis under low nitrate conditions (Zhang *et al.*, 2015). Luo *et al.*, (2010) demonstrated the GATA TFsforms key signaling molecules in light integrated brassinosteroid pathways. 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 (Sunkar *et al.*, 2009). 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 (Yamasaki *et al.*, 2007). Conversely, under copper deficiency, miR398 is induced down regulating the CSDs, ROS scavenging is taken care 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 (Yamasaki *et al.*, 2009). 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 (Gielen *et al.*, 2012). Similarly, in the French bean, leaves exhibited lowered miR398b transcripts while it is accumulated inn roots and nodules during Manganese toxicity (Valdes-lopez *et al.*, 2010). This implies down regulation of miRNAs would render plant to activate ROS scavengers as excess production of ROS is a consequence of B toxicity and disturbs photosynthetic activity (Nagesh babu *et al.*, 2012).

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 (Jones-Rhoades *et al.*, 2006). 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, Cotton (Jones-Rhoades and Bartel, 2004). Similarly, salt stress and severe cold conditions also induce miR396 expression (Zhou *et al.*, 2008) while hypoxic conditions were shown to repress miR396 (Moldovan *et al.*, 2009). 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 B responsive miRNA includes MYB (miR845) and BTB & TAZ domain proteins (miR812). 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. 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 2 folds

evidences the species specific nature of miRNA expression (Zhou *et al.*, 2010). The miR159- MYB101 network was established to be important for modulation of vegetative growth whilst controlling the salt stress induced premature transition to reproductive phase in potato (Kitazumi *et al.*, 2015). miR159 forms the key enzyme involved in hormonal signalling. 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 signalling (Tuteja, 2007).

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 signalling pathways (Du and Poovaiah, 2004). Recent evidences imply their role in gametophyte formation in Arabidopsis (Robert *et al.*, 2009), light signals, nutrient status and hormonal responses. The TFs are induced under no light conditions and involve in sugar mediated inhibition of germination. Over expression of BT2, a BTB-TAZ protein, potentiates auxin responses in post germination and vegetative development stages (Mandadi *et al.*, 2009)

The other genes identified as putative targets of B responsive miRNAs include, aminopeptidases, hydrolases, lipoyl transferase, glucosyl transferases etc., and are actively involved in cellular metabolism. Thus, we proclaim, the B 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 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 plant thus offering 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. 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 (Cui *et al.*, 2009).

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 (miR396) might fall into the class termed TATA-less (promoters) generally reported for housekeeping and developmental genes; similar observations were reported in Arabidopsis and rice. A total of 110 distinct transcription factor binding sites 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. The high occurrence of MYB may represent cross talk between disease and drought response mechanism, accounting for characteristic high inducibility of miRNAs in poplar (Guleria *et al.*, 2011). The expression of these targets is modulated by a negative feedback loop that buffers small changes in the level of their mRNAs. 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

This is the first report to investigate small RNAs in French bean associated to boron toxicity, 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. Functional analysis confirmed the putative miRNAs engaged in a network of growth and plant stress signalling. This study will provide useful information to deepen our understanding of the function and regulatory mechanisms of miRNAs in boron toxicity.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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