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ORIGINAL ARTICLE

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Unravelling Cadmium induced noncoding RNAs and their validations from Finger millet

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MicroRNAs (miRNAs) play important roles in plant responses to abiotic stress. Numerous studies have been increasing in respect to miRNA identification under stress conditions. In this study we analysed the expression patterns of seven miRNAs (miR156, 159, 169, 396, 397, 398 and 399) from cadmium stressed seedlings of Finger millet by RT-qPCR. Further these miRNAs were cloned and sequenced, which conformed its presence. Predicted targets and GO analysis of the miRNAs were found to be involved in diverse cellular processes in plants, development, apoptosis, detoxification, catalysis, protein modification. Cis-regulatory elements identification suggested their involvement in regulatory networks. This is the first study to demonstrate differentially expressed miRNA in Finger millet under cadmium stress. Findings in the present study prominence the role played by miRNAs in Finger millet under cadmium stress.

Key words: cis-regulatory element, GO analysis, NB-ARC domain, RT-qPCR

Abbreviations: GO-Gene ontology; MFEI- Minimum Free Energy Index; MATH- Mephrin and TRAF homology domain; SPL- squamosa promoter binding protein-like; miRNA- microRNA; TSS- Transcription start site; TFB-Transcription Factor Binding site; NB-ARC- Nucleotide Binding domain with ARC motif

In the recent years, abiotic stress has emerged as a most challenging environmental factor that negatively influences the crop growth and production worldwide. Heavy metal toxicity is one of them which is an increasing concern worldwide as it alters numerous physiological and biochemical processes of the plants leading to significant crop loss (Hossain *et al.,* 2010; Rascio and Navari-Izzo, 2011; Villiers *et al.,* 2011). Cadmium stress in plants can be caused by various activities, such as mining, industrial activities and direct application of phosphate fertilizers (Pinto *et al.,* 2004; Zhang and Wang, 2007). Accumulation of cadmium may lead to reduced biomass, leaf chlorosis, and inhibition of root growth, morphological alterations and plant death (Yadav, 2010). The metal not only affects crop productivity but also brings risk to food safety (McLaughlin *et al.,* 1999).Understanding of heavy metal-responsive gene expression and regulation is the first step to dissect the genetic and molecular basis of metal accumulation.

MicroRNAs (miRNA) are a class of riboregulator, non-coding RNAs (approximately 21 nt long) that bind complementary sequences in target mRNAs to specifically regulate gene expression through either mRNA degradation or translational inhibition (Bartel, 2004). Studies have reported that miRNAs were involved in regulating a range of essential cellular and biological processes, and abiotic stress, such as drought (Wang *et al.,* 2011), salinity (Macovei and Tuteja, 2012), and toxic metal stress (Zeng *et al.,* 2012). Recently, evidence showed that miRNAs functioned as a key regulator in alleviation of plant metal stresses (Sunkar *et al.,* 2006; Ding and Zhu, 2009; Yamasaki *et al.,* 2007; Gielen *et al.,* 2012). miRNAs involved in cadmium stress has been studied in wide variety of plant species *Typha angustifolia* (Xu *et al.,* 2015), Soyabean (Fang *et al.,* 2013), Rice (Tang *et al.,* 2014), Radish (Xu *et al.,* 2013) using high-throughput sequencing.

The precise role of miRNAs along with their targets in signaling, transportation and ion sequestration in response to cadmium stress remain to be explored. In an experiment, miR156, miR171, miR393 and miR396a were reported to have constitutive expression with reduced target accumulation in roots of seven day old *B. napus* when exposed to 80 µM Cd for 8 h while miR399 was unaffected (Zhao *et al.,* 2012). A set of Cd-responsive novel and known miRNAs have been cloned and validated in *B. napus* using reverse transcriptase PCR (RT-PCR) (Huang *et al.,* 2010).Using microarray assay, Ding *et al.* (2011) identified 19 miRNAs in 7 day old rice seedlings exposed to 60 µM CdCl2 for 24 h of which miR162a, miR166m, miR171b, miR390, miR168b and miR156l were further validated experimentally.

Finger millet (*Eleusine coracana* L) is a cereal grass grown mostly for its grain, ranks fourth in terms of production and cultivation all over the world and belongs to the family Poaceae. In the present work, to understand the regulatory network modulating the response of Finger millet under cadmium stress, we investigated the expression pattern of seven conserved miRNAs. We present the first data related to alterations of their expression pattern in Finger millet together with an analysis of miRNAs cis-regulatory promoter elements and the computational prediction of miRNA target genes and Gene ontology studies.

MATERIALS AND METHODS

Plant material and stress treatment

Seeds of Finger millet (*Eleusine coracana MR1*) were surface-sterilized with 4% (v/v) sodium hypochlorite and were grown hydroponically on half strength hoogland media at 28 °C day/25 °C night with 12 h light/12 h dark photo period. After 6 day of germination, seedlings were exposed to cadmium stress (100nM for 48h). Tissues were harvested immediately and stored at -80 °C for further analysis.

miRNA extraction, cDNA synthesis and RT-qPCR

Total RNA was isolated from control and cadmium stress tissue using TRizol (Invitrogen) according to the manufacturer's instructions and treated with RNAase-free DNAase I (Promega). Small RNAs were PEG precipitated and were separated on a denaturing 15 % polyacrylamide gel. Molecules ranging from 18 to 26 nt were excised and recovered. The miRNAs were transcribed into cDNA by Super-Script II Reverse Transcriptase (Invitrogen). Reverse transcription-

quantitative PCR (RT-qPCR) assays were performed in 20 µl reaction volume, containing 1 µl of cDNA template in 1 × SYBR® Premix Ex Taq (Takara, Japan), forward and reverse primers at a final concentration of 0.5 µM. MiRNAs sequence specific primers were used as forward primers (Table 2), with universal reverse primer. The assays were carried out in CFX Manager (Biorad), programmed for 35 cycles of 15 s at 95 °C and 30 s at 55 °C. Two biological replicates were tested and melt-curve analysis was performed to verify the specificity of amplified products. Fold changes in miRNA expression were calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), the relative miRNA expression level was normalized to the U6 endogenous reference.

miRNA cloning and sequencing

To determine the sequence of the seven miRNAs (Eco-miR156, 159, 169, 396, 397, 398 and 399) evaluated by RT-qPCR analysis, 50 µl of RT-qPCR products were purified using PCR Purification Kit (Thermo scientific) according to the manufacturer's instructions. The purified DNA was then analysed and quantified on a 3 % agarose gel. The purified RT-qPCR products were subsequently cloned and sequenced. The cloning reaction was performed using pMD19 Vector System (Takara): 5 µl ligation mix, 1 µl pMD19 vector (50 ng), and 0.1 - 0.3 pmol control insert were added in a final volume of 10 µl. The reactions was incubated at 16℃ for 30 minutes then used to transform using *E.coli* competent cells. Plasmids were isolated from individual colonies and sequenced.

miRNA data analysis and prediction of potential miRNA target genes

Putative origins of the cloned sequences were identified by BLASTN search against Foxtail millet genome sequence (www.phytozome.net). The sequences with perfect (0-3) matches with small RNA sequences were used for fold back secondary structure prediction using MFOLD (Zuker, 2003). Sequence was considered a valid miRNA candidate if its secondary structure met the criteria according to Meyers *et al.* (2008). To identify target genes for the candidate miRNAs, we used psRNATarget, a small RNA target analysis server (http://plantgrn.noble.org/psRNATarget/) (Dai and Zhao, 2011) with default parameters. The Setaria italica transcript dataset, downloaded from www.phytozome.net version 10 was used to determine the potential target mRNA candidates. Sequences with 3.0 points were considered as miRNA targets. Functional annotations of predicted targets were analysed using BGI WEGO (Web Gene Ontology Annotations) platform (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) (Ye *et al.,* 2006).

Analysis of miRNAs gene promoters

cis-regulatory elements present in the miRNAs were analysed by taking 1500bp upstream pre-miRNA sequence, these sequences were derived from Foxtail millet genome. The motifs were identified using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) database.

RESULTS

miRNAs expression levels

The relative abundance of seven miRNAs (Eco-miR156, 159, 169, 396, 397, 398 and 399) was studied using RT-qPCR with U6 as endogenous reference in seedling stage with 100nM cadmium. Results were expressed as nFold ($2^{-\Delta\Delta CT}$), Eco-miR156, Eco-miR397 and Eco-miR399 were down-regulated by 6.78, 0.23 and 2.53 folds respectively. Eco-miR159, Eco-miR169, Eco-miR156, Eco-miR396 and Eco-miR398 were up-regulated by 2.76, 5.16, 2.36 and 3.92 folds respectively (Figure 1). This results show that expression of Eco-miR156 and Eco-miR169 was greatly enhanced under cadmium stress in Finger millet. A significant association between the seven differentially expressed miRNAs was found by Pearson correlation analysis. A negative co-relation resulted between the expression trends of seven miRNAs, r and p values were found to be -0.6709 and 0.09961 respectively.

miRNA identification

The RT-qPCR amplified products obtained for each miRNA forward primer (Eco-miR156, 159, 169, 396, 397, 398 and 399) were sequenced. Subsequent alignment with sequences in the miRbase (http://www.mirbase.org/) confirmed homology of the amplified products. These sequences were aligned on to Foxtail millet whole genome and secondary structures

were predicted using Mfold. Precursor length of the miRNAs ranged from 90 to 202nt and the average minimum free energy index was found to be -0.84 which is in consistent with earlier reports (Table 1) (Rajagopalan *et al.,* 2006; Yao *et al.,* 2007).

Identification of miRNAs target genes and Gene ontology studies

Setaria italica transcripts (www.phytozome.net) were used to computationally identify miRNA targets by psRNA-Target (http://plantgrn.noble.org/psRNATarget/), with an expectation value of 3.0. Sixty targets were identified showing multiple hits; no hypothetical targets were found for Eco-miR399 with the criteria used, all the identified targets and their sequences are listed in Additional File 1. Eco-miR156 targets SPL protein, plant specific DNA binding domain which is found to regulate APETALA1 (AP1) and control the timing of flower formation in Arabidopsis (Yamaguchi *et al.,* 2009). Eco-miR159 and Eco-miR169 targeted protein of unknown function (DUF674) and G2484-1 protein respectively. miR396 showed 21 different targets which include BTB-POZ and MATH domain, ubiquitin carrier protein, DNAJ heat shock family, cytochrome P450, NB-ARC domain, FAR1, nuclear RNA polymerase etc., this emphasize that miR396 may play a crucial role under cadmium stress in finger millet. Eco-miR397 showed three targets laccase, glutamate receptor, P1F1 helicase; glutamate receptor acts as non-selective ion channel which mediates leaf-to-leaf wound signalling in Arabidopsis (Mousavi *et al.,* 2013). Eco-miR398 had Beta-glucosidase, GBA2 type family protein as its target. A Gene ontology study was performed with WEGO web portal to annotate target genes. Target annotations revealed that they were involved in molecular function, biological process and cellular component (Figure 2). Majority of the target genes were involved in binding, metabolic and cellular process, GO terms for targets are listed in Additional File 2.

Analysis of cis-acting elements in miRNAs genes promoter sequences

The 5' upstream sequences of the miRNAs precursor were analyzed using PlantCare database web tools (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to identify known cis-regulatory elements that could control their expression. The analysis revealed the presence of generic known transcription promoter and enhancer sequences, light responsive elements, stress-responsive elements, cis-regulatory elements for phytohormone response and cis-acting regulatory element related to tissue and organ development (Additional File 3). Interestingly Eco-miR396 has the highest number of cis-acting elements identified including circadian motif (CAANNNNATC) which acts as biological clock in plants which regulate leaf movement, growth, germination, stomatal/gas exchange, enzyme activity, photosynthetic activity, and fragrance emission. This results show that further studies may help to elucidate the overall gene regulation under cadmium stress in Finger millet.

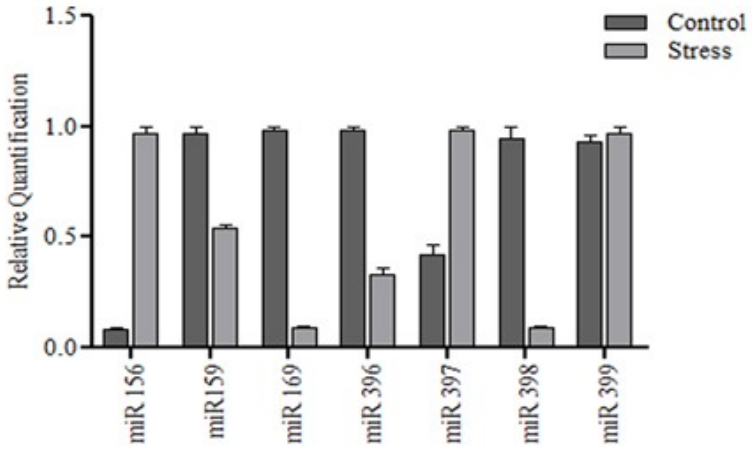

Figure 1 RT-qPCR analysis of miRNAs under cadmium stress at 100nM for 48h. Data were normalized to U6 endogenous reference and results expressed as nFold ($2^{-\Delta\Delta CT}$) change ± SE.

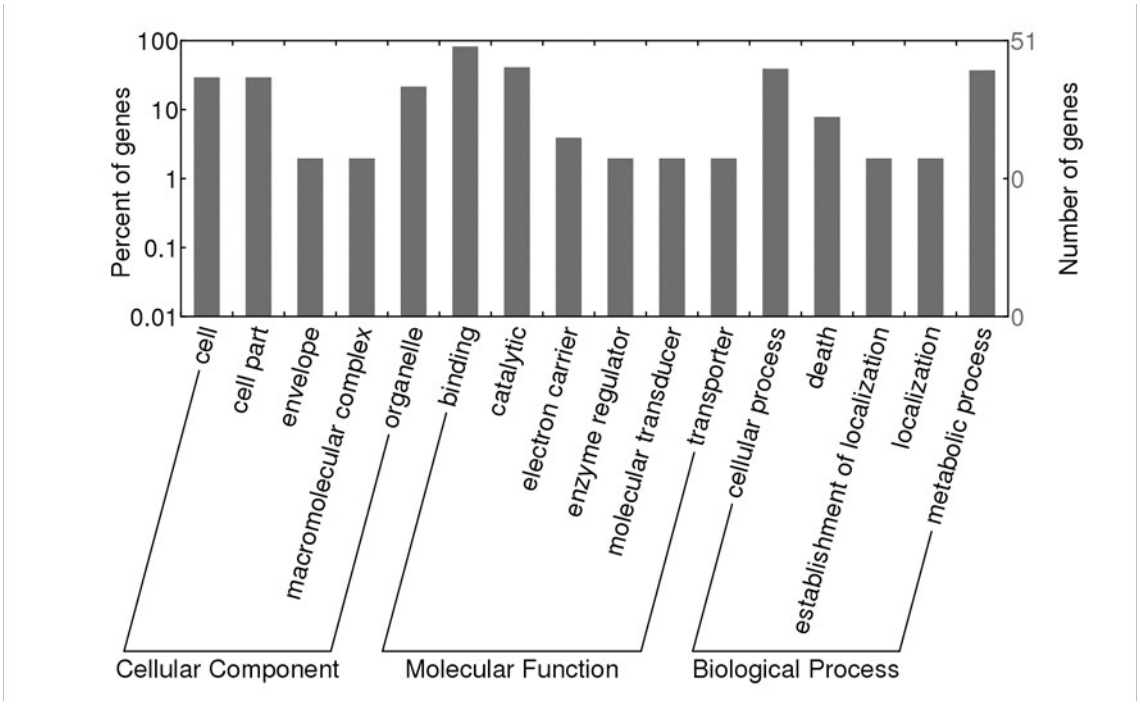

Figure 2 Gene ontology classifications of potential target genes in Finger millet under cadmium stress

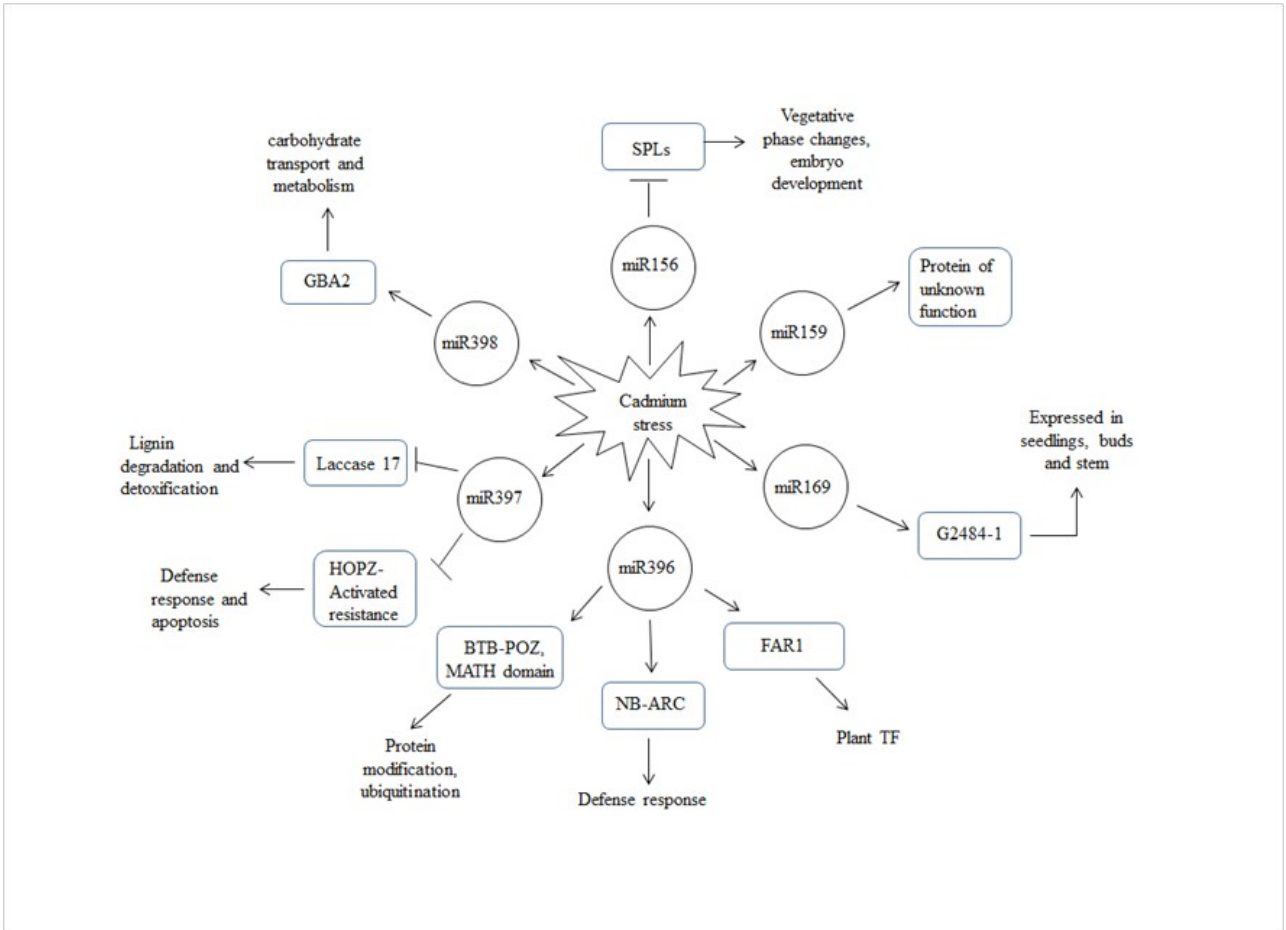

Figure 3 Proposed model of miRNA-target regulatory network under cadmium stress in Finger millet

Table 1 Conserved miRNAs in finger millet under Cadmium Stress

miRNA	Sequence	Length	Precursor length	GC%	MFEI*
Eco-miR156	UGACAGAAGAGAGUUAGCAC	20	105	52.33	-0.89
Eco-miR159	UUUGGAUUGAAGGGAGGCCUU	21	202	41	-0.76
Eco-miR169	GGCAUCCAUUCUUCGCUAGG	20	101	45.5	-0.96
Eco-miR396	UUCUUGAAGUUCUUUUUCGUC	21	167	49.9	-0.7
Eco-miR397	UCAUUGAGUGCAGCGGUGAUG	21	90	47.7	-0.7
Eco-miR398	UGUGUUCUCAGGUCAGCGCU	20	106	50.9	-0.89
Eco-miR399	CUGACAUAGGAGAGGCGCC	19	154	37.6	-1.03

* MFEI-minimal folding energies index in kcal mol^{-1}

Table 2 Forward primer sequences and annealing temperature used to amplify U6 (endogenous reference), miR156, miR159, miR169, miR396, miR397, miR398, miR399 by RT-qPCR

Oligo miRNAs	Sequence 5'-3'	Annealing temperature (ºC)
U6	GAGAAGATTAGCATGGCCCCT	56
miR156	GCGCCACAGAAGAGAGTGAGCAC	60
miR159	AGCTGCTGACTCGTTGGTTC	58
miR169	CAGCAAAAGGAAGTCGAGGA	56
miR396	TGAAGAAGATAGTCCCCTTAACACC	56
miR397	TACAAGCACACCACAATCATCACCA	58
miR398	GTTGGAGGTTGCTTGTGGAAT	56
miR399	GCGCCACGGGATCGCATTGATCC	60

DISCUSSION

Evidences show that miRNAs regulate biological processes and stress responses (Sunkar *et al.,* 2007; Lu *et al.,* 2008a; Barrera-Figueroa *et al.,* 2012; Zhao *et al.,* 2012), yet miRNA biology in Finger millet remains unknown. Certainly, no miRNAs under cadmium stress has been identified till date thus, their potential role in response to cadmium stress remains to be elucidated. In the present study we examined the expression patterns of seven cadmium responsive miRNAs (Eco-miR156, 159, 169, 396, 397, 398 and 399) through RT-qPCR. These miRNAs were further cloned and sequenced which evidenced that the miRNAs were present under cadmium stress. At present, genome data of Finger millet are unavailable, which largely limits the study in this plant including miRNA research. Hence, the secondary structures of the miRNAs were predicted using Foxtail millet and other monocots as reference.

RT-qPCR results showed that the miRNAs were differentially expressed under cadmium stress, miR156, 397 and 399 were down-regulated and miR159, 169, 396 and 398 were up-regulated. Zhoa *et al.* (2012) using RNA blot analysis showed that miR156 was abundantly expressed in shoot than roots and miR397 expressions was induced by the presence of cadmium in brassica. Microarray profiling of rice under cadmium stress showed that miR169 was up-regulated which is in consistent with the present study and targeted CCAAT-binding (TF), which plays role in flowering timing and photosynthesis (Yanfei *et al.,* 2011). miR396 and miR398 is also found to exhibit different expression patterns in different plant species (Taylor *et al.,* 2011). Seven miRNAs cloned have preference for the 5′-U, which is in accordance with the defined structures of mature miRNAs, with their precursor length range from 90-202nt. The candidate pre-miRNAs were predicted by exploring the secondary structure, MFE and minimal folding free energy index (MFEI) using Mfold with an average MFEI of -0.897 kcal/mol, which is similar to the free energy values of other plant miRNA precursor and

are apparently lower than other types of RNAs such as tRNAs, rRNAs and mRNAs (Bonnet *et al.,* 2004).

Target prediction was performed using psRNATArget (http://plantgrn.noble.org/psRNATarget/) and Foxtail millet transcripts, a total of 60 targets were predicted. As expected most of the targets were similar to the previously described (Lu *et al.,* 2005) however, Eco-miR159 and Eco-miR398 showed varied targets this may be due to non-availability of Finger millet genome. Eco-miR396 had 21 different targets such as BTB-POZ and MATH domain, kinases, heat shock proteins, zinc finger transcription factor, disease resistant protein, RNA polymerase and DNA topoisomerase, NB-ARC domain. In Arabidopsis AtCUL3b can with BTB/POZ-MATH and AtRBX1 proteins to form functional E3 ligases (Weber *et al.,* 2005). NB-ARC domain-containing disease resistance protein is a functional ATPase domain, and its nucleotide-binding state is proposed to regulate activity of the R protein and act as molecular switch depending on the nucleotide bound. In rice NB-ARC domain binds to the promoter OsWRKY13 and regulate its expression to achieve disease resistance (Qiu *et al.,* 2005). A putative model for miRNA-target interactions under cadmium stress was elucidated which is depicted in Figure 3. miR159, 169, 396 and 398 played important regulatory roles in the network by regulating the expression of genes encoding anti-stress proteins (DUF674, G2484, NB-ARC, BTB-POZ MATH, HOPZ), these play important roles in defense response, degradation, detoxification, protein modification, expression in seedlings and buds. While the down-regulated SPL and GBA2 protein were involved in developmental process, carbohydrate transport and metabolism. Of the target genes examined, NB-ARC domain might be the most important genes in the entire network as depicted. GO annotation showed that these putative target genes appeared to be involved in a broad range of biological processes such as cell death, localization, and metabolic process so on. Molecular function was enriched with binding and catalytic activity. Careful analysis of these potential targets will contribute to our understanding of the role of miRNAs in Finger millet under cadmium stress.

Presence of several known cis-regulatory elements that could regulate expression of miRNAs was identified using PlantCare database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Core promoter sequences necessary for transcription initiation and common cis-elements and enhancer regions (CAAT-box) were found in the promoter regions of all miRNA genes analyzed, confirming that miRNA genes transcription is regulated by RNA Polymerase II (Lee *et al.,* 2002). In addition, several cis-regulatory elements involved in light responsiveness or circadian control were found in upstream sequences of all miRNAs gene sequences suggesting the importance of light, and in particular, day length in miRNAs regulation. Several phytohormone responsive, stress responsive and cis-regulatory elements involved in tissue/organ development were also identified which were differentially distributed in each miRNAs. Elicitor-responsive element (TTCGACC) motif was only identified in miR397 which can elicit the numerous biological processes by binding to transcription factors.

CONCLUSION

In the present study, we report miRNAs under cadmium stress in finger millet for the first time. Differential expression patterns were observed between seven miRNAs giving its importance in Finger millet. Cloning and sequencing of these miRNAs conformed their presence; target and GO analysis suggested that these miRNAs might work in a regulatory network. Cis-regulatory motifs identified in the study also emphasise the role of miRNAs and their biogenesis. These findings can provide new information for further characterization of Cd-responsive miRNAs in Finger millet.

CONFLICTS OF INTEREST

The authors declare that they have no competing interest. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript

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