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Original Article

Identification and expression profiling of microRNAs and their corresponding targets related to phytoremediation of heavy metals in jute (*Corchorus olitorius* var. *O-9897*)

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ABSTRACT: A cost effective plant-based approach for the remediation of contaminated soil and water that pose a major environmental and human health problem, is termed phytoremediation. It takes advantage of the remarkable ability of plants to concentrate heavy metals and polluted compounds from the environment and to metabolize the same in their tissues. Industrialization and irrigation with heavy metal (i.e. As) polluted water is a source of contamination of the surface environment in Bangladesh. In the present study, a microRNA (miR319) and a gene (ATP-binding cassette transporter/ABC), known to be involved in heavy metal stress tolerance were identified in jute. Moreover profiling of heavy metal responsive microRNAs (miR159 and miR167) and their target genes (ABC and auxin responsive factor 8/ARF8) under three heavy metal stressors (As, Mn and Cr) indicated jute to be an accumulator of manganese (Mn) and chromium (Cr) but not arsenic (As). Furthermore, the experimental results suggest that down-regulation of jute-miR159 and jute-miR167 may confer better heavy metal tolerance in jute.

KEYWORDS: Phytoremediation, heavy metals, jute, microRNA, ATP-binding cassette transporter gene, auxin responsive factor 8 gene.

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INTRODUCTION

Phytoremediation is an integrated multidisciplinary approach to the cleanup of contaminated soils, which combines the disciplines of plant physiology, soil chemistry, and soil microbiology [1]. Phytoremediation has been applied to a number of contaminants in small-scale field and/or laboratory studies. These contaminants include heavy metals, radionuclides, chlorinated solvents, petroleum hydrocarbons, polychlorobiphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), organophosphate insecticides, explosives, and surfactants [2]. Certain species of higher plants can accumulate high concentrations of metals in their tissues without showing toxicity [3, 4]. Such plants can be used successfully to clean heavy metal polluted soil if their biomass and growth period is suitable to complete remediation within a reasonable period [5].

Since the time scientists searched for biodegradable fiber, they chose jute as one of their probable candidates. It is one of the cheapest and the strongest of all natural bast fibers (collected from bast or skin of the plant) and considered as the "fiber of the future". However, shortage of arable land causes jute to be pushed to unwelcoming terrains. Thus, understanding the cellular and molecular mechanisms associated with heavy metals are desirable for the development of a heavy metal-tolerant jute variety that is capable of growing in inhospitable lands and that can also act as a phytoremediator.

Rapid advancements in molecular biology and biotechnology have overcome the limitations of conventional breeding to design stress tolerant plants. Several studies have been reported in which single genes were genetically transferred or modified in plants to check for heavy metal tolerance property of the same [6-9]. However, analysis of the total gene regulatory system can ease the way of finding potential candidate genes that can be used to make transgenic plants which will be able to cope with multiple stresses rather than counteracting a single one [10].

A class of small endogenous nonprotein-coding RNAs, 20–24 nucleotides (nt) in length called microRNAs (miRNAs) [11] control the expression of target genes [12]. It is reported that a transcription factor, TCP targeted by the miRNA, miR319, is implicated in growth during abiotic stressed conditions like salinity [13], drought [13], cold [14] and heavy metal [15]. On the other hand tolerance to metal toxicity requires the expression of several metal transporters, like the ABC-transporter (ATP-binding cassette) family which mediates the transport of various heavy metals [16, 17] and is regulated by miR159. Most importantly regulation of miR167 and ARF8 (auxin responsive factor 8/ARF8) gene plays an important role in the survival of plants under various stresses [18]. Taken together, these information led us to analyze the expression pattern of miRNAs and their corresponding target genes in jute under heavy metal stress. In this study, presence of miR319 and ABC transporter was identified in jute and the expression pattern of two heavy metal responsive miRNAs (miR159 and miR167) [19] together with their corresponding targets (ABC and ARF8) were analyzed.

MATERIALS AND METHODS

Plant materials

Seeds of *Corchorus olitorius* var. O-9897 jute species were collected from the Physiology Department, Bangladesh Jute Research Institute (BJRI), Dhaka. For germination the seeds were incubated in petri dishes containing filter paper and watered for 3-5 days at room temperature in the absence of light. Once enough seedling growth was obtained, all developing seedlings were frozen in liquid nitrogen for collection. Genomic DNA was extracted from fresh seedlings using the CTAB procedure [20]. This is a slightly modified version of the ideal CTAB method [21-24]. Total RNA was isolated using TRIZOL reagent [25-27] according to the users' manual.

Primer deigning

The specificity of stem-loop RT (reverse transcriptase) primers to individual miRNA is conferred by a six nucleotide extension at the 3' end; this extension is a reverse complement of the last six nucleotides at the 3' end of the miRNA [28]. Forward primers are specific to miRNA sequence but exclude the last six nucleotides at the 3' end of the miRNA. A 5' extension of 5–7 nucleotides is added to each forward primer to increase the melting temperature; these sequences were chosen randomly and are relatively GC-rich. Standard primer designing software was used to assess the quality of the forward primers **(Table 1).**

In case of target genes, degenerate primers were designed by considering highly conserved sequences from *Arabidopis thaliana*, *Poplus trichocarpa*, *Glycine Max*, *Vitis vinifera* and *Ricinus communi* using T-Coffee Multiple Alignment server (http://www.ebi.ac.uk/Tools/msa/tcoffee/). The primers were validated with OligoAnalyzer 3.1. After sequencing, gene specific primers **(Table 2**) were designed from the sequences so obtained.

Identification of miR319 and target gene of miR159

miR319: Following stem loop RT PCR [29, 30], cloning into TOPO cloning vector, transformation, screening of transformants, plasmid isolation, lysate PCR, gel extraction (**using QIAGEN Gel Extraction Kit)** and sequencing were done and the sequences were subjected to blast analysis against miRNA sequences deposited in the miRBase Database (http://www.mirbase.org/).

Table 1: Primers designed for miR319, miR159 & miR167.

miRNA Name	Primers	Sequence (5'-3')
miR319	Stem loop RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGAGGT
	Forward	GGCGGTTGGACTGAAGGGAG
	Reverse	GTGCAGGGTCCGAGGT
miR159	Stem loop RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGAGC
	Forward	CGGCGGTTTGGATTGAAGGGA
	Reverse	GTGCAGGGTCCGAGGT
miR167	Stem loop RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGAAGC
	Forward	GGCGGTAGATCATGCTGGCA
	Reverse	GTGCAGGGTCCGAGGT

Table 2: Primer sequences along with their melting temperature for ABC & ARF8 genes.

Gene Name	Forward primer sequence	Reverse primer sequence	Tm
ABC	5′GAAGAGCACAATTCAAGCC3′	5′GGTTCCAAGAATGAGAAGGG3′	59.0°C
ARF8	5′CTTGTTGCTGGAGATTCTG3′	5′CACTAATGCCAGTTATHGTACC3′	51.5°C

ABC gene: Degenerate primers were designed and optimized for amplifying genomic DNA. The amplified bands were excised and extracted from the gel followed by sequencing which led to the designing of gene specific primers.

Analysis expression pattern of miRNAs and their transcripts through semi-quantitative PCR. Expression profiling analysis was performed in order to understand how *Corchorus olitorius* (col) miR159 and miR167 and their corresponding targets in jute respond to heavy metal stress. For this purpose, 3 day old fresh seedlings grown on a petri-dish were taken as the study subject and 250µM sodium meta arsenate (As Stress), 25mM potassium dichromate (Mn stress) and 25mM potassium permanganate (Cr stress) were applied to the seedlings individually for 48 hours (2 days). Total RNA was next isolated for further analyses.

For differential display cDNAs were prepared from all the RNA samples. For miRNAs, stem loop RT PCR and for analyses of gene expression oligo-RT PCR followed by end point PCR were used.

RT product of miRNA synthesis was incubated initially for 30 min at 16°C, followed by a pulsed RT of 60 cycles at 30°C for 30 s, 42°C and 50°C for 1s each. As the final step, reverse transcriptase was inactivated by incubating the same at 85°C for 5 min. For target genes, PCR reaction was initiated with an initial incubation at 25°C for 5 min. Then the temperature was raised to 55°C and kept for 50 min to allow SuperScript™ III RT to function. Heating at 70°C for 15 min inactivated the enzyme activity and finally the reaction was held at 37°C.

For miRNAs, end-point PCR protocol was initiated with an early incubation at 94°C for 3 min, followed by 25-35 cycles of 94°C for 15 s and 60°C for 1 min. In case of gene amplification, 25-35 cycles (28 cycles for ABC, 25 cycles for ARF8 and 35 cycles for UBC) of gene specific PCR included denaturation for 30s at 94°C, 40s for annealing (Tm in Table 2) and elongation for 40s at 72°C.The reactions were held at 4°C on completion of the amplifying cycles.

RESULTS

The first step of this study included identification and confirmation of the presence of miR319 and the target gene of miR159 (ABC) in jute which are known to play a crucial role in heavy metal phytoremediation. After confirming the presence of this miRNA and gene in jute, the next step was to study the profile of two miRNAs (miR159 and miR167) and their target genes (ABC and 8/ARF8). Variations in the expression pattern of these genes were analyzed in the second part of the study.

Identifying miR319.

Stem loop RT PCR with degenerate primers followed by cloning, gel extraction and sequencing showed the sequence **(Supplement 1)** to be similar to miR319 sequence of other plants viz. *Arabidopsis thaliana, Glycine max, Vitis vinifera*.

ABC gene identification.

Sequence of the PCR product obtained using degenerate primers showed the same **(Supplement 1)** to be highly similar to the ABC gene sequence of *Theobroma cacao, Arabidopsis thaliana, Glycine max* and others.

Semi quantitative PCR.

Each of the cDNA samples was at first tested by performing PCR with primers of two constitutively expressed housekeeping genes called U6 for miRNAs and ubiquitin-C (UBC) for the target genes. For expression profiling of each miRNA and their targets,

the amount of cDNA used was equal to that used for the normalization reaction. The relative intensity of each band was measured by GelScan software **(Supplement 2).**

An overall polar expression pattern is observed under Mn and Cr stress. Under As stress expression of this miRNA-mRNA pair was found to be similar in the early hours but later the miRNA expression increased with corresponding down regulation of its target.

Table 3: Mature microRNA sequence

microRNA name	Mature sequence
col-miR319	UUGGACUGAAGGGAGCUCCCU

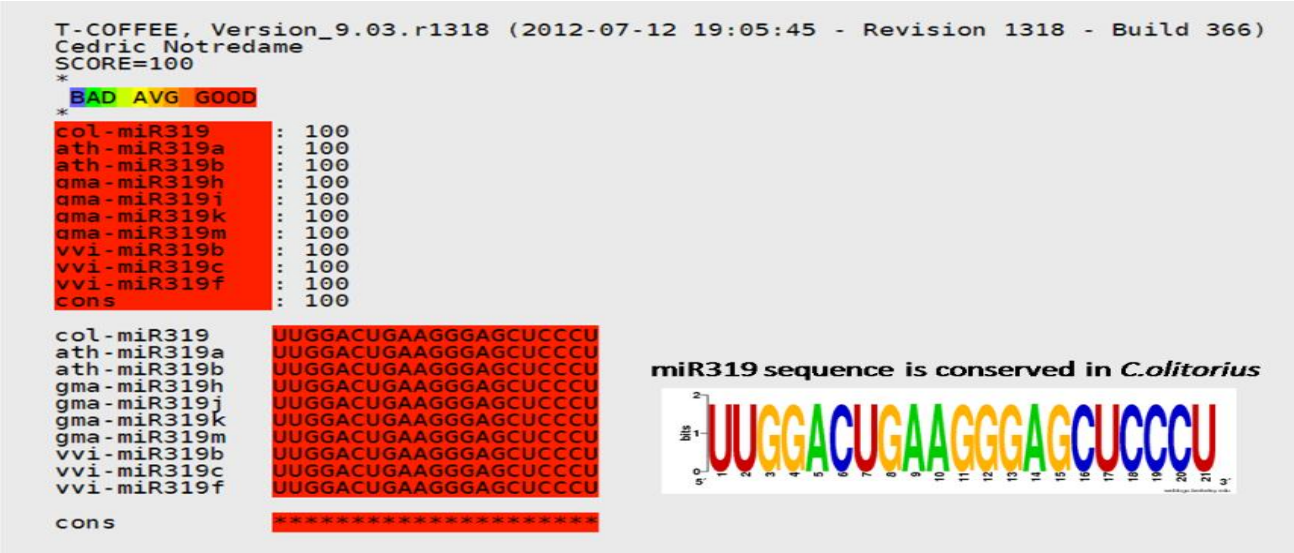

Figure 1: Conservancy analysis of the microRNA: **miR**319 **sequences from *Arabidopsis thaliana*, *Vitis vinifera* and *Glycine max* were retrieved from miRBASE and aligned with sequenced col-miR319 from *Corchorus olitorius*. col-miR319 sequence was found to have conservancy with all the plants mentioned. Among the different subtypes of microRNAs in plants it was found to be conserved with miR319a, miR319b from *Arabidopsis thaliana*; miR319h, miR319j, miR319k and miR319m from *Glycine max*; miR319b, miR319c, miR319f from *Vitis vinifera*. A web logo shows the conservancy along the 21 nucleotides of these microRNAs**

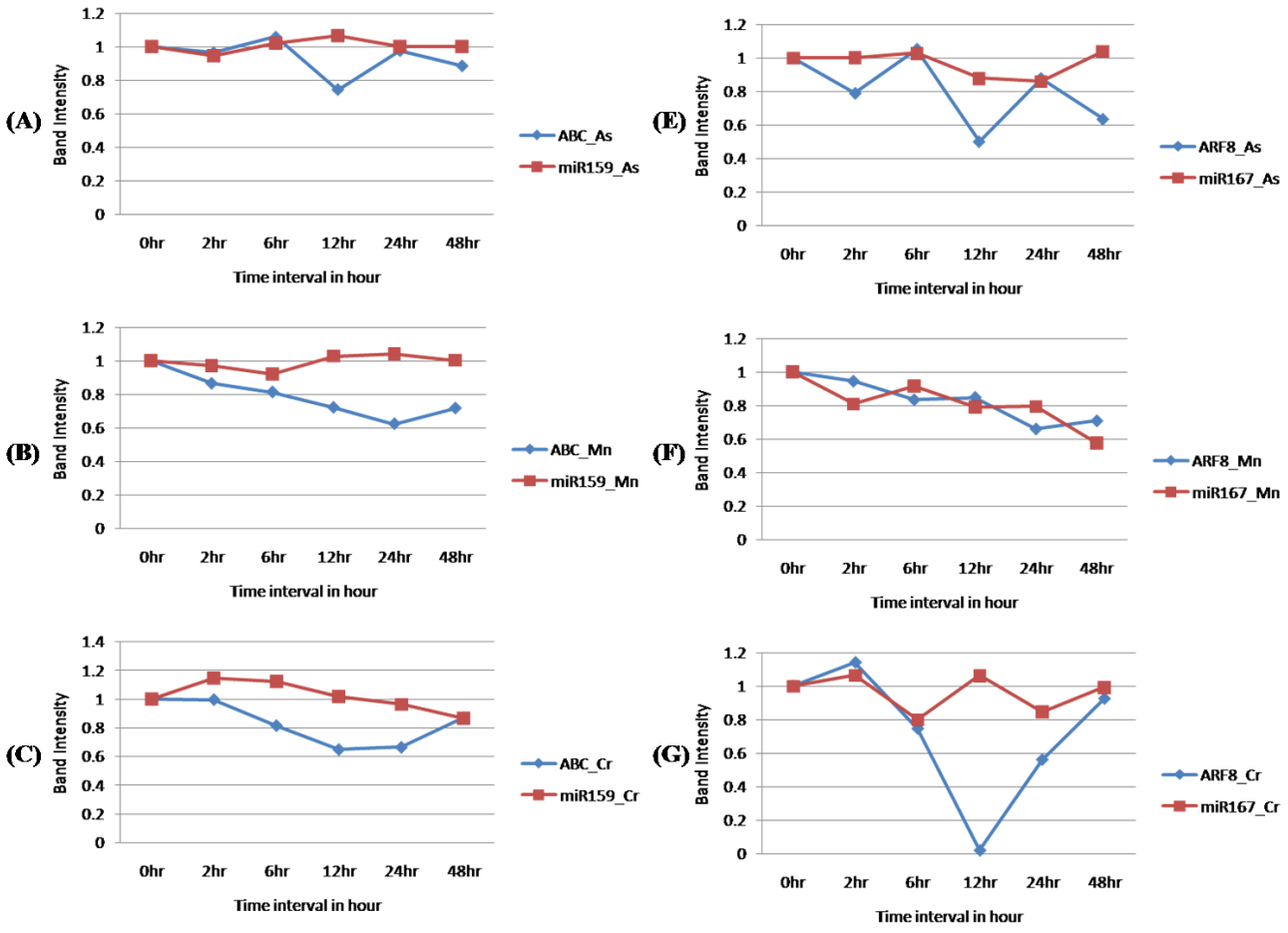

Figure 2: Expression profiling of miR159, miR167 and their target genes ABC, ARF8 (A-C): miR159 and ABC gene in As (A), Mn (B) & Cr (C) stress and **(E-F):** miR167 and ARF8 gene in As (E), Mn (F) & Cr (G) stress

DISCUSSION

Under normal conditions the range of arsenic (As) concentrations in water varies from less than 0.5 to more than 5000µg L-1 and the latter can even exceed in mining and geothermally active regions [31]. It has been reported that 250µM As stress shows toxic effect on various plants including rice [32], so this concentration of arsenic was maintained for generating the stress condition in jute. This strategy was followed for the two other heavy metal stressors (25mM Mn and 25mM Cr) as well. **miR319**, identified originally in a genetic screen regulates transcription factors of the TCP family [33]. The balance between miR319 and its targets controls leaf morphogenesis and several other plant developmental processes.

ABC proteins are modularly organized membrane proteins ("ABC transporters") that mediate Mg-ATP-energized transmembrane transport and/or regulate other transporters. Although it is often possible to predict the likely function of a plant ABC transporter on the basis of its subfamily membership, there are many whose capabilities are different from what would be predicted from the properties of even their most sequence-related counterparts [34].

Along with miR319, miR159 and miR167 are two critical regulatory players, functional during heavy metal stress in plants. Computational approaches have identified the potential target genes for miR159 and miR167 in *Arabidopsis thaliana*, *Poplus trichocarpa*, *Vitis vinifera* and *Glycine max*. The predicted genes in these plants are ABC, MYB (myeloblastosis transcription factor) for miR159 and ARF6, ARF8 (auxin response factors) for miR167. As ABC and ARF8 are more responsive than MYB and ARF6 in heavy metal phytoremediation, their expression profiling under heavy metal stress (As, Mn and Cr) was considered for investigation in jute.

Comparative analysis of semi-quantitative PCR revealed an interesting pattern of expression. Instead of direct inverse expression pattern as expected for the microRNAs and their targets a combination of reverse and similar expression were observed at different time points. At early hours similar expressions were observed but at late hours the pattern was reverse.

These expression pattern can be explained according to the findings of Mukherji, S., et al. MiRNAs can generate thresholds in target gene expression below which expression of target gene is greatly repressed [35].

According to Mukherji, S., et al., a miRNA at its basal level can repress the expression of a gene by targeting its mRNA to a certain concentration of that mRNA. When the target mRNA concentration passes that threshold, the miRNA concentration is needs to be increased for effective down-regulation which in turn results in corresponding decrease in the expression of the target mRNA. As such, the relative mRNA-miRNA expression can be found to be similar in the early stages of regulation while a conventional, polar expression is observed at the later stages when the mRNA crosses the threshold concentration. This was seen in the case of col_ miR159-ABC and col_ miR166- ARF8 expression under heavy metal stressors. Transition between miRNA mediated down-regulation and translation of target genes depend on the active pool of miRNA and mRNA. If free mRNA pool concentration increases and can pass a threshold level set by miRNAs, then it can bypass the repression mediated by miRNAs. On the other hand, after cleaving the target mRNAs, miRNAs can re-enter into the active miRNA pool to start another cycle of inhibition of the target gene expression [35].

Stress responsive factors possibly bind *cis*-regulatory elements in the promoter region of miR159 and miR167 genes to initiate their expression [10, 36]. Mature col-miR159 and col-miR167 may then target the corresponding mRNAs of ABC and ARF8 genes and inhibit them either by cleavage or by inhibition of translation.

CONCLUSION

This study gave an insight into the physical properties as well as the molecular mechanisms of jute as a heavy metal tolerant plant. The results indicate that jute is an accumulator of Mn and Cr but not As. Jute would therefore be a good candidate in the remediation of soil rich in Mn and Cr. Differential expression of heavy metal responsive microRNAs in jute suggests the involvement of diverse mechanisms. Down-regulation of miR159 and miR167 may confer better heavy metal tolerance in jute. As a future course of action this could be checked through further investigation.

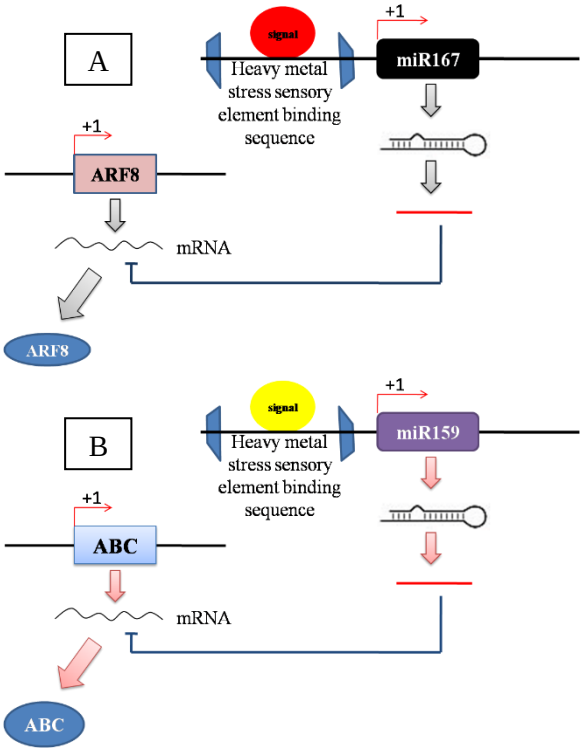

Figure 3: Proposed molecular mechanism for induction of col-miR159 and col-miR167 genes and their down-stream effects under heavy metal stressors at late hours. (A) col-miR159 represses ABC expression whereas (B) col-miR167 represses ARF8 gene expression.

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