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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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Corresponding e-mail: haseena@du.ac.bd**Abstract**

The cost effective plant-based approach to remediation of contaminated soil and water that pose a major environmental and human health problem, is termed phytoremediation technology that take advantage of remarkable ability of plants to concentrate heavy metals and polluted compounds from the environment and to metabolize various molecules in their tissues. In Bangladesh not only the industrialization but also the irrigation with heavy metal (i.e. As) polluted water is also a source of contamination in the surface environment. In this study a microRNA (miR319) and a gene (ATP-binding cassette transporter/ABC) that are known to be involved in heavy metal stressors tolerance are identified. Profiling of heavy metal responsive microRNAs (miR159 and miR167) and their target genes (ABC and auxin responsive factor 8/ARF8) in three heavy metal stressors (As, Mn and Cr) reveals that jute is a manganese (Mn) and chromium (Cr) accumulator but not arsenic (As). Moreover, the experimental results assume that down-regulation of col-miR159 and col-miR167 may confer better heavy metal tolerance in jute.

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]. Certain species of higher plants can accumulate very high concentrations of metals in their tissues without-showing toxicity [2,3]. Such plants can be used successfully to clean up heavy metal polluted soils if their biomass and metal content are large enough to complete remediation within a reasonable period [4].

Shortage of arable land causes cash crops (i.e. jute) to be pushed to these inhospitable terrains. So, understanding the cellular and molecular mechanisms associated with heavy metal is earnestly needed for development of a novel heavy metal-tolerant jute variety that can be cultivated in heavy metal polluted field as a phytoremediator.

Rapid advancements in the 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 the plants to check the heavy metal tolerance property of the plant. But analysis of the total gene regulatory system can ease the way of finding potential candidate genes that can be used to make plants transgenic which will not only be able to counteract one stress but also will enable plants to cope with other stresses [5]. As microRNA control the gene expression so a study of the expression pattern of miRNAs under heavy metal stress conditions would improve our understanding of their functions in the adaptation of heavy metal stress adaptation which would help to fine tune molecular mechanism for possible phytoremediation for heavy metals.

So, the objective of the present study was to identify the presence of crucial microRNAs and their target genes in jute along with determination of the expression pattern of microRNAs and their targets under heavy metal stress in jute.

MATERIALS AND METHODS**A. Genomic DNA isolation from jute**

Genomic DNA was extracted from fresh seedlings using CTAB procedure [6]. This is a slightly modified version of the ideal CTAB method [7-10].

B. Isolation of total RNA

Total RNA was isolated using TRIZOL Reagent [11-13] according to the users' manual.

i. Identification of miRNA and gene: miR319:

Stem loop RT PCR [14]; [15] using degenerate primer was done followed by cloning using TOPO cloning vector, transformation, screening transformant, plasmid isolation, lysate PCR, gel extraction (using QIAGEN Gel Extraction Kit)

and sequencing was done and the sequence was blasted against the miRNAs database deposited in miRBase Database (<http://www.mirbase.org/>).

ABC gene: Degenerate primers were designed and optimized from isolated genomic DNA and then the band excision and gel extraction followed by sequencing leads to the gene specific primer designing.

ii. Expression pattern analysis of miRNAs and their transcripts through Semi-Quantitative PCR

To understand how the col-miR159, col-miR167 and their corresponding targets in Jute show response to heavy metal stress, expression profiling analysis was performed. For this purpose, 3 day old fresh seedlings grown on a petri-dish were taken as the study subject and 250µM As, 25mM Mn and 25mM Cr stresses were applied to the seedlings. Then total RNA was isolated for further proceedings.

For differential display cDNAs were prepared from all the RNA samples. For miRNAs stem loop RT PCR and for gene RT PCR followed by end point PCR was employed.

Thermal Cycle for RT product of miRNA synthesis was incubated initially for 30 min at 16°C, followed by pulsed RT of 60 cycles at 30°C for 30 s, 42°C and 50°C for 1s. At the final stage, to inactivate the reverse transcriptase activity it was incubated at 85°C for 5 min.

The reaction of End-point PCR protocol started 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. When the cycles were completed then the reactions were hold at 4°C.

RESULTS

In this study the first step included the identification and confirmation of the presence of a miRNA and a gene in jute that play crucial roles in heavy metal phytoremediation, namely miR319 and ATP-binding cassette (ABC) transporter. After confirming the presence of this miRNA and gene in jute, the next step was to profile two miRNAs (miR159 and miR167) and their target genes (ABC and auxin responsive factor 8/ARF8). The variation in the expression pattern of these genes was analyzed in the second part of the thesis. To validate results **duplicate biological samples** along with **technical triplicates** were taken.

C. miRNA and Gene Identification**i. miR319 identification**

Stem loop RT PCR with degenerate primer followed by cloning, gel extraction and sequencing showed that the sequence has similarity with miR319 of other plants like *Arabidopsis thaliana*, *Glycine max*, *Vitis vinifera*.

ii. ABC gene identification

Sequencing of the PCR product using degenerate primer showed the sequence is highly similar with the ABC gene sequence

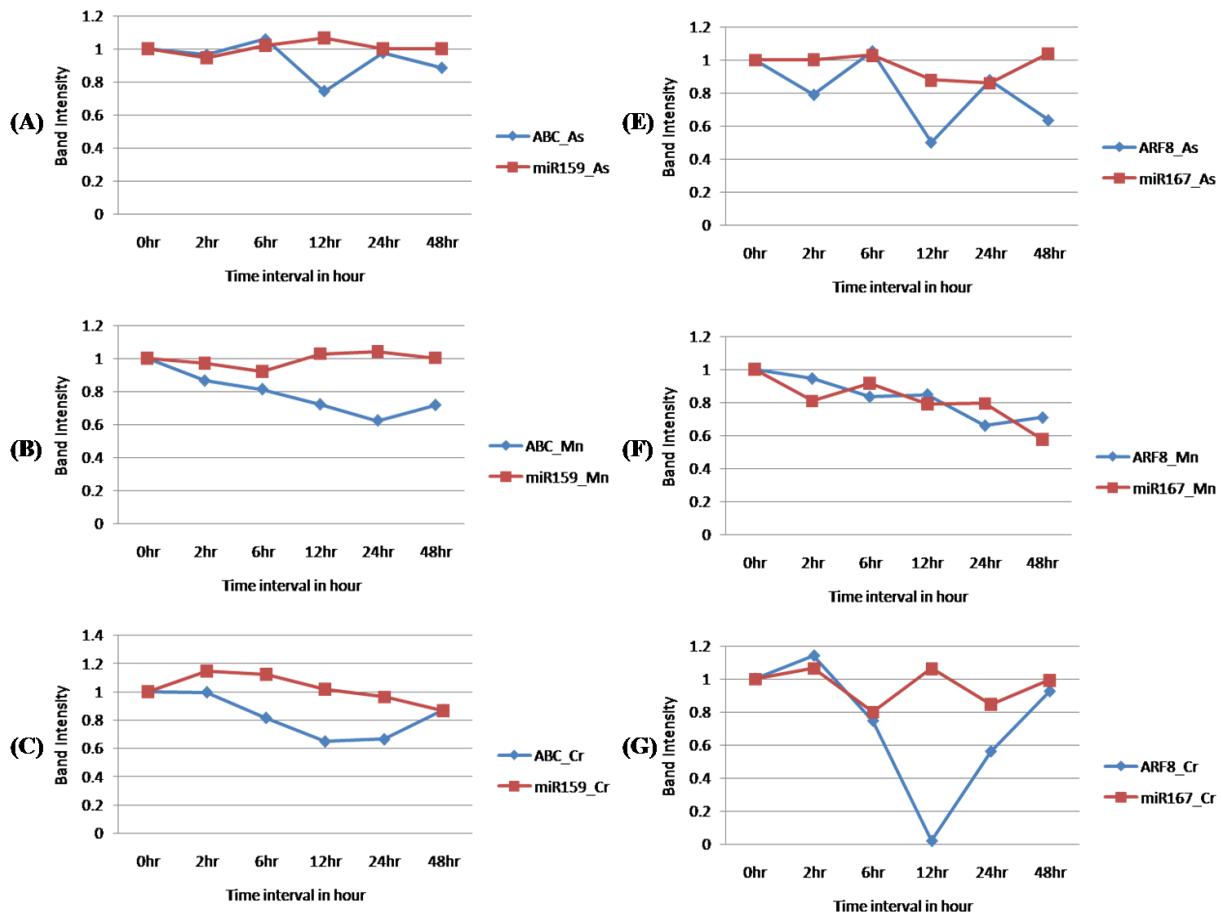


Fig. 1: Expression profiling of miR159, miR167 and their target genes ABC, ARF8.

(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.

of *Theobroma cacao*, *Arabidopsis thaliana*, *Glycine max* and others.

D. Semi quantitative PCR displays how the expression of each miRNA and its target gene is regulated in jute during heavy metal stress

Each of the cDNA samples was at first tested by performing PCR with primers of two constitutively expressed housekeeping genes called U6 for miRNA and Ubiquitin-C (UBC) for gene. For expression profiling of each miRNA and gene, the amount of cDNA used was equal to that used for normalization reaction. The relative intensity of each band was measured by GelScan software.

The expression profiling of miR159 and miR167 and their corresponding targets are summarized here (Fig. 1).

Table 1. Mature microRNA sequences

microRNA name	Mature sequence
col-miR319	UUGGACUGAAGGGAGCUCCCU

DISCUSSION

A. Identification of microRNA and gene

i. miR319

It was identified originally in a genetic screen; microRNA miR319 regulates transcription factors of the *TCP* family [16]. The balance between miR319 and its targets controls leaf morphogenesis and several other plant developmental processes.

ii. ATP-binding cassette transporter

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 deviate from what would be predicted from the properties of even their most sequence-related counterparts [17].

B. Comparative expression analysis of microRNAs and target genes

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*, *Populus trichocarpa*, *Vitis vinifera* and *Glycine max*. The predicted genes were ABC, MYB (myeloblastosis transcription factor) for miR159 and ARF6, ARF8 (auxin response factors) for miR167 in these plants. 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) has been studied here.

Comparative analysis of semi-quantitative PCR revealed an interesting pattern of expression. Instead of the 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 are seen but at late hours reverse expressions are noticed.

Two hypotheses can be implied to explain these patterns of expression.

Hypothesis 1: miRNAs can generate thresholds in target gene expression below which expression of target gene is greatly repressed. 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 [18].

Hypothesis 2: According to the second hypothesis based on two conventional mechanisms of miRNA action, parallel and reverse expression patterns for col-miR159 and col-miR167 and their target genes ABC and ARF8 may happen, if both target gene repression mechanisms of miRNAs that is (i) translational inhibition on endoplasmic reticulum and (ii) cleavage in the cytoplasm, act simultaneously.

Stress responsive factors can bind at the *cis*-regulatory elements in the promoter region miR159 and miR167 to initiate their gene expression [5,19]. Mature col-miR159 and col-miR167 then may target their corresponding targets ABC and ARF8 mRNAs and inhibit them through a combination of cleavage and translation inhibition mechanisms.

and profiling of heavy metal responsive microRNAs, MS thesis). In arsenic stress the seedlings almost died whereas under manganese and chromium stressors they survived (Fig. 2).

To account for this phenotypic change we have proposed a molecular mechanism (Figure 3). Inhibition (in arsenic stress) or stimulation (in manganese and chromium stress) of ABC and ARF8 by miR159 and miR167 may bring a marked phenotypic change of jute seedlings under heavy metal stress condition. Inhibition of ABC and ARF8 may lead to decreased PCs-mediated metal detoxification and lateral root length, whereas their stimulation may lead to increased heavy metal stress tolerance of seedlings.

So, down-regulating the col-miR159 and col-miR166 genes in transgenic jute may confer better heavy metal tolerance in this crop plant in future.

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 (found in industrial area) but not As (field irrigated with arsenic contaminated water). 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 in response to heavy metals. Down-regulation of miR159 and miR167 may confer better heavy metal tolerance in jute. As a future course of action this should be checked through developing transgenic jute variety.

ACKNOWLEDGEMENT

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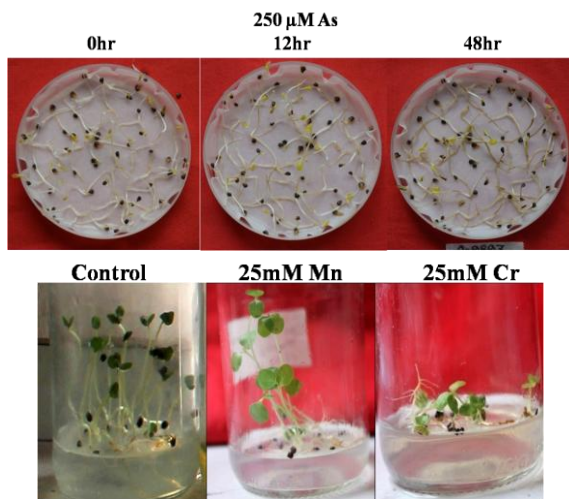


Fig. 2: Morphological changes.

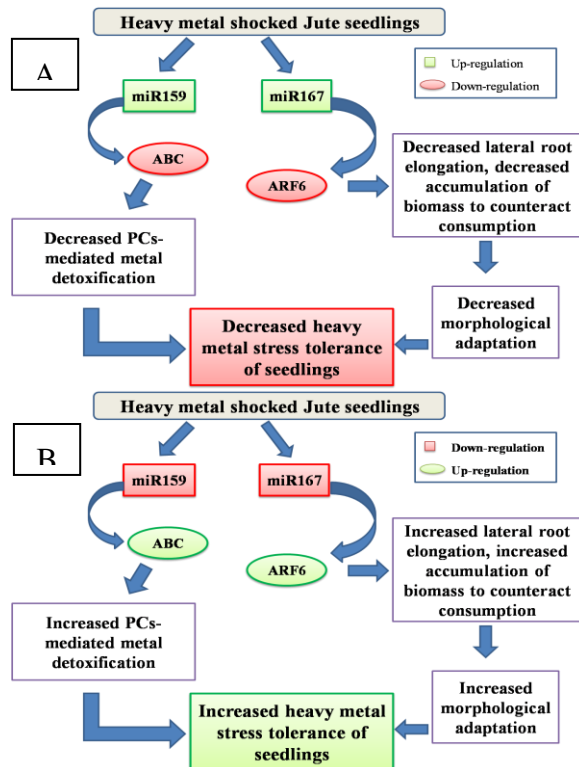


Fig. 3: Proposed molecular mechanism for the observed phenotypic effects on jute seedlings during heavy metal stress.

Considering the phenotypic analysis of the jute seedlings (data not published), lateral root elongation, total length of the plants were found to be affected greatly under heavy metal stress (Jute for the phytoremediation of heavy metal pollution

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