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IDENTIFICATION OF ABIOTIC STRESS-RESPONSIVE MIRNA TARGET NETWORK IN PHOENIX SP, A MANGROVE SPECIES

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ABSTRACT: A very tiny non-coding RNA molecule with a length of 20 to 24 nucleotides, called microRNAs or miRNAs. They control gene expression by targeting mRNAs with sequence-specificity. Depending on the degree of complementarity with the target mRNA sequences, this might result in translational repression or mRNA destruction. They are very crucial for controlling growth and development in plants. Researchers also agreed with the point that miRNAs are responsive to stress in plants. Growing in tropical and subtropical estuaries, mangrove is a very significant ecosystem both environmentally and commercially. But several natural processes, like sea level rise, salinity rise, and global warming, are disastrous for this flora, and some species are disappearing at a startling rate. Studying the function of these miRNAs in controlling mangrove homeostasis in that situation would be of utmost significance. In the most recent research, we discovered three miRNAs: ph-miR10630, ph-miR11471, and ph-miR10341 in Phoenix sp, that react to abiotic stress. This finding unmistakably indicates that miRNAs play a regulatory function in preserving cellular homeostasis. When compared to plants that are close to extinction, the experimental validation and molecular characterization of these miRNAs might provide important insights into their role in battling biotic and abiotic stress. This information would then be extremely helpful for planning the proper conservation of those plant species.

INTRODUCTION: MicroRNAs (miRNAs) are a group of tiny, non-coding RNAs that play important regulatory roles in plants as well as in animals. They have persisted throughout evolution and are endogenous in nature [1]. miRNAs were first discovered in 1993 by Victor Ambros and colleagues, who noticed that the lin-4 gene, known to regulate the development efficiency of C. elegans, produced two small RNA molecules instead of a protein. One of the RNAs, which was roughly 22nt in length, had antisense complementarity to multiple sites in the 3' UTR of the lin-14 gene, which led to translational repression of the lin-14 mRNA as part of the regulatory pathway that causes the change from the first larval stage's cell divisions to the second [2]. Subsequently in the following years, more than 100 additional genes for small noncoding RNAs were discovered and thus were referred to as microRNAs.

The discovery of miRNAs has greatly expanded our understanding of gene regulation and their importance in various biological processes. Eventually, miRNA had also been found in animals, plants, and certain viruses which are all eukaryotes that contain DNA as their genetic material. By selecting particular mRNAs for

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cleavage or translational suppression, miRNA control gene expression [3]. Numerous current scientific investigations have demonstrated that microRNAs are crucial for several biological activities such as the cell cycle, cell differentiation, leaf and flower formation, fat metabolism, immunity, and cell death in plants. Researchers also agreed that microRNAs play an important role in plant adaptation to harsh environmental conditions [4].

Furthermore, in the plant, miRNA's biogenesis typically begins with the transcription of miRNA genes by RNA Polymerase II (Pol II). The resulting primary miRNA (pri-miRNA) transcript is a long, hairpin-like RNA molecule that contains the miRNA sequence flanked by short single-stranded regions [5]. The recruitment of Pol II to the miRNA promoters involves the interaction of several transcriptional activators and various sequence motifs in the miRNA promoters [6]. Primary miRNA transcripts (pri-miRNAs) are produced as a result of this process. These pri-miRNAs fold into hairpin shapes that are identified by enzymes from the Dicer-like (DCL) family [7]. Levels of the DCL family vary across several plant species and different members of the family generate miRNAs of different lengths [8].

The production of the miRNA/miRNA* duplex takes place in the nucleus in specific spaces known as Dicing-bodies. The miRNA/miRNA* duplex is subsequently transported to the cytoplasm, where Hua Enhancer 1(HEN1) methylates the 3′ ends. In the cytoplasm, the miRNA/miRNA* duplex splits, and the guide strand is then loaded into the RNA-induced silencing complex (RISC) by entangling with Argonaut (AGO) proteins [9, 10]. The RISC, which also consists of other proteins including Heat Shock Protein 90 (Hsp90), attaches to its target by having a complementary sequence, which allows it to either direct mRNA cleavage or translational inhibition. Plant miRNAs carry out translational inhibition to regulate certain important developmental processes, including floral determin [11, 12]. Mangroves are intertidal forested wetlands found in tropical and subtropical regions, covering an estimated 18.1 million hectares globally [13]. Although mangroves have been exploited for centuries, our understanding of these wetland forests has greatly improved in recent decades, with a growing number of publications focusing on the ecology, management, and conservation of mangroves [14]. The biodiversity of mangroves is of increasing interest due to the threat they face from global climate change, particularly sea level rise [15]. The flora of mangroves consists of 65-69 species of vascular plants adapted to the dynamic coastal environment, while the fauna includes fish and crustacean resources [16]. The highest species richness of mangrove plants occurs around the equator, while the highest concentrations of mangroves occur in the Eastern Hemisphere between 90º E and 135º E. The mangrove ecology is also seen to be a very economically successful ecosystem. The mangrove ecology is also seen to be a highly economically prosperous ecosystem. According to one estimate, the economic value is worth around US$186 million each year [17]. The largest contiguous mangrove system in the world is the Sundarbans in the Ganga-Brahmaputra-Meghna Delta, shared by India and Bangladesh, which covers an area of one million hectares [14, 18]. Sundarbans mangrove forest is the most diverse among all mangrove forests and has many species, but some of them are facing the threat of extinction due to factors such as global warming, sea level rise, and increased salinity in the estuaries [15]. It also mentions that the constant stress faced by mangroves generates high levels of reactive oxygen radicals (ROS), which can cause damage to the plants, but the plants have a defense mechanism to scavenge off these ROS [18, 19]. However, studies on the role of miRNAs in regulating homeostasis in plants are very rare in the context of Sundarbans mangroves.

In the ongoing project, possible targets for putative miRNA precursor sequences in the mangrove species Phoenix species were identified. Since miRNA controls gene expression by cleaving their targeted mRNA, its discovery is predicted to result in a better understanding of its potential involvement in plant growth and development.

MATERIALS AND METHODS:

Retrieval of Data and Trimming: We started our study with Phoenix species. The 10898 miRNA sequences are collected from the publicly available database PMRD database [21]. After that redundancy checks have to perform by using the PRINSEQ tool to filter redundant sequences [20].

After removing the redundant sequence EST database of Phoenix species of NCBI (Taxonomy Id: 4791) is used to find homologs for the remaining 7240 sequences [22].

Potential miRNA Identification: All of the aforementioned 7240 sequences were aligned to the publically accessible Phoenix Sp EST (expressed sequence tag) database using the Basic Local Alignment Search Tool (BLAST) on the NCBI website [23]. The nucleotide match size was set to 15 with an expectation value of 1.7, which means that only sequences with a significant match were considered. The chosen ESTs (expressed sequence tags) were then run *via* blastx against the NCBI non-redundant protein sequence collection to remove sequences that code for proteins to prevent redundancy. Since miRNAs are non-coding RNAs, which do not code for proteins, that's why this step is crucial. By analyzing the remaining ESTs using BLAST against the NCBI nucleotide collection, any RNAs like tRNA, rRNA, snRNA, or snoRNA were ruled out. This step was necessary because these types of RNAs are not miRNAs and could interfere with the identification of true miRNAs.

RNA Secondary Structure Prediction: The selected sequences are examined for secondary structure by the Mfold web server [24]. The Mfold web server's default settings are used for this inquiry. For this investigation, the configurations are employed. The following criteria are used to identify potential miRNAs. This screening is done by keeping the miRNA's placement on a hairpin. The miRNA should have at least 15 residues.

The Maximum number of unpaired residues should not exceed 9. miRNA should only include a maximum number of 5 G-U pairs. A bulge in a miRNA sequence should not be larger than 5nt. The minimal folding energy (MFE) should be low. The MFEI, or minimal folding energy index, needs to be high. To determine the possible target, the predicted miRNAs from Phoenix spare examined [25].

MFEI = (MFE/Size of Nucleotide) ×100 / (G+C) %

Prediction of Target of Potential miRNA: The predicted miRNAs from Phoenix sp are analyzed to get the potential target. To find out the potential target, the psRNA target web server is used [23]. miRNAs of Phoenix sp are not reported to date. That's why a database of Arabidopsis thaliana is used for this space.

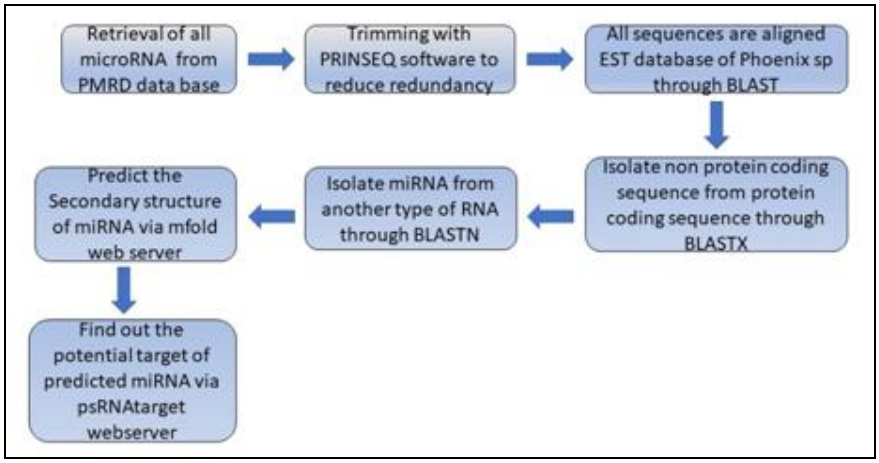

FIG. 1: FLOWCHART OF THE IDENTIFICATION OF ABIOTIC STRESS-RESPONSIVE MICRORNA OF *PHOENIX SP*

RESULT AND DISCUSSION: All previously reported miRNAs of all plant species are downloaded from the PMRD database. In this study, all the 10898 miRNA sequences are used to find out their homologs in mangrove species, Phoenix sp. Due to the lack of research on the Phoenix species so far, this step is crucial. Therefore, one of the best methods for predicting miRNAs that could be present in the species is by *in-silico* screening of their homologs with known species. According to several studies, miRNAs are thought to be vital in plant development and growth. They are associated with plants' stress reactions as well. As a result, in this study, we used

the Phoenix sp. expressed sequence tag database to predict possible miRNAs. The next step is checking the redundancy of the 10898 sequences which we collected from the publically available database of the plant i.e. PMRB database. The redundancy check has to perform by PRIENSEQ 0.20.4. This software decreased the total no of sequences from 10898 to 7240 by eliminating the redundant sequences. The homologous of these sequences, which are contained in the Phoenix sp. est database, are discovered using them as a query sequence. To align ESTs with the query miRNA sequence, Nucleotide Blast was used. The Nucleotide Blast result produced 445 hits. For nucleotide BLAST, the E value was set to 1.7. Then we have to check whether these sequences are protein coding sequences or nonprotein coding sequences. To isolate non-protein-coding sequences from protein-coding sequences BLASTX has to be performed with the shortlisted ESTs. At least 17 sequences are found here that are thought to be miRNAs.

The resulting sequences were then analyzed for their potential to be or contain miRNAs by predicting their secondary structure using the mfold software. This step is important because miRNAs must be in a specific conformation to function correctly, so it helps to filter out any sequences that are unlikely to be functional miRNAs. A specific conformation is required for an RNA or nucleotide strand to function as a miRNA; otherwise, miRNA activity would not occur**.** The seven previously described criteria were used to choose and inspect the nucleotides that were homologous to the miRNAs for this conformation. Among all the ESTs taken under consideration, the criterion got matched for three ESTs. They are named ph-miR10630 **Fig. 2**, ph-miR11471 **Fig. 3**, and ph-miR1034 **Fig. 4**. The minimal folding energy was -0.640 and minimal folding energy was calculated to be -0.640. The Minimal Folding Energy (MFE) and Minimal Folding Energy Index are mentioned in the **Table 1.**

TABLE 1: MINIMAL FOLDING ENERGY (MFE) AND MINIMAL FOLDING ENERGY INDEX

Name of the miRNA	Length (nucleotides)	MFE (Kcal/mol)	MFEI (Kcal/mol)
ph-miR10630	20	-6.40	-0.640
ph-miR11471	20	-4.30	0.430
ph-miR10341	20	-8.50	-0.70

MFE: Minimal Folding Energy, MFEI: Minimal Folding Energy Index.

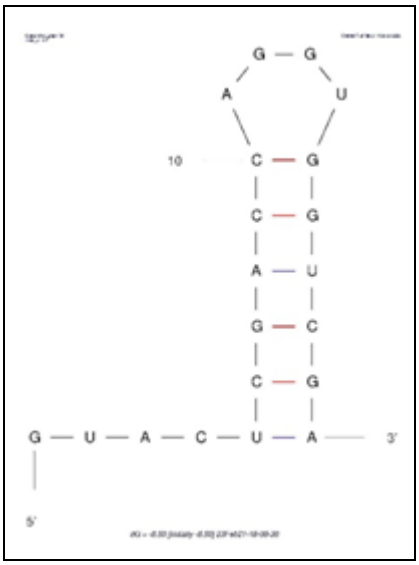

FIG. 2: PH-MIR10630

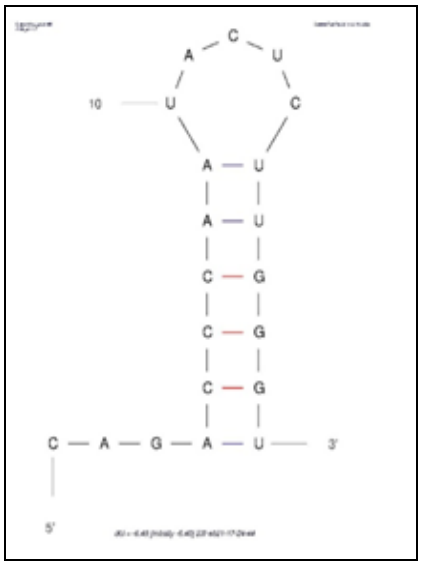

FIG. 3: PH-MIR11471

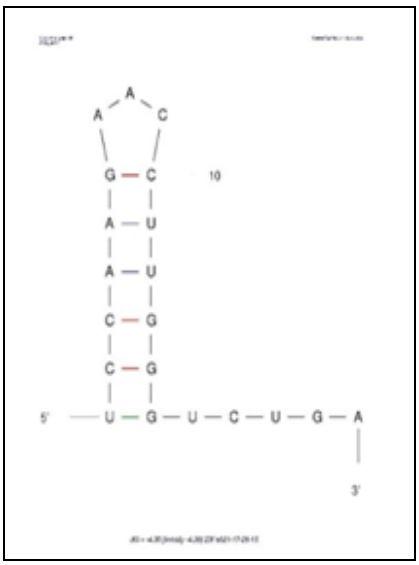

FIG. 4: PH-MIR10341

Researchers have found various roles of miRNA in plants. Among them, one of the major roles of miRNAs is to be responsive against abiotic stress[26].We looked for the potential target genes for the three newly predicted miRNAs to determine their involvement in assisting the mangrove species in such unreceptive conditions. psRNA web server was used for this study. To date, the cDNA library of Phoenix sp is available. Thus the cDNA library for A. thaliana was used instead because it has been

widely investigated as a model organism [27, 28]. The findings of this study were assessed to comprehend the function of these miRNAs in preserving cellular homeostasis in the plant. After analyzing by the psRNA target webserver it is found that the predicted miRNA ph-miR10630 produces 152 hits. The no of hits essentially demonstrates the targeted genes for this miRNA. After analyzing the hits we found the targeted proteins are F Box protein, phytochrome C protein, chloroplast RNA-binding protein 29, Plant regulator RWP-RK family protein, CDC27 family protein, protein kinases %3 Bubiquitin-protein ligase, respiratory burst oxidase-like protein, Galactose oxidase/kelch repeat superfamily protein, B3 domain-containing protein REM13, Ankyrin repeat family protein, acyl activating enzyme 1, heme oxygenase 2, HSP20-like chaperones super family protein, CCCH-type zinc finger family protein with RNA-binding domain-containing protein, trichome birefringence-like protein and so on. Plant morphology and development are greatly influenced by all of these proteins.

Scientists Lijuan Wang *et al.* state that stress toxicity frequently happens as a result of increased NO and ROS generation and the up-regulation of Heme Oxygenase 2 (HO2) brought on by environmental pressures [27]. Our studies have shown that ph-miR-10630 deduce the overexpression by translational inhibition. It is reported by researchers that F Box protein is induced by abiotic stress [28]. It is reported that the overexpression of F Box Protein is directly correlated with the abiotic stress of plants [29]. Our study also shows that miR10630 can regulate the expression of the protein by translational inhibition [30].

We discovered that the ph-miR11471 generates 161 hits when it is analyzed by the psRNA target website. This miRNA's predicted genes included a few of the following: 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase super family protein, Phosphotyrosine protein phosphatases superfamily protein, F-box and associated interaction domains-containing protein, myb domain protein 60, cellulose synthase-like B6, cellulose synthase-like B4, chloroplast RNA-binding protein 29, zinc finger CCCH domain protein, F-box associated ubiquitination effector family protein, Zinc-binding dehydrogenase family protein, hypothetical protein, guanylate kinase, TSK-associating protein 1, Leucine-rich repeat protein kinase family protein and many more. Protein phosphatases are essential parts of several signaling pathways where that control different biological reactions in plants [32]. Reversible protein phosphorylation, which is mediated by the enzymes protein kinases and protein phosphatases and regulates several biological processes, such as cell cycle events, growth factor response, hormone and other environmental stimuli, metabolic regulation, and developmental events, is a significant event in signal transduction[31, 32]. According to Hong et al. (2007), RING finger family proteins have a function in the control of the response to stress as well as the vulnerability to illness[35]. In plants, there is a sizable superfamily of proteins known as the leucine-rich repeat receptor-like kinase proteins (LRR-RLKs) [34]. After analyzing the potential target of ph-miR10341 *via* the psRNAtarget webserver we found a total of 112 hits.

Some of the anticipated genes for this miRNA included: Leucine-rich receptor-like protein kinase family protein, Subtilase family protein, Mannose-binding lectin superfamily protein, Protein kinase superfamily protein, Mannose-binding lectin superfamily protein, F-box family protein, cysteine synthase D1 and many more. Serine/threonine protein kinases sometimes referred to as RLKs, are conserved signaling elements that control plant growth, disease resistance, hormone sensing, and self-incompatibility [35]. Increased ionizing radiation resistance in Arabidopsis is caused by the overexpression of the rice jacalin-related mannose-binding lectin (OsJAC1) [36]. Abiotic stress tolerance is reduced by overexpression of an F-box Protein Gene. Only a tiny subset of F-box proteins in plants have been examined, but it has been found that by integrating nearly all phytohormone signaling pathways, they play significant roles in regulating a variety of developmental processes and stress responses [37].

CONCLUSION: The salinity in the Sundarbans estuary has been rising constantly over the past few decades, as this can have a significant impact on the ecosystem and the species that rely on it [38]. The three species, Heritiera fomes, Xylocarpus granatum, and Nypafruticans, are all important mangrove species that are adapted to live in

environments with varying levels of salinity. However, as the salinity levels continue to rise, these species may struggle to adapt, which can lead to negative impacts on their populations [39]. For example, studies have shown that increased salinity can lead to reduced growth and reproductive success in mangrove trees, as well as increased susceptibility to pests and disease. Furthermore, changes in the mangrove ecosystem can also have ripple effects on other species that depend on it, such as fish and shellfish that use the mangrove roots for shelter and feeding. It is important to monitor and address the rising salinity levels in the Sundarbans estuary to protect the diverse species that depend on it and maintain the overall health and resilience of the ecosystem [40].

This study discusses the need for genetic analysis of Sundarbans mangroves, which are at risk of extinction. The author suggests that a lack of genetic data is hindering conservation efforts and that in-depth genetic analysis could provide insight into the reasons for the mangroves' decline. The author notes that miRNAs in the species Phoenix may be targeting genes involved in growth and regulation and that further experimental validation could shed light on the genetic composition of the mangroves. This data could then be compared to distressed species to help develop restoration strategies. Overall, the passage emphasizes the importance of genetic data in conservation efforts and highlights the potential benefits of in-depth genetic analysis.

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