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Research Article

Identification and Evaluation of Regulatory Role of miR164b in Malaysian Rice Variety (MR303) under Drought Stress

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ABSTRACT

MR303 is a newly released Malaysian rice cultivar that comes with special traits as it can be cultivated in less fertile soils. Previous studies stated that miR164b is a key molecule that is responsible for regulating drought stress tolerance in many rice varieties. Thus, this study aims to identify the presence and regulatory role of miR164b in the MR303 variety using both computational and experimental approaches. The stem-loop structure of miR164b (pre-miR164b) was identified through reverse-transcriptase PCR (RT-PCR) with a size of ~ 100 bp. The target prediction by psRNA target revealed that the target gene of miR164b is the NAM protein of the NAC transcription factor. A gene expression study by RT-PCR followed by Image J analysis in both control and drought-treated plants demonstrated low expression of miR164b was observed in the drought sample, which led to the accumulation of its target, NAM1. This study provides preliminary knowledge of the presence of miR164b and its regulatory role in the MR303 rice variety.

Keywords: Drought, miR164, MR303, NAM 1, Rice

Introduction

Rice (*Oryza sativa* L.) is widely consumed by a majority of the world's population, especially in Asia [1]. According to FAO data in 2016–2017, average rice production is estimated at 5.0×10^8 tonnes and is expected to increase to 2.0×10^9 tonnes by the year 2030 due to a rise in population, which leads to an increase in demand [2]. Sufficient water supply, salt concentration, light intensity, temperature and nutrients are the important factors that support ideal plant growth. The present and foreseen worldwide food shortages urge a significant enhancement in crop productivity in less favorable rainfall areas. Nevertheless, most rice varieties are susceptible to various abiotic factors that arise due to climate change. The phenomenon affects the regularity and number of hydrological fluctuations, particularly in developing nations. Drought stress has been identified as a major threat to rice production [3].

Drought happens when there is no rainfall or insufficient rainfall in a particular area, leading to high water demand. The severity of drought is complex and depends on different factors like the frequency of rainfall, soil moisture and evaporation rate [4, 5]. Drought stress has caused morphological, anatomical and physiological changes in rice crops. Early findings revealed drought stress significantly limits seed germination and reduce growth and leaf size [6, 7]. Next, proline concentration is increased during drought stress to maintain the cell membrane integrity and stabilize the enzyme within the rice plant [8].

MiRNA is a small non-coding RNA that is involved in the post-transcriptional process. miRNA can bind to target mRNA, leading to degradation and translational repression. Over the past few years, numerous microRNAs (miRNAs) have been identified in plants and mammals. At the same time, our knowledge and understanding of this topic have been significantly expanded due to the contributions of fast and accurate

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computational approaches. Basically, the non-coding MIR gene is responsible for synthesizing the miRNA that is able to form the stem-loop structures through transcription. Once in its mature form, miRNA plays vital roles in regulating root elongation, osmotic stress regulation, photosynthesis regulation, senescence, antioxidative stress modulation, changes in hormone regulation and the plant development process [9].

Several miRNAs that are related to drought stress in plants have been studied. However, in rice, different varieties may have different gene expressions. For instance, the transcriptome analysis revealed a different set of genes were expressed exclusively in the drought-tolerant cultivar (Dhagaddeshi) compared to the drought-susceptible cultivar (IR20) [10]. In Malaysia, breeding work started in the year 2000, which gave rise to many rice varieties, including MR219, MR220, MRQ74, MR232, MR220CL1, MR220CL2, MRM16, MR253 and MR263. After that, the breeding project has been expanded from 2010 to 2018 with the objectives of high yield, good resistance, eating quality, phenotype, commercialization and pest and disease resistance breeding. In 2018, Malaysian Agricultural Research & Development (MARDI) released a new rice cultivar known as MR303 [11]. The variety was produced through a breeding program involving MR253 and MR256 varieties. Interestingly, this variety is known to be resistant to environmental stress and has a high yield [11]. This study aims to detect the presence of miR164b in the MR303 rice variety. In addition, the regulatory role of miR164b will be elucidated as this specific miRNA plays an important role in drought stress.

Material and Methods

Plant materials and treatments

The MR303 rice variety was used in this study. The rice was pregerminated in water overnight. After pre-germination, the rice seeds were washed with 20% Clorox and rinsed with distilled water. The rice seeds were moved to organic soil in pots. Two sets of rice plants were prepared for approximately 4-5 weeks: control and drought-treated plants. For control plants, the plants were watered regularly to ensure proper growth.

On the other hand, the drought-treated plants were watered sufficiently until the third week. Drought stress was imposed on the plants by discontinuing watering for 7 days until leaf rolling was observed as an indication of drought stress. After the treatment, leaves from the control and drought-treated plants were harvested and immediately kept at -80°C for further use [12].

RNA Extraction

RNA extraction was carried out using Trizol reagents according to the manufacturer's protocol [13]. About 0.1 g of fresh rice leaves were frozen, ground to a fine powder, and then transferred into new 2 mL microcentrifuge tubes. Immediately, 1 mL of Trizol Reagent was added to the tubes. The sample was incubated horizontally for 2 minutes at room temperature. Next, 0.2 mL of chloroform was added to the sample. The sample was incubated for 2 to 3 minutes at room temperature. The sample was centrifuged for 15 minutes at 4°C at 12,000 × g. The mixture was separated into a lower red phenol-chloroform, the interphase, and a colorless upper aqueous phase. The upper aqueous phase was collected and transferred into new tubes.

Next, about 0.5 mL of isopropanol was added to the aqueous phase for lysis. The sample was incubated for 10 minutes. The sample was centrifuged for 10 minutes at 12,000 × g at 4°C. The total RNA was precipitated at the bottom of the tube in the form of a pellet. The supernatant was discarded. The pellet was resuspended in 1 mL of 75% ethanol for lysis. The mixture was mixed gently and centrifuged for 5 minutes at 7500 × g at 4°C. The supernatant was discarded. The RNA pellet was left for 5 minutes for air drying. Then, 20 µL of RNase-free water was added to resuspend the pellet. The RNA samples were subjected to a Nanodrop spectrophotometer and 1% gel electrophoresis to determine the RNA quantity and quality, respectively. For further analysis, only high-purity RNA was used (ratio A260/A280 and A260/A280 above 1.8). The sample was stored overnight at -80 °C for further use.

cDNA synthesis

cDNA synthesis was carried out using RevertAid Reverse Transcriptase (Thermofisher) according to the standard manufacturer's protocol [14]. In summary, 1 µL of oligo(dT) was added to 1 µg of the RNA sample. Then, the following reagents were added to the mixture: 4 µL

of 5× Reaction Buffer, 1 µL of RiboLock RNAse Inhibitor (20 U/µL), 2 µL of 10 mM dNTP Mix and 1 µL of RevertAid RT (200 U/µL). The sample was incubated for 1 h at 42 °C in a thermocycler. The reaction was stopped by heating the sample at 70 °C for 5 min.

Pre-miR164b Validation in MR303 Rice by RT-PCR

The stem-loop precursor of the miR164b (pre-miR164b) sequence was retrieved from the miRBase database version 22.1 (https://www.mirbase.org/) [15]. The forward and reverse primers for stem-loop miR164b were designed by using Primer3 with default settings (https://primer3.ut.ee/) [16]. The forward primer sequence was: 5'-TGCACGGTGGAGAAGCAG-3' whereas the reverse primer was: 5'- TGGA-GAAGACGGGCA CATGA-3', respectively. RT-PCR was conducted by using New England Biolabs' One Taq 2X Master Mix with Standard Buffer according to the manufacturer's protocol (New England Biolab) [17]. The reaction was set up as follows: 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 2 µL template DNA, and 25 µL One Taq 2X Master Mix with Standard Buffer. Nuclease-free water was added until the total volume is 50 µL. Additionally, PCR cycle conditions were as follows: an initial denaturation step at 94 °C for 30 s, 40 cycles of 94 °C for 30 s, 56 °C for 30 s, and 68 °C for 1 minute/kb, followed by a final extension at 68 °C for 5 min.

Target Prediction for miR164b in MR303

The psRNATarget bioinformatic tool (https://www.zhaolab.org/psRNATarget/) was used to predict the targets of miR164b by using the default parameter [18]. The cDNA library was set to *Oryza sativa* (rice) transcript, MSU Rice Genome Annotation, version 7.

Relative gene expression analysis by RT-PCR

RT-PCR was performed to analyze the relative expression of miR164b and its target. For miR164b, the stem-loop primer was used. For the target sequence, the primer was 5'-GACAGCAG-CAACTACGGC- 3' and the reverse primer sequence was 5'-CGAACGAGGCATCCATGTG-3. The reaction was set up as follows: 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 2 µL template DNA and 25 µL One Taq 2X Master Mix with Standard Buffer were added into a sterile thin-walled PCR tube. Nuclease-free water was added until the total volume was 50 µL. The reaction was mixed gently. The PCR process was carried out with the following conditions: an initial denaturation step at 94 °C for 30 s, 40 cycles of 94 °C for 15-30 s, 45-68 °C for 15-60 s, and 68 °C for 1 minute/kb, followed by a final extension at 68 °C for 5 min. The intensity of the band was measured by Image J [19].

Results and Discussion

Stem-loop Identification of Pre-miR164b

Stem-loop structure identification is crucial to identify the bioavailability of miR164b in the rice variety MR303. Figure 1 shows the detection of pre-miR164b through RT-PCR. Based on Figure 1, the PCR result on gel electrophoresis indicates the stem-loop structure of miR164b, which has a size of approximately 100 bp. This result showed a similar finding with the stem- loop miR164b, which was retrieved from miRBase and consists of 109 bp. The precursors detected were smaller than predicted because the primers were designed to avoid the bulge region to prevent inefficient primer annealing [14]. Hence, we could probably confirm the bioavailability of miR164b in the MR303 rice variety. The miR164 family is a conserved miRNA that can be found in higher plants. In *A. thaliana*, three members of miR164 have been identified, including miR164a, miR164b,

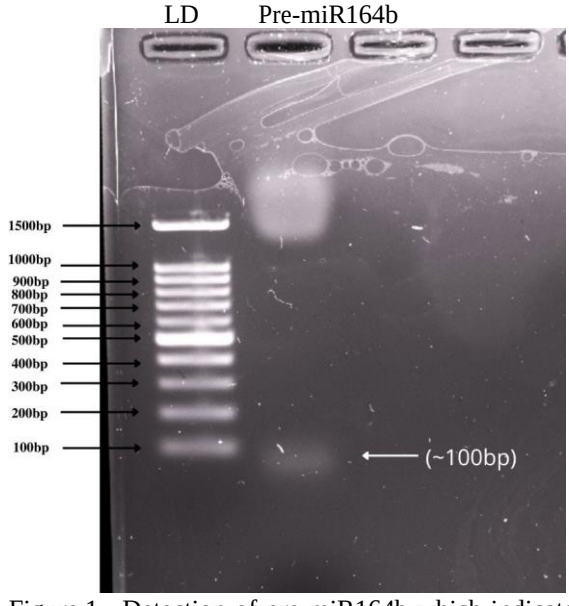


Figure 1. Detection of pre-miR164b which indicate the presence of the miRNA in the MR303 variety. From left: Ladder (LD), the precursor of miR164b.

Table 1. Top 10 genes of possible targets for miR164b

Target Accession	Expect	Target Annotation	Inhibition mode
LOC_Os06g23650.1	1.0	cDNA|no apical meristem protein, putative, expressed 1	Cleavage
LOC_Os06g46270.1	1.0	cDNA|no apical meristem protein, putative, expressed 1	Cleavage
LOC_Os12g41680.1	1.0	cDNA|no apical meristem protein, putative, expressed 1	Cleavage
LOC_Os02g36880.3	1.5	cDNA|no apical meristem protein, putative, expressed 1	Cleavage
LOC_Os02g36880.1	1.5	cDNA|no apical meristem protein, putative, expressed 1	Cleavage
LOC_Os02g36880.4	1.5	cDNA|no apical meristem protein, putative, expressed 1	Cleavage
LOC_Os02g36880.2	1.5	cDNA|no apical meristem protein, putative, expressed 1	Cleavage
LOC_Os04g38720.1	1.5	cDNA|no apical meristem protein, putative, expressed 1	Cleavage
LOC_Os08g10080.1	2.0	cDNA|no apical meristem protein, putative, expressed 1	Cleavage
LOC_Os03g47310.1	2.0	cDNA|transposon protein, putative, CACTA, En/Spm sub-class, expressed 1	Cleavage

and miR164c. Of them all, miR164a and miR164b have the same sequence [20]. In rice, the miR164 family was found to be conserved in mature sequences only but not the precursors. [21].

Target Prediction of miR164b

Due to the short sequence of miRNA, it can bind to multiple mRNA targets, and several miRNAs can regulate a single mRNA [9, 22]. A total of 165 genes were predicted by psRNA targets to be the targets for miR164b. Table 1 shows the top 10 possible target genes of miR164b.

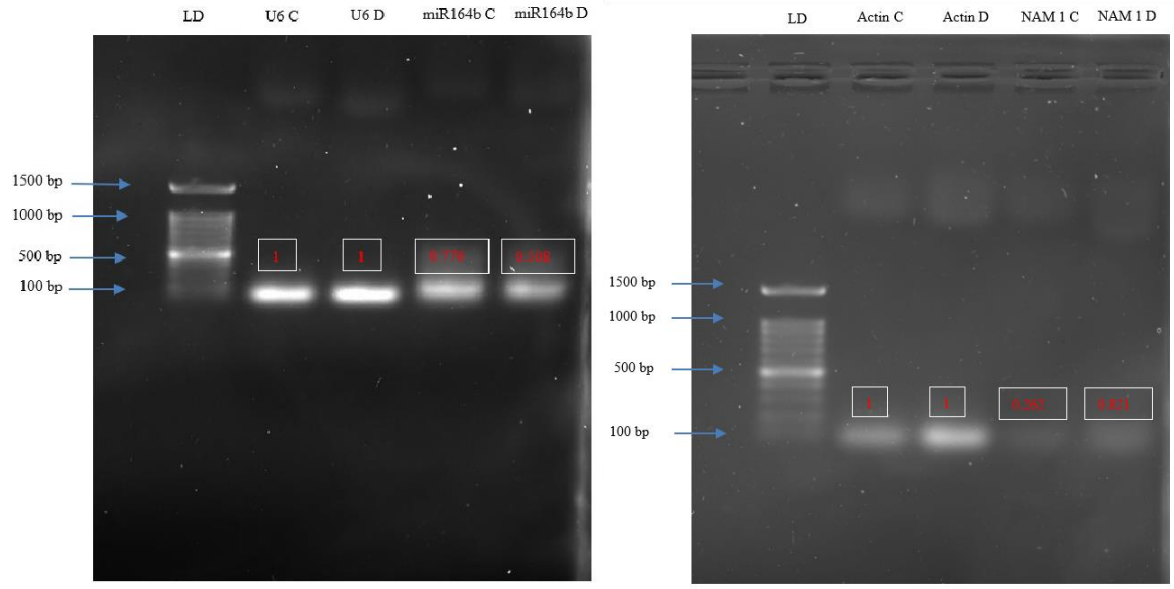
Based on the target prediction result (Table 1), miR164b may target up to two genes; No Apical Meristem (NAM) and CACTA transposon proteins, respectively. Interestingly, in the NAM target gene, miR164b is able to bind to 9 different locations with a different degree of complementarity. The Expect (E) values show the degree of complementarity between miRNA and the target gene. The lower the E value, the higher the degree of complementarity. Then, LOC_Os06g23650.1, LOC_Os06g46270.1 and LOC_Os12g41680.1 of NAM genes showed the lowest E value which indicates the highest complementarity binding with miR164b compared to the rest of the targets. In plant miRNA, high complementarity of binding will result in the cleavage of target genes [9, 23]. NAM is a part of structural domains in NAC genes that are made up of two other domains, including *Arabidopsis* transcription activation factor (ATAF1/2) and cup-shaped cotyledon (CUC2) [24]. For the second target, transposons of the CACTA superfamily (cut-and-paste) are among the most abundant. *Suppressor-mutator* (*Spm*) is the first CACTA element identified in the *Zea mays* (*Poaceae*, *Poales*) genome. The key role of CACTA elements and other transposable elements is their capability to affect the evolution of the host genome [25].

Expression analysis of miR164b towards the target gene

As NAM showed a better degree of complementarity with miR164b, the target gene has been selected for expression study under drought stress. RT-PCR has been performed to measure the expression level of miR164b and NAM genes (Figure 2a and b).

Figure 2a shows the expression analysis of miR164b in control and drought-treated plants, respectively. The expression was normalized to the U6 gene. miR164b expression showed down-regulation under drought treatment where the expression of miR164b slightly decreased from 0.776 to 0.508. Meanwhile, the expression of the target gene, NAM 1, increased from 0.262 to 0.821. This finding is supported by the previous finding that revealed miR164b was downregulated in rice (japonica variety) under drought conditions since it negatively regulates drought tolerance [26]. The down-regulation of miR164b caused the accumulation of NAM 1 targets.

In many plant species, the miR164 family is highly conserved [20]. In rice, the miR164 family consists of six members that produce four mature sequences, which are miR164a/b/f, miR164c, miR164d, and miR164e [27]. These six miR164-targeted NAC genes (*OMTN1–OMTN6*) in which four of the targets may play a role as negative regulators in drought resistance [26]. In other plants such as *A. thaliana* and tomato, the overexpression of miR164 affected the morphology of Arabidopsis and tomato, and it affected the transition from the vegetative to the reproductive phase in Arabidopsis [28]. These changes that change the morphological and physiological characteristics



a b

Figure 2. miR164b expression under control and drought-treated plants. From left; LD, ladder; U6 C, U6 control; U6 D, U6 drought-treated; miR164b C, miR164b control; miR164b D, miR164b drought (a) and NAM 1 expression under control and drought-treated plants. From left; LD, ladder; Actin C, Actin control; Actin D, Actin drought-treated; NAM 1 C, NAM 1 control; NAM 1 D, NAM1 drought (b).

of the plants may be due to the importance of the target gene itself, the NAC gene.

In this study, miR164b was predicted to target NAM 1 of the NAC transcription factor. The NAM 1 expression under drought stress can be observed in Figure 2b. The expression value for control and drought-treated plants is 0.262 and 0.821, respectively. The slight increase in the expression in the treated plant may probably be due to the decreased expression of miR164b. The NAC transcription factor is a plant-specific transcription factor. Currently, 328 members of the NAC are in rice [29]. The rice NAC TFs play important roles in various aspects of plant growth, development, and stress responses [9]. NAC TFs in rice abiotic stress responses have been extensively studied in response to abiotic stress. For instance, the overexpression of OsNAC5 [30], OsNAC9 [31] or OsNAP [32] confers drought and salinity tolerance in transgenic rice. In this study, the accumulation of the target gene may indicate the importance of NAM 1 in regulating drought-stress tolerance. Nevertheless, genetic manipulation study is required to further verify the fact. Similarly, overexpression of NAC TF from wheat and pepper, TaSNAC4 and CaNAC46, promoted drought tolerance in transgenic *A. thaliana*, respectively [33, 34]. This showed that the functions of some NAC members are conserved across plant species.

Conclusion

Our study confirmed the bioavailability of miR164b in the MR303 rice variety by detecting the precursor structure. Besides, the regulatory role of miR164b towards its target has been analyzed under drought stress. The down-regulated miR164b provides information on the importance of its target, the NAM 1 gene, in regulating drought tolerance. Perhaps, this finding could pave the way to generating drought- tolerant rice by knocking down miR164b.

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