VNU Journal of Science: Natural Sciences and Technology
Journal homepage: https://js.vnu.edu.vn/NST

Original Article

In Silico Analysis of *Osa-miR164* Gene Family in Rice (*Oryza Sativa*)

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Received 12 August 2021
Revised 06 September 2021; Accepted 06 September 2021

Abstract: MicroRNA (miRNA) is a small (~ 22 nucleotides) non-coding RNA molecule, which functions in post-transcriptional regulation of gene expression. Previous reports have shown that miRNA plays an important role in the resistance ability of plants to adverse conditions. In this research, we focused on miR164 family in rice (*Oryza sativa)*, a major food crop. By using bioinformatics approach, we analyzed sequences of all *osa-miR164s* belonging to rice miR164 family, evaluated the expression profile of *osa-miR164* under different stress conditions, predicted cis-regulatory elements on *osa-miR164* gene promoters, and simultaneously predicted miR164-targeted genes and their expressions. The results show high conserve in mature *osa-miR164* sequences but not in the precursor sequences, different expression patterns of *osa-miR164* gene members under stress conditions and various cis-regulatory elements present in *osa-miR164* gene promoters, which might explain the diverse expression patterns of *osa-miR164* genes. Some potential target genes of *osa-miR164* were identified and their expressions under different stress conditions were analyzed.

Keywords: miR164, microRNA, non-coding RNA, rice, *Oryza sativa*.

1. Introduction

MicroRNAs are small non-coding RNA molecules containing about 22 nucleotides that function in post-transcriptional regulation of gene expression [1]. miRNAs play key roles in animals and plants, by promoting cleavage or translation inhibition of targeting mRNAs [1]. Lin-4 was the first miRNA detected in *Caenorhabditis elegans* [2], and in plant, early miRNAs were detected in *Arabidopsis thaliana* [3]. miRNAs negatively regulate mRNA by guiding the cleavage while being active in RISC complex (RNA-induced silencing complex) and suppressing protein synthesis or degrading targeting mRNAs [4].

MiR164 family in plants consists of miRNAs with conserved sequences which were found in several species and were one of the first cloned miRNAs in Arabidopsis [3] In Arabidopsis, miR164s mark cleavage site of mRNAs

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https://doi.org/10.25073/2588-1140/vnunst.5304

corresponding with targeting NAC genes [NAC genes include: no apical meristem (NAM), Arabidopsis transcription activation factor (ATAF) and cup-shaped cotyledon (CUC)] [5]. In Arabidopsis, miRNA164 family has 3 members (ath-miR164a/b/c) which target five NAC genes (*CUC1/At3g15170, CUC2/At5g53950, NAC1/At1g56010, At5g07680,* and *At5g61430*). Some studies showed that these NAC genes are required for lateral root development, expansion of the boundary domain, regulation of the age-dependent cell death, and formation of vegetative and floral organs [6-8].

In rice, there are 6 gene members in miR164 family, consisting of osa-miR164a/b/c/d/e/f [9]. It is suggested that miR164-targeted NAC (*OMTN*) genes may act as negative regulators of drought tolerance in rice with overexpression of *OMTN2*, *OMTN3*, *OMTN4*, and *OMTN6* caused increased drought sensitivity in transgenic plant [10].

In this study, we aim to investigate the rice miR164 genes and their targets in response to stress conditions by employing the bioinformatic approach to research on expression profile of *osa-miR164* genes under different stress treatments, expression regulation of *osa-miR614* genes by cis-regulatory elements present in gene promoters, and miR164-targeted genes' expressions under different stresses.

2. Methodology

2.1. Genomic Analysis

All stem-loop, mature sequences and locations of *osa-miR164* family were obtained from the miRBase database (http://www.miRBase.org/) [11]. These sequences were aligned by CLUSTALW [12] using MEGA7 [13].

2.2. Identification of Potential Promoters of Osa-miR164s

For promoter prediction, the upstream sequences pre-microRNAs of each *osa-miR164* gene were downloaded from the rice genome database (http://rice.plantbiology.msu.edu/). The 1500 bp sequence of 5' to the pre-miRNA was retrieved as the promoter region. The TSSs (Transcription Starting Site) were predicted by the YAPP Eukaryotic core promoter predictor (http://www.bioinformatics.org/yapp/cgi-bin/yapp.cgi), a tool to scan for canonical core promoter elements - BREs (B recognition element), TATA boxes, INRs (initiator motif) and DPEs (downstream promoter element), and synergistic combinations of these elements. The obtained promoters were the ones closest to the 5' of the pre-miR164.

2.3. Analysis of Cis-regulatory Elements of Rice Osa-miR164 Family

Potential promoter regions (the 1500 bp of 5' region upstream of the pre-miRNAs) were used to predict the cis-regulatory elements and motifs. The SOGO NEW PLACE database (https://sogo.dna.affrc.go.jp/cgi-bin/sogo.cgi?lang=en), a Database of Plant Cis-acting Regulatory DNA Elements, was used to analyze the cis-regulatory elements of the osa-miR164 family.

2.4. Genes Target Prediction and Their Functions

Mature *osa-miR164* sequences of *Oryza sativa* were downloaded from miRBase. The target genes of *osa-miR164a/b/c/d/e/f* were identified by using psRNATarget (Version 2017) with the default parameters input, except a more stringent cut-off threshold of expectation ≤ 2. The RAP-DB database was used to confirm the information of targeted genes. Uniprot database was used to determine functions of *osa-miR164* targeted genes.

2.5. Expression of Osa-miR164 in Abiotic Stress Conditions

The expression profiles of *osa-miR164a/b/c/d/e/f* were extracted from PmiRExAt@NABI database [14]. The relative expression level of the gene was presented as the log2 fold change between the control conditions and the different treatments or stress conditions.

2.6. Expression of Osa-miR164 Targeted Genes under Abiotic and Biotic Stresses

To show the expression level of targeted genes under abiotic and biotic stresses, online TENOR and RiceXPro databases were employed to get data experiments under stress conditions of *osa-miR164* targeted genes.

3. Results and Discussion

3.1. Sequence Comparison of Osa-miR164 Gene Family

Based on miRBase database, there are six genes in rice miR164 family, including *osa-miR164a, osa-miR164b, osa-miR164c, osa-miR164d, osa-miR164e, osa-miR164f.* *Osa-miR164a osa-miR164b, osa-miR164c, osa-miR164d, osa-miR164e, osa-miR164f*, locate in Chr7: 28523341-28523496, Chr5: 15896163-15896271, Chr5: 17327440-17327558, Chr2: 33143567-33143660, Chr3: 10542157-10542288 and Chr5: 23343908-23344117, respectively. Mature sequences of *osa-miR164a, osa-miR164b, osa-miR164f* are 100% identical (Figure 1A). However, *osa-miR164c* and *osa-miR164d* have one different nucleotide from *osa-miR164a, b, f* (14^{th} base of *osa-miR164c* is U, and 21^{st} base of *osa-miR164d* is U), and *osa-miR164e* has 2 different nucleotides (20^{th} base is A and 21^{st} base is G) (Figure 1A).

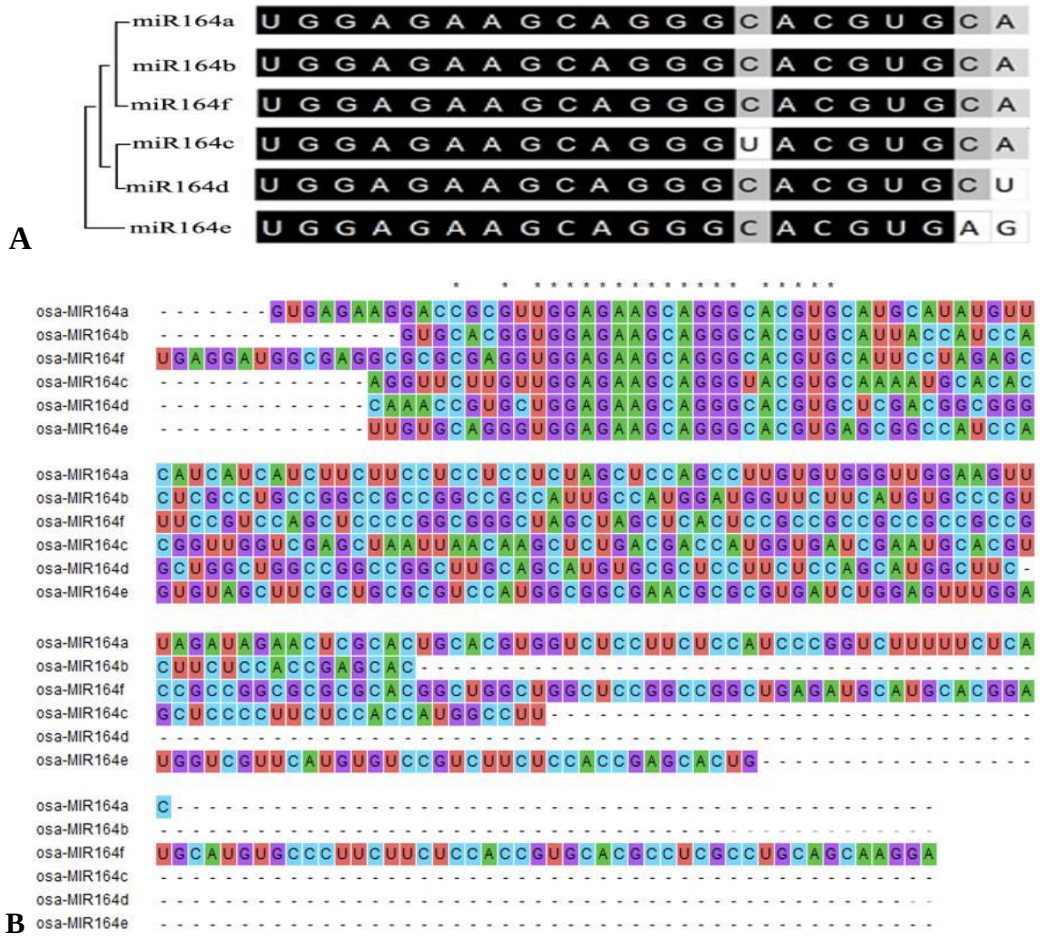

Figure 1. A - Alignment of the mature *osa-miR164* sequences in rice. Black color indicates identical nucleotides among six *osa-miR164s*, while fading ones show lower similarity among six *osa-miR164s*.
B - Alignment of the precursor *osa-miR164* sequences. Asterisk marks identical nucleotide.

Since the sequences of mature *osa-miR164s* are highly conserved, we further analyze the sequences of *osa-miR164* precursors. The results show that the *osa-miR164* precursor sequences were completely different (excluding mature sequences). They shared only 1 nucleotide C (5^{th} base upstream mature sequences) and 1 nucleotide G (2^{nd} base upstream mature sequences) (Figure 1B).

3.2. Analysis of Cis-regulatory Elements of Osa-miR164 Gene Promoters

The *cis*-regulatory elements present in *osa-miR164* gene promoters are shown in Figure 2 and Table 1. MYC, MYB and CuRe motifs (involved in response to abscisic acid, regulation of drought and copper, respectively) are common and can be found in all genes in large numbers, indicating that *miR164* gene family is highly regulated by drought, abscisic acid and copper conditions. DRE motif (salt/drought response) was common in *osa-miR164c* and *osa-miR164f* genes. They are found at the distal part of 5' regulatory region in *osa-miR164c* whilst spread across the *osa-miR164f* gene. SuRe motif (sulfur responsive element) was found in all genes in small amounts, whereas Erd-1 (required for early response to dehydration) was found in all the regulatory regions of the *osa-miR164* genes at varying frequencies. LTRE (Low temperature responsive element) was only distributed in *osa-miR164b, c, f*. I-box and ASF-1 motif (light regulation element) were widespread in all regulatory regions. Sugar-repressive elements (TATCCAY, A-box, Pyrimidine box, GARE) were distributed differently between six genes, in which they were absent in *osa-miR164e*. GARE motif was found in *osa-miR164d* and *osa-miR164f* whereas A-box was present only in *osa-miR164f*. Regarding hormone related cis-regulatory elements, ABRE motif (abscisic acid responsive element) was found in 5 genes except *osa-miR164b*. S-box (important in ABA response) was located only in *osa-miR164c*. ARF (auxin response) was presented at varying frequencies in all genes and highest in *osa-miR164f* (Figure 2).

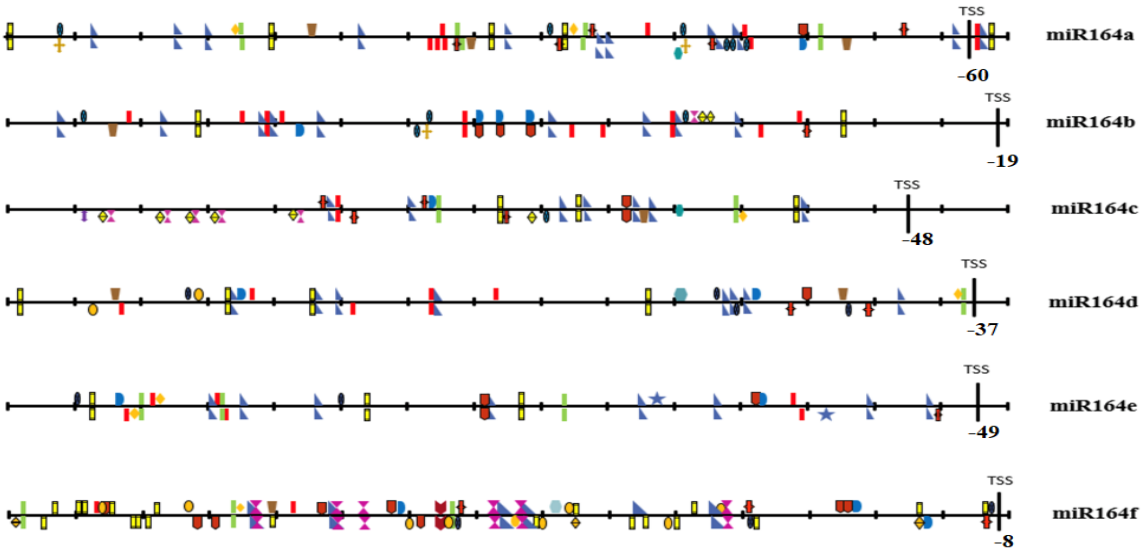

Figure 2. Map of promoter regions of *osa-miR164* genes. The corresponding cis-regulatory elements are described in Table 1. Position of transcription start side (TSS) is predicted using YAPP database. The first nucleotide of *osa-miR164* precursors is considered as +1.

Table 1. Potential cis- regulatory elements identified in the 5' regulatory sequences of *osa-miR164* gene family. The 1.5 kb of 5' regulatory region was analyzed using PLACE and YAPP databases

Cis-regulatory elements	Sequence	Symbol	Function	Reference	MiR164					
					a	b	c	d	e	f
DRE	A/GCCGAC		Salt/drought response element	[15]	0	1	5	0	0	12
ABRE	(C/A)ACG (T/C) G(T/C/G)		Abscisic acid response element	[16]	2	0	1	1	2	1
MYC	CATGTG; CACATG; CANNTG		Early response to drought and abscisic acid induction	[17]	22	18	14	20	22	17
GARE	TAACAA (G/A)		Gibberellin Responsive element	[16]	0	0	0	4	0	9
CuRE	5′-TTTGC (T/G)C(A/G)-3′		Copper responsive element and also involved in oxygen response	[18]	10	4	6	8	10	19
SuRE	GAGAC		Sulphur responsive element	[19]	1	4	1	2	2	3
ARF	GGTCCAT; TGTCTC		Auxin response element	[16]	1	3	2	1	3	8
MYB	WAACCA; TAACTG; CNGTTR; YAACKG; GGATA; CAACTG		Involved in regulation of drought	[20]	9	13	2	7	6	2
Erd 1	ACGT		Early response to dehydration	[15]	8	0	4	2	8	6
Pyrimidine box	TTTTTTCC; CCTTTT		Gibberellin-response cis-element	[21]	3	1	1	2	0	1
TATCCAY motif	TATCCA		Involved in sugar repression	[22]	1	0	1	1	0	1
SRE	TTATCCA		Sugar-repressive element	[19]	2	1	0	0	0	0
LTRE	ACCGACA; CCGAAA; GTCGAC		Low temperature responsive element	[23]	0	2	6	0	0	3
A-box	TACGTA		Sugar repression element	[24]	0	0	0	0	0	2
S-box	CACCT(C/T) (C/T)A		Sugar and ABA response	[25]	0	0	1	0	0	0
CMSRE-1	TGGACGG		Carbohydrate Metabolite Signal Response	[26]	0	0	0	0	2	0
ASF-1 motif	TGGACG		Relevant to light regulation.	[27]	5	1	4	2	1	3
I-box	GATAAG		Light box Element	[28]	6	4	1	4	2	3

3.3. Identification of Oa-miR164 Targeted Genes

Nine *osa-miR164* targeted genes were predicted by using psRNATarget, including NAC-domain proteins (*OMTN1-OMTN6*), BURP domain protein 4 (*OsBURP* 4), proteophosphoglycan ppg4 and LOC_Os03g47310. (Table 2). Five genes (*OMTN1-OMTN5*), BURP domain-containing protein 4 (*OsBURP04*) and proteophosphoglycan ppg4 are regulated by all six *osa-miR164* genes. The other genes (*OMTN 6* and LOC_Os03g47310) are regulated by five of *osa-miR164s*, except *osa-miR164c*. Functions of *osa-miR164* targeted genes are identified by using UniProt database, except *OsBURP04*, *proteophosphoglycan ppg4* and *LOC_Os03g47310* that cannot identify the functions (Table 2).

Table 2. Potential target genes of *osa-miR164* genes with their IDs and functions

Micro RNA	Target genes ID	Target genes name	Target gene function	Previous research
Osa-miR164a /b/c/d/e/f	LOC_Os02g36880	NAC1(*OMTN1*)	Controls the rate of transcription of genetic information from DNA to mRNA, by binding to a specific DNA sequence	Expression of OsNAC family genes in drought stress [10]
	LOC_Os04g38720	*OMTN2*		
	LOC_Os12g41680	*OMTN3*		
	LOC_Os06g46270	*OMTN4*		
	LOC_Os06g23650	*OMTN5*		
	LOC_Os02g18690	BURP domain-containing protein 4	Expressed in stamen	Identify BURP domain-containing genes and response levels to abiotic stresses [29]
	LOC_Os09g37700	proteophosphoglycan ppg4	Flowering related gene in wild rice	Expression profiles of the flowering related genes in common wild rice [30]
Osa-miR164 a/b/d/e/f	LOC_Os08g10080	*OMTN6*	Controls the rate of transcription of genetic information from DNA to mRNA, by binding to a specific DNA sequence	Expression of OsNAC family genes in drought stress [10]
	LOC_Os03g47310		transposon protein, putative, CACTA, En/Spm sub-class	Locates in QTLs for Zn, Fe, Al toxicity tolerance in rice [31, 32]

3.4. Expression Levels of Osa-miR164 Genes in Several Abiotic Stresses

As shown in Figure 3, the expression patterns of *osa-miR164* genes were diverse in response to stresses. While *osa-miR164c* decreased in expression level, other *osa-miR164a/b/f/d* expression levels showed slightly increase in all stress conditions. The *osa-miR164e* changed its expression level differently between stress conditions, in which it decreased under drought and salt conditions while slightly increased under cold treatment.

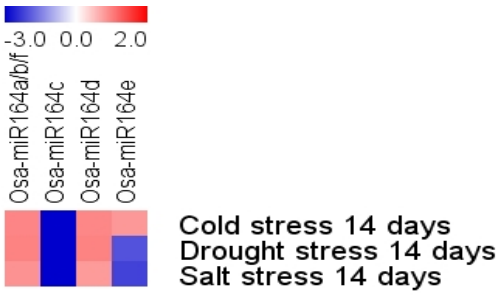

Figure 3. Heatmap representing expression level of *osa-miR164s* in several abiotic stresses.

3.5. Expression Levels of Osa-miR164 Targeted Genes in Different Stress Conditions

The expression profiles of eight *osa-miR164* targeted genes except *LOC_Os03g47310* gene in the shoot and the root under different stress conditions were obtained in TENOR and RiceXPro databases and were visualized as heatmap in Figure 4.

The gene expression in the roots was shown in Figure 4A. In the NAC gene family (*OMTN1-OMTN6*) *OMTN1* up regulated in all stress conditions and showed highest under osmosis, ABA and JA stresses; *OMTN5* also up regulated in almost all stress conditions, except after 3 h of dry treatment; *OMTN4* had slightly changes in expression levels; *OMTN3* down regulated under dry, cold, osmotic stress ABA and JA treatments, but up regulated under high salinity and flood conditions; and *OMTN6* down regulated in all stress conditions. *OsBURP04* up regulated in all stress conditions, whilst *PPG 4* down regulated in most of stress conditions, except in high salinity.

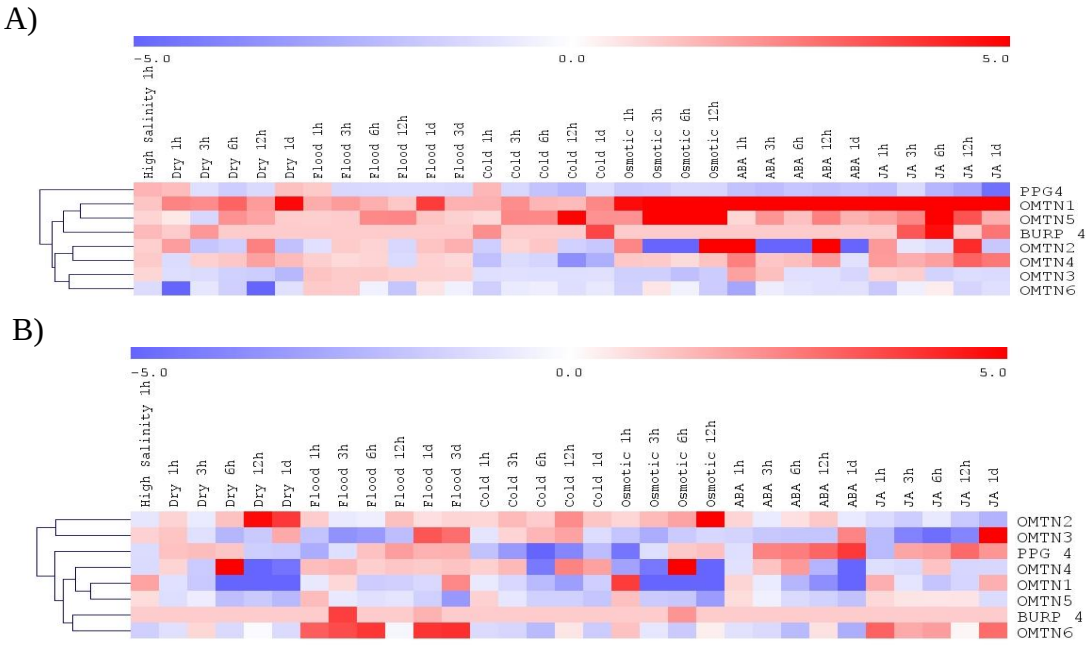

Figure 4. Heatmap representing expression pattern of *osa-miR164* targeted genes in adverse environmental conditions in the root samples (A) and the shoot samples (B).

On the other hand, in the shoot (Figure 4B), most of the NAC gene family were down regulated under drought, osmosis and ABA stress conditions, except *OMTN2* that was differently up regulated. *OsBURP04* up regulated in all stress conditions. *PPG 4* down regulated in high salinity, dry, cold treatments, but up regulated in flood, osmotics stresses, ABA and JA treatments (Figure 4B).

Comparing the gene expression between the shoot and the root shows organ-specific opposite responses suggesting an organ-specific regulators. *OMTN 1* up regulated in the root in all stresses, but down regulated in the shoot under many stresses. In the root, *OMTN 2* up regulated in high salinity, down regulated in cold condition, but not in the shoot. *OMTN 4* showed up regulation in high salinity, dry, osmotic, JA treatments and down regulation in cold condition in the root, but in the shoot the expression of *OMTN 4* was in opposite direction. *OMTN5* up regulated in all stresses in the root, but in the shoot its expression decreased in dry, flood, cold, osmotic and ABA treatments. On the contrary, *OMTN 6* down regulated in all stresses in the root, but up regulated under flood and JA treatments in the shoot. *PPG4* gene expression also showed opposite responses between the root and the shoot under high salinity, flood, ABA and JA treatments.

4. Discussion

MicroRNAs are small non-coding RNA molecules that play key roles in growth, development and stress responses of plants. The miRNAs function by selectively regulating the expression of specific target genes. Thus, identification of the potential target genes of miRNAs provides an effective way to investigate the complex mechanisms responsible for stress adaptation.

By using *in silico* bioinformatics approach in this study, we analyzed *miR164* family in rice. The sequences of mature *osa-miR164 a/b/c/d/e/f* are highly conserved as in agreement with the finding of Fang et al., [10], while the sequences of precursors are highly variable. The diversified sequences of *miR164* precursors might be of interest for designing primers for quantification of *miR164* gene expression levels, especially for *osa-miR164 a/b//f* which were shown completely identical in sequences of the mature ones.

In order to understand how stress stimuli regulate the expression of *osa-miR164* genes, we analyzed the regulatory regions of *osa-miR164* genes. The results show the different combinations of the cis-regulatory elements present in the promoter of each *osa-miR164*, suggesting the diverse expression pattern of *osa-miR164* genes in response to different environmental conditions. However, the expression data of *osa-miR164s* in database is still limited, in which the expression levels of *osa-miR164a/b/f* cannot be found separately (Figure 3). Some key cis-regulatory elements responsive for abiotic stress (DRE, MYC, MYB, etc.) and hormone related (GARE, ABRE, ARF) were found in *osa-miR164* gene promoters, indicating the functional roles of *miR164* in stress responses of rice. In the previous study of Zhang et al., (2011), *osa-miR164* was shown to up-regulate under radiation stress [33]. The expression of *miR164* gene in response to salt stress is different between plant species, in which the up-regulation was observed in *Arabidopsis thaliana* [34] and *Populus trichocarpa* [35] but the down-regulation was found in *Zea mays* [36] and *Panicum virgatum* [37]. Previous study has reported that auxin signaling by *miR164* is important for normal lateral root development [8]. *MiR164* expression was consistent with auxin level and its target NAC1 to regulate auxin signaling [38]. To our knowledge, this is the first report on the cis-regulatory elements of *osa-miR164* genes.

In the previous research, Fang et al., (2014) showed that rice *miR164s* target six NAC genes designated as *OMTN1-OMTN6* and other 3 genes named *OMT7-OMT9* [10]. In our study, we could find nine *osa-miR164* target genes, in which some of them had been reported to be involved in stress responses in rice such as genes encoding

NAC-domain proteins (*OMTN1-OMTN6*), (Table 2 and references herein). The expression levels of *osa-miR164* targeted genes under different stress conditions were variable between the root samples and the shoot samples and showed organ-specific opposite responses (Figure 4). Thus, it might be meaningful to carry out experiment to investigate the expression levels of *osa-miR164* genes separately in the roots and in the shoots of rice plants.

Overall, rice *miR164* family has six gene members which are regulated by different stress related *cis*-regulatory elements and subsequently they regulate the expression of target genes involved in stress responses of rice plants. The in-depth understanding of the miRNA-guided regulation mechanisms responsible for stress may help unravel the regulatory networks of stress response and may also help in developing new strategies to manipulate rice plants with improved stress tolerance.

Acknowledgements

This work was financially supported by the L'Oréal-UNESCO For Women in Science National Fellowship.

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