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Characterization of Three Key MicroRNAs in Rice Root Architecture under Drought Stress using In silico Analysis and Quantitative Real-time PCR

Behnam Bakhshi[1]*, Ghasem Hosseini Salekdeh[2], Mohammad Reza Bihamta[3] and Masoud Tohidfar[4]

[1]Department of Plant Breeding, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[2]Department of Genomics, Agricultural Biotechnology Research Institute of Iran, Karaj, Tehran, Iran.
[3]Department of plant Breeding, Agricultural and Natural Research College,
University of Tehran, Karaj, Tehran, Iran.
[4]Department of tissue culture and genetic engineering,
Agricultural Biotechnology Research Institute of Iran, Karaj, Tehran, Iran.

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Root is the main plant organ for water uptake and is the first organ that percept drought stress. Under drought stress conditions, plants change its root forming to cope with stress. MicroRNAs (MiRNAs) are small 19-24 nt regulatory RNAs and have important roles in biotic and abiotic stress. In this study, we evaluated characteristics, features, and differential expression of miR160, miR164, and miR167 under drought stress in rice root. These miRNAs have important role in root architecture especially under drought stress. We evaluated expression of these miRNAs in roots using qRT-PCR under normal and drought stress conditions. Results showed that miR160, miR164, and miR167 expression decreased in roots under drought stress. Target prediction showed important genes such as ARFs, F-Box and NAC1 are targeted by these miRNAs. In addition, we observed important regulatory elements in the upstream regions of these three MIRNA genes that confirmed their role under drought stress.

Key words: MicroRNA, *Oryza sativa*, *Arabidopsis thaliana*, Drought stress, root, regulatory elements, phytohormone.

One of the major abiotic stresses that plants face is drought stress. Drought stress has harmful effects on plant metabolic processes such as regulation of stomatal closure, nutrient absorption and production of Photosynthetic assimilates that result to reduction of yield (Sunkar 2010; Khraiwesh *et al*. 2012).

Under the normal condition plants develop its lateral roots (Xiong *et al*. 2006). Upon plants percept drought stress signals changes their roots forming to absorb more water. Usually under drought stress, water is not available in the surface layer of the soil and plant increase its main root in-depth to cope with drought stress through absorbing more water (Xiong *et al*. 2006). On the other hand, lateral roots development is inhibited under drought stress (van der Weele *et al*. 2000; Malamy 2005; Xiong *et al*. 2006). Phytohormones could change regulation of root forming under different conditions (Osmont *et al*. 2007). Furthermore, phytohormones are responsible in control growth,

* To whom all correspondence should be addressed.
Tel.: +98-0912-2505817; Fax: +98-021-22072220;
E-mail: behnam.bakhshi@gmail.com

development and also have important roles in responding to environmental stress.

Among phytohormones, Auxin is an important component in plant life (Wang *et al*. 2005), and plays a key role in root architecture (Millner 1995). Auxin have been identified as major contributing factor for lateral roots developments (Khan *et al*. 2011). Plants tends to decrease Auxin signaling to cope with abiotic stress (Blomster *et al*. 2011). Plant responses to Aux/IAA, ARF and some other transcription factors (Dharmasiri and Estelle 2004). Various ARFs show diverse response to abiotic stress; in a way that, transcript profiling indicated combination of up-regulation and down-regulation of ARFs in response to abiotic stresses (Jain and Khurana 2009). Likewise, diverse response of ARFs to Auxin was reported (Guilfoyle and Hagen 2007). ARFs could bind to the promoters of early Auxin response genes (Guilfoyle and Hagen 2007) and act as suppressor or inducer of responsive genes (Mallory *et al*. 2005).

One of the most significant current discussions in phytohormones is ABA signaling from root to shoot. Roots can measure water availability in the soil and transfer information to shoot by biosynthesis of ABA in root under stress and release it to xylem and leaves of plants and control stomatal closure (Davies *et al*. 2005). Furthermore, under stress conditions, ABA promotes growth by preventing ethylene effects (Davies *et al*. 2005). A strong antagonistic interaction between ABA and Auxin has been reported before. In a way that, Auxin has important role in inducing lateral root (Casimiro *et al*. 2003) but conversely, ABA inhibit lateral root development (De Smet *et al*. 2003; Xiong *et al*. 2006).

Phytohormones signaling are regulated during plant growth and development using positive and negative regulators (Huq 2006). In recent years, there has been an increasing interest in phytohormones and small RNAs relationship. Small RNAs like microRNAs (miRNAs) act as regulators in phytohormones signaling (Liu *et al*. 2009). *Hyl1* mutant that showed failed responses of phytohormones such as Auxin, ABA and Cytokinin was the first report that indicated relationship between miRNAs and phytohormones (Lu and Fedoroff 2000).

MiRNAs are small 19-24 nt regulatory RNAs that are encoded by endogenous MIRNA genes (Voinnet 2009). MiRNA biogenesis includes three steps. At first, RNA Polymerase II produces pri-miRNA from MIRNA genes. The next step is processing of pri-miRNA to pre-miRNA completed by DCL1 with the association of proteins involved in the biogenesis of miRNAs like HYL1and SE. pre-miRNA has stem-loop structure that reform to mature miRNA via further processing by DCL1 (Voinnet 2009). This mature miRNA could inhibit genes expression by either cleavage or translational inhibition of mRNAs (Brodersen *et al*. 2008).

miRNAs involved in plant developments and physiology as well as phytohormones (Bartel 2004). miRNAs have important roles in biotic and abiotic stress (Khraiwesh *et al*. 2012). MiRNAs usually targets transcription factors and involved in adaptation to stress via their positive or negative regulatory roles in plants (Ding *et al*. 2013). These features situated miRNAs in the center of regulatory expression networks (Ding *et al*. 2013). Apart from *hyl1* mutant that indicated failed responses of phytohormones, other mutants such as *dcl1*, *hen1*, *se* and *hasty* also showed hypersensitivity to ABA (ZHANG *et al*. 2008). This data highlight the importance of miRNAs in phytohormone response.

Cis-acting Regulatory elements in the upstream regions of genes are involved in the induction of phytohormones responsive genes. For example, existence of (TAACAG/AA) and (py-ACGTGGTC) is necessary for GA and ABA inducibility, respectively (Sutoh and Yamauchi 2003; Furihata *et al*. 2006). Although, existence of important phytohormone regulatory elements in the upstream regions of some MIRNA genes could associate with miRNA functions but there were some cases in which there is no association (Liu *et al*. 2009).

Some of miRNAs could reform root architecture by targeting ARFs and adjust Auxin homeostasis (Khan *et al*. 2011). Recent evidences suggest that mir160, miR164, and miR167 have important roles in root architecture (Wang *et al*. 2005; Liu *et al*. 2009; Khan *et al*. 2011). Up to date, studies have tended to focus on expression of these miRNAs in shoot tissue rather than root. So far, however, there has been no discussion about differential expression of these three miRNAs in

rice roots under drought stress yet. In this study, we are aimed to evaluate characteristics, features, and differential expression of miR160, miR164, and miR167 under drought stress of rice root.

MATERIALS AND METHODS

Plant materials

IR64 genotype used in this study was derived from the international rice institute. Rice seeds germinated in petri dishes and then were transferred to Yoshida hydroponic culture solution. After two weeks, seedlings were moved to pots containing two parts sand and one part clay and well watered for two weeks. For drought treatment, irrigation was stopped for two weeks while for normal treatments irrigation was continued. After two weeks, sampling was conducted and all root tissues were stored at -80.

qRT-PCR

All evaluated miRNAs were selected from miRBase (http://mirbase.org). Designed primers are described in table1. Also, 18S rRNA selected as references gene with 5'ATAACTCGACG GATCGCAAG3' and 5'CTTGGATGTGGT AGCCGTTT 3' sequences as forward and reverse primers, respectively. Total RNA was extracted using Trizol reagent. Quality of extracted RNA was evaluated by Nano drop. Later on, *DNase* I treatment was conducted using Invitrogen kit. In addition, cDNA synthesis was carried out using Invitrogen kit. qRT-PCR was conducted in 25µl volume including 12.5 µl of SYBER, 1 µl forward primer, 1 µl of reverse primer and 3 µl of cDNA. Calculation of miRNA Relative frequency and their differential expression was conduct according to Schmittgen *et al.* published method for relative quantification (Schmittgen *et al*. 2008).

Bioinformatics analysis

3000 bp upstream regions of all MIR160, MIR164 and MIR167 genes of *Oryza sativa* and *Arabidopsis thaliana* species were searched for the existence of important regulatory elements. Up-stream regions of these species were downloaded from plant miRNA database (Zhang *et al*. 2010) and were evaluated by NSITE-PL version 2.2004(http://www.softberry.com/cgi-bin/programs/promoter/nsite.pl). Homology level between regulatory elements and evaluated sequence were 100%. Selected motifs were completely matched with up-stream regions of selected *Oryza sativa* and *Arabidopsis thaliana* MIRNA genomes.

In addition, we used Clustal_W multiple alignment methods for identification of miRNA conserved regions using MEGA5. Furthermore, Mfold software was used for drawing miRNAs stem-loop structures. In order to predict miRNA target genes, we used OSA1 TIGR genome cDNA version 5 (OSA1R5) of psRNATarget (http://plantgrn.noble.org/psRNATarget). We have also used Rice Genome Annotation Project Database (rice.plantbiology.msu.edu) for identification of target genes ontology.

RESULTS

Response of miR160, miR164, and miR167 to drought stress

The present study was focused on miR160, miR164, and miR167 because of their significant role in rice root growth and development especially under drought stress. miR160, miR164, and miR167 containing 6, 6 and 10 known members, respectively; represent 3, 4 and 2 different mature miRNAs with an extremely little difference in their mature sequence accordingly. Several studies

Table 1. Designed primers for evaluated miRNAs

MiRNA	Selected members	Reverses primer	Forward primer	Stem-loop RT PCR primer
miR160	miR160 a, b, c, d, e, f	5'GTGCAGG GTCCGAGGT 3'	5'CCGTTGCCTGG CTCCCTGT 3'	5'GTCGTATCCAGTGCAGGGTCC GAGGTATTCGCACTGGATACGA CCGGCAT 3'
miR164	miR164 a, b, c, d, e, f	5'GTGCAGGGT CCGAGGT 3'	5'GCAGTGGAGA AGCAGGGCA 3'	5'GTCGTATCCAGTGCAGGGTCC GAGGTATTCGCACTGGATACGA CTGCACG 3'
miR167	miR160 a, b, c, d, e,f, g, h, i, j	5'GTGCAGGG TCCGAGGT 3'	5'GTCTGGAAGG GGCATGCA 3'	5'GTCGTATCCAGTGCAGGGTCC GAGGTATTCGCACTGGATACGA CCTCCTC 3'

to investigate differential expression of miR160, miR164, and miR167 were carried out earlier under drought stress. These studies have focused on *Arabidopsis thaliana* (Liu *et al*. 2008), *Glycine max* (Li *et al*. 2011b), *Medicago truncatula* (Wang *et al*. 2011),*Oryza sativa* (Barrera-Figueroa *et al*. 2012), *Populuse uphratica* (Li *et al*. 2011a; Ren *et al*. 2012) and *Vigna unguiculata* (Barrera-Figueroa *et al*. 2011). However, far too little attention has been paid to study differential expression of miR160, miR164, and miR167 in root under drought stress. We evaluated expression of these miRNAs in roots using qRT-PCR under normal and drought stress conditions. For the normal condition, all plants were placed on flooding pots in contrast to drought condition in which all plants endured 14 days without water irrigation. Results showed that miR160, miR164, and miR167 expression decreased in roots under drought stress. Wherein miR160 and miR167 showed approximately 3 times reduction of expression, miR164 showed more than 6 times reduction of expression, compared with normal condition (Fig. 1). In addition, differential expressions of these three miRNAs were significant

Table 2. Target prediction and their ontologies for evaluated miRNAs

miRNA	Targets	Ontology
miR160	Auxin response factor 16 Argonaute-like protein ATTPS6 B3 DNA binding domain containing protein Calreticulin family protein D-mannose binding lectin family protein GAST1 protein precursor Signal transducer TPR repeat nuclear phosphoprotein	anatomical structure morphogenesis , cell differentiation, growth, hydrolase activity, kinase activity, membrane, nuclease activity,regulation of gene expression, epigenetic, response to abiotic stimulus, response to biotic stimulus, response to endogenous stimulus, response to stress, signal transduction, transferase activity
miR164	Aminotransferase, class IV family protein B3 DNA binding domain containing protein Calmodulin Cryptochrome 2 apoprotein CUC2 F-box domain containing protein MYB-like DNA-binding domain NAC domain-containing protein 21/22 No apical meristem (NAM) protein Phytosulfokines 5 precursor Ubiquitin family protein UDP-glucuronic acid decarboxylase 1	anatomical structure morphogenesis, catalytic activity, cell growth, cellular homeostasis, growth, membrane, response to abiotic stimulus, response to biotic stimulus, response to endogenous stimulus, response to stress, signal transduction
miR167	Auxin response factor 6 Auxin response factor 8 BPM, putative, expressed F-box domain containing protein NBS-LRR disease resistance protein Regulator of chromosome condensation, RCC1 Retinol dehydrogenase 14 Serine/threonine-protein kinase receptor precursor Sialin	kinase activity, membrane, protein modification process, response to endogenous stimulus, response to stress

at 0.05 level using t-Student test.

MIRNA gene copies of MIR160, MIR164 and MIR167

In this study we screened all of gene copies of MIR160, MIR164 and MIR167 in *Oryza sativa* and *Arabidopsis thaliana* genomes. We observed that this miRNA are distributed in all chromosomes of *Arabidopsis thaliana* and 9 chromosomes of *Oryza sativa* (Fig 2). Wide distribution of these gene copies shows their

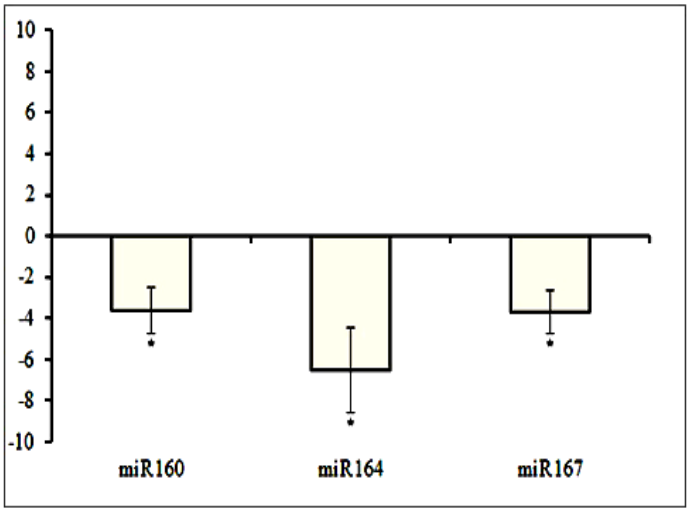

Fig. 1. Fold changes of miR160, miR164 and miR167 under drought stress relative to normal condition. "*" sign shows differential expression is significant at 0.05 level.

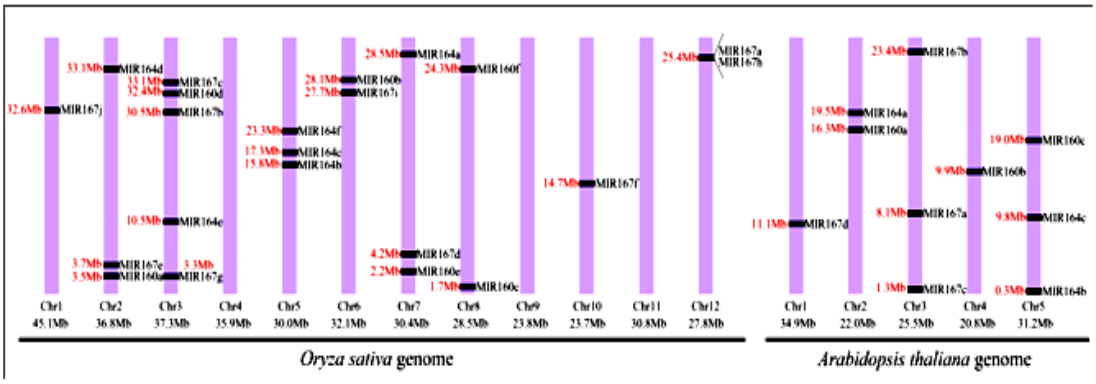

Fig. 2. Distribution of MIRNA gene in *Oryza sativa* and *Arabidopsis thaliana* genome

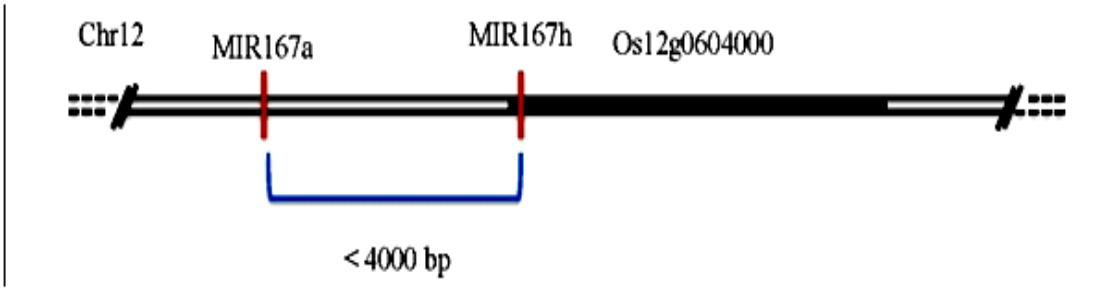

Fig. 3. Positions of MIR167a and MIR167h genes in chromosome 12 of *Oryza sativa* genome

important role in these two species. We didn't found any MIRNA gene cluster in Arabidopsis, but there was one MIRNA cluster in *Oryza sativa* genome. In a way that, MIR167h is present within Os12g0604000 gene and MIR167a clustered with MIR167h in a region of less than 4000 bp (Fig 3). it was Recently proposed that duplication of protein-coding genes has the most part in generating new MIRNA genes (Fahlgren *et al*. 2010). Positioning these two MIRNA genes in a closed region may indicate that MIR167a and MIR167h are the result of a local duplication event. Despite that these two members of miR167, show conservation in their mature miRNA regions, they showed no similarity in their stem-loop precursor structures, which is duo to less conservation in stem-loop regions of them. Thus, in the phylogenetic three miR167h is far from miR167a (Fig 4.a) and it is probable that this duplication is an ancient evolutionary event. Furthermore, Some of MIRNA genes in this study were originated from segmental duplication which can be exemplified by osa-MIR164c and osa-MIR164f which are present in the same chromosome and their distance in phylogenetic tree was small however their distance on chromosome are more than 10 kb (Fig 4.b).

Stress related cis-acting elements exist in the up-stream of MIR160, MIR164 and MIR167 genes

Several studies reported elements present in the up-stream of stress responsive MIRNA genes (Zhao *et al*. 2007; Liu *et al*. 2008; Zhao *et al*. 2009). In this study we analyzed 3000 bp upstream sequence of MIR160, MIR164 and MIR167 family genes of *Oryza sativa* and *Arabidopsis thaliana* species. We identified 614 and 203 motifs in the upstream regions of *oryza sativa* and *Arabidopsis thaliana* species, respectively. From 614 detected motifs of *Oryza sativa*, 202 of them were located in less that 1kb and 247 of them were located between 1kb and 2kb upstream of MIRNA genes and the rest were located between 2kb and 3kb. In this research, we found several stress responsive elements, such as the ABRE, ARE, AuxRE, GARE, GCC Box, MYB, DRE, and ERE. Existence of these regulatory elements indicates that these miRNA are effective in stress response. Some of important stress responsive regulatory elements like GCC-box, ABRE, MYB, and ERE were observed broadly in the upstream of all these three families. Furthermore, some of these regulatory elements repeated more than once in a MIRNA gene like ABRE that located at -34bp and -971bp from transcription start site of osa-MIR167d and GARE that located at -237bp, -270bp, -1908bp and -1941bp from transcription start site of osa-MIR164a. We observed many of these similar cases in the upstream of MIRNA genes that some of them are presented in figure 5. Existence of repetitive phytohormones responsive elements in the upstream of some MIRNA genes shows these miRNAs probably have important roles in phytohormones signaling. Furthermore, we compared existence of important regulatory elements between two *Oryza sativa* and *Arabidopsis thaliana* species. As shown in figure 5, results indicated that distribution of stress related regulatory elements are different and probably cause to diverse response of miRNAs between these two species under stress.

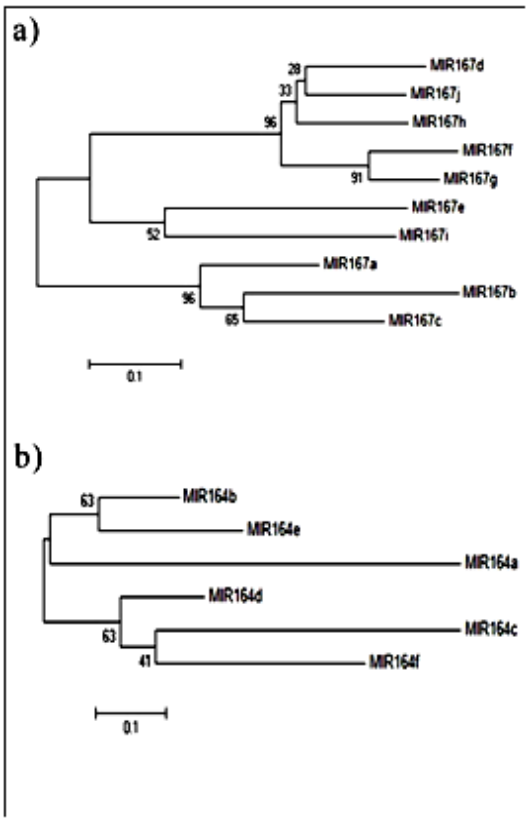

Fig. 4(a). Phylogenetic tree of miR167 members. (b) phylogenetic tree of miR164 members

Regulary elements / MIRNA genes	ABRE		ARE		AuxRE		DRE		ERE		GARE		GCC box & GCC consensus		HSE		MYB		MYC		P-box		TGA	
	osa	ath	osa	ath	osa	ath	osa	ath	osa	ath	osa	ath	osa	ath	osa	ath	osa	ath	osa	ath	osa	ath	osa	ath
ath & osa-MIR160a		1								1												1		
ath & osa-MIR160b	3	1							1											1				
ath & osa-MIR160c	4		1				1												1	1				
osa-MIR160d																			2					
osa-MIR160e									3				1				1							
osa-MIR160f																	3						1	
ath & osa-MIR164a	1	1						1			4					1		1						
ath & osa-MIR164b									2		1		2		1		1							
ath & osa-MIR164c	2	2												1			3							
osa-MIR164d																								
osa-MIR164e					1				1		2		2				1							
osa-MIR164f																								
ath & osa-MIIR167a									1				3					3		1				
ath & osa-MIR167b												1												
ath & osa-MIR167c		1		1														2		1				1
ath & osa-MIR167d	4						1													2				1
osa-MIR167e																	1				1			
osa-MIR167f	1												1		1		1							
osa-MIR167g	1														1		3							
osa-MIR167h	1								2				10		1		4							
osa-MIR167i									1												1			
osa-MIR167j											2										1			

Fig. 5. Distribution graph of regulatory elements in the 3000 bp upstream regions of MIR160, MIR164 and MIR167 genes of *Oryza sativa* (osa) and *Arabidopsis thaliana* (ath). Color intensity is correlated with number of regulatory elements

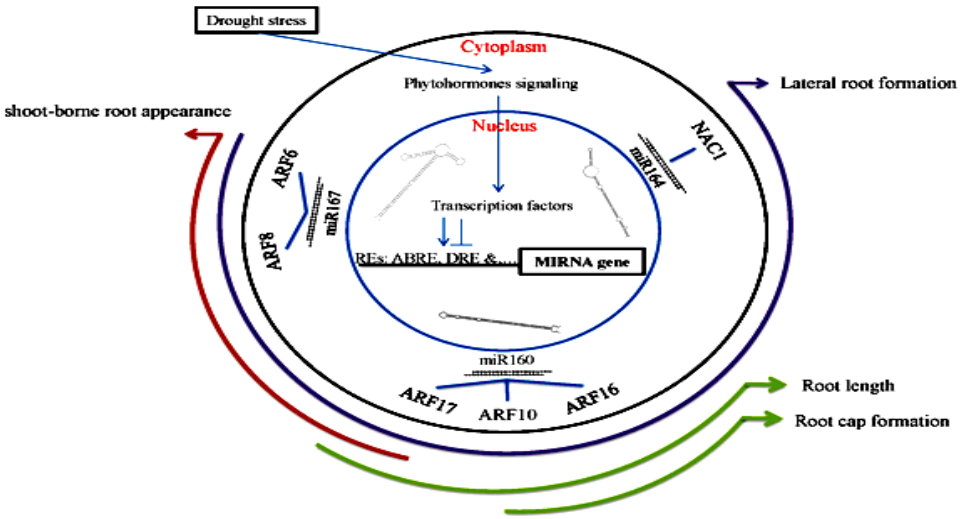

Fig. 6. Overview of mir160, miR164 and miR167 functions in root architecture.

Target prediction for miR160, miR164, and miR167

In this study, we predicted targets of miR160, miR164, and miR167 using online database of miRNA target genes psRNATarget (http://plantgrn.noble.org/psRNATarget). The results of Target prediction indicated that ARF families are targeted by miR160 and miR167 families. Likewise, F-box predicted as target of miR164 and miR167 families. Furthermore, miR164 target other important genes such as CUC2, MYB, NAC and NAM. All predicted target is presented in table 2. Another finding was that most of these targets are inhibited through cleavage (72%) and less than of them are inhibited through Translation inhibition (24%). All of important targets that noticed here are inhibited by cleavage except F-box that is inhibited at translational level by mir164 parallel to F-box cleavage by miR167. In addition, we investigated ontology of predicted targets using Rice Genome Annotation Project Database that some of them are presented in table 2. As presented in table 2, all of these miRNAs are involved in stress response.

DISCUSSION

Drought stress is the most important abiotic stress affecting about 20% of the total rice planting area (Pandey and Bhandari 2008). Root is the main plant organ for water uptake and is the first organ that percept drought stress. Under drought stress conditions, plants change its root forming. Therefore, improving our understanding in molecular mechanisms of root forming under drought stress is necessary.

The differential expression of miR160, miR164, and miR167 showed down-regulation in rice roots under drought stress. Recently, Barrera-Figueroa *et al* showed up-regulation of miR167a,b,c and miR160f in rachis, branches and spikelets of rice under drought stress (Barrera-Figueroa *et al*. 2012). Li *et al* reported up-regulation of miR167d in roots of abiotic stress sensitive soybeans under drought (2% PEG) stress (Li *et al*. 2011b). No other research has been found for differential expression of these miRNAs in roots of any species under drought stress. In addition to mentioned studies that reported up-regulation of these miRNAs, there are some studies that reported down-regulation of these miRNAs (Barrera-Figueroa *et al*. 2011; Li *et al*. 2011a; Wang *et al*. 2011; Ren *et al*. 2012). This inconsistency may be due to difference in duration of stress, developmental stages, stress severity, growth conditions, used tissues, and methods used for study of differential expression. For example, li *et al*, reported inconsistent results for miR164c expression under drought stress in *Populus euphratica*. In a way that, li *et al* observed up-regulation of miR164c using deep sequencing but conversely they observed down-regulation of this miRNA using microarray (Li *et al*. 2011a).

In this research, we studied miRNAs with important role in root architecture under drought stress. MiR160, miR164, and miR167 induced or repressed by phytohormones fluctuations and have important roles in root growth and development such as main root length, formation of lateral roots, root cap development, and shoot-borne root appearance (Wang *et al*. 2005; Liu and Chen 2009; Liu *et al*. 2009; Meng *et al*. 2010; Khan *et al*. 2011; Ding *et al*. 2013). Down-regulation of these miRNAs indicates importance of their targets genes up-regulation and their potential role under drought stress.

MiR160 was found to target three important ARFs genes including ARF10, ARF16, and ARF17 (Wang *et al*. 2005). MiR160 is the most important regulator in root growth and development (Khan *et al*. 2011). It has been reported that miR160 has important role in cell division, elongation and differentiation by regulating ARF10 and ARF16 (Khan *et al*. 2011). Furthermore, MiR160 could control Auxin homeostasis and formation of shoot-borne root by regulating ARF17 (Bartel 2004; Sorin *et al*. 2005). Likewise, miR160 positively regulate lateral root density with its downstream gene ARF16; in a way that, down-regulation of miR160 or up-regulation of ARF16 resulted to low lateral root density (Wang *et al*. 2005). Functional study focused on miR160 showed over-expression of miR160 which caused down-regulation of ARF10 and ARF16 with short length of plant roots (Wang *et al*. 2005). These data indicate, probably under drought stress plants need to decrease miR160 in their roots in order to increase its root length and decrease its lateral root density. This may explain our results in miR160 expression under drought stress.

Previous studies have reported that Auxin

signaling by miR164 is necessary for normal lateral root development (Guo *et al*. 2005). MiR164 expression is consistent with Auxin level and target NAC1 to regulate Auxin signaling (Liu and Chen 2009). NAC1 have been identified as a transcription activator in Auxin signaling and regulate lateral root development (Zhang *et al*. 2006).

In addition, miR167 target two important Auxin response factor including ARF6 and ARF8 (Khan *et al*. 2011) and regulate shoot-born root creation (Gutierrez *et al*. 2009). It has been demonstrated that, shoot-born root formation needs appropriate and continuous expression of miR167 and its up-regulation or down-regulation cause defect in Shoot-born root formation (Khan *et al*. 2011).

Along with miR164, miR167 expression is consistent with Auxin level (Yang *et al*. 2006). It has been suggested that, Auxin by inducing these two miRNAs could control level of free Auxin in an auto regulatory loop (Guo *et al*. 2005; Yang *et al*. 2006). Conversely, miR167 expression is inconsistent with ABA level and down-regulation of miR167 expression by ABA leads to ARF8 up-regulation (Liu and Chen 2009). ARF8 concentration resulted to prematurity of plants under stress (Liu and Chen 2009). Furthermore, miR160 is responsible to ABA (Ding *et al*. 2013). These results indicate that miR160 and miR164 are responsible to ABA and Auxin, respectively; but, miR167 is responsible to both ABA and Auxin phytohormones. Therefore, there is an Auxin-ABA cross talk in root architecture formation under stress that these miRNAs are in the main part of this regulation (Fig. 6).

Regulatory elements in the upstream regions of MIRNA genes play critical roles in miRNA differential expressions. Our investigation for regulatory elements in the upstream regions of these three miRNA showed existence of phytohormones responsive regulatory elements like ABRE in the upstream of these three MIRNA and AuxRE in the upstream of miR164. Existences of AuxRE in the upstream region of miR164 confirm its responsibility to Auxin. MiR160 and miR167 have been reported as responsive miRNAs to drought and ABA (Ding *et al*. 2013). Likewise, we observed DRE and ABRE regulatory elements in the upstream regions of these two MIRNA genes that confirm responsibility of these miRNAs to drought and ABA. DRE and ABRE are two major cis-acting elements that act in ABA-independent and ABA-dependent pathways, respectively (Shinozaki and Yamaguchi-Shinozaki 2007). In addition, we observed existence of MYB and ERE regulatory elements in the upstream of these three MIR genes. Likewise, GCC-box and GARE was observed in both MIR164 and MIR167 genes. Therefore, existence of drought responsive regulatory elements in the upstream regions of these MIRNA genes emphases these miRNAs roles under drought stress.

CONCLUSION

The purpose of the current study was to determine characteristics of three miRNAs that have been reported as essential regulators of root architecture. The most obvious finding to emerge from this study is down-regulation of miR160, miR164 and miR167 under drought stress. It seems possible that, these down-regulations are due to phytohormone activities like ABA and Auxin. These findings demonstrate necessity to increase the expression of miRNA target genes such as ARF, NAC1 and F-box under drought stress that plays important roles in root forming under drought stress. It was also shown that, there are important regulatory elements in the upstream regions of MIRNA genes and induced transcription factor through phytohormones signaling could bind them and regulate MIRNA genes expression. However, far too little attention has been paid to evaluate differential expression of miRNAs under drought stress in roots. Because of essential roles of root under drought stress, further research should be done to investigate the important miRNAs in root forming under drought stress.

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