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Comparative Temporal Expression Analysis of MicroRNAs and Their Target Genes in Contrasting Wheat Genotypes During Osmotic Stress

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Abstract MicroRNAs (miRNAs) are important nonprotein-coding genes involved in almost all biological processes during biotic and abiotic stresses in plants. To investigate the miRNA-mediated plant response to drought stress, two drought-tolerant (C-306 and NI-5439) and two drought-sensitive (HUW-468 and WL-711) wheat genotypes were exposed to 25 % PEG 6000 for 1, 12 and 24 h. Temporal expression patterns of 12 drought-responsive miRNAs and their corresponding nine targets were monitored by quantitative real-time PCR (qRT-PCR). The results showed differential expression of miRNAs and their targets with varying degree of upregulation and downregulation in drought-sensitive genotypes. Likewise, in drought-tolerant wheat genotypes, maximum accumulation of miR393a and miR397a was observed at 1 h of stress. In addition, nearly perfect negative correlation was observed in four miRNA and target pairs (miR164-NAC, miR168a-AGO, miR398-SOD and miR159a-MYB) across all the temporal period studied which could be a major player during drought response in wheat. We, for the first time, validated the presence of miR529a and miR1029 in wheat. These findings gives a clue for temporal and variety-specific differential regulation of miRNAs and their targets in wheat in response to osmotic shock and could help in defining the potential roles of miRNAs in plant adaptation to osmotic stress in future.

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Keywords Wheat · Osmotic stress · Drought · miRNA target pair

Introduction

Wheat is one of the most crucial staple food crops across the globe. It is highly challenged by various abiotic stresses such as drought during different growth and developmental stages leading to decreased total production. To meet the increasing demand of food from the ever-increasing population of the world, wheat production has to be increased with the limited available area whose major portion is water-short drought-prone rainfed. Drought/osmotic stress is one of the most common abiotic stresses limiting overall performance of the plants. It continues to be an important challenge to agricultural scientists to develop high-performing genotypes. Plant response to drought/osmotic shock is highly negotiated by genotype, growth and developmental stage, duration of exposure, physiological, biochemical and molecular process leading to differential reprogramming of gene expression [1, 2]. Understanding regulatory behaviour of altered gene expression at posttranscription level via microRNAs (miRNAs) is of utmost importance to unravel the mechanism of tolerance [3, 4]. miRNAs are short (~21 nt), endogenous noncoding RNAs that regulate the expression of genes at posttranscriptional in eukaryotes. In plants, they evolved naturally to fight viral infections. Their regulatory role in plants to tolerate interplay biotic and abiotic stress is well established [5–7]. In the recent past, by using various techniques such as sequencing, microarray, gel blot and qPCR, several works have shown the differential expression behaviour of miRNAs and their targets to cope up with drought/osmotic stress in many plants such as *Arabidopsis* [8], *Oryza sativa* [9, 10], *Phaseolus vulgaris* [11, 12], *Medicago truncatula* [13], *Saccharum* spp. [14], *Triticum dicoccoides* [15], *Gossypium hirsutum* [16], *Populus trichocarpa* [17], *Solanum tuberosum* [18] and *Triticum aestivum* [6]. These studies indicate that miRNAs are parts of a broad network, which regulates the plant growth and development during drought/osmotic stress in plants.

In spite of various reports on differential expression schema of drought/osmotic stress-related miRNAs and their targets in various crops, studies in wheat is underperformed due to its inadequate genome access. Therefore, in the present study, we exploited the regulatory mechanism to characterize miRNAs that could be involved in mitigating osmotic shock tolerance in wheat at seedling stage. Based on the available literature on miRNA-target pair [19–28], we selected 11 conserved drought/osmotic stress-related miRNAs (miR159a, miR164, miR168a, miR171, miR172a, miR319, miR393a, miR397a, miR398, miR408, miR529a) and one *Pysicometrella*-specific miR1029 along with their nine target transcripts (Fig. 1) with a view to capture miRNAs that might be controlled differentially under osmotic stress at different time intervals (1, 12 and 24 h) in two drought-tolerant (C-306, NI-5439) and two drought-sensitive (HUW-468 and WL-711) wheat genotypes.

Materials and Methods

Plant Material and Osmotic Treatment

In this study, we selected four wheat (*T. aestivum* L.) genotypes, viz. C-306, NI-5439 (drought tolerant), HUW-468 and WL-711 (drought sensitive) (Table 1). Seeds of all the genotypes were sterilized in 1 % sodium hypochloride for 10 min, rinsed with distilled water three times

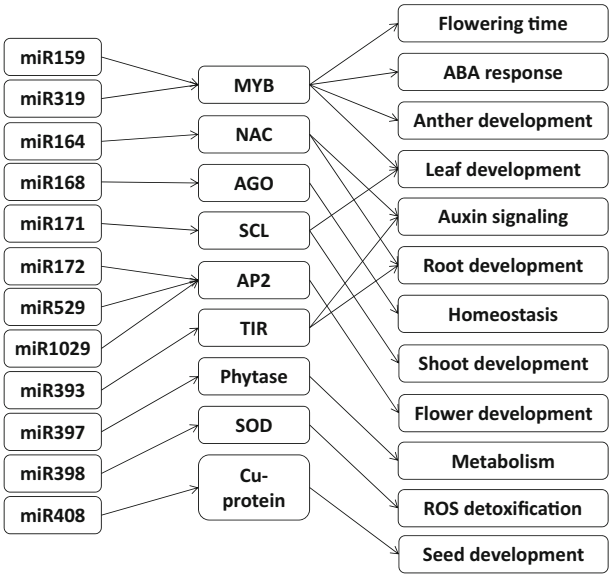


Fig. 1 List of drought-responsive miRNAs and their targets used in this study along with their physiological responses in plants

and germinated in petriplates at 22 °C temperature under controlled conditions. After 5 days of germination, seedlings were transferred to the culture bottle filled with full-strength Hoagland's solution and allowed to grow for 1 week. Each genotype was sown in two sets each having three biological replications. After 7 days of growth in Hoagland's solution, one set of seedlings of all the genotypes was exposed to osmotic shock temporally by using 25 % (*v*/*v*) polyethylene glycol (PEG) 6000 for a time period of 1, 12 and 24 h, while the other set was used as untreated control. Leaf samples from control and stressed seedlings were harvested at above mentioned time intervals and immediately frozen in liquid nitrogen and stored at −80 °C prior to small RNA isolation.

Selection of miRNAs and Their Corresponding Targets and Designing of Primers

In this study, based on available literature, we selected a total of 12 drought/osmotic stress-related miRNAs for validation at seedling stage in wheat. Amongst them, 11

Table 1 Features of wheat genotypes used in this study

Genotype	Characteristic features	Pedigree	References
C-306	Tolerant to drought stress, high tillering, popular cultivar grown in rain-fed situations of NWPZ/NEPZ#	RGN/CSK3//2*C591/3/C217/N14//C281	[29]
NI-5439	Short-duration genotypes for Peninsular India	REMP 80/3*NP710	[30]
HUW-468	Drought susceptible, grown in NEPZ Irrigation	CPAN-1962/TONI//LIRA'S'/PRL'S'	[30]
WL-711	Drought susceptible grown for NWPZ, irrigation	S308/CHR//KAL	[29]

Note: NWPZ North-West Plain Zone, *NEPZ* North-East Plain Zone

miRNAs, viz. miR159a, miR164, miR168a, miR171, miR172a, miR319, miR393a, miR397a, miR398, miR408 and miR529a were conserved, while miR1029 was specific to *Pysicometrella* along with their nine target transcripts (MYB, NAC, AGO, SCL, AP2, TIR, Phytase, SOD and Cu protein). The miR1029 was selected because it was predicted to target AP2 transcription factor in *Pysicometrella* whose role in drought stress is quite obvious [27]. Target genes of these miRNAs were selected based on previous publication [28]. The mature miRNA sequences were retrieved and downloaded from miRNA Registry database (http://miRNA.sanger.ac.uk). miRNA-specific forward primers and a universal reverse primer along with RTQ primer and U6 small nuclear RNA (snRNA) primer were designed by using BioEdit software version 5.09.04 (Supplementary Table 1). U6 snRNA was used as a reference gene for data normalization of miRNAs. Primers for target genes of miRNAs were designed by using PrimerBlast (http://www.ncbi.nlm.nih.gov/tools/primer-blast) with a product size of around 150 bp (Supplementary Table 2). β-Actin was used as a reference gene for data normalization of target genes. These primers were then used for analysis of hairpin structure, homodimer and heterodimer formation by using OligoAnalyser (http://eu.idtdna.com/calc/analyzer), and primers were made final if they were having ΔG less than −9 kcal/mol. All the primer sequences were validated by using gel electrophoresis of PCR amplicons and by the presence of only single peak on the thermal dissociation (Tm) curve generated by the thermal denaturing protocol, which followed each real-time PCR run.

Isolation of Small RNA and Total RNA

Small RNAs were extracted from 100 mg of leaf tissues by using *mir*Vana™ miRNA Isolation Kit (Ambion, USA) according to the manufacturer's instruction. For expression analysis of target transcripts, total RNA was isolated by using TRIzol® Reagent (Ambion, USA) following the manufacturer's protocol. The concentration of small RNA and total RNA was determined by Nano-Drop spectrophotometer, ND-1000 (NanoDrop Technologies, USA).

Small RNA cDNA Synthesis

Small RNA cDNA (srcDNA) library was constructed as described earlier [31]. Briefly, small RNAs isolated from leaf tissues were polyadenylated at 37 °C for 45 min in 50 μl reaction volume containing 0.3 μg RNA and 5 U *Escherichia coli* poly(A) polymerase (NEB). The poly(A) tailed small RNA was purified from sample by using a purification cartridge provided in *mir*Vana Probe and Marker Kit (Ambion) as per manufacturer's protocols. The srcDNA library was generated by mixing 500 ng of poly(A) tailed RNA and 1 μg of RTQ primer in a 26 μl reaction volume. The reaction mixture was incubated at 65 °C for 10 min followed by addition of reverse transcriptase 200 U (M-MuLV Reverse Transcriptase, NEB), 1 μl dNTP mix (10 mM) and 10× buffer in a final reaction volume of 40 μl. Reverse transcription was carried out at 37 °C for 60 min followed by inactivation of the enzyme at 70 °C for 15 min. RNase H (5 U) (NEB) was added to remove small RNAs. The samples were purified by using the QIAquick spin PCR purification kit (QIAGEN) in a final volume of 50 μl. The srcDNA concentration and purity were quantified by using Nano-Drop spectrophotometer.

cDNA Synthesis

cDNA library from total RNA was constructed by using Novagen® first-strand cDNA synthesis kit (Merck, Germany) following the user's manual. In short, cDNA library was generated by mixing 1 μg of total RNA and 0.5 μg of oligo(dT) primer in a 12.5 μl reaction volume, incubated at 70 °C for 10 min followed by addition of reverse transcriptase 100 U M-MuLV Reverse Transcriptase, 1 μl dNTP mix (10 mM), 2 μl of 100 mM DTT and 4 μl of 5× buffer in a final reaction volume of 20 μl. The reaction mixture was incubated at 37 °C for 60 min and stored at −20 °C. The concentration and purity of cDNA were quantified by using Nano-Drop spectrophotometer, ND-1000 (NanoDrop Technologies, USA).

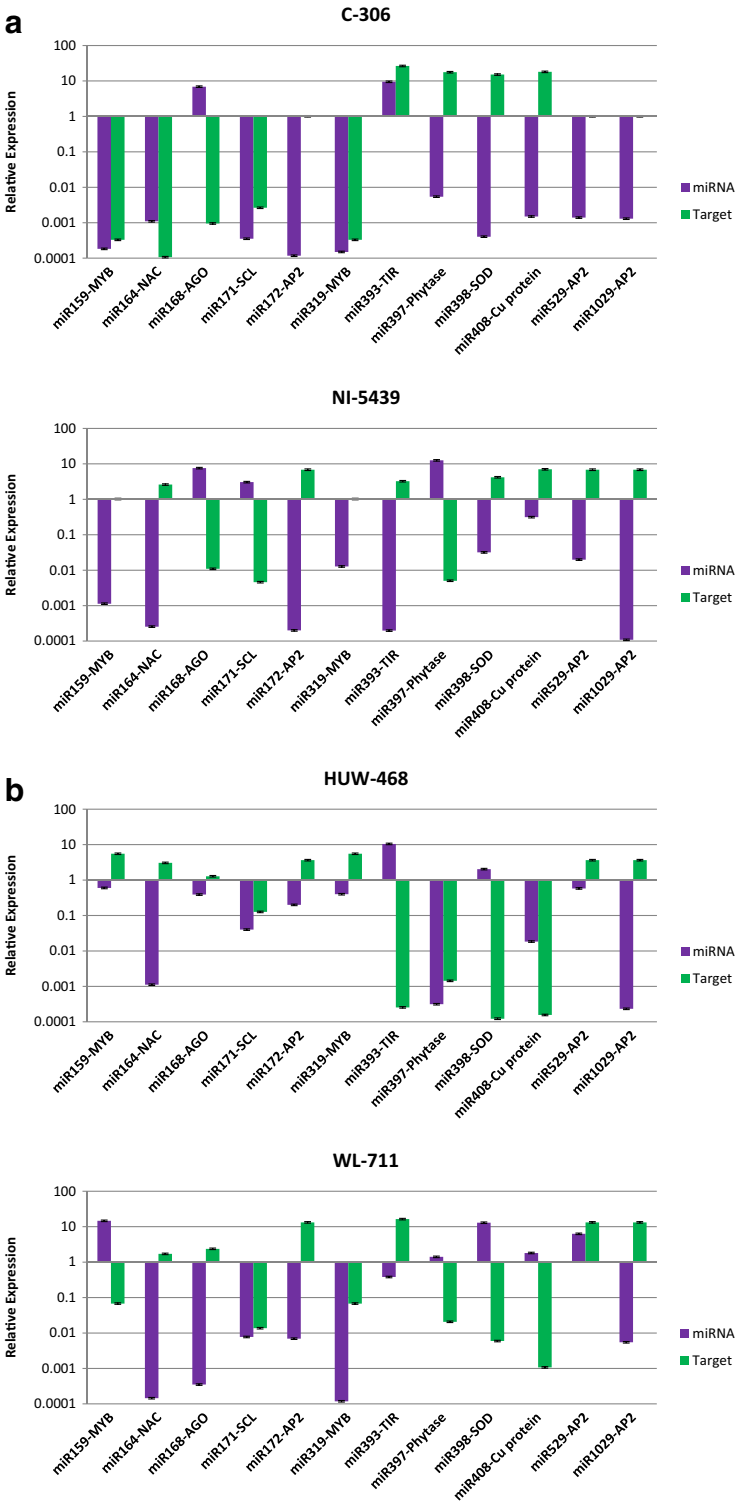
Validation of miRNAs and Their Target Genes Using Quantitative Real-Time PCR

Before proceeding to qPCR, we first checked the amplification of all the miRNAs and their corresponding target genes by using routine PCR. After PCR confirmation, qPCR was run in a reaction volume of 10 μl containing 10 ng/μl of srcDNA, 5 μl of 2× SYBR Green Master Mix (Thermo Scientific) and 1 μl each of forward and reverse primer. The qPCR protocol was programmed at 95 °C for 5 min, then 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s and a final melt curve step from 65 to 95 °C with a rise of 0.5 °C for 5 s. U6 snRNA and β-actin were used as internal control in qRT-PCR reaction for data normalization [32]. The reactions were performed in three biological replicates on CFX96™ Real-Time System (Bio-Rad, USA). The threshold cycle (Ct) value of the technical triplicates was averaged, and standard deviation was calculated. The relative expression level of all the miRNAs was calculated by using the comparative $2^{-\Delta\Delta Ct}$ method [33].

Results

Differential Expression of miRNAs and Their Targets in Drought Tolerant and Sensitive Wheat Genotypes at 1 h of Osmotic Exposure

In order to deepen our understanding during induced osmotic shock in tolerant and sensitive wheat genotypes, we performed expression-based array of miRNA and target genes in drought-tolerant (C-306, NI-5439) and drought-sensitive (HUW-468, WL-711) wheat genotypes. We carried out quantitative PCR-based expression analysis of 12 miRNAs (miR159a, miR164, miR168a, miR171, miR172a, miR319, miR393a, miR397a, miR398, miR408, miR529a, miR1029) and their corresponding nine target genes (MYB, NAC, AGO, SCL, AP2, TIR, Phytase, SOD and Cu protein). Observed data showed differential landscape pattern of miRNA and targets in leaf tissues of 1 h osmotically shocked wheat genotypes compared to control plants. Amongst 12 miRNAs, only 2 miRNAs [miR168a (~7-fold) and miR393a (~9-fold)] were found to be significantly upregulated in tolerant C-306 genotypes while 3 miRNAs [miR168a (~7.5-fold), miR171 (~5-fold) and miR397a (~12-fold)] were upregulated in tolerant NI-5439 genotypes (Fig. 2a). Interestingly, expression pattern of three miRNAs and their corresponding targets, viz. miR168a-AGO, miR398-SOD and miR408-Cu protein, was found to be similar in both the tolerant genotypes (C-306 and NI-5439) with varying degree of accumulation (Fig. 2a). Similarly, two miRNAs, viz. miR393a and miR398, were observed to


a
C-306
Relative Expression
100
10
1
0.1
0.01
0.001
0.0001
miRNA
Target
miR159-MYB
miR164-NAC
miR168-AGO
miR171-SCL
miR172-AP2
miR319-MYB
miR393-TIR
miR397-Phytase
miR398-SOD
miR408-Cu protein
miR529-AP2
miR1029-AP2
NI-5439
HUW-468
b
WL-711

◀ **Fig. 2** Relative expression of miRNA and their corresponding miRNA targets in **a** drought-tolerant genotypes C-306 (*up*) and NI-5439 (*down*) and **b** drought-sensitive genotypes HUW-468 (*up*) and WL-711 (*down*) exposed to 1 h of drought stress. *Bars* represent mean and standard deviation of values obtained from three biological replicates

be upregulated and their targets were downregulated, while five miRNAs, viz. miR159a, miR397a, miR398, miR408 and miR529a, were upregulated and their targets were downregulated in drought-sensitive genotypes HUW-468 and WL-711, respectively (Fig. 2b). In addition, five miRNA-target pairs, viz. miR164-NAC, miR168a-AGO, miR171-SCL, miR172a-AP2 and miR398-SOD, showed similar expression pattern in both the sensitive genotypes (HUW-468 and WL-711).

Differential Expression of miRNAs and Their Targets in Drought Tolerant and Sensitive Wheat Genotypes at 12 h of Osmotic Exposure

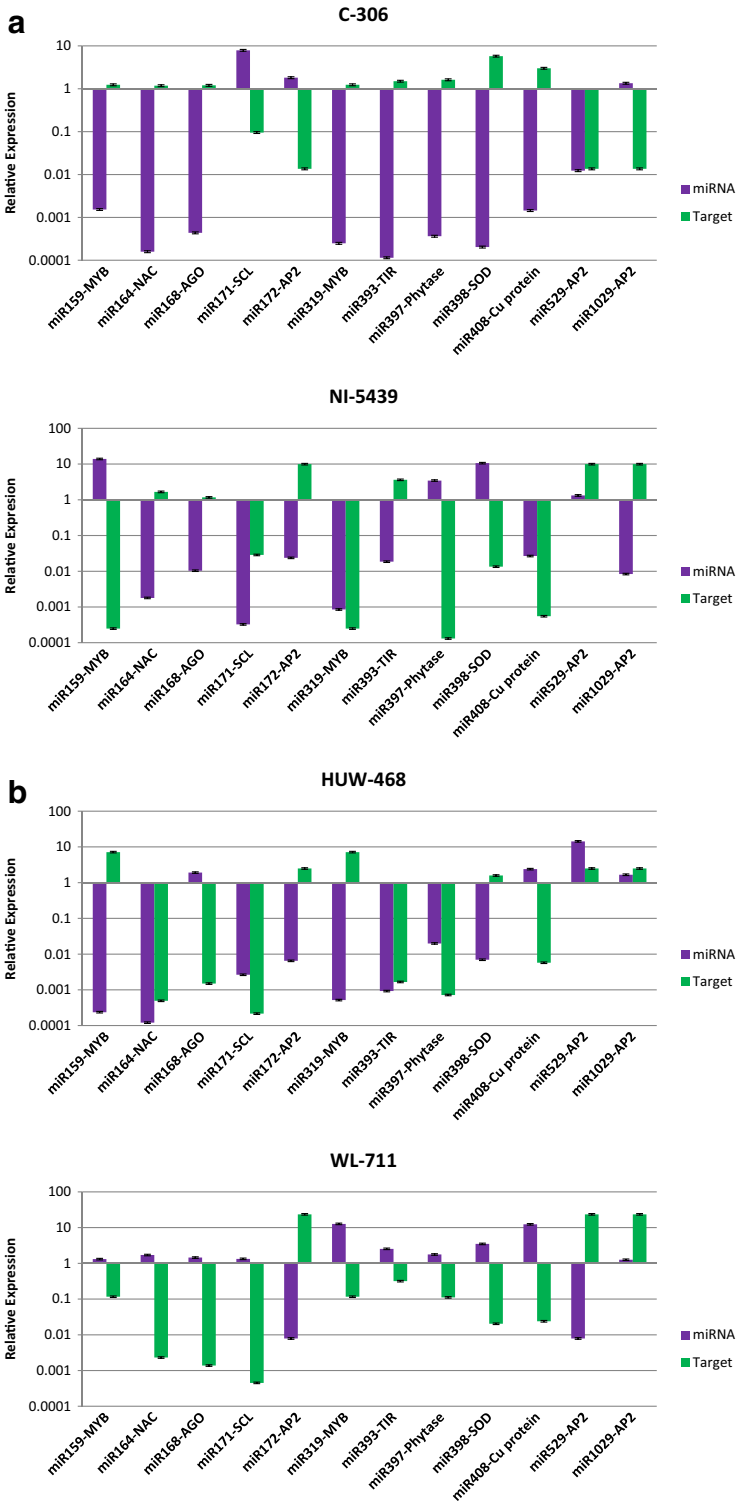
Similar to 1 h of osmotic shock treatment, 12 h of shock have also resulted into varying expression landscape of miRNA targets in both tolerant and sensitive wheat genotypes. Amongst all the differentially expressing miRNAs and their targets, we could find only three miRNA-target pairs, viz. miR164-NAC, miR168a-AGO and miR393a-TIR, whose expression pattern was similar in both the drought-tolerant wheat genotypes C-306 and NI-5439 (Fig. 3a). Highest accumulation of ~13-fold was observed for miR159a in NI-5439 genotype. Additionally, drought-sensitive genotypes, viz. HUW-468 and WL-711, showed similar expression pattern for four miRNA-target pairs, viz. miR168a-AGO, miR172a-AP2, miR408-Cu protein and miR1029-AP2 (Fig. 3b). Maximum accumulation of miR319 (~12-fold) was observed in WL-711 genotype while miR529a (~12-fold) in HUW-468. Alternatively, the three target genes, viz. NAC, AGO and TIR, indicated an osmotic-specific expression pattern as it is evident from its upregulation in drought-tolerant and downregulation in drought-sensitive wheat genotypes.

Differential Expression of miRNAs and Their Targets in Drought Tolerant and Sensitive Wheat Genotypes at 24 h of Osmotic Exposure

Furthermore, to better understand the effect of osmotic shock, the plants were exposed to dehydration for a prolonged period (24 h) which could mimic to natural condition. qPCR expression data suggests that, similar to 1-h treatment, miR319 showed downregulation in both drought-tolerant (C-306 and NI-5439) and drought-sensitive (HUW-468 and WL-711) genotypes along with miR159a and miR1029 (Fig. 4a, b). Surprisingly, five miRNAs (miR164, miR171, miR393a, miR398 and miR408) had higher levels in drought-tolerant genotype C-306. A lower accumulation level of AGO (~0.001-fold) was examined in drought-tolerant genotypes and a higher level (~5-fold) in drought-sensitive genotypes. The target gene MYB was found to be upregulated in all the genotypes of wheat.

Significant Correlation Between miRNAs and Their Target Genes

Generally, stress-induced miRNAs regulate the expression of a gene by cleaving its mRNA transcript. Therefore, a negative correlation is always expected in miRNAs and their targets. In our study, 3 out of the 12 miRNA-target pairs, miR164-NAC, miR168a-AGO and miR398-


a
C-306
Relative Expression
10
1
0.1
0.01
0.001
0.0001
miRNA
Target
miR159-MYB
miR164-NAC
miR168-AGO
miR171-SCL
miR172-AP2
miR319-MYB
miR393-TIR
miR397-Phytase
miR398-SOD
miR408-Cu protein
miR529-AP2
miR1029-AP2
NI-5439
Relative Expresion
100
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0.1
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miRNA
Target
miR159-MYB
miR164-NAC
miR168-AGO
miR171-SCL
miR172-AP2
miR319-MYB
miR393-TIR
miR397-Phytase
miR398-SOD
miR408-Cu protein
miR529-AP2
miR1029-AP2
b
HUW-468
Relative Expression
100
10
1
0.1
0.01
0.001
0.0001
miRNA
Target
miR159-MYB
miR164-NAC
miR168-AGO
miR171-SCL
miR172-AP2
miR319-MYB
miR393-TIR
miR397-Phytase
miR398-SOD
miR408-Cu protein
miR529-AP2
miR1029-AP2
WL-711
Relative Expression
100
10
1
0.1
0.01
0.001
0.0001
miRNA
Target
miR159-MYB
miR164-NAC
miR168-AGO
miR171-SCL
miR172-AP2
miR319-MYB
miR393-TIR
miR397-Phytase
miR398-SOD
miR408-Cu protein
miR529-AP2
miR1029-AP2

◀ **Fig. 3** Relative expression amount of miRNA and their corresponding miRNA targets in **a** drought-tolerant genotypes C-306 (*up*) and NI-5439 (*down*) and **b** drought-sensitive genotypes HUW-468 (*up*) and WL-711 (*down*) exposed to 12 h of drought stress

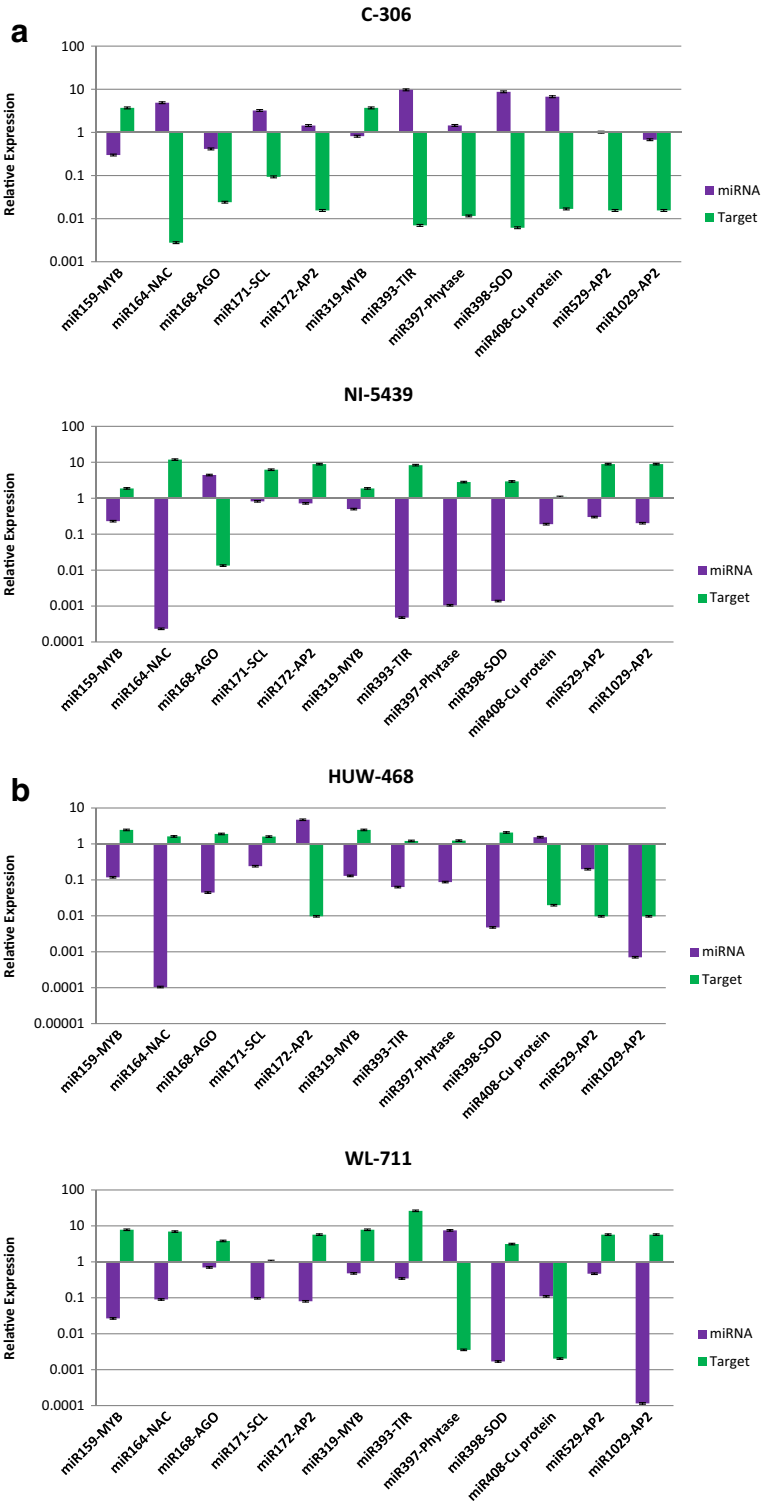
SOD, showed a perfect negative correlation in all the genotypes of wheat indicating their active regulatory role in drought stress (Fig. 5). A reverse correlation was also found in miR159a and MYB in all the genotypes except in C-306 where they were in a positive correlation with each other. Additionally, seven miRNA-target pairs, miR171-SCL, miR172a-AP2, miR319-MYB, miR393a-TIR, miR408-Cu Protein, miR529a-AP2 and miR1029-AP2, showed negative correlation in at least one genotype across all the studied stage which could signify their variety-specific osmotic response which needs further validation.

Discussion

Crop production is rigorously affected by biotic and abiotic stresses. By enlarge biotic stresses could be overcome with the help of man-made chemicals such as pesticides, while abiotic stresses may not be touched with such efforts. Drought/osmotic is one of the most puzzling abiotic stress as it instigates serious damage to crop growth vis-a-vis production.

Since decade, many miRNAs had been known for their governing role during drought/osmotic stress [6–9, 11–13, 15, 34]. Therefore, these miRNAs are crucial and being extensively explored as a newly identified gene resource for the genetic improvement of crops. In the current work, we selected 12 drought/osmotic stress-related miRNAs along with their nine target transcripts with a view to understand their involvement during induced osmotic shock temporally (1, 12 and 24 h) in two drought-tolerant (C-306, NI-5439) and two drought-sensitive (HUW-468 and WL-711) wheat genotypes.

Induced osmotic shock resulted in differential expression of miRNA-target genes temporally (1, 12 and 24 h). The miRNA168 is characterized for its significant role towards drought stress by cleaving the transcripts of AGO [32]. In this study, miRNA168 showed an osmotic shock-specific response between drought-tolerant and drought-sensitive genotypes at 1 and 12 h, whereas its target gene AGO was quite evident in all the treatment. A perfect negative correlation of miR168a with its target gene AGO might suggest its proactive role in dissecting the osmotic shock response in wheat (Fig. 5). Amongst all the three time periods, expression of miR159a-MYB pair was negatively correlated at 24 h in all the genotypes (Fig. 5). This result is in accordance with *Arabidopsis* [19], French bean [35], maize [36] and cotton [37] indicating its active role in osmotic stress tolerance and signal transduction under drought stress. Same pattern of expression of miR159-MYB pair in both tolerant and sensitive wheat genotypes might be due to their different genetic constitution which can further be confirmed by studying expression schema on RIL population having same genetic background. Additionally, miR319 showed downregulation in both drought-tolerant genotypes and one drought-sensitive HUW-468 genotype in all the treatments and is in agreement with the finding in cotton [37]. The role of miR164-NAC protein is to attenuate auxin signals for lateral root development in *Arabidopsis* [26] and *M. truncatula* [13]. Our results showed negative correlation of miR164-NAC transcript in NI-5439 and WL-711 at 24 h which might be playing some role in root development in response to osmotic stress in wheat. Similarly, accumulation of miR529a in all the genotypes after 24 h was in accordance with the findings in rice [11], while inverse relation was observed in cowpea [38]. Therefore, it could be clearly


a
C-306
Relative Expression
100
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0.1
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miR159-MYB
miR164-NAC
miR168-AGO
miR171-SCL
miR172-AP2
miR319-MYB
miR393-TIR
miR397-Phytase
miR398-SOD
miR408-Cu protein
miR529-AP2
miR1029-AP2
miRNA
Target
NI-5439
Relative Expression
100
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0.001
0.0001
miR159-MYB
miR164-NAC
miR168-AGO
miR171-SCL
miR172-AP2
miR319-MYB
miR393-TIR
miR397-Phytase
miR398-SOD
miR408-Cu protein
miR529-AP2
miR1029-AP2
miRNA
Target
b
HUW-468
Relative Expression
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0.1
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miR159-MYB
miR164-NAC
miR168-AGO
miR171-SCL
miR17Z-AP2
miR319-MYB
miR393-TIR
miR397-Phytase
miR398-SOD
miR408-Cu protein
miR529-AP2
miR1029-AP2
miRNA
Target
WL-711
Relative Expression
100
10
1
0.1
0.01
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0.0001
miR159-MYB
miR164-NAC
miR168-AGO
miR171-SCL
miR172-AP2
miR319-MYB
miR393-TIR
miR397-Phytase
miR398-SOD
miR408-Cu protein
miR529-AP2
miR1029-AP2
miRNA
Target

◀ **Fig. 4** Relative expression of miRNA and their corresponding miRNA targets in **a** drought-tolerant genotypes C-306 (*up*) and NI-5439 (*down*) and **b** drought-sensitive genotypes HUW-468 (*up*) and WL-711 (*down*) exposed to 24 h of drought stress

observed that the expression of different miRNAs in response to osmotic shock differs from species to species and even within the species with varying treatment conditions.

The target of miR393a, TIR, is a positive regulator of auxin signalling by degrading Aux/IAA proteins [22]. Previous studies have shown that miR393 was upregulated in response to drought in rice [9, 10] and *Arabidopsis* [8, 39]. Thus, induced level of miR393a in drought-tolerant wheat genotype C-306 might be contributing in adapting osmotic stress by retarding plant growth and development. Accumulation of miR398 in drought-tolerant genotype C-306 after 24 h of shock was quite in line with the results of *M. truncatula* [40] and *T. dicoccoides* [15] to increase oxidative stress tolerance in the plants. In response to drought stress, miR408 inhibits seed germination and root growth in transgenic tobacco and rice [41, 42]. We found a differential expression pattern for miR408 across the genotypes temporally which might be regulating root development. In addition, we studied AP2 as a target for three miRNAs, viz. miR172a, miR529a and miR1029. Result indicated an inverse expression pattern only in miR172a-AP2 pair in all the shock treatments while same was absent in miR529a-AP2 and miR1029-AP2 pairs. Therefore, it could be stated that miR172a is significantly regulating AP2 under osmotic shock in wheat genotypes compared to others. Additionally, we for the first time validated expression of Ppt-miR1029 in wheat under osmotic stress temporally. Expression level of miR164, miR171 and miR398 in NI-5439 and WL-711 during 24 h was in line with the findings in *M. truncatula* [13] which might be playing similar response in wheat. But

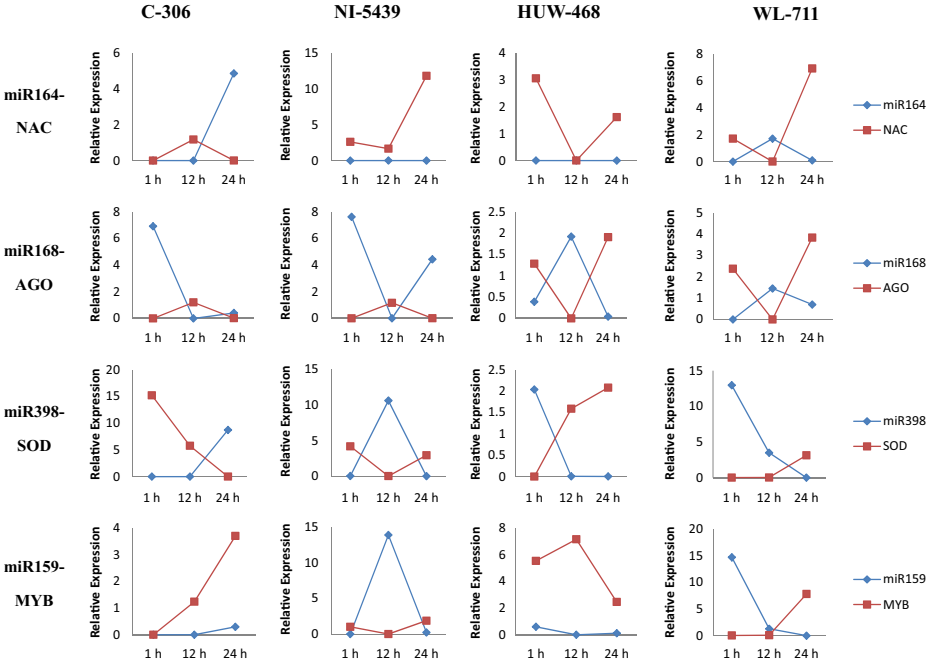

Fig. 5 Negative and positive correlations of miR164-NAC, miR168a-AGO, miR398-SOD and miR159a-MYB in the drought-tolerant (C-306 and NI-5439) and drought-sensitive (HUW-468 and WL-711) wheat genotypes

surprisingly miR164, miR393a and miR398 were downregulated in early stages of osmotic shock in C-306 while upregulated after 24 h of shock treatment. This type of response suggests that these miRNAs are more proactive during later stage of shock. The wheat genotype C-306, which is known for its drought tolerance characteristics, possesses a regulatory network of relationship between miRNAs and their targets in drought/osmotic stress in wheat (Supplementary Fig. 3). This finding is in agreement with the study conducted by Kumar [42]. miR159 was inhibited by the stress condition which, in turn, induces the expression of MYB gene. Additionally, miR-164, miR-171, miR172, miR397 and miR398 along with their targets were found to participate in the drought stress tolerance mechanism in wheat.

Small RNA-based strategies are being widely exploited for crop improvement. In order to get a clear picture of miRNAs and their targets, the correlation was analysed between them. A negative correlation was observed in miR164, miR168a and miR398 with their corresponding target genes NAC, AGO and SOD, respectively, which could be the major player in osmotic shock response in wheat. In conclusion, we analysed temporal and variety-specific differential expression profile of miRNAs and their corresponding target genes in wheat genotypes exposed to osmotic shock which would deepen our understanding of regulatory cross talk amongst them during such stress. However, further investigations by using reverse genetic approach are required to draw any firm conclusion.

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References

1. Chaves, M. M., Maroco, J. P., & Pereira, J. S. (2003). Understanding plant responses to drought from genes to the whole plant. *Functional Plant Biology, 30*, 239–264
2. Denby, K., & Gehring, C. (2005). Engineering drought and salinity tolerance in plants: lessons from genomewide expression profiling in *Arabidopsis*. *Trends in Biotechnology, 23*, 547–552.
3. Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell, 116*, 281–297.
4. Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., & Jones, J. D. (2006). A plant miRNA contributes to antibacterial resistance by repressing auxin signalling. *Science, 312*, 436–439.
5. Khraiwesh, B., Zhu, J. K., & Zhuc, J. (2012). Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. *Biochemistry and Biophysics Acta, 1819*(2), 137–148.
6. Gupta, O. P., Meena, N. L., Sharma, I., & Sharma, P. (2014). Differential regulation of microRNAs in response to osmotic, salt and cold stresses in wheat. *Molecular Biology Reports, 41*(7), 4623–4629.
7. Pandey, B., Gupta, O. P., Pandey, D. M., Sharma, I., & Sharma, P. (2013). Identification of new stress-induced microRNA and their targets in wheat using computational approach. *Plant Signal Behaviour, 8*(5), e23932.
8. Liu, H., Tian, X., Li, Y., Wu, C. A., & Zheng, C. (2008). Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. *RNA, 14*, 836–843.
9. Zhao, B., Liang, R., Ge, L., Li, W., Xiao, H., Lin, H., Ruan, K., & Jin, Y. (2007). Identification of drought-induced microRNAs in rice. *Biochemistry and Biophysics Research Communication, 354*, 585–590.
10. Zhou, L., Liu, Y., Liu, Z., Kong, D., Duan, M., & Luo, L. (2010). Genome-wide identification and analysis of drought-responsive microRNAs in *Oryza sativa*. *Journal of Experimental Botany, 61*(15), 4157–4168.
11. Arenas-Huertero, C., Perez, B., Rabanal, F., Blanco-Melo, D., De la Rosa, C., Estrada-Navarrete, G., Sanchez, F., Covarrubias, A. A., & Reyes, J. L. (2009). Conserved and novel miRNAs in the legume *Phaseolus vulgaris* in response to stress. *Plant Molecular Biology, 70*, 385–401.
12. Kulcheski, F. R., de Oliveira, L. F. V., Molina, L. G., Almerao, M. P., Rodrigues, F. A., Marcolino, J., Barbosa, J. F., Stolf-Moreira, R., Nepomuceno, A. L., Marcelino-Guimaraes, F. C., Abdelnoor, R. V., Nascimento, L. C., Carazzolle, M. F., Pereira, G. A. G., & Margis, R. (2011). Identification of novel soybean microRNAs involved in abiotic and biotic stresses. *BMC Genomics, 12*, 307.

13. Wang, T., Chen, L., Zhao, M., Tian, Q., & Zhang, W. H. (2011). Identification of drought responsive microRNAs in *Medicago truncatula* by genome-wide high throughput sequencing. *BMC Genomics, 12*, 367.
14. Ferreira, T. H., Gentile, A., Vilela, R. D., Costa, G. G., Dias, L. I., Endres, L., & Menossi, M. (2012). MicroRNAs associated with drought response in the bioenergy crop sugarcane (*Saccharum* spp.). *PloS One, 7*(10), e46703.
15. Kantar, M., Lucas, S. J., & Budak, H. (2011). miRNA expression patterns of *Triticum dicoccoides* in response to shock drought stress. *Planta, 233*, 471–484.
16. Wang, M., Wang, Q., & Zhang, B. (2013). Response of miRNAs and their targets to salt and drought stresses in cotton (*Gossypium hirsutum* L.). *Gene, 530*, 26–32.
17. Shuai, P., Liang, D., Zhang, Z., Yin, W., & Xia, X. (2013). Identification of drought responsive and novel *Populus trichocarpa* microRNAs by high throughput sequencing and their targets using degradome analysis. *BMC Genomics, 14*, 233.
18. Zhang, N., Yang, J., Wang, Z., Wen, Y., Wang, J., He, W., Liu, B., Si, H., & Wang, D. (2014). Identification of novel and conserved microRNAs related to drought stress in potato by deep sequencing. *PloS One, 9*(4), e95489.
19. Reyes, J. L., & Chua, N. H. (2007). ABA induction of miR159 controls transcript levels of two MYB factors during *Arabidopsis* seed germination. *Plant Journal, 49*, 592–606.
20. Allen, R. S., Li, J. Y., Alonso-Peral, M. M., White, R. G., Gubler, F., & Millar, A. A. (2010). MicroR159 regulation of most conserved targets in *Arabidopsis* has negligible phenotypic effects. *Science, 1*, 1821.
21. Chen, Z. H., Bao, M. L., Sun, Y. Z., Yang, Y. J., Xu, X. H., Wang, J. H., Han, N., Bian, H. W., & Zhu, M. Y. (2011). Regulation of auxin response by miR393-targeted transport inhibitor response protein 1 is involved in normal development in *Arabidopsis*. *Plant Molecular Biology, 77*, 619–629.
22. Aukerman, M. J., & Sakai, H. (2003). Regulation of flowering time and floral organ identity by a microRNA and its Apetala2-like target genes. *Plant Cell, 15*, 2730–2741.
23. Zhu, Q. H., Upadhyaya, N. M., Gubler, F., & Helliwell, C. A. (2009). Over-expression of miR172 causes loss of spikelet determinacy and floral organ abnormalities in rice (*Oryza sativa*). *BMC Plant Biology, 9*, 149.
24. Zhu, Q., & Helliwell, C. A. (2010). Regulation of flowering time and floral patterning by miR172. *Journal of Experimental Botany, 62*(2), 487–495.
25. Sunkar, R., Kapoor, A., & Zhu, J. K. (2006). Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by down regulation of miR398 and important for oxidative stress tolerance. *Plant Cell, 18*, 2051–2065.
26. Guo, H. S., Xie, Q., Fei, J. F., & Chua, N. H. (2005). MicroRNA directs mRNA cleavage of the transcription factor NAC1 to down regulate auxin signals for *Arabidopsis* lateral root development. *Plant Cell, 17*(5), 1376–1386.
27. Axtell, M. J., Snyder, J. A., & Bartel, D. P. (2007). Common functions for diverse small RNAs of land plants. *The Plant Cell, 19*, 1750–1769.
28. Yao, Y., Guo, G., Ni, Z., Sunkar, R., Du, J., Zhu, J. K., & Sun, Q. (2007). Cloning and characterization of microRNAs from wheat (*Triticum aestivum* L.). *Genome Biology, 8*, R96.
29. Kadam, S., Singh, K., Shukla, S., Goel, S., Vikram, P., Pawar, V., Gaikwad, K., Khanna-Chopra, R., & Singh, N. (2012). Genomic associations for drought tolerance on the short arm of wheat chromosome 4B. *Functional & Integrative Genomics, 12*(3), 447–464.
30. Tomar, R. S., Vinod, Tomar, S. M. S., Sai Prasad, S. V., Naik, K. B., Jha, G. K., Singh, N. K., & Chand, S. (2012). Development of mapping populations and their characterization for drought tolerance in wheat. *Indian Journal of Genetics and Plant Breeding, 72*, 195–207.
31. Seungil, R. O., Park, C., Jin, J., Sanders, K. M., & Yan, W. (2006). A PCR-based method for detection and quantification of small RNAs. *Biochemical and Biophysical Research Communication, 351*(3), 756–763.
32. Yan, J. W., Yuan, F. R., Long, G. Y., Qin, L., & Deng, Z. N. (2012). Selection of reference genes for quantitative real-time RT-PCR analysis in citrus. *Molecular Biology Reports, 39*, 1831–1838.
33. Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)). *Methods, 25*, 402–408.
34. Nageshbabu, R., Jyothi, M. N., Sharadamma, N., Sarika, S., Rai, D. V., & Devraj, V. R. (2013). Expression of miRNA regulates growth and development of frenchbean (*Phaseolus vulgaris*) under salt and drought stress conditions. *International Research Journal of Biological Science, 2*(1), 52–56.
35. Jing-Sheng, L. I., Feng-Ling, F. U., Ming, A. N., Shu-Feng, Z., Yue-Hui, S., & Wan-Chen, L. I. (2013). Differential expression of microRNAs in response to drought stress in maize. *Journal of Integrated Agriculture, 12*(8), 1414–1422.
36. Li, Y., Li, C., Ding, G., & Jin, Y. (2011). Evolution of MIR159/319 microRNA genes and their post-transcriptional regulatory link to siRNA pathways. *BMC Evolutionary Biology, 11*, 122.
37. Barrera-Figueroa, B. E., Gao, L., Diop, N. N., Wu, Z., Ehlers, J. D., Roberts, P. A., Close, T. J., Zhu, J. K., & Liu, R. (2011). Identification and comparative analysis of drought associated microRNAs in two cowpea genotypes. *BMC Plant Biology, 11*, 127.

38. Sunkar, R., & Zhu, J. K. (2004). Novel and stress-regulated microRNAs and other small RNAs from *Arabidopsis*. *The Plant Cell, 16*, 2001–2019.
39. Trindade, A., Capitao, C., Dalmay, T., Fevereiro, M. P., & Santos, D. M. (2010). MiR398 and miR408 are up regulated in response to water deficit in *Medicago truncatula*. *Planta, 231*, 705–716.
40. Mutum, R. D., Balyan, S. C., Kansal, S., Agarwal, P., Kumar, S., Kumar, M., & Raghuvanshi, S. (2013). Evolution of variety-specific regulatory schema for expression of Osa-miR408 in indica rice varieties under drought stress. *FEBS Journal, 280*, 1717–1730.
41. Feng, X. M., Qiao, Y., Mao, K., Hao, Y. J., & You, C. X. (2010). Overexpression of *Arabidopsis* atmiR408 gene in tobacco. *Acta Biologia Cracoviensia Series Botanica, 52*(2), 26–31.
42. Kumar, R. (2014). Role of microRNAs in biotic and abiotic stress responses in crop plants. *Applied Biochemistry and Biotechnology, 174*, 93–115.