FUNCTIONAL GENOMIC PROFILING OF DROUGHT RESPONSIVE Micro-RNA IN WHEAT

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Hexaploid bread wheat (*Triticum aestivum* L.) is considered the most important crop in Egypt and one of the main staple crops worldwide. From economical point of view, it is the most world trade crop in the world. (faostat.fao.org). Its yield is affected by several biotic and abiotic stresses. One of the major abiotic stresses that affect wheat production is drought. It can cause a serious negative effect on plant metabolism, and these changes always begin at the molecular level. Plants deal with these kinds of stresses by expressing some genes to deal with stress or by regulating their genes expression levels by different regulatory mechanisms.

One of the most powerful mechanisms of gene regulation is post transcription regulation. Plant microRNAs (miRNAs) are a highly-conserved class of small (21-24 nucleotides), non-coding RNAs that regulate gene expression by post-transcriptional degradation or translational repression (Liu *et al*., 2008; Unver *et al*., 2009; Eldem *et al*., 2013; Zhang, 2015). Plant miRNAs have been reported to play an important role in post-transcriptional gene regulation.

This study aimed to assess the impact of 10 miRNA on some drought implicated genes in wheat plants. By using stem-loop real time PCR, we can accurately measure the expression level of these miRNA in the first shock to drought stress. For further declaration of their role, their targets were predicted and functionally enriched. Also, pathway analysis and transcription factors binding to these miRNA and their targets have been studied. This study provides a comprehensive analysis of these miRNAs expressed in leaves of bread wheat in response to drought stress.

MATERIALS AND METHODS

Plant material and stress treatment

Egyptian wheat cultivar Giza 168 was used in this study. It was provided by Agriculture Genetic Engineering Research Institute, Agriculture Research center, Giza, Egypt. This genotype is considered as tolerant cultivar to drought stress.

Wheat grains were grown in green house. Three pots were used in three replicates. Seedling plantlets were exposed to PEG 6000. The final concentration of

Egypt. J. Genet. Cytol., 46: 363-370, July, 2017
Web Site (www.esg.net.eg)

PEG was 20% w/v, while the control was irrigated with fresh water. Samples from leaves were taken of control seedlings and stressed plants after two and 12 hours from treatment. Samples were directly subjected for RNA extraction.

RNA extraction and stem-loop qRT-PCR

Total RNA was extracted from both the control and the treated wheat leaves by TRI Reagent® RNA Isolation Reagent (Sigma-aldrich corp., Cat. No. T9424). Five hundred ng of total RNA was used for the first strand cDNA synthesis using 10 miRNA-specific stem-loop primers with Superscript reverse transcriptase III (Invitrogen) according to manufacturer's recommendations. Primers used in this study are listed in Table (1). The reverse transcription reaction was done at 15°C for 45 min followed by 60 cycles of 30°C for 30 s, 42°C for 30 s, and 50°C for 1 s, and the reaction was terminated at 85°C for 5 min.

A pulsed RT reaction was performed in a thermal cycler as follows: 30 min at 16°C, 60 cycles at 30°C for 30 s, 42°C for 30 s and 50°C for 1 s. RT enzyme was inactivated by incubating the reaction at 85°C for 5 mins, 1ml of direct cDNA was used for PCR using miRNA specific forward primer and universal reverse primer to get a 63 bp amplification product. 18S rRNA was used as an endogenous control. The PCR- based expression analysis was performed using three replicates for each set and the calculated standard deviation for each set is shown as error bars on the graphs.

Target prediction and GO annotation

Putative targets for selected miRNAs were predicted by psRNAtarget webserver (Dai and Zhao, 2011) using the wheat genes ensemble plant database. Default parameters were employed and mRNA sequences with a score 3 were considered as potential targets. All the predicted mRNAs were searched in Plant Transcription Factor database, PlantTFDB 3.0 for gene set enrichment analysis (Jin *et al*., 2014). GO enrichment was done using Singular Enrichment Analysis by employing Plant GO slim with p-value cut-off score of 0.05.

RESULTS AND DISCUSSION

Stem-Loop real time PCR

Eight miRNA showed up- regulation expression after the treatment of seedlings with PEG 6000. These miRNAs were osa_miR319a-3p.2-3, miR5048, gma-miR5783, hci-miR156a, bdi-miR159b-3p, zma-miR164g-3, ssl-miR398 and ptc-miR482c-5p (Fig. 1). Only osa-miR172b was down-regulated.

There were two miRNAs (hci-miR156a, and osa-miR319a-3p.2-3) showed sudden decrease of their expression after 12 hours from the treatment. Although their expression decreased after the 12 hours, they still up-regulated in comparison with the control (zero time). This pattern is a strong evidence on how the cell regulate its resources based on time and environment.

In the last ten years, several studies focused in the role of miRNA in biotic and abiotic stresses. Several miRNA families were found to be differentially expressed in response to drought stress in various plant species, including rice (Zhao *et al*., 2007), *Arabidopsis* (Liu *et al*., 2008), *Medicago truncatula* (Trindade *et al*., 2010), peach (Eldem *et al*., 2013), barley (Kantar *et al*., 2010), and wheat (Gupta *et al*., 2014; Pandey *et al*., 2014). Also several works have been done before in *T. aestivum* L. (Xin *et al*., 2010; Inal *et al*., 2014; Pandey *et al*., 2014), but none of these miRNAs were studied in the early exposure to drought stress.

Target prediction

It is essential for complete figuring out the biological role of the differentially expressed miRNAs, identifying target genes that are post translationaly regulated by them. Target prediction was done using psRNAtarget web server with default parameters. All miRNA family members under study were identified. One of the most important features of miRNA, is that one miRNA can regulate more than one gene. There were 47 target genes predicted with some genes that regulated by more than one miRNA. This differential expression associated with the variability of the number of target genes indicated that miRNA plays an effective role in drought stress tolerance and can significantly change the plant response at both the physiological and molecular levels.

Gene set enrichment analysis was carried out using Plant TFDB. The three main categories (biological process, molecular function and cellular component) were studied (Fig. 2). The functional annotation of differentially expressed genes showed that DNA binding, nucleic acid binding, and cyclic compound binding were the most represented Go terms in the molecular function domain. While in the biological process domain, the organic substance metabolic process, macromolecule metabolic process and cellular metabolic process Go terms were the highest represented terms, compared to other terms in the domain. Regarding cellular component Go terms were represented in similar percentage for nucleus and intracellular component.

Out of all families that were studied before, miR398 was the first miRNA reported to be linked with stress tolerance in plants (Sunkar *et al*., 2007). It is directly associated with stress regulatory networks with varied responses to drought, salinity, ABA, oxidative stress & metal ion deficiency etc., (Zhu *et al*., 2011). In our study ssl-miR398 showed up-regulation compared with non-treated seedlings especially, after two hours from the treatment. In rice, miR398 showed leaf-preferential expression in young seedlings (Mittal *et al*., 2013) and this could explain its high levels in the wheat seedlings. Its levels were induced in response to drought stress except, in Arabidopsis, miR398is known to target Cu/Zn superoxide dismutase (CSD1/CSD2),which help in ROS detoxification, an important process for stress resistance and plant surviv-

al (Beauclair *et al*., 2010); a reduced level of miR398 improves tolerance to oxidative stresses (Sunkar *et al*., 2007). ROS is produced under all kinds of abiotic stresses to fight adverse conditions, including water deficit. In *Medicago trunculata*, miR398 is up-regulated under water deficit (Trindade *et al*., 2010). MiR398 is a drought inducible miRNA in *Triticum astevium* with respect to tissue and drought duration.

In conclusion, it is evident from the aforementioned discussion that miRNA has a vital role in the differential expression of several genes implicated in drought tolerance in wheat. This indicated that miRNA could be considered an effective marker for targeting a bunch of genes involved in drought stress in a single shot. This could be used as a dependable tool for improving wheat and other cereal crops for abiotic stresses.

SUMMARY

MicroRNAs are small non-coding RNAs that play a major regulatory role in post translation regulation either by translation inhibition or mRNA cleavage. Recent studies proved that miRNAs have a regulatory role in both abiotic and biotic stress in plants. In our study, we measure the expression profile of 10 known abiotic stress miRNAs in hexaploid bread wheat (*Triticum aestivum* L.), under drought stress in the seedling stage. Significant up-regulation was observed with osa_miR319a-3p.2-3, miR5048, gma-miR5783, hci-miR156a, bdi-miR159b-3p, zma-miR164g-3, ssl-miR398 and ptc-miR482c-5p. Among these, hci-miR156a, and osa-miR319a-3p.2-3 showed down-regulation after 12 h compared to 2 h treatment. Only osa-miR172b was down-regulated. This variant and dynamic expression patterns is good evidence and certain indicator of miRNA correlation to drought tolerance mechanisms present in wheat. This is the first data that provides accurate measurement of some drought related miRNA in wheat in the early exposure to drought stress and in the identification of several genes that could be implicated in drought tolerance and could be used for improving wheat and other cereal crops in this respect.

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Table (1): Primer sequences used for miRNA qRT-PCR experiment.

MiRNA	Forward primer	Stem-loop primer
osa-miR172b	5'-ggaatcttgatgatgctgcat-3'	5'-gttggctctggtgcagggtccgaggtattcgcaccagagccaacatgcag-3'
osa_miR319a-3p.2-3p	5'-ttggactgaagggtgctccc-3'	5'-gttggctctggtgcagggtccgaggtattcgcaccagagccaagggagc-3'
cre-miR1169-3p	5'-tgtggatgttgcttgctggat-3'	5'-gttggctctggtgcagggtccgaggtattcgcaccagagccaaatccag-3'
hvu-miR5048	5'-tcgcttatttgcaggtttt-3'	5'-gtcgtatccagtgcagggtccgaggtattcgcactggatacgacttagac-3'
gma-miR5783	5'-gacgacgacggggaggacgcgc-3'	5'-gttggctctggtgcagggtccgaggtattcgcaccagagccaagcgcgt-3'
hci-miR156a	5'-tgacagaagagagtgagtac-3'	5'-gttggctctggtgcagggtccgaggtattcgcaccagagccaacgtactc-3'
bdi-miR159b-3p	5'-cttggattgaagggagctct-3'	5'-gttggctctggtgcagggtccgaggtattcgcaccagagccaaagagct-3'
zma-miR164g-3p	5'-cacgtgctccccttctccacc-3'	5'-gttggctctggtgcagggtccgaggtattcgcaccagagccaaggtgga-3'
ssl-miR398	5'-tgtgttctcaggtcacccctc-3'	5'-gttggctctggtgcagggtccgaggtattcgcaccagagccaagagggg-3'
ptc-miR482c-5p	5'-tatgggagaggcgggaatgact-3'	5'-gttggctctggtgcagggtccgaggtattcgcaccagagccaaagtcat-3'
Universal reverse primer: 5'-gtgcagggtccgaggt-3'		

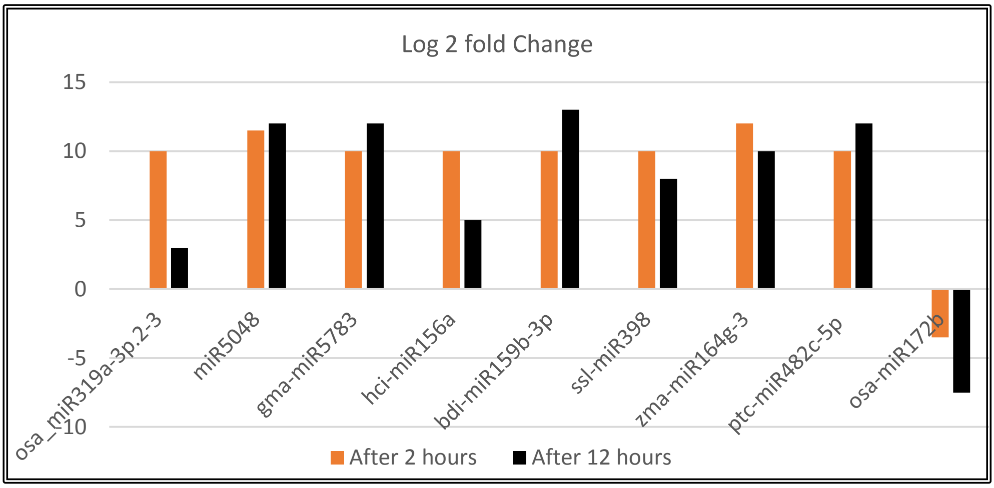

Fig. (1): Log 2 fold change of the nine differentially expressed miRNA after treatment with PEG 6000 compared to control samples without treatment.

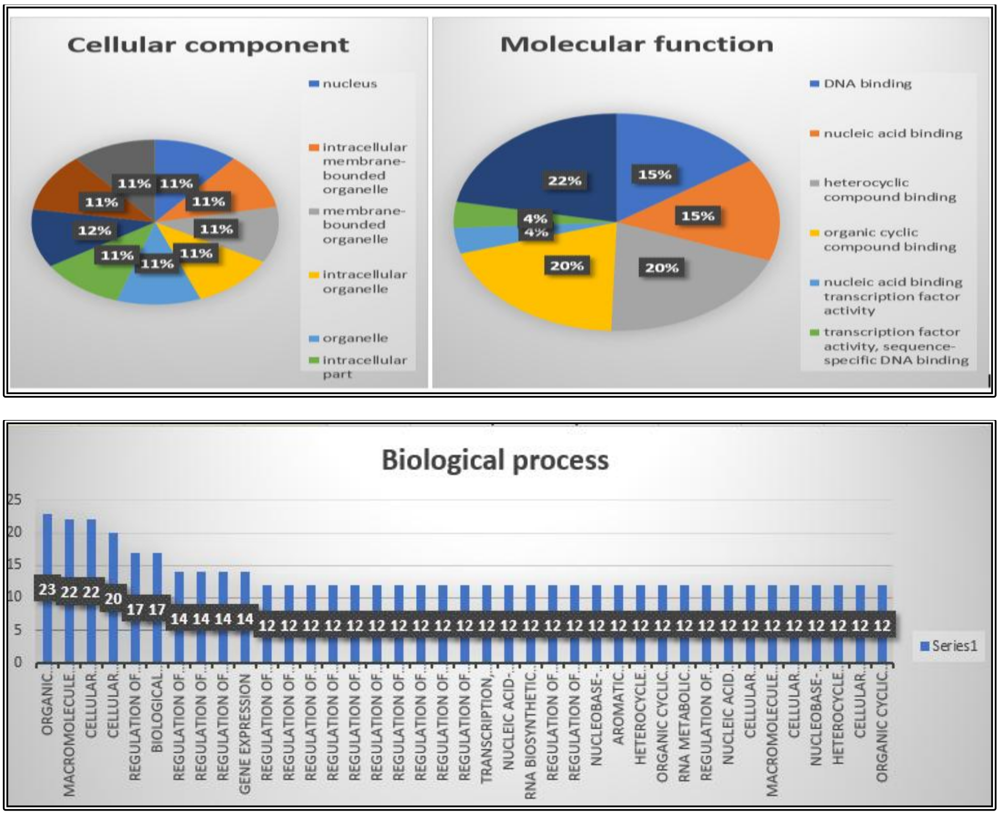

Fig**.** (2): Functional annotation of the 300 potential targeted genes in the wheat genes to the studied miRNA under drought stress.