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Effect of drought stress on expression of HSP70 protein and miR398 in *Echinacea purpurea* L.

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ABSTRACT

Background & Aim: Drought is a natural and recurrent climatic characteristic in most parts of the world and plays an important and restrictive role in crop yield. One of these defense mechanisms is the reprogramming of gene expression using microRNAs. MiRNAs regulate the gene expression more by inhibiting the translation of mRNA and reducing the expression of target protein expression.

Experimental: In the present study, the expression pattern of miR398 and its target gene (NtTG5b) in the leaf tissue of purple coneflower under controlled conditions and four levels of drought stress (85, 75, 50 and 25% Field Capacity (FC) were investigated using qRT-PCR method. To ensure the applying the stress on plants, the heat shock protein (HSP) expression was evaluated as a criterion.

Results: The results showed increased expression of HSP in leaf tissue, therefore the selected levels for drought stress were confirmed. The expression of miR398 at each stress level was often the same, and the process of expression of the target genes in most cases revealed an inconsistent process that could be due to the difference between the target cell and the cell in which the miRNA was expressed, so this shows the complex regulatory network of miRNAs.

Recommended applications/industries: Finally, it can be concluded that miR398 is a drought-responsive miRNA that may play its effects through leaf development control. This could be an important aspect for future studies, because increasing leaf biomass in conditions that have water constraints can be an incentive to use purple coneflower as a plant for medicine.

1. Introduction

Purple coneflower (*Echinacea purpurea* L.) belonging to the Asteraceae family is a diploid, herbaceous, perennial and cross pollinated plant with a height of 1 to 1.5 m. This plant is one of the most valuable plants that has been considered for its components and extract applications in the preparation of herbal medicines. The whole organs of this plant, including root and shoot, contain valuable materials such as alkylamide compounds, isobutylamide and cichoric acid. . It also has essential oils that the most important ingredients of its essence are Humolin, Caryophyllene and Caryophylline oxide (Gruenwald *et al.*, 1998). Drought is a natural and recurrent climatic feature in most parts of the world and plays an important and limiting role in crop yields. Drought is one of the most popular abiotic stresses that affect the growth, development and yield of the crops (Ceccarelli, 1997). Understanding the

tolerance of plants to drought stress is important for improvement of efficiency in crops (Lawlor, 2012). This is largely due to the fact that each internal miRNA controls several genes and each gene is controlled by several miRNAs (Reinhart *et al.*, 2002). It is expected that miRNAs as gene regulators have a role in regulating these genes that responsible for drought. Studies have shown that the expression of miRNAs varies in response to drought stress. It is common that the levels of expression or responsiveness of miRNA to drought are depends on the type of plant species (Kulcheski *et al.*, 2011).

It is possible that regulators of miRNA genes alter their expression under drought conditions, which results in changes in the expression of miRNAs and ultimately changes in the expression of miRNA targets (Reyes and Chua, 2007, Trindade *et al.*, 2010). Although miRNAs are protected among plant species but their targets may not be same (Lu *et al.*, 2005). Hence, miRNA targets need to be identified in any plant. Target evaluation can help provide the performance evidence of protected and specific miRNAs in plant species.

MiR398 has a special position in plant miRNAs, because it is the first reported miRNA related to plant resistance in plants (Sunkar *et al.*, 2006). This miRNA is directly involved to the regulatory network of stress and in response to various stresses such as salinity, ABA, oxidative stress, metal ion deficiency, etc. (Zhou *et al.*, 2013). In rice, the expression of miR398 was high in young seedlings (Mittal *et al.*, 2013). The reduced levels of miR398 expression improve the resistance to oxidative stress (Sunkar *et al.*, 2006). Free radicals are increased to the abiotic stresses (Trendade *et al.*, 2010). In Arabidopsis, miR396 controlled the development of the leaf by regulating the cell proliferation and through regulator factor of growth (Liu *et al.*, 2009).

When the cells and tissues of the plant are exposed to sudden stress, they begin to express temporary HSPs (Heat Thermal Shock). It has been shown that the original structure of HSP proteins, and in fact the response to thermal shock in organisms is strongly protected, so it is assumed that HSPs may have a close relationship in protecting organisms against thermal stress and maintaining homeostasis. HSP70, a member of the family of HSPs, is simulated by rapid temperature rise. This protein is coded by a protected multi-genic family in eukaryotes and prevents changes in the nature of proteins this protein participates in the translation, transfer and function of steroid receptors (Liu *et al.*, 2012).

miRNAs are involved in many biological activities of plants, including the response to biotic and abiotic stresses. Considering the great importance of the purple coneflower in the production of herbal drugs and since that abiotic stress is one of the most important factors affecting the production and enhancement of secondary metabolites of this medicinal plants (Amiri *et al.*, 2011), and so far there is not a report to find out the involved miRNAs in response to drought stress in purple coneflower, the present study carried out for identifying the involved miRNAs in response to drought stress in purple coneflower.

2. Materials and Methods

2.1. Detection of target gens and primer designing

Studied miRNAs included mir398 were selected from the miRBase database. The primers designed for miRNAs are shown in (Table 1). 18SrRNA was also used as reference gene. Prediction of target genes was done by bioinformatics method. Detection of target genes for microRNAs is very simple, because their sequences are approximately or fully complementary to the sequence of their target genes. This is particularly prominent among plant miRNAs (Bartel *et al.*, 2004). The miRNAs generally connect to the 3'-UTR region, or the transcription region of the mRNA, and prevent its expressions. Therefore, it is possible to predict the transcripts of the target, the same miRNA complement sequences, by the Blast algorithm. To find the target genes of miR169, miR172 and miR398, an online database of miRNA target genes (http://plantgrn.noble.org/psRNATarget) was used.

Table1. The sequence of primers designed for the synthesis reaction of cDNA and qRT-PCR in miR398.

Reverse primer miR398	GTGCAGGGTCCGAGGT
Stem-loop primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGC CAACAAGTTC
Forward primer	GGGGTTCCACAGCTTTCTT

2.2. Assessment of HSP70 Gene Expression in Leaf of Echinacea purpurea L.

Investigation of the expression pattern of HSP70 gene as a molecular scale was investigated to ensure the applying of drought stress on plants. After obtaining the gene's HSP70 sequence from the NCBI site, the design of the gene was initiated using the online software of IDTDNA (Integrated DNA Technologies). The primer sequence was shown in the (Table 2).

Table 2. The sequence of primers designed for the synthesis reaction of cDNA and qRT-PCR in HSP70.

GTGGAGAGGGTGGTGATGAA	Forward primer of HSP70-related protein
GGTTGGGATGACGGTGTTTC	Reverse primer of HSP70-related protein

2.3. Plant materials and applying the drought stress

The experiment was carried out in a Complete Randomized Design with five treatments and three replications. The treatments consisted of different levels of drought stress including: Control (irrigation at 100% Field Capacity (FC)), mild stress (irrigation at 85% and 75% FC), medium stress (irrigation at 50% FC) and severe stress (irrigation at 25% FC). In order to apply irrigation stress, we first calculated the FC using a simple method that is detailed below:

A pot containing field soil was prepared and completely filled with water and then enclosed with plastic to completely saturate the soil. After 24h, when the water of the soil porous was removed by gravity, plastic was removed; the soil samples were weighed and placed in oven at 105°C for 24h. The samples were weighted with a precise weight scale and evaporated moisture content was calculated. When the plastic was taken out of the pot, the FC was measured with a hand humidifier, so that the two methods were compared and don't have much differences.

The application of stress treatment was carried in this way that irrigation of any treatments was done after moisture content of soils reached the desired level (humidity controlled by humidifier). In stress treatments, 85, 75, 50 and 25% FC were applied for irrigation at approximately 2, 4, 8 and 12 days respectively. All operations were performed identically for all treatments (except irrigation). For stress treatments sampling was carried out at seedling stage and for non-stress control was at seedling stages. Irrigation of the pots is carried out every three days until the soil water reaches 70% F.C.

2.4. RNA extraction and synthesis of cDNA

100 mg of leaf tissue was powdered with liquid nitrogen in mortar. RNA extraction was performed according to the RNA extraction kit protocol, RNAX-PLUS manufactured by Sinagen Co... Determination of the quantity of RNA samples carried out by spectrophotometric method using a biometric apparatus (according to the 260 nm wavelength that expresses the quantity of RNA in terms of nanogram per microliter). Also, the absorbance ratio of the RNA samples in the wavelength of 260 to 280 nm, which is the RNA purity index, was read from the device. The best purity for RNA (in the range of 260 to 280) is 1.7 to 2, and in the range of 260 to 230, the range is 1.4 to 2. (Accerbi *et al.,* 2010). The values that outside this range indicate contamination and presence of impurities in the RNA samples. Also, to determine the quality of RNA, two micrograms of each sample were electrophoresed on a 1% agarose gel. After assuring the quality of RNA and observing the 18S and 23S, the DNaseI enzyme treatment was performed according to the proposed method of Fermentas Co. for all specimens. Tubes containing RNA were stored at -80 ° C.

To create the cDNA of the microRNA, first, the stem-loop primers were designed according to Varkoni-Gasic *et al.,* (2007) and Chen *et al.* (2014). Following the design of the primers, to construct the cDNA using Varkonyi-Gasic *et al.* (2007), stem-loop PCR method and reverse transcriptional enzyme were

used. 1 µl of RNA at 200ng concentration with 1 µl RT primer and 0.5 µl (10 mM) dNTP was mixed and the volume of the reaction was reached to 4.8µl to with DEPC. To remove the secondary structure, the samples were placed at 65 ° C for five minutes and then quickly transferred to ice for two minutes. Then, 2 µl of the reaction buffer (5X), 0.5 µl reverse transcriptase enzyme (200 u/µl) and 0.25 µl enzyme (40 U/µl) of RNase inhibitor were added to each tube. The final volume of the reaction was 10 µl. To create the cDNA, the Stem-loop PCR heating program was performed with the following conditions: 30 minutes 16 ° C, 30 seconds at 30 ° C, 30 seconds at 42 ° C, 50 ° C for one second and 85 ° for 5 seconds.

To amplify cDNA, 1 µl of cDNA with 2 µl reaction buffer (5X), 0.5 µl dNTP (10 mM), 0.4 µl of forward primer (10 µM), 0.4 µl of reverse primer (10 µM) and 0.4 µl of Taq polymerase enzyme was added and the final volume of the reaction was adjusted to 20 µl using 15.3 µl sterilized distilled water. The endpoint PCR program was performed under the following conditions: for two minutes at 94 ° C, 15 seconds at 94 ° C, and one minute at 60 ° C.

2.5. Synthesis of cDNA for target genes

The design of primers for target genes was performed using the NCBI site and the Primer3 software. In the design of the primers for target genes, it was important to note that the supplemented region should be amplified with microRNA. Given this necessity, specific primers were designed for target genes. The cDNA was synthesized using polyA oligonucleotides and reverse transcriptase enzyme. 1 µl of RNA at 200 ng concentration with 1 µl of OligodT primer was mixed and the reaction volume was reached to 12.5 µl by adding the DEPC. To remove the secondary structure, the samples were placed at 65 ° C for five minutes and then quickly transferred to a container containing ice. Then, 4 µl of buffer (5X), 2 µl (10 mM) of dNTP, 1 µl of reverse transcriptase enzyme (100 U/µl) and 0.5 µl of RNase inhibitor enzyme (40 U / µl) were added to each tube. The final volume of 20 µl well mixed and then the samples were exposed to a temperature of 72 ° C for 60 minutes and were placed at 72 ° C for 5 minutes to complete the reaction. To ensure the proper amplification of the cDNA of target gene, PCR program was the same as the program for microRNA.

2.6. Investigation the miR398 expression and its target genes using qRT-PCR reaction

In order to investigate the changes in the expression of studied microRNAs and their target genes, the Real-Time RT PCR was used. The reaction of Real-Time RT PCR was performed in ABS device using a single reaction mixture containing SYBR Green and a specific primer. 12 µl of the reaction mixture, containing 6 µl of a single reaction mixture containing SYBR Green, 1 µl cDNA, 1 µl of forward primer, 1 µl of the reverse primer and 3 µl of sterile distilled water.

This reaction was carried out using three technical replications and two biological replications. Gene reference of 18srRNA as internal control was used to normalize qRT-PCR results. Estimates of the relative frequency of miRNAs in normal and stress samples, their expression variables were calculated using CT and $2^{-\Delta\Delta CT}$ formula (Schmittgen *et al.*, 2008).

3. Results and discussion

3.1 Effect of drought stress on expression pattern of miR398 and NtGT5b gene

According to the obtained results, it can be concluded that miR398 may contribute to reducing the energy consumption for photosynthesis in the pathway of sugar biosynthesis and increase plant tolerance to abiotic stress. The expression of miR398 is significantly increased in leaf tissue and during drought stress, which is consistent with previous studies (Guan *et al.*, 2013). Different expressions of miR398 have been reported in response to drought stress, salinity, heat, and cadmium in several plant species such as wild wheat, alfalfa, tobacco, brassica and Arabidopsis (Trindade *et al.*, 2010; Frazier *et al.*, 2011; Kantar *et al.*, 2011; Zhou *et al.*, 2013). The regulation of the mRNA of target genes by multiple miRNA or in contrast the regulation of multiple target mRNAs by a single miRNA, the interaction between the miRNA and the target genes is very flexible (Zandkarimi *et al.*, 2015).

The results of the analysis by the Ct method (Figure 1) showed that miR398 expression has been reduced in comparison with the control in four levels of drought. This decline was higher in severe stress (25% FC). Data from Real-Time RT PCR were analyzed by t-test and showed that expression of NtGT5b gene in all treatments was significantly decreased in comparing to

the control, except at 85% FC (Table 3). In general, it can be stated that expression of the miR398 and its target gene was heterogeneity. MiR398 has a special place in plant miRNAs because the first reported miRNA is related to plant resistance in plants (Sunkar *et al.*, 2006). This miRNA directly participates in the regulation network for stress and in response to various stresses such as salinity, ABA, oxidative stress, metal ion deficiency, etc. (Zhu *et al.*, 2011).

Table 3. Results of changes in expression of miR398 and NtGT5b gene in drought stress conditions and t-test at two levels of p-value <0.05 and p-value <0.01 for comparison with control treatment.

Stress intensity	25% FC	50% FC	75% FC	85% FC
miR398	↓-9.72**	↓-2.17ns	↑1.41ns	↓-4.31**
NtGT5b	↓-9.02*	↓-5.51**	↓-5.80**	↓-1.75ns

* and ** represent the significant difference between the expression in stress and control conditions in the level of probability of 5% and 1%, respectively, and ns is non-significant. The ↑ and ↓ mark represent an increase and decrease in the expression of the miRNA and the target gene.

The results of this study showed that the reduced expression of miR398 in comparison to the control in the four levels of the studied drought, so that the reduction was more in severe stress level (25% FC). Also, the expression of its target gene, NtGT5b, was significantly reduced in all treatments in contrast to the control except 85% FC (Table 3 and Figure 1). Based on these results, NTGT5b gene could be probably introduced as a target gene for miR398 under drought stress. Similar expressions between target gene and miRNAs can indicate the inconsistent regulation of miRNA, in which this type the regulation of miRNA and its target gene occurs in a cell at the same time, and most likely this is due to the expression of other genes and unknown transcription factors and activation of other metabolic networks in the drought stress resistance network. Among miRNAs responding to stress, miR398 directly influences regulatory stress response systems and regulates the plant responses to biotic and abiotic stresses such as drought, salinity, oxidative stress and bacterial contamination (Zhu *et al.*, 2011).

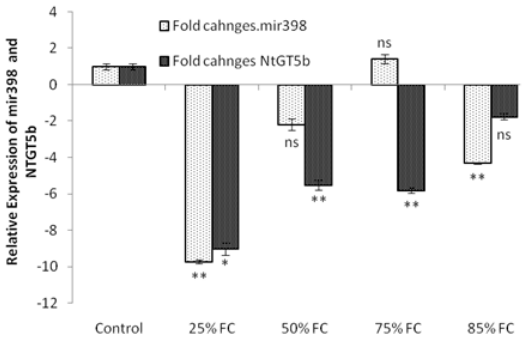

Fig.1. The relative expression of miR398 and its target gene (NtGT5b) in the control and stress (85%, 75%, 50% and 25% FC) conditions in the leaves of Echinacea purpureaL.* and ** represent the significant difference between the expression in stress and control conditions in the level of probability of 5% and 1%, respectively, and ns is non-significant.

In the present study, the NtGT5b gene was selected as the target gene for miR398. Until today, any gene has not been reported as a target gene for miR398. Also, NtGT5b gene was not studied in drought stress in purple coneflower. Blasting at the NCBI site revealed that the UGT85A5 gene was homologous to the NTGT5a and NTGT5b genes, but no physiological role was reported for the two NTGT5a and NTGT5b genes. The UGT85A5 gene in Arabidopsis is significantly induced in drought stress. Ebrahimi *et al.* (2013) reported that the effect of heat stress on sunflower plant increased the expression of miR398 and reduced its target gene expression (NtGT5b) in leaf tissue. These researchers also stated that miR398 has the ability to change the gene expression pattern in many growth, developmental, and stress-response systems in plants. The results of Esmaeeli *et al.* (2013) showed that in peach and during mild and severe drought stress, significant increase in expression of miR398 will be observed, and in almonds, the expression of this microRNA was increased during mild stress and decreased during severe stress. These researchers also stated that the reduction of miR398 expression in severe stress in almonds has led to an increase in the expression of superoxide dismutase encoding genes to fight ROS. On the other hand, increasing in the expression of miR398 during drought stress in photosynthetic tissues is to maintain copper to continue the photosynthetic activity of enzymes such as plasticianine that use the copper as a cofactor

(Esmaeeli *et al.*, 2013). Safarzadeh (2012) reported that miR1118, miR1120, miR1125, miR1127, miR1135, miR156, miR159a, miR159b, miR160, miR164, miR167a, miR399, and miR398 miR1118, miR1120, miR1125, miR159a, miR119, miR119, miR398, and miR398 were up regulated in a study of wheat leaf miRNA in response to water stress. Khalaji *et al.*, (2013), in examining the pattern of expression of miR398 in response to salinity stress in sunflower plant, showed a significant increase in both leaf and root tissues and both levels of salinity stress. Mirlohi, (2013) in identification of the microRNAs involved in drought stress resistance in a sunflower plant, stated that the miR398 and its target gene (NTGT5b) are incorporated in the plant defense system and the network respond to hormones of auxin and abscisic acid.

3.2 Effect of drought stress on expression pattern of HSP gene

Changes in the expression of HSP protein gene in response to different levels of drought stress were also studied and the results showed that the stress caused significantly increase in the expression of HSP in all treatments, which also demonstrates all changes are resulting from the drought stress. In other words, it can be concluded with certainty that any increase or decrease in the expression of the miR398 and its target gene is due to drought stress in the present study. One of the most respected plant responses in dealing with abiotic stresses is the production of a group of proteins known as HPSs. The results of changes in the expression of HSP protein gene in response to different levels of drought stress are presented in (Table 4 and Figure 2). Accordingly, and as observed, drought stress caused a significant increase in the expression of HSP in all studied treatments, which also proofed the changes that caused by drought stress. In other words, we can say conclusively that any increase or decrease in the expression of the miR398 and its target gene is due to drought stress in the purple coneflower. It should be noted that the highest expression of HSP was observed in 50% FC.

Table 4. Results of changes in expression of HSP gene in drought stress conditions and t-test at two levels of p-value <0.05 and p-value <0.01 for comparison with control treatment .

Stress intensity	25% FC	50% FC	75% FC	85% FC
HSP	↑5.76**	↑9.42**	↑7.12**	↑8.97**

* and ** represent the significant difference between the expression in stress and control conditions in the level of probability of 5% and 1%, respectively, and ns is non-significant. The ↑ and ↓ mark represent an increase and decrease in the expression of the miRNA and the target gene.

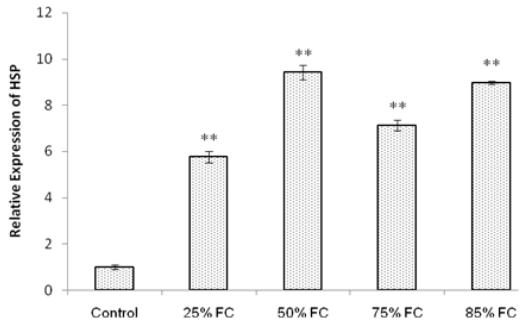

Fig. 2. The relative expression of HSP in the control and stress (85%, 75%, 50% and 25% FC) conditions in the leaves of *Echinacea purpurea*L.* and ** represent the significant difference between the expression in stress and control conditions in the level of probability of 5% and 1%, respectively, and ns is non-significant.

The purpose of producing of these proteins is to play a role in folding, assembling, locating and destroying proteins in the natural processes of cells, and under stress conditions in protecting plants caused to maintain the cell homeostasis. Also, HSPs interact with other mechanisms of stress response (such as osmolites) (Mandeh and Moali Amiri, 2011). With increases in expression of HSPs in rice, Sato and Yokoya (2008) produced transgenic plants that tolerant to the drought stress. Although no significant difference was found between transgenic lines and control plants in terms of the water potential of buds at the end of the drought period, but only transgenic buds with high expression of this protein, were able to resume their growth after irrigation. Kotak *et al.* (2007) reported that the expression of HSP in response to the salinity stress in barley leafs. It is stated that increases in HSP amounts may be incorporated in the repairing or contributing of denatured proteins under stress or re-establishing the

natural formulation of cytosolic proteins (Mittler and Zilinskas, 1992).

4. Conclusion

Investigating the expression of the HSP70-dependent protein reveals various stimulating mechanisms in plants in stress. The measured changes in the metabolic and physiological status of *Echinacea purpurea* have led to corrective reactions in response to stress conditions. Finally, it can be concluded that miR398 is a drought-responsive miRNA that may play its effects through leaf development control. This could be an important aspect for future studies, because increasing leaf biomass in conditions that have water constraints can be an incentive to use purple coneflower as a plant for medicine.

5. References

Accerbi, M., Schmidt, S. A., De Paoli, E., Park, S., Jeong, D.H. and Green, P. J. 2010. Methods for isolation of total RNA to recover miRNAs and other small RNAs from diverse species. *Methods in Molecular Biology*, 592:31-50.

Ceccarelli, S. 1997. Adaptation to low/high input cultivation. Adaptation in plant breeding: Springer, Dordrecht.

Frazier, T.P., Sun, G., Burklew, C.E. and Zhang, B. 2011. Salt and drought stresses induce the aberrant expression of microRNA genes in tobacco. *Molecular Biotechnology*, 49: 159-165.

Gruenwald, J., Brendler, T. and Jaenicke, C. 1998. PDR for herbal medicines. Medical Economics Co, New Jersy, USA.

Guan, Q., Lu, X., Zeng, H., Zhang, Y. Zhu, J. 2013. Heat stress induction of miR398 triggers a regulatory loop that is critical for thermotolerance in Arabidopsis. *The Plant Journal*, 74: 840-851.

Kantar, M., Lucas, S.J. and Budak, H. 2011. miRNA expression patterns of *Triticum dicoccoides* in response to shock drought stress. *Planta*, 233: 471-484.

Khalaji, F., Shiran, B. and Rafiei, F. 2013. Investigation of microRNA398 expression pattern in response to salinity stress in sunflower plant. 1th National Conference on Salinity in Plants and Agricultural Development Strategies. Tabriz, Shahid Madani University of Azerbaijan (In Persian).

Kotak, S., Larkindale, J., Lee, U., von Koskull-Döring, P., Vierling, E. and Scharf, K.D. 2007. Complexity of the heat stress response in plants. *Current Opinion in Plant Biology*, 10: 310-316.

Lawlor, D.W. 2012. Genetic engineering to improve plant performance under drought: physiological evaluation of achievements, limitations, and possibilities. *Journal of Experimental Botany*, 64: 83-108.

Liu, H., Jiang, D., Chi, F. and Zhao, B. 2012. The Hippo pathway regulates stem cell proliferation, self-renewal, and differentiation. *Protein Cell*, 3: 291-304.

Liu, D., Song, Y., Chen, Z. and Yu, D. 2009. Ectopic expression of miR396 suppresses GRF target gene expression and alters leaf growth in Arabidopsis. *Physiologia Plantarum*, 136: 223-236.

Lu, S., Sun, Y.H., Shi, R., Clark, C., Li, L. and Chiang, V.L. 2005. Novel and mechanical stress–responsive microRNAs in *Populus trichocarpa* that are absent from Arabidopsis. *The Plant Cell*, 17: 2186-2203.

Mirlohi, S.H. 2013. Identification and investigation of microRNAs involved in drought stress and related genes in sunflower. Master's thesis, Faculty of Agriculture, Shahrekord University, Iran (In Persian).

Mittal, D., Mukherjee, S.K., Vasudevan, M. and Mishra, N.S. 2013. Identification of tissue-preferential expression patterns of rice miRNAs. *Journal of Cellular Biochemistry*, 114: 2071-2081.

Mittler, R. and Zilinskas, B. 1992. Molecular cloning and characterization of a gene encoding pea cytosolic ascorbate peroxidase. *Journal of Biological Chemistry*, 267: 21802-21807.

Reinhart, B.J., Weinstein, E.G., Rhoades, M.W., Bartel, B. and Bartel, D.P. 2002. MicroRNAs in plants. *Genes and Development*, 16: 1616-1626.

Reyes, J.L. and Chua, N.H. 2007. ABA induction of miR159 controls transcript levels of two MYB factors during Arabidopsis seed germination. *The Plant Journal*, 49: 592-606.

Safarzadeh, M. 2012. Wheat leaf miRNA analysis in response to water stress. Master's thesis, Faculty of Agriculture and Natural Resources. University of Zanjan. (In Persian).

Sato, Y. and Yokoya, S. 2008. Enhanced tolerance to drought stress in transgenic rice plants overexpressing a small heat-shock protein, sHSP17. 7. *Plant Cell Reports*, 27: 329-334.

Schmittgen, T.D. and Livak, K.J. 2008. Analyzing real-time PCR data by the comparative CT method. *Nature Protocols*, 3: 1101-1108.

Sunkar, R., Kapoor, A. and 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. *The Plant Cell*, 18: 2051-2065.

Trindade, I., Capitão, C., Dalmay, T., Fevereiro, M.P. and Dos Santos, D.M. 2010. MiR398 and miR408 are up-regulated in response to water deficit in *Medicago truncatula*. *Planta*, 231: 705-716.

Varkonyi-Gasic, E., Wu, R., Wood, M., Walton, E.F. and Hellens, R.P. 2007. Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. *Plant Methods*, 3: 12-23.

Zhou, M., Li, D., Li, Z., Hu, Q., Yang, C., Zhu, L. and Luo, H. 2013. Constitutive expression of a miR319 gene alters plant development and enhances salt and drought tolerance in transgenic creeping bentgrass. *Plant Physiology*, 161:1375-1391.

Zhu, H., Hu, F., Wang, R., Zhou, X., Sze, S.H., Liou, L.W., Barefoot, A., Dickman, M. and Zhang, X. 2011. Arabidopsis Argonaute 10 specifically sequesters miR166/165 to regulate shoot apical meristem development. Cell, 145: 242-256.