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Expression Patterns of Drought-related miRNAs in Chickpea (*Cicer arietinum* L.) under Drought Stress

Reda M. Gaafar#, Maha M. Seyam, Adel R. El-Shanshory
Botany and Microbiology Department, Faculty of Science, Tanta University, Tanta, 31527, Egypt.

DROUGHT is a prominent abiotic stress that has a global impact on crop yields. MicroRNAs have been shown to be significant modulators of plant drought tolerance. The impact of drought stress on three chickpea varieties (Giza 1, Giza 2, and Giza 3) and the expression profiles of miRNA genes were evaluated at various levels of polyethylene glycol (PEG) (0, 10%, 20%, and 30%). Increased PEG levels reduced germination percentage, root and shoot length, relative water content (RWC%), and photosynthetic pigments. The highest PEG level (30%) resulted in a significant decrease in germination percent, root, and shoot lengths. The largest reduction in germination percentage and root length was detected in Giza 2, whereas Giza 3 exhibited the highest germination percentage and root length. In addition, the highest RWC% was detected in Giza 3, whereas the lowest was recorded in Giza 2. These results suggest that Giza 3 is the most drought-resilient, whereas Giza 2 is the most drought-sensitive. The expression profiles of five microRNA (*miRNA*) genes in Giza 2 and Giza 3 revealed that at high drought stress (30% PEG), five *miRNA* genes were significantly reduced in both the roots and shoots of Giza 2, except *miR408b*, which was increased by 1.17-fold in the Giza 2 shoots. In contrast, they showed a significantly higher fold-change in both the roots and shoots of Giza 3, except for *miR104*, which was down-regulated in the roots of Giza 3. These findings suggest that up-regulated miRNA genes play a significant role in the drought tolerance of the chickpea Giza 3 variety.

Keywords: Abiotic stress, Drought tolerance, miRNA, Growth, Polyethylene glycol (PEG).

Introduction

Chickpea (*Cicer arietinum* L.) is among the first leguminous plants to be domesticated by man and it is grown widely in arid and semi-arid regions of the Americas, Asia, Australia, East Africa, the Middle East, and the Mediterranean area (Jayashree et al., 2005). Chickpea seeds are abundant in protein (20.6%) and carbohydrates (61.2%), and they also contain 2.2% fat (Gupta, 1987). The dramatic increase in global population requires an increase in the total yield of legume crops to satisfy the demand for protein, especially in areas of chickpea cultivation, such as recently reclaimed lands and in drought and salinity environments (Kandil et al., 2012). Chickpeas have been categorized as a low water-demanding legume with an ability to adapt to dry climates; however, for crop establishment, it is largely dependent on seed germination potential because of the scarcity of water (Arjenaki et al., 2011; Koskosidis et al., 2020).

Drought, like salinity, has an impact on germination rates and seedling growth (Van den Berg & Zeng, 2006). As drought stress increases, the availability of water decreases, affecting seed germination, and the growth of seedlings. The establishment of seedlings is an important undertaking in dry environments, and the scarcity of moisture in the soil is often a key factor in the death of seedlings (Schütz et al., 2002). Throughout their lifecycle, drought stress adversely affects crops and causes yield losses, the degree of which varies greatly depending on

#Corresponding author email: redagaafar@science.tanta.edu.eg Mobile phone: 01062922133
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the duration and intensity of the stress, genetic history, and growth phase. Drought stress reduces the yield and biomass of chickpeas (Leport et al., 2006) and decreases the relative water content (RWC%) (Chandrasekar et al., 2000). Polyethylene glycol (PEG) is a non-ionic water polymer that does not permeate quickly into plant tissues and is extensively used to produce water stress (Nepomuceno et al., 1998).

MiRNAs are a group of non-coding short RNAs with an average length of 21 nucleotides that influence many plant biological processes including growth, environmental stress response, and protein catabolism (Vaucheret, 2006; Hu et al., 2013). The first plant miRNA was detected in *Arabidopsis thaliana* in 2002 using a cloning approach (Reinhart et al., 2002; Morad et al., 2016). Subsequent studies have revealed that miRNAs affect target genes by inhibiting transcription or translation (Chen, 2004; Kim, 2005) and interact with growth regulating signaling networks (Wang et al., 2005; Liu & Chen, 2010; Chen et al., 2011). Stress-responsive miRNAs regulate plant growth and development under stress, which suggests that these miRNAs play a pivotal role in plant adaptation to stressful situations. Multiple stress-specific miRNAs have been discovered in model plants under a range of biotic and abiotic stress conditions including high salinity (Zhao et al., 2009), drought (Zhao et al., 2013, Zhang et al., 2011; Ferdous et al., 2015), cold (Zhou et al., 2008), and nutrient deficiency (Fujii et al., 2005; Liu et al., 2014; Zhou et al., 2007). In the present study, we identified the most drought-tolerant and drought-sensitive chickpea varieties, and established expression profiles for five miRNAs linked to PG-induced drought stress.

Materials and Methods

The seeds of three Giza 1, Giza 2, and Giza 3 chickpea varieties were procured from the Agriculture Research Center, Giza, Egypt.

Germination percentage

Prior to germination, homogeneous seeds of three Giza 1, Giza 2, and Giza 3 chickpea plants were selected, surface-sterilized in 30% (v/v) H_2O_2 for 20min, then rinsed several times with distilled water. The seeds were incubated in distilled water at room temperature overnight. The next day, the seeds were germinated in Petri-dishes (100mm in diameter) on Whatman filter paper (90mm) moistened with 10mL of various concentrations (10%, 20%, and 30%) of polyethylene glycol (PEG, 4000) for six days at room temperature (27°C ± 1). Then, the germination percentage was calculated for each variety at different PEG concentrations.

Plant growth parameters

To estimate root and shoot length, seeds were germinated in sterilized Petri-dishes on Whatman filter paper moistened with 10mL distilled water for six days at room temperature (27°C ± 1) as described above, then 10 seedlings of each variety were transferred into Petri-dishes containing Whatman filter paper moistened with either distilled water (control) or three different concentrations of PEG (10%, 20%, and 30%) and allowed to grow for four days at room temperature (27°C±1). Three replicates were performed for each PEG level per variety. Finally, the root and shoot lengths were recorded using a ruler to identify the most tolerant variety of chickpea under drought stress conditions.

RWC%

According to Turner (1981), RWC% was calculated using the following equation:

$$RWC\% = [(FW-DW)/(TW)]\times 100$$

where, FW represents the fresh weight of the shoots, TW is the weight of full turgor shoots, measured after floating for 24h in distilled water. DW refers to dry weight, which was determined after drying the shoots at 70°C until reaching a constant weight.

Photosynthetic pigments

The content of chlorophyll a and b, total chlorophyll (a + b), and carotenoids was estimated according to Arnon (1949). Total chlorophyll was extracted from 200mg of fresh leaf tissue after grinding in five ml (80%) of acetone using a mortar and pestle. The extract was then centrifuged for 10min at 5000g using a cooling centrifuge (Sigma 3-30KS, Germany). The supernatant was then collected and its absorbance (OD) was recorded spectrophotometrically at 663 and 646nm using a LaboMed Spectro UV-VIS Dual beam-UVS-2700 (USA). Chlorophyll A, B, and total chlorophyll content was determined by the following equations:

$$\text{Chlorophyll a } (\mu g\ g^{-1}\ FW) = 12.21\ OD_{663} - 2.81\ OD_{646}$$

$$\text{Chlorophyll b } (\mu g\ g^{-1}\ FW) = 20.13\ OD_{646} - 5.03\ OD_{663}$$

Total chlorophyll (µg g^{-1} FW) = Chlorophyll a + Chlorophyll b

Carotenoids (µg g^{-1} FW) were quantified at 470nm according to Lichtenthaler & Wellburn (1983) using the following formula:

Carotenoids (µg g^{-1} FW)= (1000 OD_{470} – 3.27 Chlorophyll a– 104 Chlorophyll b) / 229

Quantitative Real-time PCR (qRT-PCR) analysis

Total RNA Extraction

Total RNA was extracted from the roots and shoots of both Giza 2 and Giza 3 after exposing them with different PEG concentrations for four days using Trizol reagent® (Sigma-Aldrich, USA). Both roots and shoots (200mg) were placed into 2mL Eppendorf tubes and stored at −80°C for 24h. Then, they were ground with a mortar and pestle in 1mL of Trizol. The samples were vortexed, incubated at room temperature (RT) for 10min, and centrifuged at 13,000rpm for 10min at 4°C. The supernatant was collected, transferred to a fresh 2mL tube, and 200µL chloroform were added. The tubes were vortexed for 15sec and centrifuged at 13,000rpm for 15min at 4°C. The upper aqueous phase was collected, 600µL of isopropanol was added, mixed gently, and incubated at RT for 10min. The mixture was transferred to a spin column and centrifuged at 13,000rpm for 10min at 4°C. The supernatant was discarded and the column was washed with 500µL of RNase-free EtOH (70%). Then, the column was centrifuged at 1000rpm for five min, washed with 500µL RNase-free EtOH (70%), and left to dry for 10min. Total RNA was eluted using 30µL of sterile RNase-free water by centrifugation at 1000rpm for five min. The total RNA level and purity were estimated using a Nanodrop Spectrophotometer (Thermo Fisher Scientific, USA).

cDNA synthesis

Using the HiSenScript™ RH(-) cDNA Synthesis Kit (INTRON Biotechnology, USA), first-strand cDNA was synthesized following the manufacturer's protocol. The reverse transcription reaction was carried out in a 20 µl reaction mixture at 42°C for one hour, then the reverse transcriptase was deactivated at 85°C for 10min. The cDNA samples were stored at −20°C until they were used.

qRT- PCR and miRNA gene expression

Quantification of gene expression was carried out using TopPreal™ QPCR 2X PreMIX (SYBR Green with low ROX) in a 20µL reaction volume. Each reaction contained the following: 1µL (10pmol) of each primer, 2µL of template cDNA, and 6µL RNase-free water. The Rotor gene 5 plex (Qiagen, Germany) was used for PCR. The cycling conditions included an initial denaturation at 98°C for 10min, annealing at 60°C for 15sec, and elongation at 72°C for 30sec for 45 cycles. The gene expression analysis was done at the Central Laboratory at Tanta University.

The expression of five miRNA genes (*miR156a*, *miR166h*, *miR408b, miR71, miR104*) specific for chickpea was determined. The *OsU6* gene was used as an internal control and the measurements were done in triplicate. Table 1 lists the primer sequences used for the qRT-PCR analysis. The expression level of the five selected miRNA genes was first normalized with the *OsU6* gene and then expressed relative to the control by calculating the fold-change in expression using the method described by Livak & Schmittgen (2001).

TABLE 1. Names (primer IDs), sequences and primer lengths of five drought-related microRNA (miRNA) genes, reference gene (OsU6) and the universal reverse primer used in Quantitative Real Time PCR (qRT-PCR) (Hu et al. 2013)

miRNA ID	Forward primer	Primer length (bp)
miR156a	5'-CGGCATGACAGAAGAGAGT-3'	19
miR166h	5'-GTAATACTTCGGACCAGGCT-3'	20
miR408b	5'-ACATATTGCACTGCCTCTTC-3'	20
miR71	5'-TATGTTGGGTCGATCGGT-3'	18
miR104	5'-ACATATTGCACTGCCTCTTC-3'	20
OsU6	5'-GGGGACATCCGATAAAATTGG- 3'	21
Universal reverse primer	5'-CCAGTGCAGGGTCCGAGGTA-3'	20

Statistical analysis

The significance of the difference between the mean values derived from three separate experiments was calculated using a one-way analysis of variance at a 95% confidence interval. The standard deviations among means were calculated using GraphPad Prism ver. 7 software. Graphical analysis was performed using Microsoft Excel and a heatmap of gene expression was constructed using RStudio. Drought tolerance indices (DTIs) were estimated for each measured trait as the percentage of plants under stress relative to the mean of the control plants.

Results

To determine the most drought-tolerant and -sensitive chickpea varieties among the three varieties (Giza 1, Giza 2, and Giza 3) in Egypt, seeds were grown under drought stress conditions generated by different PEG levels (10%, 20%, and 30%). Different growth parameters including germination percent, root, and shoot lengths, and RWC% were measured. In addition, photosynthetic pigment, chlorophyll a, b, total chlorophyll (a + b), and carotenoid content were estimated.

Germination percentage

The germination percentage of three Egyptian chickpea varieties under the influence of different PEG concentrations is shown in Fig. 1. Seeds were considered to be germinated when approximately two mm of the radicle had spread out. The results showed that the germination of all varieties began on the 10th day of sowing and reached 100% germination for the control seeds. In contrast, the germination percentage of the three chickpea varieties decreased with increasing PEG concentrations (10, 20, and 30%). Giza 3 exhibited the highest germination percentages at 80% (0.8 DTI), 43.33% (0.43 DTI), and 6.67% (0.066 DTI), whereas Giza 2 had the lowest percentages at 56.67% (0.56 DTI), 26.67% (0.26 DTI), and 0% (0.0 DTI). This indicates that Giza 3 is the most drought-tolerant variety among the chickpea varieties.

Plant growth

An increase in PEG concentration resulted in a gradual decrease in root and shoot lengths of the three examined varieties compared with the control plants (Fig. 2). The highest PEG level (30%) resulted in a significant reduction in the length of both roots and shoots in all treated varieties. However, an insignificant decrease in root length was observed in the three treated chickpea varieties at 10% and 20% PEG, except for the Giza 1 variety. Also, an insignificant decrease in shoot length was observed at 10% and 20% PEG in Giza 2 and Giza 3, respectively. It was obvious that shoot length was decrease markedly when compared with root length in all chickpea varieties compared with control plants.

RWC%

As shown in Fig. 3, a steady decrease in RWC percentage in all treated varieties after four days of PEG treatment was observed with increasing PEG concentration. The decline in RWC percentage was highly significant ($P< 0.01$) in all treated chickpea varieties compared with the controls. At 30% PEG, the highest RWC percentage was observed in the Giza 3 variety (70.7%), followed by Giza 1 (68.7%), whereas the lowest RWC percentage was observed in Giza 2 (65.1%). These results also suggest that Giza 3 is the most drought-tolerant variety among these chickpea varieties.

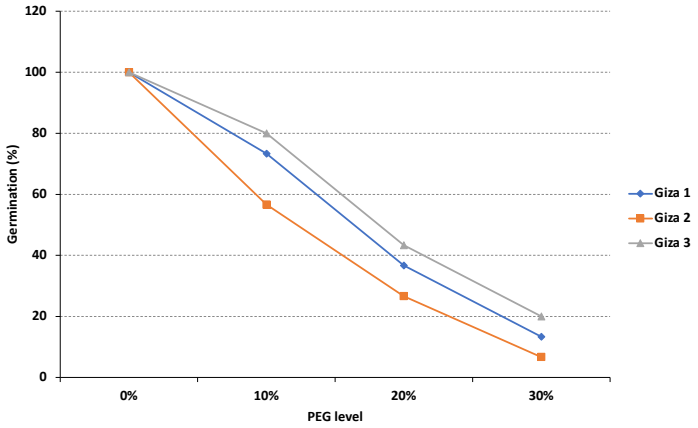

Fig. 1. Impact of different levels of PEG on the germination percentage of the three Egyptian chickpea varieties

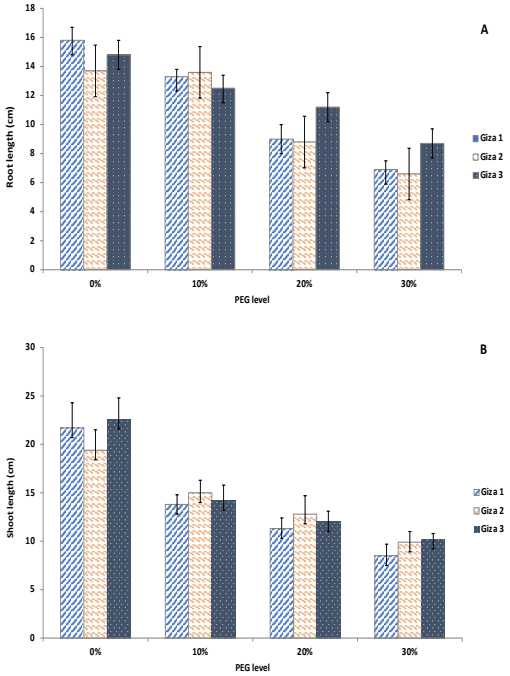

Fig. 2. Impact of drought stress induced by various PEG levels on root length (A) and shoot length (B) of the three Egyptian chickpea varieties

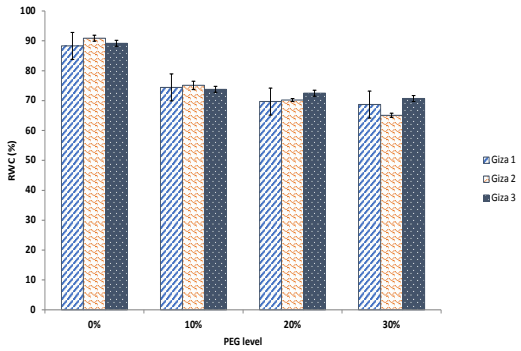

Fig. 3. Impact of drought stress induced by various PEG levels on the relative water content (RWC %) of the three Egyptian chickpea varieties.

Photosynthetic pigments

After treatment with different concentrations of PEG for four days, chlorophyll a, b, total chlorophyll, and carotenoids were estimated for the three chickpea varieties. Table 2 shows that at 10 % PEG, Giza 1 exhibited a significant ($P< 0.01$) augmentation in chlorophyll A and total chlorophyll, whereas Giza 2 and Giza 3 exhibited an insignificant increase in chlorophyll A, B, and total chlorophyll. The Giza 3 control showed the highest levels of chlorophyll A, B and total chlorophyll (27.12, 11.05, and 38.9µg g^{-1} FW, respectively), followed by Giza 2 (20.1, 5.9, and 24.9µg g^{-1} FW, respectively), whereas Giza 1 exhibited the lowest (18.3, 4.9 and 23.2µg g^{-1}, respectively).

At high PEG concentrations (30%), there was a significant decrease ($P< 0.01$) in chlorophyll A, B, and total chlorophyll content for Giza 1, Giza 2, and Giza 3. The highest level of chlorophyll A at 30% PEG was observed in Giza 3 (21.35µg g^{-1} FW), followed by Giza 1 (8.17µg g^{-1} FW), and Giza 2 (5.4µg g^{-1} FW). Conversely, a high content of chlorophyll B was detected in Giza 3 (7.47 µg g^{-1} FW), followed by Giza 1 (1.72µg g^{-1} FW), and Giza 2 (1.58µg g^{-1} FW). Similarly, total chlorophyll content was highest in Giza 3 (29.2 µg g^{-1} FW), followed by Giza 1 (10.2µg g^{-1} FW), and Giza 2 (7.2µg g^{-1} FW).

We also observed that Giza 1 seedlings showed a decrease in carotenoid content (1.81µg g^{-1} FW) which was significant ($P< 0.05$) at 20% PEG, whereas an increase (3.24µg g^{-1} FW) at high PEG levels (30%) was observed (Table 2). At high PEG levels (30 %), Giza 2 showed a decrease in carotenoid content (2.03µg g^{-1} FW). The highest carotenoid content at high PEG levels (30%) was detected for Giza 3, followed by Giza 1 and Giza 2 (4.83, 3.24 and 2.03µg g^{-1} FW), respectively, when compared with the control.

qRT-PCR analysis of miRNA genes

The relative expression (fold-change) of five miRNAs (*miR71, miR104, miR156a, miR166h, and miR408b*) associated with drought stress in two Egyptian chickpea varieties, Giza 2 (drought-sensitive) and Giza 3 (drought-tolerant) in the roots and the shoots are shown in Figs. 4 and 5, respectively.

The relative expression of the five genes was significantly ($P< 0.01$) down-regulated in the roots of Giza 2 at all PEG concentrations (10, 20, and 30%) compared with the control plants (Fig. 4**)**, whereas in Giza 3 only, the expression of *miR104* was significantly ($P< 0.01$) decreased (Fig. 4B). In addition, the expression of *miR71*, *miR104*, *miR156a*, *miR166h*, and *miR408b* was significantly ($p \leq 0.01$) down-regulated at 20% PEG in Giza 3 by 0.11 and 0.95, 0.89, 0.86, and 0.17 fold, respectively. Interestingly, the expression of *miR71*, *miR156a*, *miR166h*, and *miR408b* was significantly increased by 5.05, 3.27, 2.45 and 1.45-fold, respectively, at 30% PEG compared with the control plants (Fig 4A, C, D, and E).

TABLE 2. Impact of drought stress imposed by different PEG levels on photosynthetic pigments (chlorophyll a, b, total chlorophyll and carotenoids) content of the three Egyptian chickpea varieties

Variety	PEG levels	Chl. a (µg g^{-1} FW)	Chl. b (µg g^{-1} FW)	Total chl. content (µg g^{-1} FW)	Carotenoids (µg g^{-1} FW)
Giza 1	0%	18.3 ± 0.4	4.9 ± 0.4	23.2 ± 0.9	4.74 ± 0.5
	10%	19.9 ± 0.4**	6.62 ± 0.6	27.1 ± 1.1**	4.38 ± 0.5
	20%	7.15 ± 0.1**	3.29 ± 0.3	10.6 ± 0.4**	1.81 ± 0.2 **
	30%	8.17 ± 0.2**	1.72 ± 0.2**	10.2 ± 0.4**	3.24 ± 0.3
Giza 2	0%	20.1 ± 0.5	5.9 ± 0.4	24.9 ± 1	4.44 ± 0.5
	10%	19.16 ± 0.5	6.62 ± 0.5	26.3 ± 1.1*	4.45 ± 0.5
	20%	12.9 ± 0.2**	3.19 ± 0.4**	16.36 ±0.6**	4.43 ± 0.5
	30%	5.4 ± 0.09**	1.58 ± 0.2**	7.2 ± 0.2**	2.03 ± 0.2*
Giza 3	0%	27.12 ± 0.7	11.05 ± 0.8	38.9 ± 1.6	3.45 ± 0.4
	10%	27.28 ± 0.7	10.9 ± 0.8	39.1 ± 1.6	3.54 ± 0.5
	20%	21.91 ± 0.2**	7.43 ± 0.9	29.6 ± 1.2**	5.01 ± 0.5
	30%	21.35 ± 0.3**	7.47 ± 0.8	29.2 ± 1.2**	4.83 ± 0.6

Each value is a mean of three replicates ± standard error of the mean

* Significant at P value < 0.05, ** Significant at P value < 0.01.

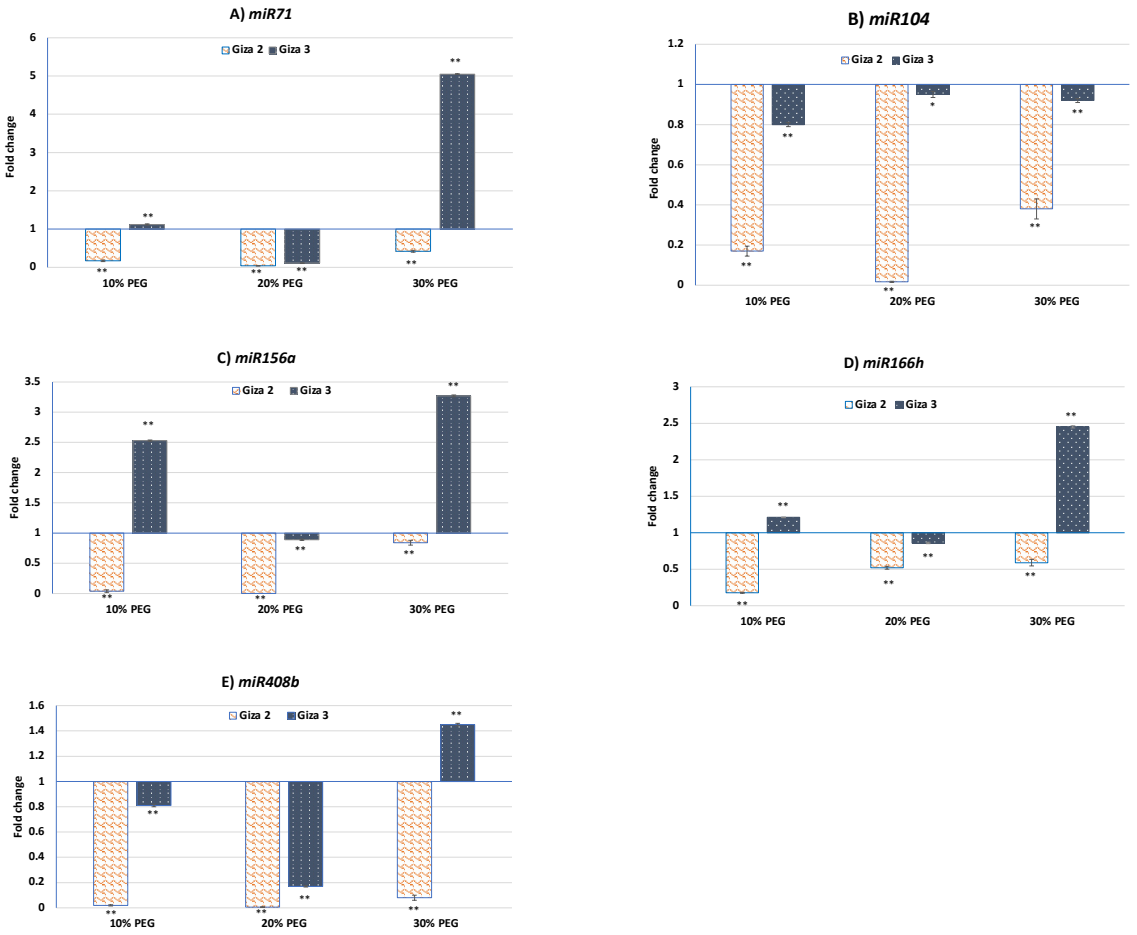

Fig. 4. The relative expression (fold change) of five microRNA (miRNA) genes in the roots of two chickpea varieties (Giza 2 and Giza 3) seedlings under drought stress imposed by different PEG levels (10, 20, and 30%) after four days of treatment. miRNA156a (A), miRNA166h (B), miRNA408b (C), miRNA71 (D), miRNA104 (E)

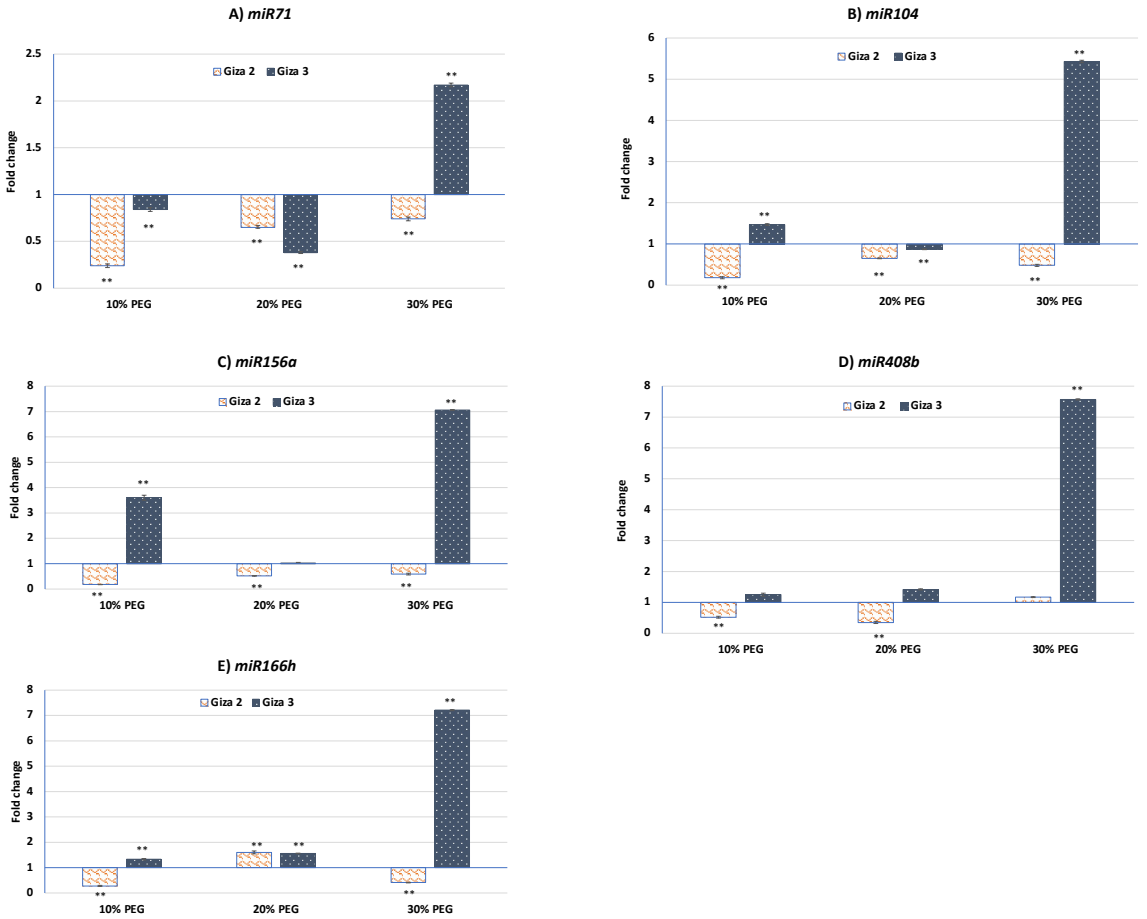

Fig. 5. The relative expression (fold change) of five microRNA (*miRNA*) genes in the shoots of two chickpea varieties (Giza 2 and Giza 3) seedlings under drought stress imposed by different PEG levels (10, 20, and 30%) after four days of treatment. *miRNA156a* (A), *miRNA166h* (B), *miRNA408b* (C), *miRNA71* (D), *miRNA104* (E)

Similar to that shown in the roots of the Giza 2 plants, the expression of drought-related miRNA genes was significantly (p £ 0.01) down-regulated in the shoots of Giza 2 plants at all PEG levels (Fig. 5), except for *miR166h* and *miR408b*, which were significantly up-regulated by 1.6 (20% PEG) and 1.17 (30% PEG) fold, respectively (Fig. 5D and E). Similarly, the relative expression of drought-related miRNA genes was up-regulated in the Giza 3 shoots at all PEG levels except for *miR71* and *miR104*, which were down-regulated by 0.84 and 0.38 (10% and 20% PEG) and 0.87 (20% PEG), respectively (Fig. 5A and B). The highest relative gene expression values (2.17, 5.43, 7.06, 7.57, and 7.21-fold) were observed in the shoots of Giza 3 plants at 30% PEG with *miR71, miR104, miR156a*, *miR166h*, and *miR408b* genes, respectively (Fig. 5A, B, C, D, and E) compared with the control plants.

Correlation analysis

Pearson's correlation analysis was done to find a relationship among the biological traits and different PEG concentrations. The high level of PEG negatively and significantly affected various morpho-physiological traits (germination %, shoot length, RWC%, and chlorophyll content) of Giza 2 and Giza 3. A heatmap of drought-related *miRNA* gene expression, with different colors showing various degrees of gene expression is shown in Fig 6. The dark blue color indicates the down-regulated genes (fold-change), whereas the yellow color displays genes with a medium fold-change. In contrast, the red color indicates the highest gene expression (highest fold-change). The relationship between various *miRNA* gene expression is also presented in Figure 6, in which clustering between *miRNA* gene expressions at 10% and 20% PEG cluster together (Fig. 6).

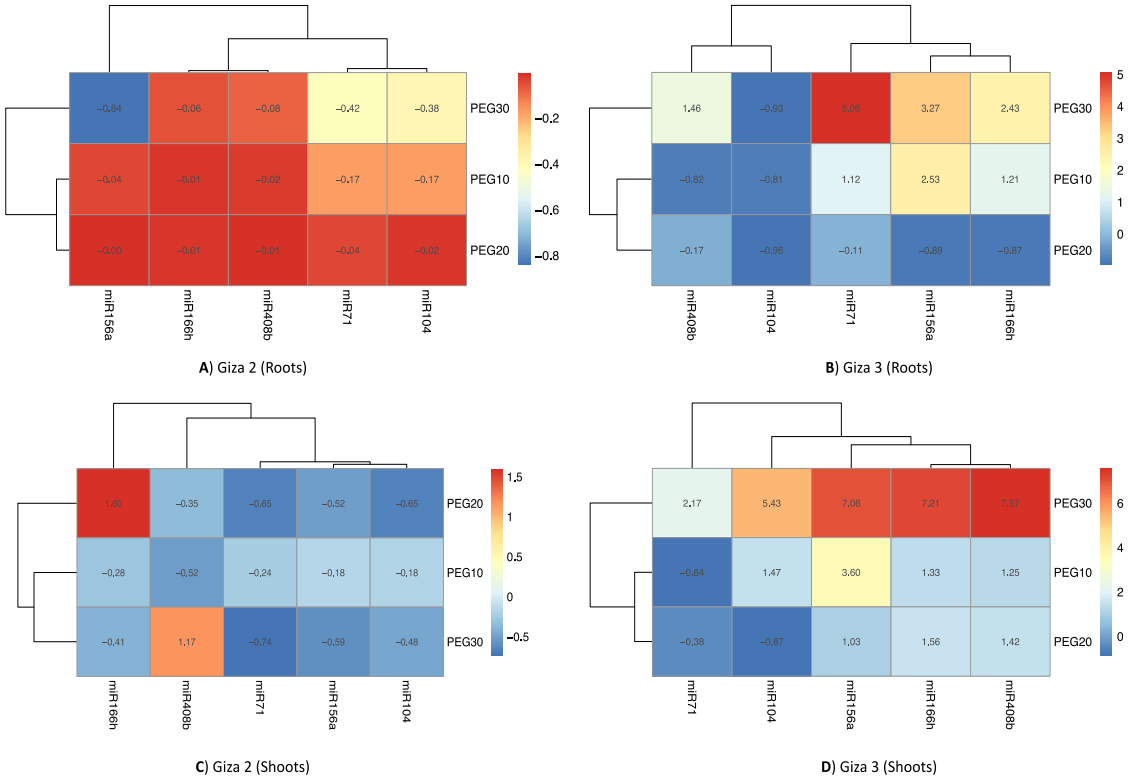

Fig. 6. Heatmap of the relative expression and relationship of five microRNA (*miRNA*) genes in the roots (A & B) and shoots (C & D) of two chickpea varieties (Giza 2 and Giza 3) seedlings under drought stress imposed by different PEG levels (10, 20, and 30%) after four days of treatment

Discussion

Drought is undeniably one of the most significant environmental stresses that limits plant yield and productivity worldwide (Bohnert et al., 1995). The morpho-physiological results of this study indicated that under drought stress conditions (PEG treatment), the Giza 3 variety is the most drought-tolerant chickpea plant, whereas Giza 2 is the most drought-sensitive. Seed germination is the most important stage in seedling establishment as it determines crop yield (Almansouri et al., 2001). It has been shown that drought stress (Wilson et al., 1985; Okçu et al., 2005) and high salinity (Perry, 1984; Sadeghian & Yavari, 2004) are the two primary factors that adversely affect seed germination. In the present study, the germination percentage of chickpea varieties was decreased concomitantly with elevated PEG levels. This is in agreement with the results of earlier studies showing that drought significantly affects plant seed germination in chickpeas. Koskosidis et al. (2020) found that seed germination of ten chickpea varieties was significantly affected by PEG-induced drought stress. In addition, Macar et al. (2009) studied the impact of water deficit produced by PEG as well as NaCl on chickpea varieties at an early seedling stage. They reported that PEG was more efficient at inhibiting germination percentage than NaCl under the drought conditions used. Moreover, the results of this study agree with those of Muscolo et al. (2014), who found a remarkable decrease and delayed germination in lentil varieties during PEG-induced drought stress.

Early and rapid elongation of the root is a well-known indicator of drought tolerance. A root system with a longer root length at a deeper depth is beneficial for water extraction in upland environments (Kim et al., 2001). In the present study, the root and shoot lengths decreased with increasing PEG levels, and the reduction in shoot length was more apparent than that of root length, particularly in drought-sensitive chickpea varieties, Giza 1 and Giza 2. This finding is consistent with that of Macar et al. (2009) who found that drought stress caused by PEG impeded epicotyl elongation more than root growth, lowering the shoot/root ratio. In

contrast, drought stress affected root length more than shoot length in pearl millet, according to Govindaraj et al. (2010), and root length decreased considerably with higher external water in all genotypes examined compared with the controls. Furthermore, our findings agree with those of Khodarahmpour (2011), who found that root length is an important indicator of plant response to drought stress and that all drought-stressed corn hybrids exhibited a distinct decrease in root, shoot, and seedling length.

Under drought stress, RWC normally decreases, but not always, as drought-resistant wheat varieties have a higher percentage of RWC (Schonfeld et al., 1988; Keyvan, 2010). The results of this study showed that RWC declined with increasing PEG levels. Conversely, at high PEG levels (30%), Giza 3 exhibited the highest RWC percentage, whereas Giza 2 showed the lowest. When studying the performance of chickpea cultivars under drought stress and non-stress conditions, Khodadadi (2013) found that drought stress lowered the RWC% in chickpea genotypes, which is similar to what we observed in this study. In the case of beans, Korir et al. (2006) reported similar results.

Our findings indicate a highly significant reduction in chlorophyll A, B, and total chlorophyll in three chickpea varieties, Giza 1, Giza 2, and Giza 3, at high PEG concentrations. The highest levels of chlorophyll A, chlorophyll B, and total chlorophyll were observed in Giza 3. Similarly, Talebi et al. (2013) found that total chlorophyll content was significantly lower in 35 chickpea genotypes during drought stress, but the decrease was insignificant in the tolerant genotypes. Our results are also consistent with those of Mafakheri et al. (2010), who reported that drought stress significantly decreased chlorophyll A and B, and total chlorophyll content at the vegetative and flowering stages in three chickpea varieties. Furthermore, our findings are similar with the results of Nyachiro et al. (2001), who found that water deficit in six *Triticum aestivum* L. varieties resulted in a reduction of chlorophyll a and b content. Carotenoids have an important function as photoprotective compounds by quenching triplet chlorophyll II and singlet oxygen derived from extra light energy, thus preventing membrane damage (Talebi et al., 2013). In the present study, a high carotenoid content was found in Giza 3 at 30% PEG compared with the control and the other two chickpea varieties. This result is consistent with that of Khan et al. (2002) and Gunes et al. (2008), who demonstrated that drought-tolerant cultivars retained higher levels of carotenoids compared with susceptible ones.

Plants have developed very intricate and sharpened tactics to adapt to drought stress including altering gene expression, adjusting metabolism, and water rationing to protect cell structures from environmental challenges (Zhu, 2002). miRNAs are a class of short non-coding RNAs that are now recognized as essential regulators of gene expression during plant growth and in response to biotic and abiotic stressors (Huang et al., 2011; Krishnatreya et al., 2021). Only a few studies have examined miRNA expression in various tissues during drought stress (Sunkar et al., 2012). In the present study, we identified differences in miRNA expression profiles in the roots and shoots of chickpeas during drought stress. The results suggest that the relative expression of five miRNAs was significantly down-regulated in the roots of Giza 2 under all PEG levels compared with control plants, whereas in Giza 3, only the expression of *miR104* was significantly down-regulated (Fig. 4B**)**. Interestingly, the expression of *miR71*, *miR156a*, *miR166h*, and *miR408b* was significantly up-regulated (overexpressed) by 5.05, 3.27, 2.45 and 1.45-fold, respectively at 30% PEG compared with the control plants (Fig. 4). Also, the expression of drought-related miRNA genes was significantly decreased in the shoots of Giza 2 plants at all PEG levels (Fig. 5), except for *miR166h* and *miR408b*, which were significantly up-regulated by 1.6-fold (20% PEG) and 1.17-fold (30% PEG), respectively (Fig. 5**)**. In contrast, the relative expression of drought-related miRNAs was increased in the shoots of Giza 3 under all PEG levels, except for *miR71* and *miR104*, which were down-regulated by 0.84 and 0.38 (10% and 20% PEG) and 0.87 (20% PEG), respectively (Fig. 5A and B).

The results of this study agree with previous studies regarding the impact of drought stress upon miRNA gene expression. Several studies have demonstrated that miR156 has agricultural importance for crop development and stress tolerance (Jones-Rhoades & Bartel, 2004; Sunkar & Zhu, 2004). Also, Cui et al. (2014) reported that *miR156* reduces plant sensitivity to salt and drought stress. The results of the above-

mentioned studies are in accordance with the results of Adalı (2015), who confirmed the roles of *miR156* and *miR171* in plant development and stress tolerance in different Turkish tobacco varieties during drought stress. He found that *miR156* was up-regulated in all wild-type tobacco cultivars under all drought stress conditions. In addition, our results are consistent with that of Kantar et al. (2010) in barley, who showed that *miR166* was up-regulated in leaves, but down-regulated in roots. While *miR156a* and *miR408* expression was increased in leaves, it remained unchanged in roots. Moreover, the expression of *miR156* and *miR408* in rice was significantly decreased in response to drought stress (Zhou et al., 2010). In contrast, Morad-Talab & Hajiboland (2016) reported that *miR156* was up-regulated under drought stress in both drought-sensitive and -tolerant cowpea genotypes. However, in this study, *miR156* was increased only in the roots and shoots of the Giza 3 chickpea variety under drought stress.

Furthermore, the *miR408b* gene was up-regulated (overexpressed) in the Giza 3 (drought tolerance) variety by 1.45- and 7.21-fold in roots and shoots, respectively, under drought stress compared with control plants. A similar finding was obtained by Hajyzadeh et al. (2015), who revealed that *miR408* overexpression enhanced drought tolerance in chickpea and up-regulation of *miR408* level was vital to drought tolerance. MiRNAs modulate copper levels in response to stress by targeting some copper-related transcripts. Similarly, a previous study found that miR408 controls the genes that code for copper proteins, suggesting that copper homeostasis and water stress response are linked (Abdel-Ghany & Pilon, 2008; Trindade et al., 2010).

Although thousands of miRNAs have been discovered in a variety of plant species, there have been few studies on chickpea microRNAs. Two conserved miR71 and miR104 microRNAs have been identified in chickpea (Hu et al., 2013). Recently, Fantao et al. (2018) found a drought stress-responsive novel microRNA (*miR71*) in Dongxiang wild rice. They confirmed that the novel *miR71* gene was the most frequently up-regulated miRNA. The results of our study indicated that *miR71* was up-regulated by 5.05-fold in the roots of the Giza 3 (drought-tolerant) variety, which is in agreement with that of Fantao et al. (2018).

Conclusions

Drought tolerance of three chickpea varieties (Giza 1, 2, and 3) was evaluated using morpho-physiological responses to PEG-induced drought. These parameters were significantly reduced with an increase in PEG concentration. The results indicated that Giza 2 is a drought-sensitive variety, whereas Giza 3 is drought-tolerant. Small non-coding RNAs (miRNAs) are known to be essential determinants of gene expression in response to biotic and abiotic stressors.

A qRT-PCR analysis of five drought-related miRNA genes, miR71, and miR104, miR156a, miR166h, and miR408b, from seedlings of two chickpea varieties, Giza 2 and Giza 3, revealed that at high PEG concentration (30%), there was an up-regulation in all miRNA genes tested in Giza 3 (roots and shoots). This demonstrates a key function of these miRNA genes in drought tolerance of the Giza 3 variety.

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التعبير الجيني لجينات الأحماض النووية الريبوسوميه الدقيقة المرتبطة بالجفاف في نبات الحمص تحت إجهاد الجفاف

رضا جعفر، مها صيام، عادل الشنشوري
قسم النبات - كلية العلوم - جامعة طنطا رقم بريدي 72513 طنطا - مصر.

يعد الجفاف أحد أنواع الإجهاد الغير حيوي البارز الذي له تأثير عالمي على إنتاجية المحاصيل. وقد أثبتت الدراسات السابقة أن الأحماض النووية الريبوسوميه الدقيقة تلعب دور هام في تحمل النباتات للجفاف. في هذه الدراسة تم أختبار تأثير إجهاد الجفاف على ثلاثة أصناف من الحمص (جيزة 1، 2 و3) والتعبير الجيني للأحماض النووية الريبوسوميه الدقيقة تحت مستويات مختلفة من البولي إيثلين جليكول (0، 10٪، 20٪، و30٪). وقد وجد ان زيادة تركيز البولي إيثلين جليكول تقلل من نسبة الإنبات، طول الجذر والساق، محتوى الماء النسبي، وأصباغ التمثيل الضوئي. وأظهر أعلى مستوى البولي إيثلين جليكول (30٪) انخفاضا كبيرا في نسبة الإنبات وطول الجذر والساق. وقد لوحظ ان أكبر انخفاض في نسبة الإنبات وطول الجذر كان في الصنف جيزة 2، في حين أظهر صنف جيزة 3 أعلى نسبة إنبات وطول للجذر. بالإضافة إلى ذلك، وجد ان أعلى معدل للمحتوى الماء النسبي كان في صنف جيزة 3، في حين تم تسجيل أقل نسبة في صنف جيزة 2. وتشير هذه النتائج إلى أن صنف جيزة 3 هو الأكثر تحملا للجفاف وأن صنف جيزة 2 هو الأكثر حساسية. وكشفت نتائج التعبير الجيني لخمسة جينات للأحماض الريبوسوميه الدقيقة أنه مع الاجهاد العالي للجفاف (30٪ بولي إيثلين جليكول) ، يقل التعبير الجيني لهذه الجينات بشكل كبير في كل من جذور وساق صنف جيزة 2، باستثناء جين *miR408b* حيث زاد تعبيره الجيني بمقدار 1.17 ضعف. في المقابل، كان هناك تغيرا مضاعف في التعبير الجيني لهذه الجينات في جذور وسيقان صنف جيزة 3، باستثناء جين *miR104* 3. وخلصت النتائج إلى أن زيادة التعبير الجيني لجينات الأحماض الريبوسوميه الدقيقة تلعب دورا مهما في تحمل الجفاف للصنف جيزة 3.