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miRNAs involved in drought stress in Italian ryegrass (*Lolium multiflorum* L.)

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Abstract: miRNAs have been characterized as a regulator of main processes in plants by silencing genes. The functions of microRNAs have been studied in various crops, hovewer, no studies have been observed about miRNAs in Italian rygrass (*Lolium multiflorum* L.) against drought stress. This experiment aimed to reveal the involvement of miRNAs against drought stress in sensitive and tolerant Italian ryegrass genotypes. Four genotypes (G1 and G2 as drought sensitive – G3 and G4 as drought tolerant) were selected. The sensitivities of these genotypes against drought stress were verified by performing growth parameters, relative water and proline contents under normal and drought conditions. The results show that the relative expression of the miRNAs revealed both similarities and differences between sensitive and tolerant Italian ryegrass genotypes. Under drought conditions, significant upregulations (miRNA156i, miRNA845a) and downregulations (miRNA2937, miRNA3980b) were observed in drought tolerant genotypes. Similarly, significant upregulation (miRNA845a) and downregulation (miRNA5636) were observed in drought sensitive genotypes under drought conditions. The results indicate that miRNA3980b, miRNA156i, and miRNA2937 are responsible for drought stress tolerance in tolerant Italian ryegrass. These miRNAs could be used to develop Italian ryegrass plants that tolerate drought stress conditions.

Key words: Abiotic stress, forage crop, transcription regulation

1. Introduction

Drought stress limits plant growth and quality, which eventually affects crop production sustainability (Khandal et al., 2017). Thus, there is a need for tolerant crops that can stand against drought stress without having any quality and yield loss (Demirkol, 2020). In recent studies, stress defense mechanisms can be explained at the molecular level based on stress factors (Kadioglu et al., 2012; Ma et al., 2019).

Small RNAs regulate posttranscriptional gene expression during plant development and various biological functions (Chen, 2009). Increasing evidence shows that plant miRNAs regulate functional genes that are important for development processes, including meristem structure, morphogenesis and responses to biotic and environmental stresses (Sunkar, 2010; Huang et al., 2014). miRNAs, important class of endogenous small RNAs, that are widely distributed in plants are one of the tools used to suppress the level of expression of the target gene under abiotic stress conditions (Liu et al., 2017). The miRNA analyses in various plant species highlight the important roles of miRNAs in regulation of plant responses to biotic and abiotic stresses (Pokoo et al., 2018). Moreover, recent evidence from genomic studies shows that miRNAs play crucial roles in regulating antioxidant defence, ABA response, phosphate synthase mechanism, chlorophyll, anthocyanin and flavonoid contents of plants, which are exposed to drought stress, by modulating the respective target genes (Ding et al., 2013; El Sanousi et al., 2016; Hamza et al., 2016; Balyan et al., 2017).

miRNAs have been shown to play a crucial role in modulation of drought tolerances of plants through controlling the expression of drought responsive genes (Ding et al., 2013). For instance, drought induced miRNAs downregulate their target mRNAs, which potentially suppresses the functional proteins involved in drought response. On the other hand, other miRNAs could be downregulated, resulting in accumulations of their target mRNAs, which can induce the expression of proteins contributing positively to stress adaption. It is noteworthy to mention here that most of the miRNAs involved in drought responsive target genes encode transcription factors, thereby placing miRNAs at the centre of gene regulatory networks (Covarrubias and Reyes, 2010; Ding et al., 2013). Characterization and understanding of the functions of the miRNAs started to increase our knowledge of the complex regulatory networks of miRNA systems. This further allows scientists to deepen their research into organism gene expression and regulation (Liu et al., 2017).

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Italian ryegrass (*Lolium multiflorum* L.) is a widely used forage crop in temperate regions due to its high nutritional value (Pan et al., 2016). It can be utilized as hay, fresh, and silage (Ozelcam et al., 2015). However, one of the drawbacks of the Italian ryegrass is its sensitivity to stress factors such as drought. The decreases in yield and quality were observed in previous studies in Italian ryegrass grown under drought stress conditions (Cyriac et al., 2018; Kemesyte et al., 2017). Due to continuous increase in water-limited areas, drought stress is becoming a most prevalent factor that globally limits agricultural productivity (Basu et al., 2016). Until today, many miRNAs have been determined in various crops; however, the information about the role of miRNAs in Italian ryegrass is insufficient. Solely, a total of 12 miRNAs were identified in perennial ryegrass by Huang et al. (2014). No research has been done on the expressing profiles of these miRNAs under drought conditions.

The aim of this study was to determine the microRNAs involved under drought stress conditions in sensitive and tolerant Italian ryegrass genotypes. This will help in choosing drought stress tolerant genotypes that could be used for further breeding programmes.

2. Materials and methods

2.1. Plant material and miRNAs

Four genotypes (G1 and G2 as drought sensitive – G3 and G4 as drought tolerant) were selected out of 32 Italian ryegrass genotypes collected from Turkey considering the preliminary drought sensitivity study. The preliminary study included drought stress that was created by adding polyethylene glycol at four different concentrations: 0%, 5%, 10%and 20% in petri dishes considering germination and seedling growth parameters. The G1 and G2 genotypes were observed to be the most tolerant against drought stress treatment, while the G3 and G4 were the most sensitive.

The miRNAs (miRNA3980b, miRNA5817, miRNA5636, miRNA396h, miRNA156i, miRNA5543, miRNA845a, miRNA5075, miRNA5021, miRNA156k, miRNA6245, miRNA2937) identified by Huang et al. (2014) were used in order to observe the involvement in drought stress responses in sensitive and tolerant Italian ryegrass genotypes, because these miRNAs have been suggested to be involved in drought stress (Huang et al., 2014; González-Villagra et al., 2017; Noman and Aqeel, 2017; Vakilian, 2020). The sequences of the primers used are listed in Table 1.

Table 1. The sequences of the miRNAs.

miRNA	Primer sequence
miRNA3980b	GUGGCCGAGGCCGUCGCCGUG
miRNA5817	GGAAAUUUGAAAGAAAAAAAUUG
miRNA5636	AUAGCUUGCAGAGCUUGACGG
miRNA396h	UCCACAGGCUUUCUUGAACGG
miRNA156i	UGACAGAAGAGAGUGAGCAC
miRNA5543	UAUGAAUGGUAUAUUUGUUGG
miRNA845a	GGGCUCUGAUACCAAUUGAAA
miRNA5075	UUCUCGUCGCCGCCGUCCGU
miRNA5021	GGAGAAGAAGAAGAAGAAGA
miRNA156k	UGACAGAAGAGAGUGAGCACA
miRNA6245	GGUAUAGGUGUCGGCUAAGCA
miRNA2937	GCCAGAGCUGUUGAAGGAGGG

2.2. Growth and stress conditions

Healthy Italian ryegrass seeds were surface-sterilized using a 70% (v/v) ethanol solution for 5 min, followed by washing at least five times with ddH_2O. Subsequently, the seeds were plated on ½MS medium [contains 1% sucrose and 1% agar (pH 5.8)] (Murashige and Skoog, 1962). The plantlets were transferred to pots grown in a growth chamber that has the following conditions: 16 h photoperiod with an intensity of 100 µmol m^{-2} s^{-1} light and 60% relative humidity at 25 °C. Twenty-eight days old plants that have visually similar were selected, followed by exposing to drought stress by withholding the water supply under the conditions of 25 °C and 100 mmol photons m^{-2} s^{-1} light intensity. Before withholding, the plants were irrigated with an equal amount of water for 7 days, as described by Turner (2019). After 4 and 7 days of withholding, the plants were allowed to recover drought conditions. Drought times (4 and 7 days) were done based on the preliminary study in which the most appropriate maximum limits of drought were determined.

After stress applications, the germination percentages were calculated of the treatments. Afterwards, the roots and shoots of the genotypes were collected. In order to determine the dry weight in addition to the length (cm) of roots and shoots, , the samples were dried in an oven at 70 °C for 2 days. After drying, the dry weights (g/plant) of these parts were also recorded.

2.3. Relative water content and free proline analyses

The water content of each treatment of the each leaf samples were determined by measuring relative water content (Farrant, 2000). RWC was calculated as 100 × (fresh weight – dry weight) / (turgid weight – dry weight). Free proline content was determined by the method described by Bates et al. (1973).

2.4. RNA isolation and real-time (RT) PCR

Total RNA was extracted using miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions (note that this particular kit also allows to

precipitate the small RNA fractions presented). QuantiMir RT Kit (System Biosciences SBI, Tokyo, Japan) was used to synthesize cDNA from the total RNA (5 ug) according to the manufacturers' instructions.

2.5. Expression analysis of the miRNAs

Expression levels of the miRNAs was determined on a semi quantitative RT-PCR using the synthesized cDNAs as templates. The RT-PCR reaction was performed as follows: 2 µL of the cDNA added to 40 µL PCR reaction mixture and amplified with specific miRNA primers (Table 1). The reaction conditions standardized were: 94 °C for 10 min, 30 cycles of 94 °C for 30 s, 58 °C for 1 min and hold at 15 °C. Finally 5 µL of PCR product was run on 10X gel electrophoresis in 5X TBE buffer for 3 h. Transcript levels of the miRNAs were measured relative to the 18S rRNA.

2.6. Expression analysis of the pectinesterase and phosphate synthesis genes

For the expression analysis and to find out the role of pectinesterase for flower development and fertility under drought stress conditions, the gene that is responsible for pectinesterase synthesis under drought conditions were selected (Zhang et al., 2020). The extracted gDNAs were coupled with convergent primers complementary to the pectinesterase gene (F: 5'-TATGCTCGTAAACCTAACCCG-3', R: 5'-TCAATGCAAAATCACCACTCC-3). For the expression analysis and to find out the role of phosphate synthase gene under drought stress conditions, the responsible in the gene that is responsible for phosphate synthase synthesis under drought conditions were selected (Benedetti et al., 2020). The extracted gDNAs were combined with convergent primers complementary to the phosphate synthase gene (F: 5'-ACAGAGGGGCTACATTGCAC-3', R: 5'- CTGCAACTGCTCCAAGTGAA-3). The PCR protocol was; 1 cycle (95 °C for 5 min), 30 cycles (93 °C for 1 min, 59 °C for 30 s), 72 °C for 1 min, and a final extension cycle of 10 min at 72 °C for both of two analyses. The actin gene was used as a reference gene for expression analysis of pectinesterase and phosphate synthesis genes.

2.7. Determination of total anthocyanin contents

Total anthocyanin content was measured by the method as previously described by Ryu and Koh (2018). Twenty-five millimolar potassium chloride (pH1.0) and 0.4 M sodium acetate (pH 4.5) were used as buffer solutions. The extracts were diluted using a buffer solution that has a pH value of either 1.0 or 4.5. Absorbance was read using an UV/visible spectrophotometer (Shimadzu, Kyoto, Japan) at 520 nm and 700 nm. Total anthocyanin content was calculated as mg cyanidin-3-*O*-glucoside equivalents per 100 g samples.

2.8. Determination of total chlorophyll contents

Total chlorophyll contents of the samples were determined according to the method of Whapham et al. (1993). Briefly, fully expanded younger fresh leaves (1.0 g) were extracted with 90% acetone and filtered. Then absorbancies were measured with a UV/visible spectrophotometer (Shimadzu) at 645 and 663 nm. The total chlorophyll content were measured as chlorophyll a+b.

2.9. Determination of total flavonoid contents

Total flavonoid contents of the samples were determined using aluminum chloride colorimetric method, as previously described (Tohidi et al., 2017). Briefly, 125 µL of the extract was added to 75 µL of a 5% $NaNO_2$ solution, followed by incubated at room temperature for 6 min. Afterwards, 150 µL of $AlCl_3$ (10%) was included and incubated for 5 min at room temperature. 750 µL of NaOH (1 M) was then added. The final volume of the solution was brought to 2.5 mL with distilled water. Then it was incubated at room temperature for 15 min. Afterwards the mixture turned pink and the absorbance of the mixture was read at 510 nm using a UV/visible spectrophotometer (Shimadzu). The total flavonoid content (TFC) was calculated as mg of quercetin equivalents (QE) per gram of the extract.

2.10. Statistical analysis

The analyses were carried out in triplicate. SPSS 22 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Tukey's test was performed at the $\alpha = 0.05$.

3. Results and discussion

The performance of G1, G2, G3, and G4 genotypes were evaluated to determine whether they have tolerance or sensitivity against drought stress. The results revealed that drought stress conditions caused more severe declines in germination rates, root lengths, shoot lengths, root dry weights and shoot dry weights in G1 and G2 genotypes, compared to G3 and G4 (Figures 1a–1e). These indicate that drought stress treatments negatively influenced the growth parameters in G1 and G2 genotypes. The declines were reported in root growth of various drought sensitive crops, resulting in low water uptake rates in both the upper and lower soil layers (Huang and Gao, 2000; Ebrahimiyan et al., 2013). However, G3 showed enhanced root dry weight and root length values under 4 days of drought treatment, compared to those measured in their counterparts growing under normal conditions (24% and 15% increase, respectively) (Figures 1b–1d). The increased root length observed in G3 genotype under drought stress conditions suggests that this genotype has the ability to use existing water more effectively. The enhanced root length and biomass are considered to be important defense responses in plants to adapt against drought stress. Similar results were reported in previous studies (Jordan et al., 1983; Kashiwagi et al., 2006; Bothe et al., 2018). The variation in root and shoot growths in response to drought stress observed in the present study strongly suggests high genetic variability among studied Italian ryegrass genotypes.

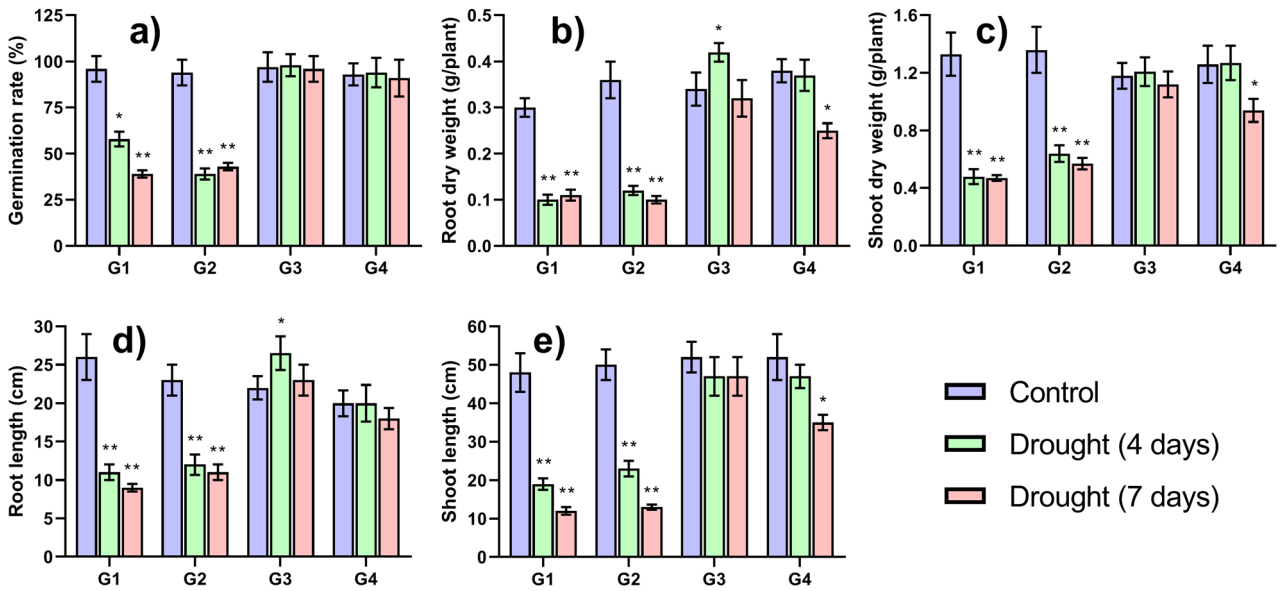

Figure 1. Drought stress tolerance analysis of G1, G2, G3, and G4 genotypes. a) Germination rates, b) root dry weight, c) shoot dry weight, d) root length, e) shoot length. Data were presented as mean ± SD (n = 3). Asterisks indicate the significant differences between control and stressed genotypes at *P < 0.05 or **P < 0.01.

In addition to growth parameters, relative water and free proline contents were measured to determine the drought stress tolerances of the genotypes (Figures 2a and 2b). The accumulation of free proline and the decrease in relative water content are considered as an early response against drought stress. The degree of proline accumulation is strongly correlated with increasing water potential and relative water content (Ranganayakulu et al., 2015). Increasing evidence demonstrates that relative water content and free proline content can be used to distinguish between plants that are tolerant or sensitive to drought stress conditions (Rosales et al., 2013; Zegaoui et al., 2017).

After either 4 or 7 days of drought stresses, G1 and G2 genotypes were found to show less relative water contents (45% and 30 % in G1, 54% and 47% in G2 after 4 days and 7 days drought conditions, respectively), compared to those growing under normal conditions (91% in G1, 88% in G2) (Figure 2a). However, no significant change was observed in G3 and G4 growing either under drought stress or normal conditions (89%, 88%, and 83% in G1, 88%, 82%, and 82% in G2 as control, 4 days and 7 days drought conditions, respectively), showing that these genotypes were able to preserve more water, which is required to maintain the growth during the drought conditions.

Similarly, after water withholding for 4 and 7 days, G3 and G4 genotypes were found to contain higher free proline amounts than those growing under normal conditions (100% and 380% increase in G3, 500% and 700% increase in G4 against 4 days and 7 days drought conditions, respectively), while no significant change was observed in G1 and G2 (Figure 2b), suggesting that increased contents of free proline contributes to better drought stress tolerance in Italian ryegrass. Proline allows the plant to tolerate stress factors by maintaining turgor and redox homeostasis (Rajasheker et al., 2019; Zegaoui et al., 2017).

These results collectively showed that G3 and G4 are tolerant against drought stress, while G1 and G2 are sensitive. This means, G3 and G4 genotypes that can tolerate water deficiency for a longer time require less irrigation, they seem to have great potential to contribute to water consumption control in agriculture.

After the determination of sensitive and tolerant Italian ryegrass genotypes, the expression levels of the miRNAs identified by Huang et al. (2014) in perennial ryegrass with and without stress were assessed in these genotypes. Firstly, the presence of the miRNAs used in the study was confirmed in all genotypes. It was observed that, the expression level of the 5 miRNAs (miRNA3980b, miRNA5636, miRNA156i, miRNA845a, miRNA2937) changed significantly (Figures 3a, 3c, 3e, 3g, 3l), while no change was observed in 7 miRNAs (miRNA5817, miRNA396h, miRNA5543, miRNA5075, miRNA5021, miRNA156k, miRNA6245) (Figures 3b, 3d, 3f, 3h, 3i, 3j, 3k). Although it has been reported in the previous studies that miRNA396, miRNA5075, miRNA5021, miRNA156k are expressed in response to stress in several crops (Liu et al., 2009; Nguyen et al., 2015; Akdogan et al., 2016), the results of this study show that the expressions levels of these miRNAs did not significantly change in the studied genotypes (tolerant vs. sensitive genotypes). This further

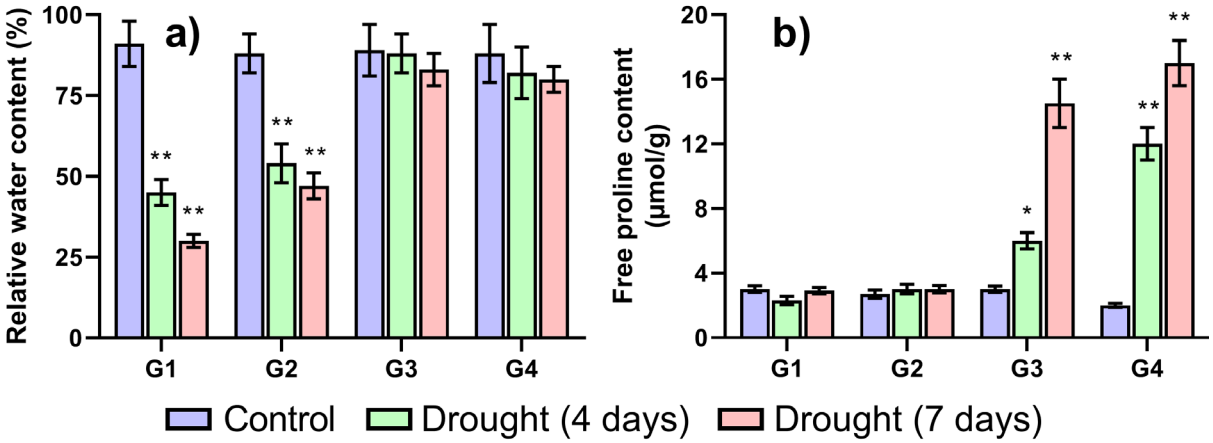

Figure 2. Drought stress tolerance analysis of G1, G2, G3, and G4 genotypes. a) Relative water content, b) free proline content. Data were presented as mean ± SD (n = 3). Asterisks indicate the significant differences between control and stressed genotypes at *P < 0.05 or **P < 0.01.

suggests that these miRNAs of Italian ryegrass do not play key roles in drought stress responses.

Under drought stresses (4 and 7 days), significant upregulation (miRNA845a) (Figure 3g) and downregulation (miRNA5636) (Figure 3c) were observed in both of sensitive genotypes (G1 and G2). Similarly, under drought stresses (4 and 7 days), significant upregulations (miRNA156i, miRNA845a) (Figures 3e–3g) and downregulations (miRNA3980b, miRNA2937) (Figures 3a–3l) were observed in both of tolerant genotypes (G3 and G4).

The drought stress downregulated the levels of miRNA3980b (Figure 3a) and miRNA2937 (Figure 3l) in drought tolerant genotypes, while no change was observed in sensitives, suggesting that these miRNAs have the potential to play key roles for determining the drought tolerances of Italian ryegrass genotypes. The downregulation of miRNA3980b significantly increased in 7 days drought compared to 4 days drought in G4, while no change was observed in stress treatments in G3. In addition, the downregulation of miRNA2937 did not change between 4 days and 7 days drought in G3 and G4 (Figure 3l). Under drought stress, similar downregulations for miRNA3980b were observed in several studies (Mahto et al., 2020; Zhang et al., 2017). It was reported that the target gene of miRNA3980b is pectinesterase, which is a key regulator in flower development and fertility (Liu et al., 2017). Therefore, it is decided to determine whether the relative expression of the pectinesterase gene in the genotypes under drought stress was changed. The results revealed that the expression levels of the pectinesterase gene was increased after 4 days and 7 days drought in drought tolerant genotypes, while no change was observed in sensitive ones (Figure 4). This is in an agreement with previous reports (Wang et al., 2014; Liu et al., 2017).

On the other hand, the miRNA2937 was indicated as a stress response in a previous study (Huang et al., 2014). It was reported that miRNA2937 targets phosphate synthase gene under stress conditions and the downregulation of this miRNA increases the expression level of phosphate synthase gene under stress conditions (Muhammad et al., 2018). Therefore, the relative expression of the phosphate synthase gene in the genotypes under drought stress was studied. The expression levels of the phosphate synthase gene was increased after 4 days and 7 days drought in drought tolerant genotypes, while no change was observed in sensitives (Figure 5), showing that the downregulation of miRNA2937 improves drought stress tolerance by increasing phosphate synthesis under drought stress.

The expression levels of the miRNA5636 (Figure 3c) were downregulated in drought sensitive genotypes, indicating that miRNA5636 functioned in a stress inducible manner in sensitive Italian ryegrass genotypes under drought stress conditions. In addition, the downregulation of miRNA5636 did not change between 4 days and 7 days drought in sensitive genotypes. These results clearly show that downregulation of miRNA5636 increases drought susceptibility in Italian ryegrass. This is the first report showing the expression of the miRNA5636 under stress conditions.

The expression levels of the miRNA156i after 7 days drought increased significantly compared to control (3.6 and 6.4 fold in G3 and G4, respectively) and also compared to 4 days drought (2.2 and 1.4 fold in G3 and G4, respectively) in drought tolerant genotypes (Figure 3e). The upregulation of miRNA3980b significantly increased in 7 days drought compared to 4 days drought in G3, while no change was observed in stress treatments in G4. The results suggest that the upregulation in miRNA156i under drought stress was a response providing tolerance

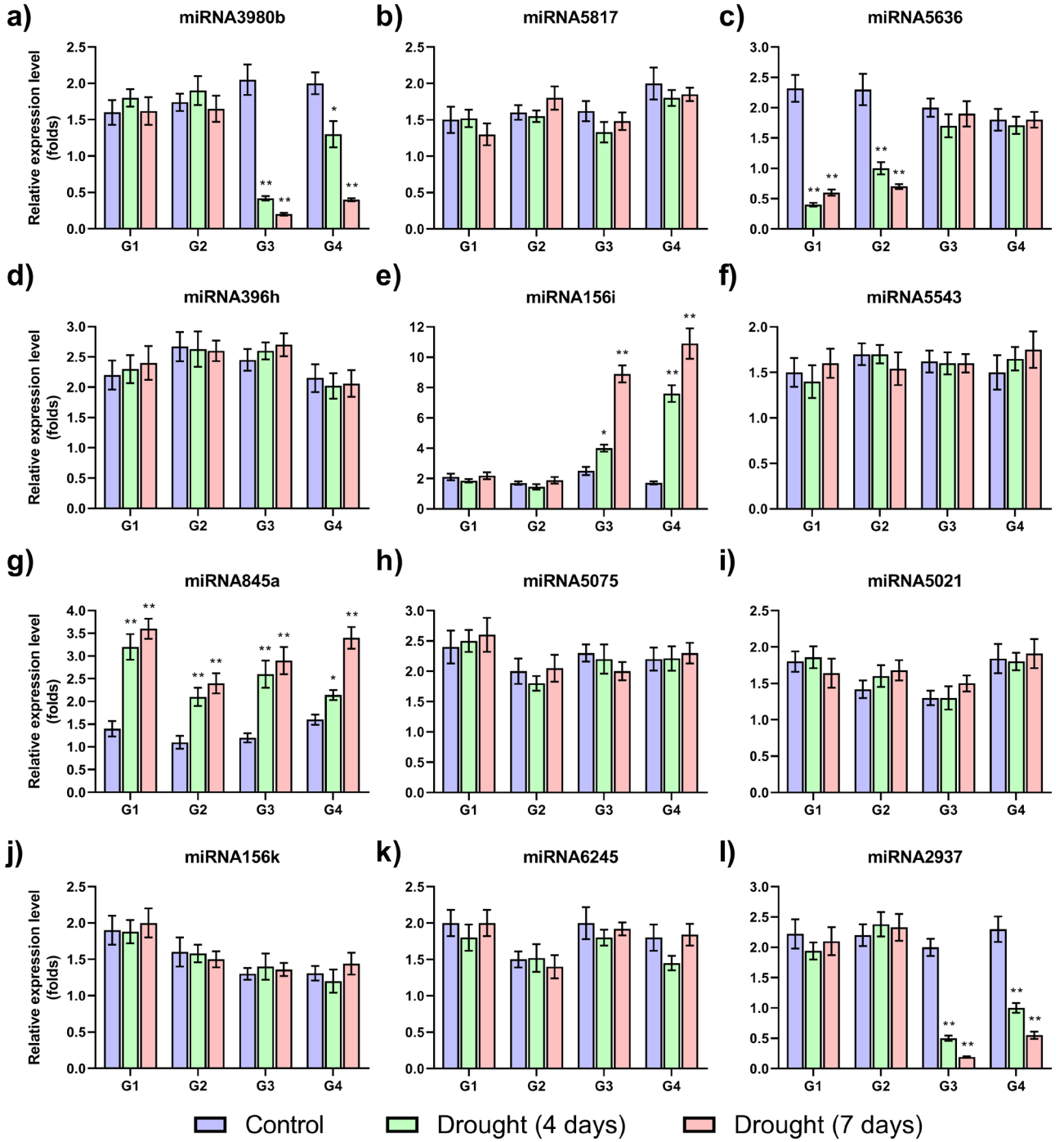

Figure 3. Relative expression analysis of the 12 miRNAs in G1, G2, G3, and G4 genotypes under control and drought stresses. a) miRNA3980b, b) miRNA5817, c) miRNA5636, d) miRNA396h, e) miRNA156i, f) miRNA5543, g) miRNA845a, h) miRNA5075, i) miRNA5021, j) miRNA156k, k) miRNA6245, l) miRNA2937. Data were presented as mean ± SD (n = 3). Asterisks indicate the significant differences between control and stressed genotypes at *P < 0.05 or **P < 0.01.

in tolerant genotypes. Therefore, miRNA156i could be accepted to be involved in drought stress tolerance in Italian ryegrass. Similar results were observed in various crops (Barrera-Figueroa et al., 2011; Kantar et al., 2010; Nageshbabu et al., 2013). Increasing evidence demonstrates that the upregulation of miRNA156i could increase anthocyanin synthesis against drought stress conditions (Boopathi, 2015; González-Villagra et al.,

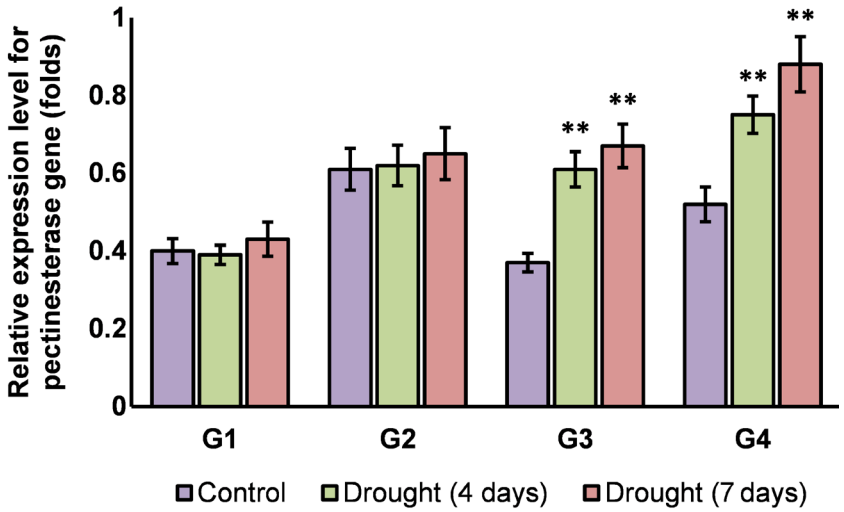

Figure 4. Relative expression levels for pectinesterase gene in G1, G2, G3, and G4 genotypes under control and drought stresses. Data were presented as mean ± SD (n = 3). Asterisks indicate the significant differences between control and stressed genotypes at *P < 0.05 or **P < 0.01.

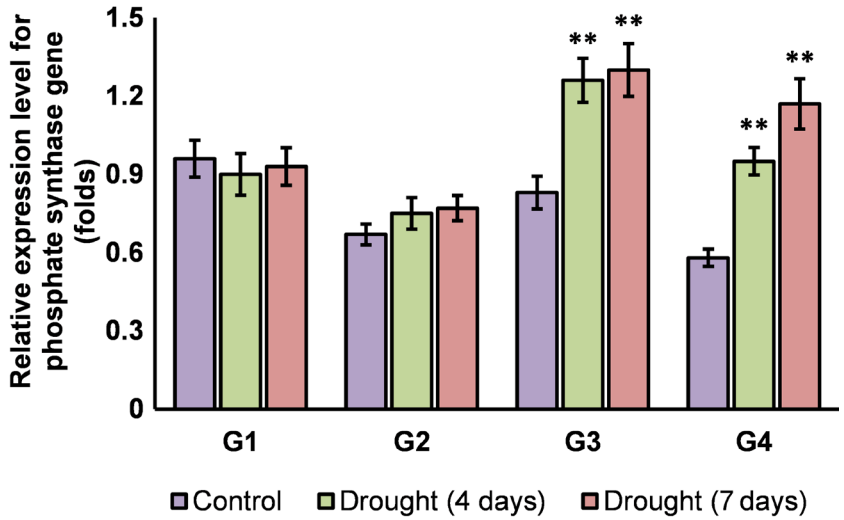

Figure 5. Relative expression levels for phosphate synthase gene in G1, G2, G3, and G4 genotypes under control and drought stresses. Data were presented as mean ± SD (n = 3). Asterisks indicate the significant differences between control and stressed genotypes at *P < 0.05 or **P < 0.01.

2017). Thus, the total anthocyanin content of the genotypes under drought stress was studied. The total anthocyanin content was increased after 4 days and 7 days drought in drought-tolerant genotypes, while no change was observed in sensitives except for 7 days drought treatment of G1 (Figure 6). This result suggests that the upregulation of miRNA156i may improve drought stress tolerance by increasing total anthocyanin content under drought stress.

The expression level of miRNA845a was upregulated under drought conditions in all genotypes (2.6, 2.2, 2.4, and 2.6 fold in G1, G2, G3, and G4 under 7 days drought condition, respectively) (Figure 3g). This means, miRNA845a could not be differentiated between sensitive and tolerant Italian ryegrass genotypes. Similar result was observed by Zhou et al. (2010) in *Oryza sativa* plants against drought stress. The miRNA845 has been reported to target a gene encoding protein S-acyltransferase involving in leaf senescence that is an important process in plant development and stress responses (Zeng et al., 2018). In addition, miRNA845 was reported to be involved in the

flavonoid and chlorophyll biosynthesis pathways during stress conditions (Liu et al., 2017). In the present study, total chlorophyll and total flavonoid contents were determined in the genotypes under drought stress conditions. The total chlorophyll contents were not changed in drought tolerant genotypes, while 7 days drought treatments were decreased in sensitives (Figure 7). This suggests that the drought tolerant genotypes maintained their chlorophyll contents under drought conditions. Several studies reported that that total chlorophyll content was strongly correlated with environmental stress tolerance in various crops (Li et al., 2006; Makbul et al., 2011). In addition, the total flavonoid contents were increased under drought stress conditions in all studied genotypes (Figure 8). This supports that miRNA845 is involved in increased total flavonoid content under drought stress.

The responses of the miRNAs against drought stress in several plants have been identified in previous studies (Table 2). As can be seen in Table 2, a small number of miRNAs have been considered to be important to plant's response against drought stress. In addition, the same miRNA can respond to drought stresses differently in different plant species (Table 2). Some of the miRNAs in this study showed altered expression patterns when compared to other researches done using different plants (Yang and Du, 2009; Kantar et al., 2011). miR396 and miR3980 was upregulated

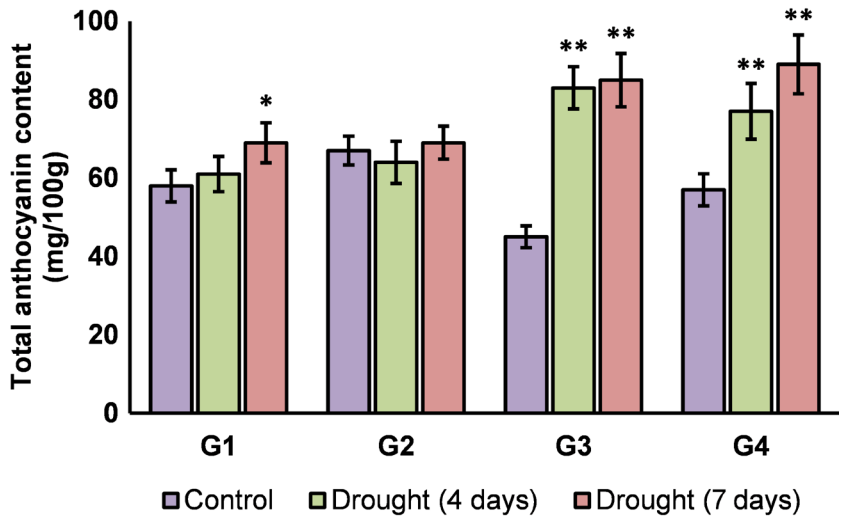

Figure 6. Total anthocyanin content in G1, G2, G3, and G4 genotypes under control and drought stresses. Data were presented as mean ± SD (n = 3). Asterisks indicate the significant differences between control and stressed genotypes at *P < 0.05 or **P < 0.01.

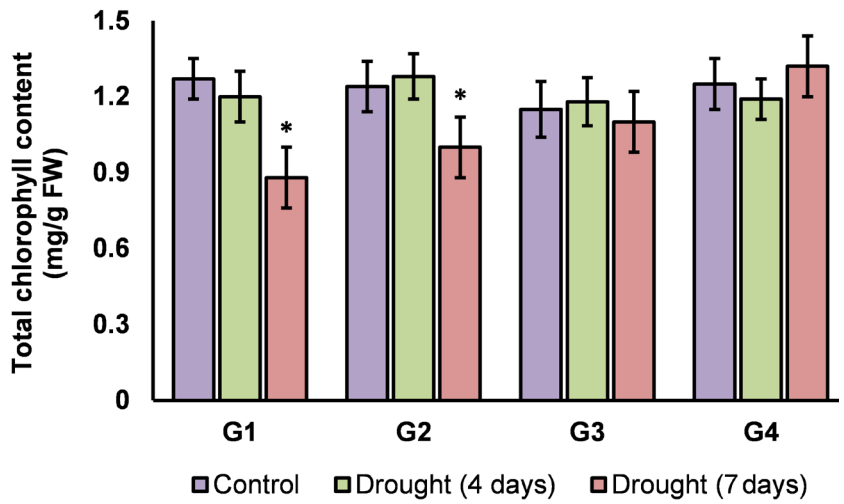

Figure 7. Total chlorophyll content in G1, G2, G3, and G4 genotypes under control and drought stresses. Data were presented as mean ± SD (n = 3). Asterisks indicate the significant differences between control and stressed genotypes at *P < 0.05 or **P < 0.01.

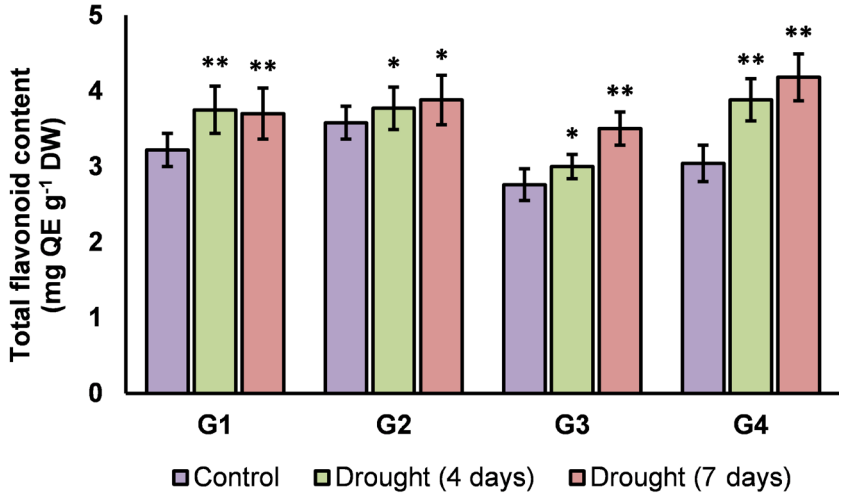

Figure 8. Total flavonoid content in G1, G2, G3, and G4 genotypes under control and drought stresses. Data were presented as mean ± SD (n = 3). Asterisks indicate the significant differences between control and stressed genotypes at *P < 0.05 or **P < 0.01.

Table 2. The identified drought responsive miRNAs in plants.

miRNA	Response	Species	References
miR168	Upregulated	*Arabidopsis thaliana*	Liu et al., 2008
	Downregulated	*Oryza sativa*	Zhou et al., 2010
	Downregulated	*Nicotiana tabacum*	Chen et al., 2017
miR159	Upregulated	*Arabidopsis thaliana*	Ding et al., 2013
miR167	Upregulated	*Arabidopsis thaliana*	Liu et al., 2008
	Downregulated	Zea mays	Wei et al., 2009
miR169	Downregulated	*Medicago truncatula*	Wang et al., 2011
	Upregulated	*Oryza sativa*	Zhao et al., 2007
miR160	Upregulated	*Arabidopsis thaliana*	Liu et al., 2015
miR393	Upregulated	*Oryza sativa*	Zhao et al., 2007
miR390	Upregulated	*Vigna unguiculata*	Ding et al., 2013
	Downregulated	*Nicotiana tabacum*	Chen et al., 2017
miR396	Upregulated	*Nicotiana tabacum*	Yang and Du, 2009
miR474	Upregulated	*Zea mays*	Wei et al., 2009
miR528	Downregulated	*Zea mays*	Wei et al., 2009
	Upregulated	*Triticum aestivum*	Akdogan et al., 2016
miR397	Downregulated	*Oryza sativa*	Zhou et al., 2010
	Upregulated	*Arabidopsis thaliana*	Sunkar and Zhu, 2004
miR3980	Upregulated	*Triticum dicoccoides*	Kantar et al., 2011
	Downregulated	*Nicotiana tabacum*	Chen et al., 2017
miR156	Upregulated	*Populus euphratica*	Bakhshi et al., 2017
miR164	Upregulated	*Triticum durum*	Liu et al., 2015
miR166	Upregulated	*Triticum aestivum*	Ma et al., 2015
	Upregulated	*Medicago truncatula*	Boualem et al., 2008

Table 2. (Continued).

	Downregulated	*Nicotiana tabacum*	Chen et al., 2017
miR172	Upregulated	*Oryza sativa*	Bakhshi et al., 2016
miR9666	Upregulated	*Hordeum vulgare*	Hackenberg et al., 2015
miR1432	Upregulated	*Triticum dicoccoides*	Kantar et al., 2011

in *Nicotiana tabacum* (Yang and Du, 2009) and *Triticum dicoccoides* (Kantar et al., 2011), respectively, under drought conditions. In contrast, miR396 was unchanged in this study under drought in all genotypes and miR3980 was downregulated in tolerant genotypes (G3 and G4). This finding further confirms that miRNA-mediated target regulation shows variations between genotypes.

The results of this study indicates that miRNA3980b, miRNA5636, miRNA156i, and miRNA2937 have potential to effect the drought senstitivities and tolerances of Italian ryegrass.

4. Conclusion

Both similarities and differences between sensitive and tolerant Italian ryegrass genotypes have been observed in gene expression levels after drought stress conditions. The study indicates that miRNA156i, miRNA2937, and miRNA3980b are responsible for drought stress tolerance in tolerant Italian ryegrass. The results of this study have advanced our understanding of miRNAs-mediated gene regulation in Italian ryegrass that could be potential targets for agricultural productivity of Italian ryegrass under water limited areas. New Italian ryegrass having upregulated miRNA156i and downregulated miRNA3980b, and miRNA2937 could be used to develop that tolerate drought stress conditions.

In addition, since G3 and G4 genotypes that can tolerate water deficiency for a longer time, they seem to have great potential to contribute to water consumption control.

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