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Article

Drought-Induced miRNA Expression Correlated with Heavy Metal, Phenolic Acid, and Protein and Nitrogen Levels in Five Chickpea Genotypes

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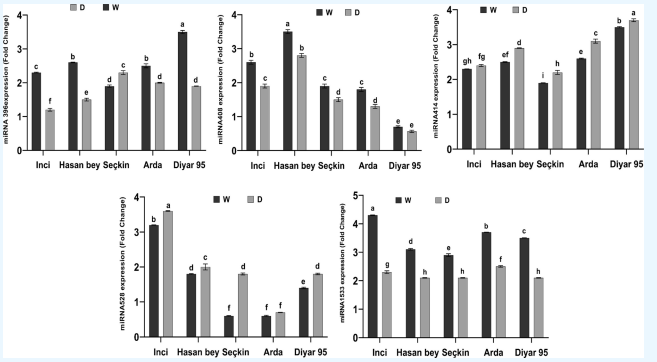
ABSTRACT: Drought is a prime stress, drastically affecting plant growth, development, and yield. Plants have evolved various physiological, molecular, and biochemical mechanisms to cope with drought. Investigating specific biochemical pathways related to drought tolerance mechanisms of plants through biotechnology approaches is one of the quickest and most effective strategies for enhancing crop production. Among them, microRNAs (miRNAs) are the principal post-transcriptional regulators of gene expression in plants during plant growth under biotic and abiotic stresses. In this study, five different chickpea genotypes (İnci, Hasan bey, Arda, Seçkin, and Diyar 95) were grown under normal and drought stress. We recorded the expression levels of microRNAs in these genotypes and found differential expression (miRNA396, miR408, miRNA414, miRNA528, and miRNA1533) under contrasting conditions. Results revealed that miRNA414 and miRNA528 considerably increased in all genotypes under drought stress, and expression levels of miRNA418, miRNA1533, and miRNA396 (except for the Seçkin genotype) were found to be higher under the watered conditions. These genotypes were also investigated for heavy metal, phenolic acid, protein, and nitrogen concentrations under normal and drought stress conditions. The Arda genotype showed a significant increase in nitrogen (5.46%) and protein contents (28.3%), while protein contents were decreased in the Hasan bey and Seçkin genotypes subjected to drought stress. In the case of metals, iron was the most abundant element in all genotypes (İnci = 15.4 ppm, Hasan bey = 29.6 ppm, Seçkin = 37.8 ppm, Arda = 26.3 ppm, and Diyar 95 = 40.8 ppm) under normal conditions. Interestingly, these results were related to miRNA expression in the chickpea genotypes and hint at the regulation of multiple pathways under drought conditions. Overall, the present study will help us to understand the miRNA-mediated regulation of various pathways in chickpea genotypes.



INTRODUCTION

Drought, salt, and high temperatures are the main abiotic stresses limiting crop growth and development.[1−5] In the 21st century, agricultural lands and overall productivity are decreasing due to changes in the global ecosystem balance and climatic conditions. Drought is one of the major abiotic factors affecting agricultural production efficiency worldwide, including in Turkey.[8−10] Drought stress affects the physiological, biochemical, and molecular systems that allow plants to respond to micro/macroenvironmental changes at the cellular level. The loss of turgor, restriction of leaf water potential, stomatal closure, and reduced cell growth and development are crucial characteristics of drought stress.[6,7] Drought has harmful impacts on plants' physiological and biochemical processes, such as transpiration, photosynthesis, carbohydrate production, ion uptake, transport, and nutrient metabolism, leading to a reduction in yield.[11,12]

Plants cope with drought stress through an osmotic adjustment mechanism. In response to a decrease in the water potential of the cellular environment, the osmotic arrangement involves the active accumulation of organic and inorganic compatible solutes known as osmolytes or osmotic preservatives in the cell.[13] These include producing and accumulating several metabolites involved in primary and secondary metabolism, such as amino acids, sugars, organic acids, and flavonoids.[14] Plant primary metabolites are

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compounds that are necessary for reproduction, growth, and development. Secondary metabolites are organic products that aid the plant with protection, defense, reproduction, and adaptation to these adverse environments.[15] Flavonoids and phenolic compounds play a critical role in the defense against drought stress.[16,17] In crops, drought stress and its interaction with growth and photosynthesis are associated with changes in nitrogen (N) and protein status.[18,19] Phenolic compounds are known to be produced in higher amounts under stress conditions as these play a significant role in the growth and development of plants.[20] During their growth and development, plants absorb essential microelements such as copper (Cu), zinc (Zn), and manganese (Mn), as well as some nonessential metal elements like cadmium (Cd), lead (Pb), and mercury (Hg).[21,22]

Plants utilize a variety of physiological and molecular mechanisms to cope with drought stress.[23] In this regard, microRNA (miRNA) is the versatile regulator of gene expression in plants and animals, and the genes associated with growth and stress in plants control expression levels.[24] Production of miRNAs and their growth and signaling as standard endogenous gene regulators in plant genomes play a crucial role in growth and stress response.[25,26] Several studies have revealed that miRNAs govern various cellular, biochemical, and molecular pathways under different abiotic stresses, including drought stress in crop plants.[27−32] Moreover, 224 conserved and 60 novel miRNAs in chickpeas have been discovered under drought and salt stress.[33]

The present study hypothesized that exploring and understanding biochemical pathways in different chickpea genotypes under drought stress using biotechnological tools could be the quickest and most reliable strategy to under-regulate biochemical mechanisms and enhance crop production under drought stress. Based on this hypothesis, the present study compared drought stress tolerance of five chickpea genotypes depending on the expression levels of miRNAs and their effect on heavy metals, phenolic acid, protein, and nitrogen concentrations under normal and drought stress conditions.

RESULTS

Impact of Water Stress on Nitrogen Contents. Under water (W) conditions, the nitrogen content of the Arda genotype was drastically reduced. Similarly, in drought (D) conditions, the amount decreased in the İnci and Diyar 95 genotypes. However, the nitrogen fluctuation detected in the genotypes exposed to drought stress was not statistically significant (Figure 1).

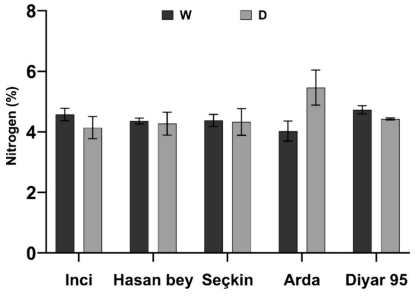

Figure 1. Total nitrogen concentration in the seeds of chickpea genotypes exposed to drought stress (W: watered, D: drought).

Impact of Water Stress on the Total Protein Content. The protein content in all of the genotypes exposed to drought stress and well-watered conditions did not show any statistical significance. The present study therefore established that there was no effect on protein levels concerning five chickpea genotypes subjected to a drought stress regime (Figure 2).

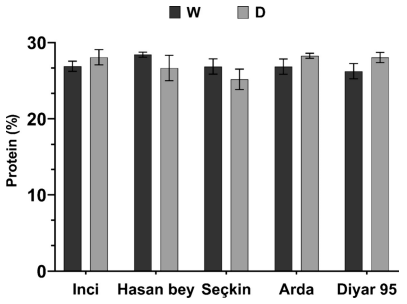

Figure 2. Total protein concentration in chickpea genotypes exposed to drought stress (W: watered, D: drought).

Impact of Water Stress on Phenolic Compounds. When the drought was applied, gallic acid concentrations were decreased in the İnci, Seçkin, and Diyar 95 genotypes; thus, decline was severe in the İnci genotype. Gallic acid concentrations increased in the Hasan bey and Arda genotypes. Compared to nonstressed plants, this increased dramatically in the Arda genotype. When gallic and chlorogenic acid concentrations were compared, both decreased in İnci and Seçkin. However, the amount of gallic acid increased in Hasan Bey and Arda genotypes under drought stress. In addition, the gallic acid amount decreased while the chlorogenic acid amount increased in the Diyar 95 genotype under drought stress (Figure 3). In İnci, Hasan bey,

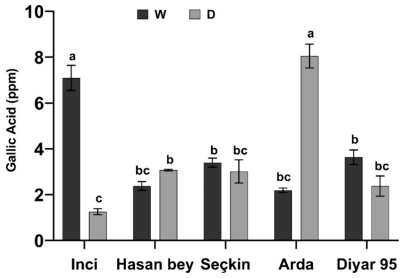

Figure 3. Effect of drought stress on phenolic compound contents in chickpea genotypes, where W means watered and D means drought. The columns denoted by different letters indicate significant differences according to the Tukey test at $p \leq 0.05$ ($n = 3$).

Seçkin, and Diyar 95 genotypes, the amount of 4-hydroxybenzoic acid was increased under drought stress. Hence, the amount of 4-hydroxybenzoic in the Arda genotype decreased significantly under drought stress (Figure 3). On exposure to drought stress, the caffeic acid concentration increased in the Arda and Seçkin genotypes, whereas its amount decreased in the Diyar 95 and Hasan bey genotypes. As a result of the statistical analysis, it was observed that the amount of caffeic acid in all genotypes was significant at the 0.05 level (Figure 3).

Similarly, the vanillic acid concentration increased in İnci and Seçkin genotypes but decreased in Arda and Diyar 95 under drought stress. However, the amount of vanillic acid in

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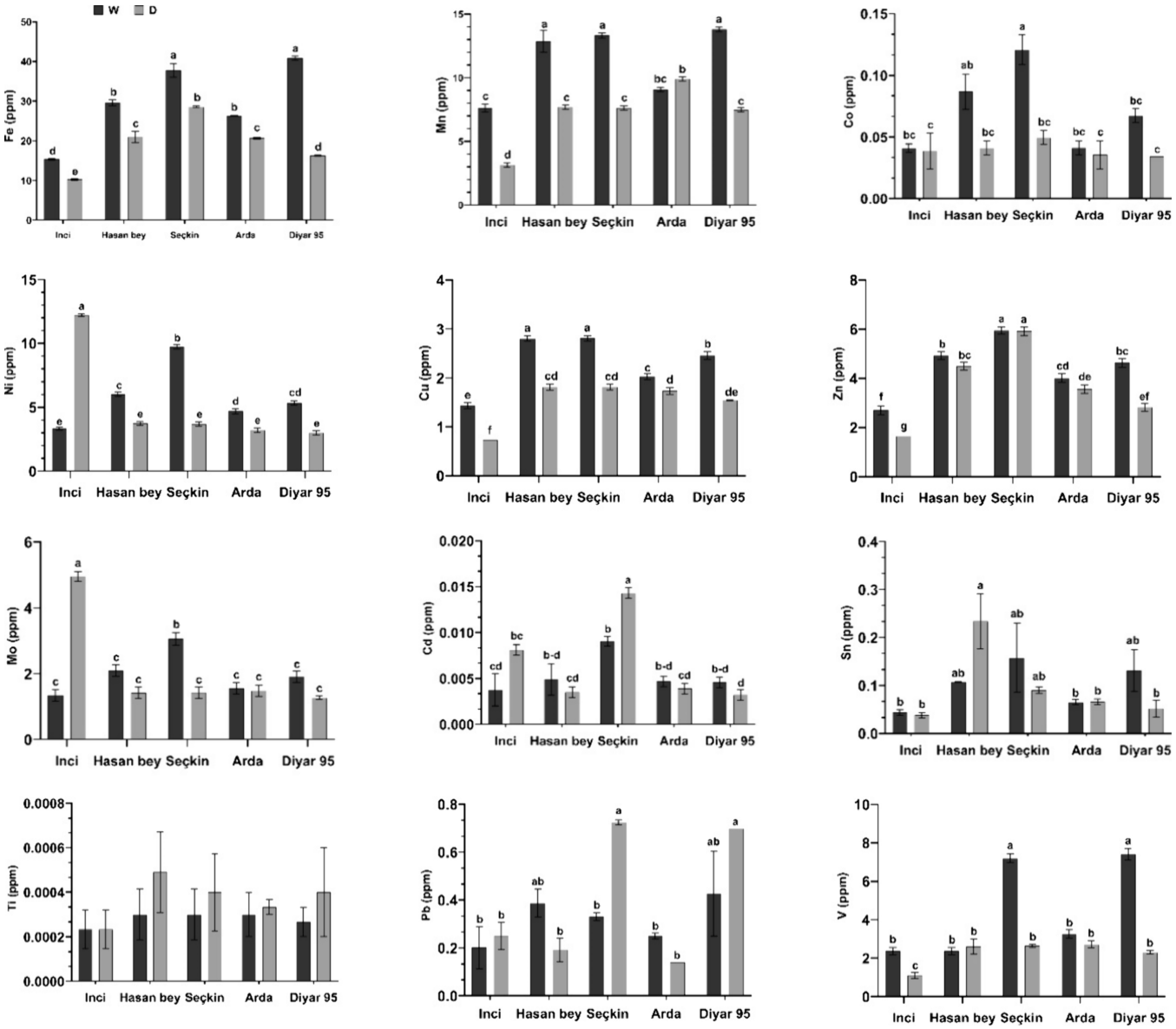

Figure 4. Effect of drought stress application on heavy metal contents in chickpea genotypes (W: watered, D: drought). The columns denoted by different letters indicate significant differences according to the Tukey test at the $p \leq 0.05$ level ($n = 3$).

all genotypes except Diyar 95 and Arda was not significantly different ($p \leq 0.05$). *trans*-Ferulic acid concentrations decreased in İnci, Hasan bey, Arda, and Diyar 95 genotypes, and a relative increase was noticed in the İnci genotype under drought stress. However, differential behavior of all genotypes for *trans*-ferulic acid was observed except in İnci and Seçkin, which did not show significant differences and performed alike (Figure 3).

Impact of Water Stress on the Heavy Metal Content. A decrease in Cu, Fe, Zn, and Mn concentrations was noticed in all genotypes except Arda. Likewise, no change in Ni contents was observed in the İnci genotype as compared to other tested genotypes exposed to drought stress, but their amounts increased under watered conditions. The profiles exhibited by Mo and Ni metals were quite similar. While the amount of Mo increased in the İnci genotype under drought stress, it decreased in the Hasan bey, Seçkin, and Diyar 95 genotypes. However, in the Arda, Hasan bey, and Diyar 95 genotypes, the amount of Mo was not statistically significant. Except for the İnci genotype, the amount of T_1 metal increased in other genotypes under drought stress, while the amount of Co metal decreased. However, no statistical significance was found in terms of T_1 metal for these genotype-specific increases and declines. When the V and Sn amounts were analyzed, it was found that the amount of both metals decreased in İnci, Seçkin, and Diyar 95 while increased in Hasan bey under drought stress. Under water-deficit conditions, the amounts of Pb and Cd metals decreased in all genotypes except Diyar 95 (Figure 4).

Impact of Water Stress on Different miRNA Expressions. Statistical analysis showed that the expression level of miRNA396 significantly decreased in all genotypes except for Seçkin under drought stress. The highest increase in miRNA 396 was observed for Seçkin under drought stress, while the lowest was detected in the İnci genotype under the same condition (Figure 5).

It is observed that the expression of miRNA408 decreased in all genotypes except for Diyar 95, which was exposed to

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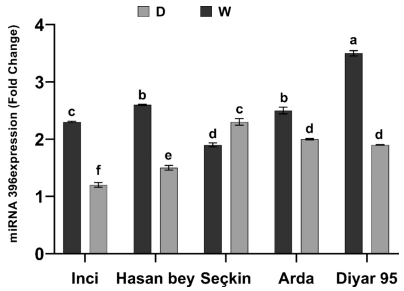

Figure 5. Total miRNA396 expression levels in chickpea genotypes with drought stress (W: watered, D: drought). The columns denoted by different letters indicate significant differences according to the Tukey test at $p \leq 0.05$ ($n = 3$).

drought stress. Results showed that the increase in miRNA 408 expression was statistically significant in the Diyar 95 genotype. Considering drought stress and irrigated conditions, except for Diyar 95, when other genotypes were compared statistically within and between each other, it was seen that the miRNA408 expression level obtained was significant in all cases (Figure 6).

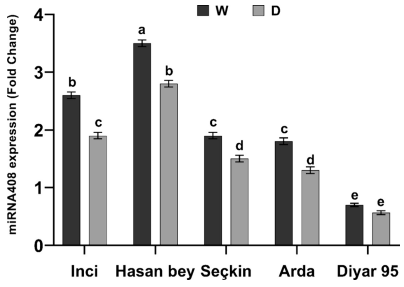

Figure 6. Total miRNA408 expression levels in chickpea genotypes with drought stress (W: watered, D: drought). The columns denoted by different letters indicate significant differences according to the Tukey test at $p \leq 0.05$ ($n = 3$).

The expression level of miRNA414 was relatively increased in all five chickpea genotypes exposed to drought. In particular, for İnci, Hasan bey, Seçkin, Arda, and Diyar 95, the expression level of miRNA was statistically significant. At the same time, an increase in miRNA expression was primarily seen in the Diyar 95, while the minimum increase was witnessed in the Seçkin genotype (Figure 7). To obtain these miRNA expression data, genotypes were compared within and between each other.

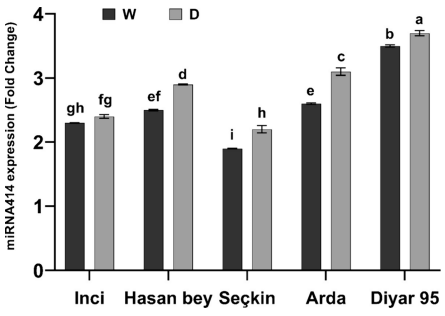

Figure 7. Total miRNA414 expression levels in chickpea genotypes with drought stress (W: watered, D: drought). The columns denoted by different letters indicate significant differences according to the Tukey test at $p \leq 0.05$ ($n = 3$).

Four genotypes (İnci, Seçkin, Arda, and Diyar 95) of chickpeas exposed to drought stress were found to have higher expression of miRNA528. In addition, the increased amount of miRNA528 was highest in the İnci genotype. However, the miRNA expression level in the Arda genotype was not statistically significant (Figure 8).

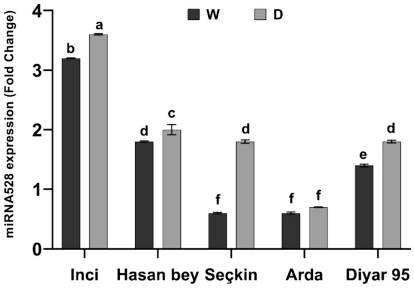

Figure 8. Total miRNA528 expression levels in chickpea genotypes with drought stress (W: watered, D: drought). The columns denoted by different letters indicate significant differences according to the Tukey test at $p \leq 0.05$ ($n = 3$).

Five chickpea genotypes were compared within and between each other in the statistical analysis to establish the miRNA1533 expression level. As a result, the expression data obtained were significant at the 0.05 level in all cases. The expression level of miRNA1533 was observed to decrease in five chickpea genotypes exposed to drought stress at a significance of 0.05 (Figure 9).

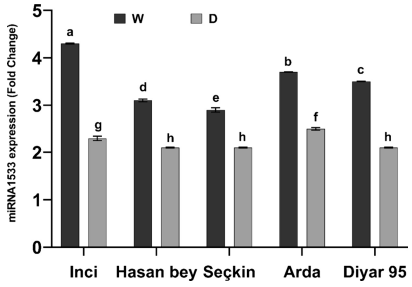

Figure 9. Total miRNA1533 expression levels in chickpea genotypes with drought stress (W: watered, D: drought). The columns denoted by different letters indicate significant differences according to the Tukey test at $p \leq 0.05$ ($n = 3$).

The miRNA396, miRNA408, and miRNA1533 expression increased in chickpeas treated with full irrigation. At the same time, Fe, Ni (except for the İnci genotype), Cu, and Mn uptake increased in these genotypes. Changes in miRNA expression levels are due to drought-induced miRNA−metal element relationships and are found to be consistent at transcript levels. The expression levels of miRNA414 and miRNA528 did not increase in chickpea genotypes treated with drought.

DISCUSSION

Plants use a variety of strategies to change physiological and molecular mechanisms in response to drought.[33−35] Numerous studies have demonstrated that regulating nitrogen (N) metabolism is closely related to drought stress in plants.[36] Therefore, optimizing the amount of nitrogen can be a crucial determinant in screening chickpea genotypes under drought stress. The physiological status of chickpeas is dependent on

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nitrogen fixation and the photosynthetic capacity. In this situation, the whole nitrogen fixation system plays a critical role in regulating the free oxygen concentration inside nodules.[37] The total nitrogen content of genotypes in this study showed different but statistically insignificant responses to drought stress. The increased N content in plants leads to an increase in protein production, while these molecules retain the capacity for osmosis under water stress. The uniformity of total N and total protein contents with each other confirms this result. Some studies show that drought reduces nitrogenase activity; this could be owing to a reduction in the availability of photosynthetic products to nodules for symbiotic nitrogen fixation as well as other causes. This condition is critical for other legumes, such as the chickpea plant, as it results in less N for protein biosynthesis and decreased grain output.[11,38] Previous studies showed that some proteins increased during abiotic stress conditions such as drought, salinity, heat, and cold.[39−41] This indicates that protein is essential in the plant response to drought stress. However, there have been different outcomes of protein accumulation in plants under stress conditions, and the increased expression of these proteins contributes to plant stress management.[42]

In this study, a decrease in the protein concentration was recorded in plants subjected to drought stress. Increasing the total protein content as osmotic defenders is critical for plant osmoregulation under water stress circumstances.[43] As a result, any increase in the total protein content aids in combating the damaging effects of drought stress. It is well-recognized that a lack of water impacts various biochemical and physiological processes ranging from photosynthesis to protein synthesis.[44] Furthermore, the protein content may decrease as a result of proteolysis due to the harmful effects of ROS.[45]

In the present study, the amounts of phenolic compounds in each genotype varied due to drought stress, which is in accordance with previous studies that reported that plants under stress produce more phenolic compounds (phenolic acids and flavonoids).[43,46] The commonly held view that phenolic compounds rise in water stress is frequently incorrect, as there may be no drop or change in the phenolic compound concentration when exposed to water stress.[47] Plant phenolic compounds respond to drought stress in various ways depending on factors such as the exposure time and volume of water given to the plant. An increased total content of flavonoids, phenolics, and multiple polyphenolics and increased radical scavenging activity were observed in plants under drought stress.[18,47] Caffeic acid levels, for example, do not follow any pattern. The decrease in the protein content can be attributed to a reduction in protein synthesis or an increased rate of protein degradation.[14,38] These findings indicate that heavy metal levels are essential in shielding plants from drought stress (Figure 4).

The miRNA396 is a conserved miRNA that might be found in both monocots and dicots. It regulates genes post-transcriptionally by inhibiting its targets, which are growth factors (GRFs). The role of miR396 in plant growth and development has been well-characterized in various crops.[48] Under salt and drought stress, the expression of tomato miR396a (Sp-miR396) was found to be upregulated.[49,50] In *Arabidopsis*, miR396 is sensitive to salinity and drought.[51] These TFs also regulate other TFs or their direct targets, such as other regulatory and functional proteins. It indicates that miR396 serves as an essential key regulator of stress-sensitive genes during the plant's abiotic stress response[52] and is associated with aluminum (Al) metal.[53] It was reported that the expression level of miRNA396 decreased in rice when exposed to water scarcity,[54] which strengthens the findings of the present study and suggests that miRNA396 plays a critical role in inducing drought tolerance in plants.

It has been reported that environmental stresses regulate the expression of miR408, i.e., miR408 expression is induced in response to dehydration, mechanical stress, and ROS.[55−57] It has been reported that the miR408 expression level decreased in *Prunus persica* and *Oryza sativa* upon drought stress.[57,58] In the present study, drought stress has played an important role in drought resistance by reducing miRNA408 expression in chickpea genotypes. The study by Hajyzadeh et al.[59] found that overexpression of miRNA408 was achieved in *Cicer arietinum* L. under a drought resistance mechanism. The miR414 is thought to have 145 targets, mainly in protein or sugar transport. Drought causes an upregulation of sucrose transporters and group 3 late-embryogenesis abundant (LEA) proteins.[60−62] Sugar transporters have been recognized as crucial targets for their regulatory role in plant distribution and allocation of carbon resources.[61] LEA proteins are assumed to be involved in drought resistance because they accumulate in plants during drought stress.[62]

The miR528 was discovered to be differentially regulated by various abiotic stressors among the differentially expressed miRNAs identified.[63,64] It has been reported that the expression level of OSA-miR528 in rice is upregulated, especially in roots, improving drought resistance.[65] Bakhshi et al.[66] investigated miRNA drought response in rice roots, and miRNA528 participated in drought stress and drought signaling. Studies have reported that drought stress upregulates miR528 expression in sugar cane and *Brachypodium* sp.[66,67] and reported that the expression level of miRNA528 was increased. Our research was corroborated by Bertolini et al.[68] who found that miRNA528 targets Cu^{2+} binding proteins (CBP), putative IAR1 proteins, and l-ascorbate oxidase. Likewise, miRNA528 regulates metal ion homeostasis and controls cellular free auxin levels and ascorbate metabolism through these targets.[69,70]

The miRNA1533 was the first to be identified in cassava with a certain number of miRNA members. The predicted targets of miR1533 indicated that this could be associated with the biosynthesis of plant hormones and starch metabolism in potatoes and wheat.[71,72] Much evidence has shown that miRNAs play a crucial role in the signal transduction of plant hormones.[73] WRKY transcription factors play an essential role in plant processes such as germination, aging, and responses to abiotic stresses such as drought and cold.[74] In Chinese cabbage, miR1533 can target WRKY proteins.[73] So, drought tolerance can be developed in chickpea genotypes using the target proteins determined in their studies using tomato and Chinese cabbage as plant materials. These transcription factors come from various families including NAC, WRKY, and transcription factors with the homeobox domain.

When we look at the protein−metal relationship, this situation can directly affect the activity of proteins by using elements such as Cu and Fe as cofactors. The miRNA−metal interactions also significantly affect the post-transcriptional regulation of proteins requiring cofactors. Changes in miRNA expression levels based on watered conditions and drought may have induced drought resistance in chickpea genotypes. Finally, the metal−gene interaction is vital in drought stress in plants as metal ions affect the miRNA expression levels of the

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plant cell, influencing the plant's defense against stress with the miRNA−arget gene regulatory network.

CONCLUSIONS

Drought stress negatively affected chickpea plants in terms of their growth, biomass, nutrient absorption, and photosynthetic pigment levels, which affected their physiological, biochemical, and molecular processes. Drought stress reduced the amounts of Fe and Mn metals and miRNA1533 and miRNA408 expression levels as compared to those with optimal water supply in plants; thus, the miRNA414 and miRNA528 expression levels were decreased in all genotypes exposed to irrigation. The expression profile obtained from this study revealed that miRNA 528−1533−414−408 can serve as a supportive mark for drought stress studies in *Cicer*. Under well-watered conditions, chlorogenic acid levels increased in all genotypes except the Diyar 95 genotype, and a similar trend was found for the N content in İnci and Diyar 95. In contrast, the total protein amount increased under drought stress in all genotypes except for the Hasan bey and Seçkin genotypes. This study provides insight into molecular agricultural data for future chickpea breeding and sustainable farming projects. In the future, it is necessary to support the efficiency values with several studies to authenticate the obtained results.

MATERIALS AND METHODS

Plant Materials, Growth, and Stress Conditions. In this study, seeds of Turkey's most commonly used five chickpea genotypes (İnci, Hasan bey, Seçkin, Arda, and Diyar 95) were obtained from the Eastern Mediterranean Agricultural Research Institute, Turkey. For planting in 2016−2017, 30 × 50 cm pots were washed and sterilized using standard methods. The seeds of different genotypes were sterilized by washing three times with deionized water after being treated with sodium hypochlorite (5%) for 10 min. The genotypes' seeds were planted in 30 × 50 cm plastic pots with 1 kg of soil and 3 kg of peat mix under 16 h light, 8 h dark, and 26 °C conditions. Later, until the germinated seeds developed 6−7 leaves, all genotypes were irrigated at field capacity. The field capacity was determined using a pressure table to determine the percentage of moisture retained in the soil at a 1/3 bar pressure. Then, irrigation was halted for the group subjected to drought treatment.[59] The irrigated group was watered at field capacity for 2 weeks until stress symptoms appeared in the control group (nonirrigated). In addition, leaf samples were collected in triplicate from control and drought-stressed plants and stored in a deep freezer to analyze gene expression, phenolic acid, protein, and heavy metals.

RNA Isolation, cDNA Synthesis, and RT-PCR. A mirVana miRNA isolation kit (Ambion, CA, USA) enriched by short RNAs was utilized to isolate RNA from leaf tissues for miRNA study.[75] The quality and amount of isolated RNA were determined by using a NanoDrop ND-2000c spectrophotometer from Thermo Fisher Scientific. The expression levels of five distinct miRNAs (miR396, miR408, miR414, miR528, and miR1533) in leaf tissues were evaluated using the qRT-PCR. To isolate miRNAs in 0.2 mL sterile tubes, 1 μL of 50 pmol μL^{-1} oligo dT (20) primer, 1−5 μg of total RNA, and a 10 mM dNTP mixture were added with sterile distilled water until the total volume reached 12 μL. The mixture was held at 65 °C for 5 min before being placed on ice. Following light centrifugation, the following components were collected at the bottom of the tube: 2 μL of 0.1 M DTT and 1 μL of RNase inhibitor tube contents were softly mixed and steeped for 2 min at 42 °C, and 1 μL (200 units) of enzyme SuperScript III reverse transcriptase was added. The reaction took 1.5 h at 50 °C and was discontinued after 15 min at 70 °C.[76] The universal reverse primer (5′-GTGCAGGGGTCCGAGGT-3′) was used to detect expression. Specific primers were designed for each miRNA as forward and stem loop primers. qRT-PCR conditions were set as follows: 95 °C for 10 min followed by 50 cycles of 95 °C for 5 s, 56 °C for 10 s, and 72 °C for 30 s. All PCR products were denatured at 95 °C and chilled at 65 °C. Reactions were repeated at least three times for robust statistical analysis.[77]

Solvents and Reagents. Gallic acid, chlorogenic acid, 4-hydroxybenzoic acid, caffeic acid, vanillic acid, *trans*-ferulic acid, methanol, cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), lead (Pb), molybdenum (Mo), nickel (Ni), thallium (TI), tin (Sn), vanadium (V), zinc (Zn), nitrogen, nitric acid, dNTP, $MgCl_2$, PCR buffer, primers, SuperScript III reverse transcriptase, SYBR green, Taq-polymerase, triazole, ethyl alcohol, propanol-2, oligo dT, methanol, RNase out, and DTT were used.

Phenolic Acid Analyses. To determine the profiles of six phenolic acids (gallic acid, chlorogenic acid, 4-hydroxybenzoic acid, caffeic acid, anillic acid, and *trans*-ferulic acid) in five chickpea genotypes, the following methods were followed: Dried leaf tissues were first crushed into flour in a mill. Then, 2.5 g was placed in a falcon tube with 50 mL of a 25% (75:25 H_2O:CH_3OH) methanol solvent. The samples were centrifuged at 6000 rpm for 5 min after shaking at 200 rpm for 25 min at 25 °C. The supernatant part of the centrifuged sample was taken into vials with the help of a 0.25 μm filter, and the prepared samples were transferred to a Thermo Scientific UltiMate 3000 HPLC (high-pressure liquid chromatography) device for phenolic acid analysis. The components were DGU-20A5 degassing units, an SIL-20A HT autosampler, an SPD-M20A diode array detector, and a CTO-20A column oven. Chromatography was accomplished using wavelengths ranging from 280 to 320 nm and a C18 column with a separation of 250 nm, 4.65 μm. The injection volume was set to 20 μL, while the flow rate and temperature were set at 0.75 mL min^{-1} and 28 °C.[78]

Nitrogen and Protein Determination with an Elemental Analysis Approach. A Thermo Scientific Flash 2000 N protein analyzer evaluated the total nitrogen and protein concentrations. Approximately 5 g of the mature seed samples were taken and dried at 100 °C, and after reaching a constant weight, they were turned into flour with the help of a glass mortar, weighed between 2 and 4 mg, and packaged into tin capsules. Following placement of the packaged samples in the device's automatic sampling unit, the samples were delivered to the device's combustion reactor. The samples were then entirely burned in the colon furnace, and the gas released was shipped to a second reactor packed with copper rods filled with helium gas. The amount of nitrogen was measured in the thermal conductivity detector with the help of the gas generated as a result of this combustion. The combustion temperature was adjusted to 950 °C, its detector temperature was 65 °C, the carrier gas (He) flow rate was 100 mL min^{-1}, and the reference flow rate was 100 mL min^{-1}.[79]

Heavy Metal Analyses Using ICP-MS. To measure the cadmium (Cd), cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), lead (Pb), molybdenum (Mo), nickel

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(Ni), thallium (TI), tin (Sn), vanadium (V), and zinc (Zn) heavy metals, the plant leaf tissues were ground in a mortar, and about 5 g of samples was placed on crucibles and weighed. The crucible was caught in the back flame for 3−4 min after four drops of 65% (m/m) nitric acid were dropped on it. The temperature was then raised by 50 °C every half-hour, starting from 300 °C, which initiated the burning process in the ash oven. Whenever the temperature hit 550 °C, it was held there for 4 h. After cooling in the oven, the sample was treated with a 10 mL of 1 M nitric acid solution before filtering it using a black tape filter paper. After the container was washed multiple times with a 1 M nitric acid solution, the filters were collected in a 25 mL measuring balloon, and the total volume was completed with a 1 M nitric acid solution. The sample solutions were deposited in lidded polyethylene containers and analyzed by using an ICP-MS (inductively coupled plasma-mass spectrometry, Thermo) apparatus. In the heavy metal determination stage, standard solutions (0−100 mg/L) were made separately and in a known amount for each element, and calibration graphs were drawn for these standard solutions and desired elements. The elements' quantities were determined using the calibration graphs, and the results were statistically evaluated and interpreted.[80]

Statistical Data Analyses. The data obtained in the study were subjected to a two-way analysis of variance at the factorial level (5 × 2), and the statistical significance between the means was calculated at 0.05 using the Tukey test. All analyses were conducted by using SAS version 9.3.[81]

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Author Contributions

B.I., M.M., E.D.T., M.R., A.K., M.A.A., M.A., M.S., A.I.A., and A.D. performed conceptualization, formal analysis, resources acquisition, software use, validation, visualization, and review and editing of the manuscript; B.I. performed data curation and writing of the original draft; M.S. performed funding acquisition; M.A. performed investigation; M.M., E.D.T., M.R., A.K., M.A.A., and M.A. performed methodologies, project administration, and supervision. All authors have read and agreed to the published version of the manuscript.

Notes

The authors declare no competing financial interest.

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