*Avrupa Bilim ve Teknoloji Dergisi*
*Sayı 19, S. 189-195, Ağustos 2020*
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***Araştırma Makalesi***

www.ejosat.com ISSN:2148-2683

*European Journal of Science and Technology*
*No 19, pp. 189-195, August 2020*
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***Research Article***

# The Effects of Salicylic Acid Applications on miRNA Expression in Wheat Cultivars under Drought Stress

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(First received 6 April 2020 and in final form 23 May 2020)

(**DOI:** 10.31590/ejosat.715266)

**ATIF/REFERENCE:** Derelli Tüfekçi, E. & İnal, B. (2020). The Effects of Salicylic Acid Applications on miRNA Expression in Wheat Varieties under Drought Stress. *European Journal of Science and Technology*, (19), 189-195.

## Abstract

Wheat cultivation in Turkey, is highly affected by environmental factors such as drought stress, which reduces yields significantly. Applying chemicals that stimulate plant growth is an easy, low-cost, low-risk and effective approach to increase stress tolerance, and salicylic acid is prominent in adapting plants to adverse environmental conditions such as drought stress, salinity, and improving plants' tolerance to stress. In the study, Gün 91 and Ankara 98 cultivars were used as drought tolerant and Ankara 98the sensitive plant material, respectively. Two different doses (0.1 mM, 0.5mM) of salicylic acid (SA) were applied to these plants. Drought stress teratment was performed one week after SA application. Total RNAs were isolated from leaf samples collected from application and control pots. The expression levels of miR156, miR169, miR172, miR319, mir398 and the genes targeted by these miRNAs were determined. According to the results, in Gün 91, the expression levels of miR156 and miR172 was found higher in leaf tissues treated with SA under drought stress conditions. On the other hand, the level of miR169 was higher in Ankara 98. In Gün 91, miR319 expression level was higher in 0.5 mM salicylic acid + drought treatment, whereas in Ankara 98 was higher in 0.1 mM SA + drought treatment.

According to the control group, mir398 expression level decreased significantly in both 91 and Ankara 98 plant groups in which SA + drought stress was applied together. As a result of this study, it was emphasized that salicylic acid is an important signal molecule in response to stress and it mitigates the effect in stress conditions, and the tolerance mechanism of drought tolerant and sensitive cultivars with salicylic acid application were different.

**Keywords:** Wheat, Drought, miRNA, Salicylic acid

# Kuraklık Stresi Altındaki Buğday Çeşitlerinde Salisilik Asit Uygulamalarının miRNA İfadesi Üzerine Etkileri

## Öz

Türkiye'de buğday yetiştiriciliği yapılan alanlarda bitki büyümesi, kuraklık stresi gibi çevresel bir faktörden yüksek oranda etkilenmektedir ve bu abiyotik stres tarımsal ürünlerin verimini önemli derecede düşürmektedir. Bitki büyümesini uyaran kimyasalların bitkilere uygulanması stres toleransını arttırmak için kolay, düşük maliyetli, düşük riskli ve etkili bir yaklaşımdır ve bunlar içerisinde salisilik asit kuraklık stresi, tuzluluk gibi olumsuz çevre koşullarına bitkilerin uyum sağlamasında ve bitkilerin strese dayanıklılık geliştirmesinde ön plana çıkmaktadır. Bu çalışmada kuraklığa toleranslı çeşit olarak Gün 91, hassas çeşit olarak Ankara 98 çeşiti kullanılmıştır ve bitkilere iki farklı doz (0.1 mM, 0.5mM) salisilik asit uygulaması yapılmıştır. Salisilik asit uygulamasından bir hafta sonra kuraklık stresi uygulaması gerçekleştirilmiştir. Kuraklık stresi uygulamasının sonunda uygulama ve kontrol saksılarından toplanan

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yaprak örneklerinden RNA'lar izole edilmiş ve miR156, miR169, miR172, miR319 ile mir398 ve bu miRNA'lara ait hedef genlerin ifade seviyeleri belirlenmiştir. Kuraklık stres koşulları altında salisilik asit ile muamele edilen yaprak dokularında; miR156 ve miR172'nin ekspresyon seviyesi kuraklık stresine toleranslı Gün 91 çeşidinde yüksek iken, miR169 seviyesi hassas Ankara 98 çeşidinde daha yüksek olarak belirlenmiştir. miR319 ifade seviyesi Gün 91 çeşidinde 0.5mM salisilik asit+kuraklık uygulamasında daha yüksek iken, Ankara 98 çeşidinde 0.1mM salisilik asit+kuraklık uygulamasında daha yüksek olarak gözlenmiştir. miR398 ekspresyon seviyesi, hem Gün 91, hem de Ankara 98 çeşitlerinde salisilik asit ile kuraklık stresinin birlikte uygulandığı bitki gruplarında miR398 ifade seviyesi kontrol grubuna göre belirgin azalma göstermiştir. Salisilik asit uygulamasının tek başına yapıldığı gruplarda ise miR398 ifade seviyesi daha yüksek olarak belirlenmiştir. Sonuç olarak; bu çalışma ile salisilik asidin strese yanıtta önemli bir sinyal molekülü olduğu ve stres koşullarındaki etkiyi azalttığı ve stres tolerans mekanizmasının uygulanan salisilik asit ile kuraklığa toleranslı ve hassas buğday çeşitlerinde farklı olduğu vurgulanmıştır.

**Anahtar Kelimeler:** Buğday, Kuraklık, miRNA, Salisilik asit

# 1. Introduction

Wheat is a plant belonging to the genus Triticum from the Poaceae family. Wheat, which is a monocotyl plant, is a self-pollinating, fringed roots, annual fruit with a caryopsis type (Erayman et al., 2016; Mehmood et al., 2014). Wheat has a rather large genome of 1.6 x $10^{10}$ bp (~17 Gb) (Flavell and Smith, 1976), genome size is 35 times higher than rice (*Oryza sativa* L.) and 6 times greater than corn (*Zea mays* L.) (Arumuganathan and Earle 1991). Hexaploid bread wheat (*Triticum aestivum* L.) was obtained by crossing tetraploid (AABB) *Triticum dicoccoides* and diploid (DD) *Aegilops tauschii* and carries three diploid genomes (A, B and D) (Erayman et al., 2016; Brenchley et al., 2012). It is considered as an indispensable product in the nutrition of the rapidly increasing world population in terms of being cultivated in diverse climates and soil conditions, containing carbohydrates, starch, protein, some vitamins and minerals in its composition, and is a valuable and inexpensive food source.

Wheat breeding in the areas of plant growth in Turkey, from environmental factors such as drought stress are affected by high rates and this abiotic stress significantly reduces the yield of agricultural products (Rahaie et al., 2010). Drought has been observed as the most common natural stress factor with a rate of 26% when available areas of the world are classified according to stress factors. In recent years, it has become an even more important meteorological phenomenon with population growth and global warming. It is a situation that occurs due to the decrease in the amount of precipitation or irregularity. Drought is mentioned as a result of the deterioration of the balance between precipitation and evaporation in a certain region. High temperature, changes in precipitation intensities, delay of the rainy season, strong wind and low humidity are among the important factors that play a role in the formation of drought. The drought is very slow and its effectiveness continues for a long time. Agricultural drought is defined as the lack of water in the soil to meet the needs of the plant. It causes a slowdown in the development of plants and a decrease in the number of crops (Miyashita et al., 2005).

Salicylic acid (SA) is a natural phenol product extracted from willow bark (*Salix sp.*) for the first time, and its use in adapting to adverse environmental conditions such as osmotic stress and salinity is among the approaches that enable plants to develop resistance to stress (Senaratna et al., 2000). There are reports that SA provides tolerance to many stress factors (such as temperature, drought and cold). In a study with tomato and bean seedlings exposed to cold and drought stress, SA caused seedlings to remain green longer (Senaratna et al., 2000), Hamada and Al-Hakimi (2001), applied 100 ppm SA to wheat seeds and then investigated the effects of applications against drought and salt stress during the seedling stage. Wheat seedlings were irrigated with water containing 160 mM NaCl for 2 weeks (salt stress) and 30% of the field capacity for 2 weeks (water stress). As a result of the experiment, they reported that SA applied wheat seedlings survived, resulting in higher photosynthesis rate, higher $K^{+}$, $Ca^{+2}$ and $Mg^{+2}$ ion uptake compared to lower $Na^{+}$ ion uptake compared to control plants. Bhupinder and Usha (2003); investigated physiological and biochemical changes caused by salicylic acid application on wheat seedlings (1-3 mM) under drought stress. They determined that the dry weight of the plants increased significantly in the SA applied plants compared to the ones not applied, and the humidity amount decreased due to drought stress, but the humidity amount of the plants in drought stress was higher with the SA application compared to the plants SA not applied. It has been reported with studies that miRNAs have a very important role in drought stress tolerance in plants. It was stated that the expression of miR172 increased 2-fold in the Arabidopsis plant, which was exposed to cold stress by keeping it at +4°C, compared to the plant that was not exposed to stress. In addition, it was found that the expression of miR172 increased in tobacco plant exposed to drought and salinity stress (Frazier et al. 2011; Xin et al., 2011). Excessive expression of miR159 increases susceptibility to abscisic acid and miR159 is also involved in drought, salinity, boron tolerance, and bacterial pathogen response by targeting transcription factor genes (Frazier et al., 2011; Xin et al., 2011; Alonso-Peral et al., 2012). It is known that miR167 also plays a role in stress response such as drought, salinity, cold, bacterial pathogen (Xue et al., 2009; Yanik et al., 2013). In addition, the SBP (SQUAMOSA promoter binding protein) transcription factor family involved in the plant's biological processes such as leaf morphogenesis, aging, flowering time and seed development is controlled by miR156. The expression of several miRNAs increased or decreased with one or more stresses applied. While miR393 NaCl is highly expressed with cold, drought and ABA application; miR397b and miR402 were slightly expressed in all stress applications while miR319c was over-expressed with cold stress; however, this was not observed in drought, NaCl or ABA stresses (Liu et al., 2008; Sunkar et al., 2012). The studies in barley in the case of drought revealed tissue-specific regulation of four miRNAs. While the regulation of miR169 increases, the regulation of the roots decreases. While miR156a, miR171 and miR408 were induced in the leaves, no change was observed in the roots. This shows the tissue-specific importance during miRNA studies in the whole plant (Kantar et al., 2010).

In this study, it was aim to determine the expression level / condition of miRNAs and target genes in the tolerance mechanism of drought stress tolerant and sensitive wheat varieties applied to salicylic acid.

## 2. Material and Method

### 2.1. Plant materials, Growth and Stress Conditions

In the selection of materials, wheat varieties that are prominent in terms of being tolerant and sensitive to drought stress were evaluated following the main objectives of the study. In this context, Gün 91 and Ankara 98 was used as drought stress tolerant and sensitive cultivars, respectively (given a reference). Within the scope of the study, the soil, which was first turned into a heap for growing plants, was filled in pots with a diameter of 25 cm and a depth of 30 cm. Fourteen seeds were planted in each pot, and the number of plants in the pot was reduced to 7 during the tillering period. Three replications were planted for drought stress application with control groups. Plants were grown for 15 days until they reached the 3-4 leaf stage, and 2 different doses (0.1 mM and 0.5 mM) of salicylic acid were sprayed onto the leaves. Drought stress was applied one week after the salicylic acid application. Three plant pots, which was randomly selected for drought stress factor, were placed in climate cabins previously set at 24ºC during the light and 14ºC at dark. In the climate cabins, light and dark times are adjusted to 16 and 8 hours, respectively, and relative humidity is 75%. In drought stress application, all pots were filled with water up to the field capacity, and no water was given to the pots from this point on the day of drought stress application. During the stress application, the amount of moisture in the soil was checked regularly, and the control pots that did not have apply salicylic acid applications were regularly irrigated. At the end of the 10th day of drought stress treatment, leaf samples were collected from the application and control pots were taken in separate transparent bags, quickly frozen in liquid nitrogen and the materials were stored in a -80°C freezer for use in RNA isolation studies.

### 2.2. RNA isolation, cDNA synthesis and RT-PCR

Total RNA was extracted by using the TRIzol reagent (Trade mark) according to the manufacturer's instructions. Total RNA libraries were enriched in terms of small RNAs by using the mirVana miRNA Isolation Kit. RNA quality was checked on 1.5% agarose gel, and the concentration of the RNA was measured via NanoDrop ND-2000c spectrophotometer. To measure the expression levels of five miRNAs and their target genes in leaf tissues of wheat, total RNA samples were exposed to synthesize first-strand cDNA primed with oligo (dT) in a 20 µl reaction mix using M-Mule Reverse Transcriptase (Trade mark) following the manufacturer's instructions. The qRT-PCR was conducted in 96-well optical plates using 100 pmol forward and reverse primers, 2 µl cDNA, 10 µl FastStart SYBR Green I Master Mix (Trade mark) and to complete the final volume to 20 µl with the nuclease-free distilled $H_2O$. The qRT-PCR was achieved on Thermo PikoReal™ Real-Time PCR System. All reactions were designated as triplicate and the 18S rRNA gene was used for gene normalization (Inal et al., 2014). To measure the expression levels of five miRNAs (miR156, miR169, miR172, miR319 and mir398), The miRNA stem-loop reverse transcription (RT) was used as follow: 1 µg of total RNA sample, 0.5 µl 10 mM dNTP mix, 1 µM stem-loop RT primer, and 11.15 µl of nuclease-free distilled $H_2O$ were mixed. The mix was incubated at 65°C for 5 min and then put on ice for 2 min. Then, 4 µl of first-strand buffer (5X), 2 µl of 0.1 M dithiothreitol, 4 U RNAseOUT, and 50 U SuperScript III were added into each tube. Three control reactions containing all components without RT primer, RNA template, or SuperScript III were also done. qRT-PCR was conducted using the SYBR Green I Master Kit on a Thermo PikoReal™ Real-Time PCR System. Three technical and biological replicates were used for each experiment. The relative fold change for each comparison was calculated by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

## 3. Results and Discussion

Highly expressed miR156, miR169, miR172, miR319 and miR398 conserved miRNAs, which are prominently expressed in drought stress tolerance and sensitivity. Expression levels of those miRNAs and their targets were measured with qRT-PCR (Table 1). The SBP transcription factor family, which is involved in biological processes of plants such as leaf morphogenesis, aging, flowering time and seed development, is controlled by miR156. In addition, abiotic stresses such as drought, salinity, boron excess, cold and biotic stresses such as fungal pathogens change the expression of miR156 in the plant (Eldem et al., 2013).

*Table 1. miRNA and target gene information*

| miRNA | Mature miRNA | Target Gene |
|---|---|---|
| **miR156** | TGACAGAAGAGAGTGAGCAC | **SquamosaPromoter Binding Like Protein** |
| **Pre-miRNA** | GGGTAAAGGAGGTGACAGAAGAGAGTGAGCACACATGGTGTTTTCTTGTATGA-TATTCTCCATGCTTGAAGCTATGGGTGCTCACCCTCTTTCTGTCACCCCACCACTCT-CTCTCTCTCTC | |
| **miR169** | UGAAGCUGCCAGCAUGAUCUU | **Nuclear factor-Y (NF-YA)** |

| | | |
|---|---|---|
| **Pre-miRNA** | ATCGTGCACCACTAGCAGATGAAGCTGCCAGCATGATCTTAACTCGCCTTT-GATCAACATCGACTCGGGGAGAGATCAGATCATGCGGCAGCTTCACCTGTT | |
| **miR172** | TGAATCTTGATGATGCTACGC | **APETALA2 (AP2)** |
| **Pre-miRNA** | AGTCGTTTATTGCCGATGCAGCATCATCAAGATTCTCACCATCAAAAGCTTTGGTA-GAGAGAGAGAGAGAGAGACTATATCAGTTTACCATCTTGCCTTTATATGTGAATCTTGATGGTGCTAC | |
| **miR319** | UUGGACUGAAGGGAGCUCCC | **TCP4 Transcription Factor** |
| **Pre-miRNA** | GAAGAGAGCTTTCTTCAGTCCAGTTATGGGAGGCCGTAGGATTCAATTTGCTGTT-GACTCATTCATCCAAATGCTGTGTAGTTGATGAGTTTTACACGGTAACTGAGTGAAT-GATGCGAGAGTCAAGTTGAATCTTAAGCTTTCTGTACTTGGACTGAAGGGAGCTCCCTT | |
| **miR398** | AAGCUCAGGAGGGAUAGCGCC | **Cu/Zn superoxide dismutases** |
| **Pre-miRNA** | AGTATAGAAGAATCTTTAAAGCTCAGGAGGGATAGCGCCGTAAGGATCTATATATTT-TACATCATTAGTGGCGCTATCTATCCTGAGTTTCATGGGTTGTTCTTGCT | |

In this study, as a result of qRT-PCR analysis, SA applications were found to increase the expression level of miR156 in Gün 91 wheat cultivar tolerant to drought stress compared to sensitive Ankara 98. In addition, although drought stress and different salicylic acid dosing responses were showed the different profile in tolerant and sensitive cultivars of wheat, it is concluded that salicylic acid has protective properties against drought stress and 0.5 mM salicylic acid dose on drought stress (Figure 1).

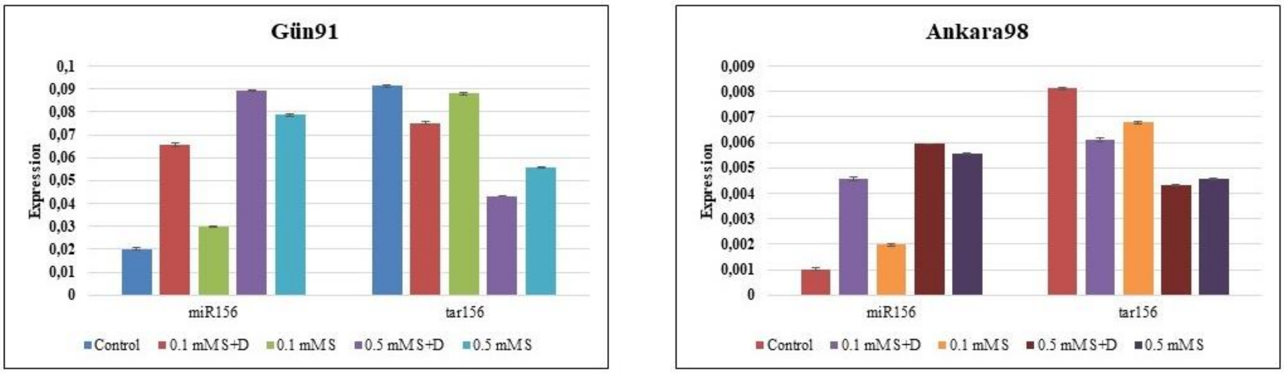

Figure 1. The expression levels of mir156 and tar156 in Gün 91 and Ankara 98 varieties

The miRNA genes target transcription factors that play a role in regulating the expression of many genes, it promises to obtain plants resistant to abiotic stress with miRNA-based RNAi technology in the coming years. Accordingly, miR169 has been found to play an important role in plant development and stress response in plants by regulating the expression of the ”Nuclear Factor Y A5 (NFYA5)” transcription factor. In this study, it was observed that miR169 expression showed a decreasing profile during drought compared with the control group and increased with a salicylic acid application. It was also determined that miR169 expression level was higher in drought stress sensitive Ankara 98 than tolerant Gün 91 (Figure 2).

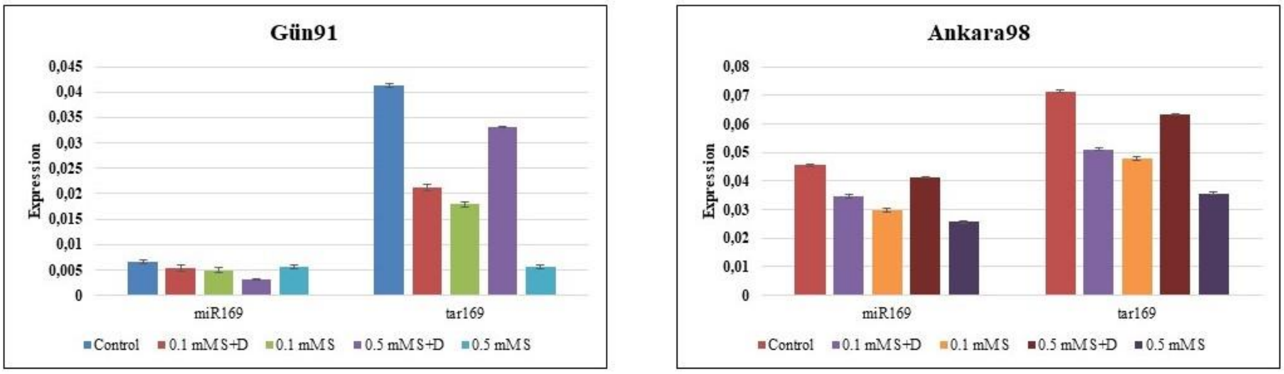

Figure 2. The expression levels of mir169 and tar169 in Gün 91 and Ankara 98 cultivars (S: SA, D: Drought)

Zhang et al. (2014) worked on drought tolerance and identified 458 known, 674 new miRNAs from the control and 471 known miRNAs in drought applied samples. Accordingly, most of the found were transcription factors and found that the expression levels of miR168 and miR166 decreased and the expression level of miR156 increased. In this study, it was determined that miR156 expression level was higher as a result of drought stress compared to the control group, and that salicylic acid applications increased the expression level. A high level of expression of miR169 increases tolerance for drought stress in tomato, while decreasing tolerance in *Arabidopsis thaliana* (Ramanjulu et al., 2012). Plant miRNAs have been showed variable results according to stress and plant species. Apetala 2 (*AP2*) carries a complementary region for miR172 and is negatively regulated by miR172. *AP2* is an important gene involved in the determination of flower organs. miR172 plays a critical role in suppressing *AP2* genes involved in determining flower organs and flowering time. In addition, it was determined by studies that the expression of miR172 increased in plants exposed to abiotic stress such as drought and salinity. In this study, as a result of drought stress application, miR172 expression increased especially in tolerant Gün 91 compared to the control group. As a result of drought stress after salicylic acid application, it was determined that miR172 expression level was higher and salicylic acid played a big role in regulating plant responses in drought stress (Figure 3).

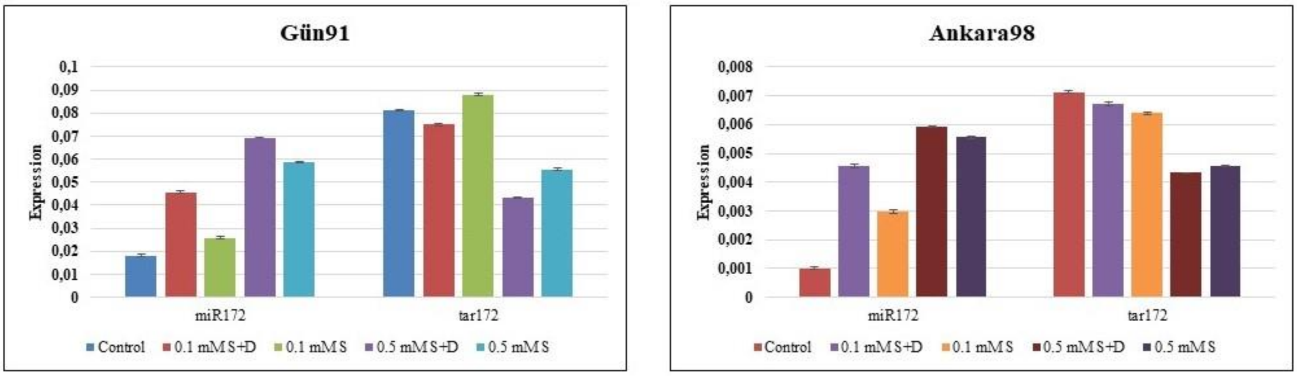

Figure 3. The expression levels of mir172 and tar172 in Gün 91 and Ankara 98 cultivars (S: SA, D: Drought)

Hwang et al. (2011) determined that the expressions of stu-miR172c, stu-miR172d, and stu-miR172e miRNAs changed under drought stress conditions. On the other hand,Yang et al. (2013) conducted a study on miRNAs in proline increase in drought stress. As a result of expression and function analysis, findings have been obtained that miR172, miR396a, miR396c and miR4233 may be involved in regulating the expression of the *P5CS* gene, one of the genes responsible for proline synthesis. In plants, miR319 has been proven by many studies that play a role in abiotic stress tolerance by targeting the "TCP (TEOSINTE BRANCHED/CYCLOIDEA/PCF )" transcription factor, and the expression of miR319 has been inversely correlated with the expression of the target gene, the TCP4 transcription factor protein. In the drought tolerant Gün 91, it was observed that miR319 expression increased significantly as a result of drought stress application especially with 0.5 mM salicylic acid. Although this situation is also observed in the sensitive cultivar, Ankara 98, miR319 expression level was found to be lower. In Gün 91, miR319 expression level was higher in 0.5mM SA+drought treatment, whereas in Ankara 98 was higher in 0.1mM SA+drought treatment (Figure 4).

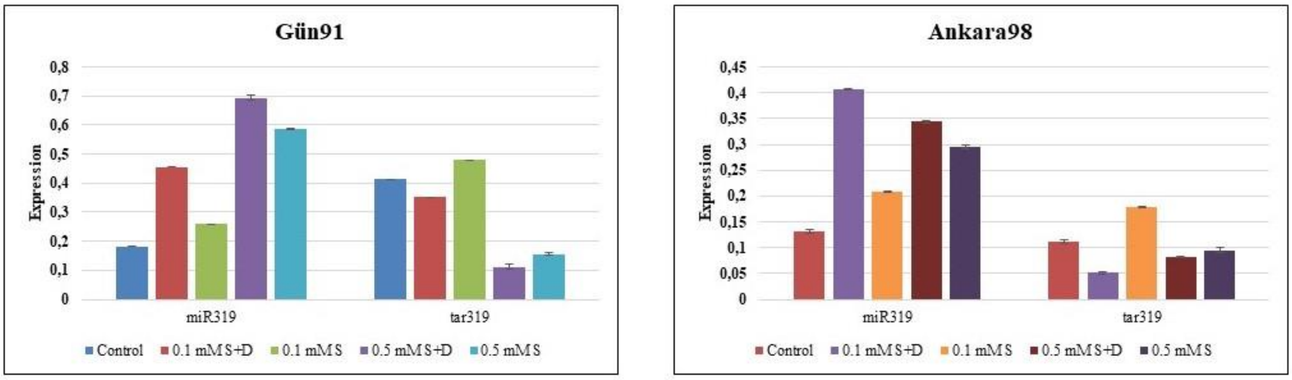

Figure 4. The expression levels of mir319 and tar319 in Gün 91 and Ankara 98 cultivars (S: SA, D: Drought)

Zhou et al. (2013) conducted a study to investigate the drought and salinity tolerance of *Agrostis stolonifera* L., a grass species, through miR319. As a result of the study, the miR319 gene has been transferred and transgenic *A. stolonifera* L. plants, which express high levels of miR319, have increased tolerance to drought and salinity. As a result of gene expression analysis, the expression of four target genes (TCP-AsPCF5, TCPAsPCF6, TCP-AsPCF8 and TCP-AsPCF14) of miR319 has been significantly reduced and increased abiotic stress tolerance may be associated with a decrease in expression of these genes. miR398 gene expression decreases with drought-like many other abiotic strains. The increased regulation of Cu/Zn superoxide dismutase (*CSD*) genes is associated with miR398.

Cytosolic CSD1 and plastidic CSD2 are both targets of miR398. In the case of oxidative stress, the expression level of miR398 decreases; thereby increasing the accumulation of CSD1 and CSD2 mRNAs. Thus, as with many drought, miR398 gene expression decreases with drought. In line with this information, both plant groups in which Gün 91 and Ankara 98 cultivars were applied with salicylic acid and drought stress, miR398 expression level decreased significantly compared to the control group. In the groups where the salicylic acid application was performed alone, the miR398 expression level was determined to be higher (Figure 5).

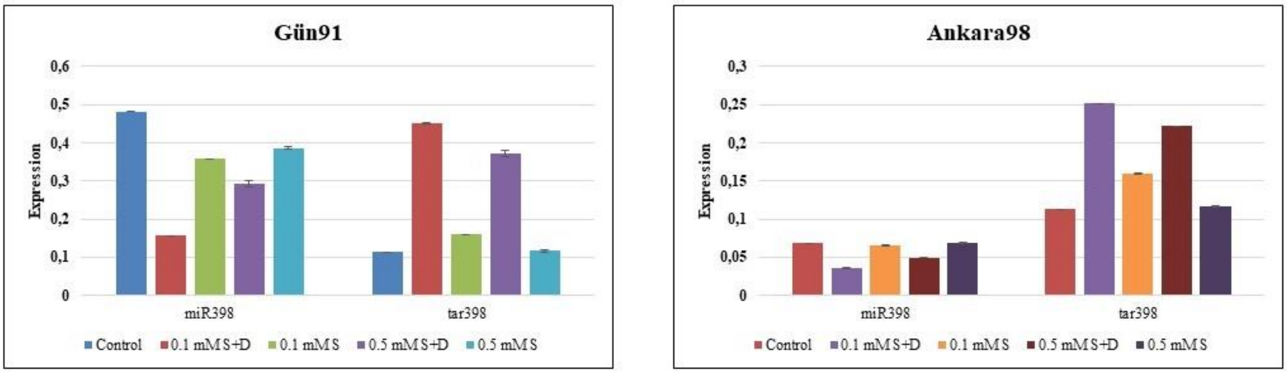

Figure 5. The expression levels of mir389 and tar389 in Gün 91 and Ankara 98 cultivars (S: SA, D: Drought)

miR398 gene expression decreases with drought-like many other abiotic strains. mir398; regulates cytoplasmic and chloroplastic Cu/Zn superoxide dismutase (CSD) genes post-transcriptionally (Zhua et al., 2011; Sunkar et al., 2012). In this study, miR398 expression level decreased significantly in both the groups of Gün 91 and Ankara 98, where salicylic acid and drought stress were applied together, compared to the control group. As a result, current study will be a guiding resource for plant physiology and nutrition and molecular studies that aim to researching the drought tolerance mechanism in plants.

## 4. Conclusions

In the current study, the tolerance mechanism of salicylic acid applications in drought tolerant and sensitive wheat cultivars was investigated in terms of expression of miRNAs and target genes. In this context, the molecular changes under the drought stress of the wheat plant were determined and most importantly, the contribution of salicylic acid to productivity in plant production and the protective effect on the plant in terms of drought tolerance were determined. In addition, it has been emphasized that salicylic acid is an important signal molecule in response to stress and mitigates the effect in stress conditions, as well as the tolerance mechanism of drought stress tolerant and sensitive wheat cultivars differed by drought stress.

## 5. Acknowledge

This study was financially supported by Çankırı Karatekin University Scientific Research Projects Coordinator (BAP) under the project numbered YM0150219B12.

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