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EGE UNIVERSITY

Graduate School of Applied and Natural Science

COLD TOLERANCE RELATED MI RNA EXPRESSION PATTERNS IN CULTIVATED AND WILD TOMATO GENOTYPES

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Plant Breeding – Second Cycle Programme with Thesis

İzmir

2020

Houtan Vafaeifard tarafından yüksek lisans tezi olarak sunulan “COLD TOLERANCE RELATED MI RNA EXPRESSION PATTERNS IN CULTIVATED AND WILD TOMATO GENOTYPES” başlıklı bu çalışma EÜ Lisansüstü Eğitim ve Öğretim Yönetmeliği ile EÜ Fen Bilimleri Enstitüsü Eğitim ve Öğretim Yönergesi’nin ilgili hükümleri uyarınca tarafımızdan değerlendirilerek savunmaya değer bulunmuş ve 06.06.2022 tarihinde yapılan tez savunma sınavında aday oybirliği/oyçokluğu ile başarılı bulunmuştur.

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EGE ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ

ETİK KURALLARA UYGUNLUK BEYANI

EÜ Lisansüstü Eğitim ve Öğretim Yönetmeliğinin ilgili hükümleri uyarınca Yüksek Lisans Tezi / Doktora Tezi olarak sunduğum "COLD TOLERANCE RELATED MI RNA EXPRESSION PATTERNS IN CULTIVATED AND WILD TOMATO GENOTYPES" başlıklı bu tezin kendi çalışmam olduğunu, sunduğum tüm sonuç, doküman, bilgi ve belgeleri bizzat ve bu tez çalışması kapsamında elde ettiğimi, bu tez çalışmasıyla elde edilmeyen bütün bilgi ve yorumlara atıf yaptığımı ve bunları kaynaklar listesinde usulüne uygun olarak verdiğimi, tez çalışması ve yazımı sırasında patent ve telif haklarını ihlal edici bir davranışımın olmadığını, bu tezin herhangi bir bölümünü bu üniversite veya diğer bir üniversitede başka bir tez çalışması içinde sunmadığımı, bu tezin planlanmasından yazımına kadar bütün safhalarda bilimsel etik kurallarına uygun olarak davrandığımı ve aksinin ortaya çıkması durumunda her türlü yasal sonucu kabul edeceğimi beyan ederim.

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İmzası

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ÖZET

BAZI YABANİ VE KÜLTÜR DOMATES GENOTİPLERİNDE SOĞUK TOLERANSI İLE İLGİLİ MİRNA İFADE ANALİZLERİ

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Haziran 2022, 61 sayfa

Kültür domatesi (*Solanum lycopersicum L.*), dünyada genetik ve moleküler ıslah çalışmalarında hedef bitki olarak kullanılan popüler sebzelerden biridir. Domates, tropik kökenli olduğu için soğuk stresine karşı hassastır. Soğuk stresi, bitkinin büyüme ve gelişmesine kolayca zarar verebilmektedir. Bu çalışmada, *Solanum habrochaites* (LA 1777) ve *Solanum pimpinellifolium* (LA 1579) yabani genotipleri ile *Solanum lycopersicum* cv. Anit F1 ve *Solanum lycopersicum* cv. Lice kültür çeşitlerinde, soğuk stresinin miRNA ifadeleri üzerine etkileri araştırılmıştır. Bu genotiplere ait genç bitkiler, 4°C'de 4, 6 ve 8 saat soğuk stresine maruz bırakıldıktan sonra miRNA 167, miRNA 169, miRNA 172, miRNA 393 ve miRNA 397'nin ekspresyon düzeyleri, Real-time PCR ile değerlendirilerek, bu miRNA'ların soğuk toleransı ile bağlantılı genlerin ekspresyonunu kontrol etmekten sorumlu olup olmadıkları araştırılmıştır. Ayrıca bu bitkilerde bazı morfo-fizyolojik özellikler de gözlenmiştir.

S. habrochaites (LA 1777), *S. pimpinellifolium* (LA 1579) ve *S. lycopersicum* cv. Anit F1 genotiplerinde farklı soğuk stres seviyelerinde miRNA 167, 169 ve 172 ve 397'de önemli artış belirlenmiştir, Buna karşın, *S. lycopersicum cv.Lice* genotipinde bu miRNA'ların ifade düzeyinde artış belirlenmemiştir. Ayrıca bu çalışmada yaprak yaş ve kuru ağırlığı, kök yaş ve kuru ağırlığı, yaprak alan indeksi, klorofil içeriği ve çiçeklenme günündeki değişimler *S. lycopersicum* cv.Lice'de soğuk stresine bağlı olarak istatistiki önemde değişiklik göstermiştir.

S. pimpinellifolium (LA 1579) genotipinde soğukla ilişkili miRNA ekspresyonu seviyeleri ve değerlendirilen morfo-fizyolojik parametrelerdeki önemli farklılıklar, bu genotipin soğuk stresine tolerant olabilceğini, buna karşın *S. lycopersicum* cv.Lice genotipinin soğuk stresine duyarlı olabileceğini göstermiştir.

Anahtar Kelimeler: miRNA, soğuk stresi, Real-time PCR, gen ekspresyonu, morfo-fizyolojik özellikler

ABSTRACT

COLD TOLERANCE RELATED MIRNA EXPRESSION PATTERNS IN CULTIVATED AND WILD TOMATO GENOTYPES

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June 2022, 61 pages

The cultivated tomato (*Solanum lycopersicum* L.) is one of the popular vegetables in the world that has been used as a target plant in genetic and molecular breeding studies. As this plant originated from the tropics, it is susceptible to chilling stress. Chilling stress can easily damage its growth and development. In the present study, The expression level of miRNA 167, miRNA 169, miRNA 172, miRNA 393, and miRNA 397 in wild genotypes of *Solanum habrochaites* (LA 1777) and *Solanum pimpinellifolium* (LA 1579) and cultivated genotypes of *Solanum lycopersicum* cv. Anit F_1 and *Solanum lycopersicum* cv. Lice were assessed at 4, 6, and 8 hours after chilling treatment (4°C) via Real-time quantitative PCR to determine if these miRNAs are responsible for controlling the expression of the genes associated with chilling tolerance. Also, some morpho-physiological traits were observed in these plants. Significant upregulation of miRNA 167, 169 and 172, and 397 were observed at different levels of chilling stress in the tolerant genotypes of *S. habrochaites* (LA 1777) and *S. lycopersicum* cv. Anit $F_{1.}$ and also, in *S. pimpinellifolium* (LA 1579) while this level of up-regulation was not seen in *S. lycopersicum* cv. Lice. Also, the changes in the fresh and dry weight of leaf and root, specific leaf weight, leaf area index, the chlorophyll content, and flowering day measured in this study were significant in *S. lycopersicum* cv. Lice compared to the other genotypes.

The observed levels of cold-related miRNA expression in *S. pimpinellifolium* (LA 1579) and significant differences in the evaluated morpho-physiological

parameters indicated the tolerance of *S. pimpinellifolium* (LA 1579) and susceptibility of *S. lycopersicum* cv. Lice to chilling stress.

Keywords: miRNA, chilling stress, Real-time PCR, gene expression, morpho-physiological responses

ACKNOWLEDGMENT

I would like to thank to Dr. Aylin KABAS, for supporting the seeds of the tomato genotypes in the thesis. All the stages of this research have been done using the equipment of the laboratories in the Seed Technology Center and the Department of the Horticulture, Ege University.

Izmir
08.05.2022
Houtan Vafaeifard

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Symbols and Abbreviations

µg	Microgram
µL	microlitre
ng	nanogram
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary DNA
DNase	deoxyribonuclease
Ribonuclease	RNase
dNTP	deoxyribonucleoside triphosphates
PCR	Polymerase chain reaction
qRT PCR	Real-Time Quantitative Reverse Transcription PCR
FC	Fold change
Cp	crossing point
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
miRNA	MiRNA
SLW	Specific Leaf Weight
Pri-mirna	Primary-miRNAs

1. INTRODUCTION

The plants originating from tropical areas such as tomato (S. lycopersicum L.) lack the ability to acclimatize to low temperatures (Ou et al., 2015). Tomato (S. lycopersicum L.) is a popular and essential vegetable crop around the world because of its nutritional, economic, and therapeutic benefits (Gerszberg et al., 2015). It is quite vulnerable to low temperatures (Weiss and Egea-Cortines, 2009). Cultivated tomato (S. lycopersicum L.) is vulnerable to chilling stress in all steps of its growth process (Liu et al., 2012). Low temperatures can lead to a deleterious effect on several molecular, physiological and morphological elements of tomato (S. lycopersicum L.) development (Wu et al., 2022; Mesa et al., 2022). This condition can reduce tomato seed germination and disturb vegetative growth by degrading hydration status and photosynthesis (Foolad and Lin, 2000, 2001; Venema et al., 2005). Furthermore, the low temperature has a negative impact on tomato reproductive development, which reduces the fruit set (Lozano et al., 1998).

Assessment of the gene expression regulation system of cold-responsive genes is critically important to decipher chilling stress tolerance mechanisms. There have been a large number of studies regarding the importance of the genes encoding chilling-regulated proteins such as anti-chill proteins in the tomato plant's reactions to chilling stress (Kyu et al., 2019; Ma et al., 2018; Zhang, 2020; Wang et al., 2020).

MiRNAs are one of the different types of small non-coding RNA molecules that are considered vital molecules in plant responses to environmental stimuli (Wang et al., 2017). The intial miRNA was explored in plants less than 20 years ago (Millar, 2020). There has been a wealth of knowledge released on the origin of miRNAs since the research of Reinhart et al. (2002) on plant miRNAs. Following their discovery, evidence of the existence of more than 2000 plant miRNAs was published (Alptekin et al., 2017). MiRNAs are abundant and variable and many biological processes in plants require the interference of miRNAs at various phases of growth (Voinnet, 2009). Recent genetic studies aimed at determining the origin, synthesis, and modes of action of plant miRNAs (Rogers and Chen, 2013). The mature miRNA performs its function during the post-transcriptional regulation (Michlewski et al., 2019). The nucleus is the first site for the primary miRNA (pri-

mRNA) to be transcribed. Following this process, the precursor miRNA (pre-mRNA) is generated by cleaving the pri-mRNA. The cleavage of pri-mRNA results in a duplex of mature miRNA. The mature miRNA needs to be loaded in an RNA-induced silencing complex (RISC) for mRNA degradation or translation inhibition (Cardoso et al., 2018).

The up or down-regulation of some miRNAs under environmental stresses was proven in many crops (Khraiwesh et al., 2012). The miRNAs that are involved in chilling defense mechanisms have recently been discovered in various plants including Arabidopsis thaliana Populus tomentosa, Oryza sativa, Prunus persica, Brachypodium, and Zea mays, and Astragalus propinquus (Sunkar and Zhu, 2004; Abla et al., 2019; Chen et al., 2012; Lv et al., 2010; Barakat et al., 2012; Zhang et al., 2009; Yang et al., 2011). The predicted target genes of mentioned miRNAs probably mediate several physiological and biochemical responses to chilling stress. It is also found that many conserved and novel miRNAs were associated with the different levels of heat stress in S.lycopersicum L. and it elucidates the miRNA-mediated regulatory mechanism in this plant in abiotic stresses (Zhou et al., 2016).

High-throughput sequencing was used to find many conserved and new miRNAs implicated in the chilling response in a tolerant genotype of tomato (S. habrochaites LA 1777), and degradome sequencing was used to examine the target genes. Significant up and down-regulation of a number of these novel and conserved miRNAs have been approved (Cao et al., 2014). In addition, some chilling-stress-related miRNA expressed differentially in tomato (S.lycopersicum L. var. H-2274) under chilling stress and their important effect on chill-related genes and chilling tolerance was approved (Koc et al., 2015).

Having said that, there is little research about the chilling-responsive miRNAs in different species of tomato treated by various levels of chilling stress at different stages of growth. It seems that more detailed information is required for a better understanding of the miRNA differential expression in the wild and cultivated species and its correlation with morpho-physiological traits. Also the level of sensitivity to chilling stress in the wild tomato of Solanum pimpinellifolium (LA 1579) and cultivated tomato of S. lycopersicum cv. Lice had not been determined

in the previous studies. The present study provided some comparable information to extend and complete previous data regarding the expression profile of these miRNAs and the relation of their expression profile with the tolerance of the mentioned genotypes.

1.1. Thesis Goal

In this thesis, the differential expression of miR167, miR169, miRNA172, miRNA 393, and miRNA 397 were evaluated in the wild tomato genotypes including *S. habrochaites* (LA1777), *S. pimpinellifolium* (LA1579), and cultivated tomato genotypes including *S. lycopersicum* cv. Anit F_1. The expression level of these miRNAs was measured in leaf samples under chilling conditions (4°C) at three distinct stress points. Quantitative real-time PCR was used in this project. Also, some morpho-physiological traits including the fresh and dry weight of leaf and root, specific leaf weight, leaf area index, the chlorophyll content, and flowering day were evaluated.

2. LITERATURE REVIEW

2.1. Chilling Stress and Physiological Traits

2.1.1. Chlorophyll Content:

Any substance or stimulus that inhibits plant metabolism, growth, development, or crop productivity, including biotic and abiotic stressors, is referred to as stress (Lichtenthaler, 1998). Abiotic stress has a negative impact on plant growth and agricultural productivity (Guo et al., 2018; Liu J. et al., 2018).

Tomato (S. lycopersicum L.) has evolved several physiological and molecular processes that allow them to adapt to chilling stress. Much progress has been made in describing the primary components related to chilling stress tolerance and investigating their regulatory mechanisms over the last two decades (Solanke et al., 2008). Many physiological parameters can be affected by chilling stress including chlorophyll (Chl) content which is a vital photosynthetic pigment for plants, largely determining photosynthetic capacity and thus plant growth. However, the information regarding the main factors involved in the chlorophyll content is very limited (Li et al., 2018). Chlorophyll biosynthesis was clearly hindered in chilling stress during growth, and chlorophyll buildup decreased. The suppression of conversion from protochlorophyllide (Pchlide) to chlorophylls (Chls) and the inhibition of aminolevulinic acid (ALA) synthesis may result in a decrease in chlorophyll synthesis (Jiwan, 2009).

Two physiological research projects were conducted to assess the effects of chilling stress on tomato plants (Lycopersicon esculentum Mill. Sunny) in 1990. Before being transferred to the field, the seedlings of tomato were subjected to cyclic cold stress in a greenhouse at 2±1°C and 29±6°C. The plants were exposed to 2°C for 3, 6, or 12 hours for 1, 3, or 6 days before transferring to the field during the first year of a 2-year research project. Prior to field planting in the following year, plants were exposed to 2°C for 4, 7, or 10 days for 6, 12, or 18 hours at a time. According to the physiological results, cold stress typically decreased seedling height, leaf area, and shoot and root dry weights during the first year. All seedling

growth characteristics decreased in the second year as a result of the chilling stress treatment. In the first experiment, different chilling-stress levels were discovered to be usually stimulators to seedling growth and development. The prolonged periods of cold stress stopped the respiration of the seedlings. Also in the second trial, excessive chilling stress prevented growth completely. Low levels of chlorophyll may be a sign that prolonged cold exposure permanently damaged photosynthetic machinery as it was reported by King et al. (1988). The results of this study demonstrated the serious impact of the short and long periods of chilling stress on tomato physiological parameters (Dufault et al., 1990).

Brüggemann and Linger (1994) evaluated the characteristics of two key Calvin-cycle enzymes, stromal fructose-1,6-bisphosphatase (sFBPase) and Rubisco, in four lines of wild tomato (L. peruvianum Mill.) and a cultivated tomato (L. esculentum Mill.). The activation energy (EA) of the process produced by sFBPase decreased by 5–10 kJ/mol in L. esculentum and the three L. peruvianum lines while exposed to chilling (10°C) for 14 days. When L. peruvianum was chilled, there was either no loss of enzyme activity or very little. This suggested that the latter species can adapt its Calvin-cycle enzymes to low temperatures. As a result, unlike cultivated tomatoes, wild tomato species recover quickly after being exposed to sub-optimal temperatures, as evidenced by increased growth capacity and decreased photosynthesis inhibition.

The findings of Cornejo-Ríos et al. (2021) demonstrated the effect of chilling stress on chlorophyll content in tomato (Solanum lycopersicum cv. Vita). The seeds were sown on 200-cell trays with sterile perlite and sterile peat. The seedlings were moved after 14 days of germination and kept in the growth chamber at 24–30 °C during the day and 18 °C at night. The photoperiod ratio was 16h / 8h for light and darkness. Up until the application of the stress treatment, irrigation with either tap water or Hoagland's nutritional solution was applied on alternate days. For all trials, plants were used at 35 days of age. The plants were treated with chilling stress (4°C) for 9 days and then the chlorophyll content was measured. Using a spectrophotometer, absorption data at 663 and 645 nm were used to analyze the chlorophyll concentration. It was concluded that chilling stress lowered the amount of chlorophyll a and b contents significantly.

The chlorophyll content showed a decrease in other members of the Solanaceae family. According to a study performed by Ahmad et al. (2014) increased resistance to a range of abiotic stressors has been achieved by genetically modifying normally non-accumulating plants with the gene(s) for glycinebetaine (GB) producing enzymes. The purpose of that study was to assess how well transgenic potato plants that synthesize GB perform under chilling stress. Transgenic and non-transgenic plants that had been in vitro rooted were put into pots and given another four weeks of growth in the growth chamber as previously mentioned. After that, plants were exposed to chilling stress for one week by being placed in containers at 4°C with a 16-hour day/night photoperiod. Each plant's topmost completely opened leaf was picked for a post-stress non-destructive analysis of chlorophyll concentration. After the chilling stress treatment, the chlorophyll concentration was tested 2, 4, and 6 days later. Plants were returned to normal growing condition after receiving the chilling stress treatment for a week so they could recuperate. The amount of chlorophyll was examined to track how well transgenic potato plants handled stress. To determine whether there were any appreciable differences, the outcomes were compared to Non-Transgenic (NT) plants and statistically examined. Although a general decrease was seen in the chlorophyll content of all samples after chilling treatment, transgenic plants displayed noticeably higher levels of chlorophyll than non-transgenic plants. Chlorophyll content remained more stable in tolerant genotype compared to susceptible one.

In a recently published study, the early response of potato plants (Solanum tuberosum cv. Russet Burbank) to chilling stress (2°C) was examined in the leaves of 5-week-old cultivated potato plants during the first 12 hours of cold treatment (2h dark and 10h light). First, certain signs of plant stress were investigated, and it was discovered that brief exposure to chilling stress significantly changed the chlorophyll content after 12 h (Orzechowski et al., 2021).

2.1.2. Flowering Time:

Jung et al., (2012) reported that a network of gene regulation that integrates environmental and developmental inputs in plants manages the time of flowering. One of the key environmental factors that control flowering time is temperature. While repeated brief chilling treatments delay flowering, persistent low nonfreezing temperature therapy stimulates flowering by stable repression of flowering locus C (FLC). The findings of Seo et al. (2009) showed that a feedback loop exists between the cold response and flowering-time regulation. This loop delays flowering by increasing FLC during transient chilling stress, such as those that occur in the fall or early spring, but it suppresses the cold response during floral induction by repressing cold-inducible genes by SOC1. By integrating chilling stress signals into the flowering genetic pathways in Arabidopsis, it was shown that CONSTANS-LIKE 4 (COL4), which is a flowering time modulator in Arabidopsis thaliana controls flowering time properly and is essential for both plant reproduction and crop productivity. Numerous biochemical pathways that react to both internal and external inputs might cause flowering. acted as a molecular hub. It was suggested that under short-term temperature circumstances, which frequently happen during local weather disturbances, the HOS1-CO module helps to fine-tune photoperiodic blooming.

Jung et al., (2013) showed that after being exposed to a brief period of low but non-freezing cold temperatures, Arabidopsis plants (A. thaliana) could develop their resistance to low temperature through a process called cold acclimation. The cold acclimation and vernalization pathways appear to be separate since cold acclimation can be induced at 12 °C but vernalization requires lower temperatures (Bond et al., 2011). Cold acclimation leads to a delay in flowering time. Long-term cold exposure stimulates blooming in Arabidopsis plants that are vernalization-sensitive. Contrarily, short-term chilling exposure or overexpression of genes responsive to cold postpones blooming by activating FLC which is a flowering locus.

2.2. Chilling Stress and Morphological Traits

Many morphological parameters can be changed while the plants are exposed to chilling stress. Productivity is one of the indexes that are measured in terms of the dry weight of material produced per unit area during a specific period. Improving dry matter production in the vegetative and flowering phases of tomato (S. lycopersicum L.) is one of the breeding goals of the breeders. Tomato plant dry weight, as determined at a given period, the vegetative weight of planting material, and growth rate are multifaceted characters and are one the most important morphological components (Sparnaaij et al., 1996).

Dufault et al., (1990) carried out two experiments to assess the effects of chilling stress on tomato plants (Lycopersicon esculentum Mill. Sunny). Before being transferred to the field, tomato seedlings were subjected to cyclic cold stress in a greenhouse at 2±1°C and 29±6°C. Plants were exposed to 2°C for 3, 6, or 12 hours for 1, 3, or 6 days before transferring to the field during the first year of a 2-year research project. Prior to plant transfer in the second year, plants were subjected to temperatures of 2°C for periods of 4, 7, or 10 days that lasted 6, 12, or 18 hours each. According to the morphological results, cold stress typically increased the shoot dry weight, leaf area, and root dry weight during the first year. All seedling growth characteristics decreased in the second year as a result of the prolonged cold-stress treatment. In the first experiment, different chilling-stress levels were discovered to be usually stimulators to seedling growth and development. The prolonged periods of cold stress stopped the respiration of the seedlings. But in the second trial, excessive chilling stress prevented growth completely. Low levels of chlorophyll may be a sign that prolonged cold exposure permanently damaged photosynthetic machinery as it was reported by King et al. (1988).

Lozano et al., (1998) conducted a research project in which tomato plants (Lycopersicon esculentum Mill.) were grown at chilling stress. After germination, plants were kept at standard temperatures of 26°C during the day and 18°C at night. Once the plants had three to four true leaves, they were moved to growth chambers where they remained until flowering under both standard and low temperatures of

17°C during the day and 7°C during the night. According to the results, flower and fruit development were substantially hampered, showing homeotic and meristic transformations as well as changes in the fusion pattern of the organs. The majority of these homeotic changes altered the identity of the stamens and carpels and intermediate organs which were produced. Meristic alterations occur extremely early in the flower's development and are associated with a large rise in the floral meristem's size, according to scanning electron microscopy. Low temperatures significantly raised the steady-state levels of transcripts belonging to a group of tomato genes that may be connected to these flower defects. Chilling stress also changed the stage-specific expression of the genes, according to in situ hybridization studies. Therefore, low temperature caused abnormalities in tomato morphology, and alterations in genes' level of expression were connected to this anomaly.

In another study done by Tepe and Kabas (2019) plant material from 9 pure lines at the F9 stage from the BATEM (Batı Akdeniz Agricultural Research Institute) was used to identify which lines were adaptable to low temperatures by measurement of two morphological parameters. As tolerant genotypes, S. habrochaites (LA 1777) and two commercial cultivars (S. lycopersicum cv.Cigdem F1 and S. lycopersicum cv.Anit F1) were employed as control samples. When the plants reached two-true leaf-stage, they were subjected to 5±1°C for five days at a light intensity of 350 mol m-2 sec-1 in the vegetative test. Eventually, it was discovered that two of these pure lines were low-temperature tolerant in terms of seed number and dry matter accumulation in both the vegetative and generative stages. Plant dry matter production and the seed quantity rates in the generative test were used to identify chilling stress-tolerant lines in vegetative and generative testing. The dry matter production of two lines was ranked first and had the same performance as the two commercial varieties of this study that are used in winter season cultivation in the market. Also, they were found to be in the same group in terms of seed quantity before and after flowering time. Identifying chilling stress-tolerant genotypes has been the subject of numerous investigations. According to these findings, the tolerant genotypes 5 and 6 performed similarly to the tomato varieties most commonly grown in the winter season by farmers, and these genotypes can be used to produce chilling-tolerant varieties.

Based on research carried out recently the effect of chilling stress on leaf fresh weight and leaf area index of tomato (Solanum lycopersicum cv. Vita) were evaluated. The seeds were sown on 200-cell trays with sterile perlite and sterile peat. The seedlings were moved after 14 days of germination and kept in the growth chamber at 24–30 °C during the day and 18 °C at night. The photoperiod ratio was 16h / 8h for light and darkness. The plants were treated with chilling stress (4°C) for 6 days for leaf area index and 9 days for leaf fresh weight. It was concluded that chilling stress lowered the amount of leaf fresh weight and area index significantly (Cornejo-Ríos et al. 2021).

The morphological changes were seen in other plant species as well. According to Sun et al. (2020), chilling stress could lead to morphological damage and decrease some important morphological parameters. The seedlings of two maize hybrids were utilized. One of these hybrids was a cold-tolerance variety and the other one was a cold-susceptible variety. The growth stage was three leaves. The group receiving low-temperature treatment was set at 4 °C. The daytime was set for 12 hours, the relative humidity was 70%. The low-temperature treatment groups were kept at 4°C for 2 days. While the normal temperature control treatment (CK) group was moved to the 25°C incubators for typical growth. Representative plants were sampled after the chilling treatment in order to determine the physiological and biochemical indices. Based on the outcomes, chilling stress decreased the dry and fresh weights of seedlings and roots in both susceptible and tolerant hybrids.

2.3. MiRNAs and Chilling Stress

The goal of some studies over the last decade has mostly been to find plant miRNAs that are responsible for responding to single or many environmental stressors, identification of their expression profiles, and elucidate their exact involvement in stress responses and plant tolerance. Many abiotic stress factors such as drought, salt, high or low temperatures, lack of enough nutrition, and metal elements have been shown to alter the expression of miRNAs involved in plant

growth advancement in a variety of plants (Shriram et al., 2016). These findings suggest that miRNAs may hold the key to engineering abiotic stress tolerance in crop plants through genetic modifications (Wani et al., 2020). The creation of crops that can withstand abiotic stress requires a deeper deciphering of the gene-regulation processes utilized against varied external changes by the plant. The study of non-protein-coding short RNAs' post-transcriptional regulation, which involves inhibiting certain messenger RNAs or altering epigenetic processes for the evaluation of transcription regulation, has gotten a lot of interest (Robles and Quesada, 2019).

Plants have remarkably developed capacities to interact to and adapt to demanding conditions through processes of evolutionary change. Plant breeders and biotechnologists have recently focused a lot of research on unraveling the complex physiological and biochemical responses of plants to various abiotic stressors. To sense and react to environmental stimuli, plants use a variety of abiotic stress-responsive processes that regulate the expression of stress-responsive genes. Recent investigations have revealed that environmental stimuli cause thousands of protein-coding genes to express themselves irregularly in a variety of plant genotypes (Khare et al., 2015). In response to stress, rice (Oryza sativa) exhibits variable expression of many of these genes, such as the genes which are involved in the biosynthetic pathways of proline protein (Kumar et al., 2010) and the genes which are related to different reductases in Arabidopsis thaliana (Lee et al., 2014), and also some transcription factors which were activated in the wheat plant (Rong et al., 2014), were up-regulated to create modified species. But, the mechanisms governing these protein-coding genes are not recognized completely yet (Sun, 2012). Therefore, it is possible that miRNAs will play a crucial role in understanding these gene-regulatory systems as well as stress responses. Recent research revealed that miRNAs can also be specified for each stress, tissue, species, or each physiological stage (Sun, 2012). Under 35 different forms of abiotic stressors, Zhang et al. (2013) discovered the differential transcript accumulation of 1062 miRNAs in 41 plant species. Varying miRNA relative expressions have been seen in main crops such as rice (Oryza sativa), common wheat (Triticum aestivum), Saccharum (Saccharum officinarum), Soybean (Glycine max), and Barley (Hordeum vulgare) under various abiotic stresses.

Liu et al., (2008) investigated a group of miRNAs using microarrays under different abiotic stresses and it was believed that cold temperatures are a typical environmental stressor and tolerance to this stress has a significant impact on the improvement of the plant around the world. This subset of small RNAs is a newly discovered class of stress response-related gene expression modifiers.

The impacts of chilling stress were studied in Arabidopsis thaliana, and ten stress-inducible miRNAs were found using microarray data. The chilling stress signaling pathways all have a lot of cross-talk, according to RT-PCR analyses. The presence of stress-related components in miRNA promoter regions added to the credibility of these findings. These results added to the recent comprehension of miRNA as ubiquitous stress regulators. On the microarray, it was discovered that miR167, miR169, miR172, miR393, and miR397 had varied expression profiles in response to abiotic stressors. During this experiment, no statistically significant down-regulated miRNAs were discovered. Among ten up-regulated miRNAs that were involved in chilling stress the expression of miR393 significantly increased. Abiotic stress responses are hypothesized to be influenced by the transcriptional regulators that miR169 and miR172 target, which are further involved in controlling the expression of genes and transferring signals (Jones-Rhoades and Bartel 2004).

The stimulation of these small RNAs seems to result in the suppression of some associated transcription factor genes, which, in turn, target a set of protein-related genes and play defensive roles against stress. The messager RNAs of F-box proteins are targeted by miR393, which have been revealed to be differently modified by environmental stresses and played important roles in the tolerance to these types of stresses (Navarro et al. 2006). ARF6 and ARF8 are two auxin-related genes that miR167 targets (Wu et al. 2006). Many important elements of plant improvement, and tolerance to abiotic stresses, are regulated by plant auxin (Achard et al. 2006). Therefore, this miRNA can affect the auxin production process and has an important role to improve tolerance. Also, MiR397 whose target genes are a group of genes that are related to hydrolase and oxidoreductase, respond to a variety of stressors (Kimura et al. 2003; Apel and Hirt 2004).

The expression profile of a group of miRNAs involved in chilling stress was analyzed in a study using the microarray technique in rice (Oryza sativa). The seedlings were maintained at 4 °C till harvest. At 0, 1, 3-, 6-, 9-, and 12-hours following treatment, seedlings were taken. Seedlings that weren't treated served as controls. Quantitative real-time PCR validated the miRNA expression patterns discovered by microarrays under chilling stress. The analysis method was QRT-PCR to measure the miRNA transcript abundance, and the outcomes were standardized to the level of U6 snRNA. 18 miRNAs were discovered that responded to chill. The expression levels mostly decreased in these miRNAs. The profiles of miR-167 and miR-319 family members were comparable. Interesting expression patterns were displayed by miR-171 family members. Three miRNAs produced from transposable element sequence were grouped together within an intron and only the chilling stress caused them to co-transcribe with the host gene. The presence of hormone-responsive components in the upstream regions of the miRNAs that responded to chill suggested the significance of hormones in this miRNA-mediated defense mechanism. Under chilling stress, two miRNA target pairings had different expression patterns. Furthermore, the findings were supported by the predicted targets of these miRNAs that were associated with stress. The importance of miRNAs as pervasive regulators in rice was confirmed by these findings (Lv et al., 2010).

Although Lv et al., (2010) had investigated the transcription level 18 chilling-related miRNAs in rice (Oryza sativa) and the bulk of which were downregulated by chilling stress (4°C for 8h), in rice plants, however, chilling stress resulted in a large increase too according to another study carried out by Jeong et al. (2011). In this study, small RNA libraries were created and utilized for high-throughput sequencing to find previously unidentified miRNAs and the miRNAs which were influenced by chilling stress and also other abiotic stresses. The libraries contained a variety of tissues from both control and plants that have undergone various environmental stress treatments. More than 16 million unique short RNA sequences were found from the more than 94 million readings that had their genomes matched. 76 novel stress-related miRNAs were explored. Members of the same miRNA family that was variably expressed in other organs and had unique sequences were among the new miRNAs. Some of these unique family members, which had distinct

targets in the high-resolution analysis were seen in other plants as well. One of these miRNAs is miR812q which originated from a sequentially distinct place and had a distinct sequence and was related to chilling tolerance. miR812 expression level increased at the start of the reproductive season. Under chilling stress, miRNA 812 was activated and downregulated a gene named CIPK10. CIPK transcripts provided tolerance against many types of abiotic stresses including chilling stress in different signaling pathways (Jeong and Green, 2013).

Tomato (S. lycopersicum L) is a crucial plant for fundamental breeding studies. Molecular studies help to find chilling-related miRNAs in this plant and broden the information about of miRNAs linked to low-temperature stress that had been previously found. The studies also prepared the way for future studies on how chilling stress can affect plants' ability to control miRNAs. Cao et al., (2014? Or 2016) conducted a study regarding the typical abiotic stress of chilling which has a significant effect on the development and photosynthetic capacity and the role of chilling-responsive miRNAs that have recently been found in a number of plant species. The study was designed for S. habrochaites (LA 1777) because it seemed that the miRNAs in the tomato model plant were not well understood, yet and also because of a number of desirable traits in this species which have demonstrated to endure chilling stress. The seeds of the tomato were sown and grown in a greenhouse. The temperatures for days and nights were 25°C and 20°C, respectively. When the seedlings transferred to the pots, they had 3 true leaves. Healthy tomato seedlings which had five true leaves were prepared with a photoperiod of 14 hours of light (25°C) and 10 hours of darkness (20°C). The plants remained in this condition for 2 days. After that, the seedlings were split into two groups for treatment; one group received low-temperature treatment at 4°C, however, another set of plants was not exposed to low-temperature stress. These two groups were grown using the same photoperiod. The treatment was applied at 1, 4, 8, 12, 24, and 48 hours. Following treatment six tomato seedlings were sampled in order to minimize individual variances. Additionally, each treatment had three biological replicates. Degradome sequencing was used to examine the target sequences, and RNA sequencing was employed to characterize many conserved and new miRNAs related to the chilling responses. Quantitative real-time PCR was used to confirm six target genes and nine miRNAs (qRT-PCR).

Many conserved and new miRNAs were explored in the two libraries after high-throughput sequencing and filtering. Many of these cold-responsive miRNAs had higher expression and also many of them had lower expression. Several conserved miRNAs were discovered as well which cleaved over 60 target genes. Also, a number of target genes were affected by new miRNAs.

Yang et al., (2020) studied one of the most important non-coding RNAs named circular RNAs (circRNAs). The function of miRNAs is reported to be significantly regulated by circular RNAs (Dori et al., 2022). However, there are few reports of circRNAs being involved in the response to abiotic stress. In this study, the role of circRNAs in tomato leaves' response to low temperature-induced stress was examined. Two groups of the fully developed and 14-day seedlings of tomato (S.lycopersicum cv. 1479) were created. For 3 weeks, the two groups were exposed to 60–70% relative humidity at either room temperature (25°C), 14-h light, or low temperature (15°C), 14–h light. Each plant's fifth leaf was taken after chilling exposure. Genome-wide profiles of different types of RNAs were created by using the RNA-seq technique and the Illumina platform was used to detect the sequencing. According to the findings, many circRNAs were found in the leaf after room temperature and how temperature exposure, a high number of which were differently activated while subjected to chilling stress. It was discovered that the detected circRNAs were primarily found at gene exons, although they were less widely dispersed at gene introns or intergenic regions. According to these findings, many circRNAs exhibited variable expression and were anticipated for many miRNAs that aim to target a huge number of mRNAs. The main roles of circular RNAs and the related targeted genes were revealed which showed that the genes of over-expressed circRNAs were triggered by low temperature. These findings indicated that circRNAs may regulate pathways related to environmental adaptation, by affecting the related proteins. Some of the circRNAs were assumed to control the transcript abundance of the genes related to transcription factors. Finally, the study demonstrated that in response to low-temperature stress, different circRNAs were identified and were controlled in diverse ways in tomato (S.lycopersicum cv. 1479) leaf tissues.

Omidvar et al., (2015) performed study on chilling tolerance for S. lycopersicum cv. Rutgers, and its wild-type. By imposing the seedlings to 4°C for short stress levels including 0 to 24 hours, the expression of miR159, miR166 as well as their targets in response to chilling treatments were explored. Two small RNA (sRNA) libraries from genetically developed tomato and wild-type seedlings treated simultaneously with chilling stress and were sequenced, and sRNA profiles were compared, in order to find sRNAs associated with stress tolerance in genetically modified (GM) tomato. From the two libraries, several families of conserved and many potentially new miRNAs were found. In stress-treated GM tomato seedlings, some of the miRNAs were upregulated, while some conserved and novel miRNAs were downregulated. Rapid amplification of 5 complementary DNA ends (5-RACE) analysis was used to confirm miRNA targets with potential involvement in stress regulation. Finally, a strong negative connection was found between the levels of these miRNAs (miR159, miR166) and the expression of their targets. As a result of the research, it was found that miR159, miR166, miR472, miR482, miR#A, and miR#D are likely to facilitate the BL-specific enhanced tolerance of 7B-1 to abiotic stress.

Gao et al., (2015) studied chilling stress and its negative effect on the amount of phosphorus (P) as one of the vital elements required for tomato plant growth and development. Chilling stress has a negative impact on plant growth, limits the accumulation or transfer of mineral nutrients like P, and reduces crop productivity and quality. By influencing phosphate uptake and transport, miR399 has an important effect to regulate systemic phosphate synthesis. According to the results of this study overexpression of miR399, induced P toxicity and slowed development in chilling stress. The authors modified a tomato (S. lycopersicum L., cv. C1) genetically with a gene controlled by a specific promoter. This gene encoded miRNA 399. Plants grown hydroponically underwent a low-temperature treatment. Seedlings that were uniformly 15 days old were put into the growth chamber, which had a 16°C temperature setting for day and night. After 4, 8, 12, 24, and 72 hours of treatment plants, the roots of transgenic tomato and wild-type were collected for RT-qPCR analysis. Using the primer pairs, RNA isolation, cDNA synthesis, and RT-qPCR were carried out in this experiment. Relative expression levels were computed and adjusted in relation to a reference gene named

α-tubulin mRNA to prevent bias. For analysis, four replications of each line at each time point were employed. The expression of a miRNA gene in the transgenic genotype displayed a stress-inducible pattern. A miRNA was slightly up-regulated against chilling stressors, according to the findings. Transgenic roots had an 8.3-fold higher level of miR399d expression than the wild types' roots after a 12-hour low-temperature treatment. Additionally, transgenic tomato roots had a higher expression level of this miRNA throughout the whole-time course than wild types.

Koc et al., (2015) experimented and assessed a group of miRNAs that were involved in chilling stress in tomato (S. lycopersicum var.H-2274). The chilling stress points included 0, 2, 4, and 16 hours after chilling treatment. Plants of the tomato (Solanum lycopersicum var. H-2274) were cultivated in the soil in a greenhouse under 16h of light and 8h of darkness at 25 °C and 2 °C. Plants were given a cold treatment with a light intensity of 20 µmol/m2s after 4 weeks of growth. Plant leaves were then promptly collected and kept at 80 C. The cold treatment was not applied to the control plants. The stem-loop quantitative reverse transcription-polymerase chain reaction was used to determine the relative expression of these miRNAs. Eventually, it was concluded that miR167, 169, 172, and 393 were expressed differentially in the early growth phases under a short period of chilling treatment. MiR172, in this research, had the highest transcript abundance. Also, the target genes of chosen miRNAs were followed, and their expression levels were investigated among cultivated tomatoes that were susceptible to chill and those that were not. Stem-loop qRT PCR was used to evaluate these certain cold-stress-related miRNAs. In addition, three tomato genotypes taken from the tomato functional genomics database were utilized to extract the expression pattern of projected target genes. Different target gene expression profiles were found in different cultivars. The findings indicated that this variation among tomatoes may emphasize tomato genotypes' chilling resistance. Under chilly temperatures, an insignificant change in miR397 was seen. According to cis-element analysis, the studied miRNAs exhibited a high frequency of biochemical and ecological responses to regulatory elements. Three genotypes showed distinct expression profiles for anticipated target genes. As a result, it was hypothesized that tomato genotypes' cold tolerance would alter the transcription level of target genes.

The differential expression of cold-related miRNAs was not limited to tomato plants and was seen in other members of Solanaceae as well. Eggplant (Solanum melongena) and tomato (S. lycopersicum) are from the same family of Solanaceae and are very similar to each other genetically (Rinaldi et al., 2016). The chemical pathways underlying Eggplants' reaction to low-temperature stress have not yet been fully understood. Yang et al., (2017) employed high-throughput sequencing to determine Solanum aculeatissimum miRNA and the target genes which were affected under chilling stress (1°C) at various time points. This wild genotype of eggplant was seeded, and the seedlings were then grown into 3–4 true leaves in a controlled environment of 25 °C. At a low temperature of 1 °C, the identical seedlings' growth capacity was examined. Other environmental characteristics and environmental processing remained constant. The seedlings grew in the control group under normal environmental conditions (0h). The seedlings were grown in a chilling condition (1 °C) for 2, 6, 12, and 24 hours in the treatment group. Finally, the differentially expressed miRNAs and the genes related to these miRNAs were identified. In S. aculeatissimum, over 50 miRNAs were expressed less and over 20 miRNAs were expressed more compared to the control samples and they were found to correspond to many up-regulation or down-regulation of many mRNAs.

3.MATERIAL & METHODS

3.1. Plant Growth

The accessions used for this study are wild genotypes; Solanium haibrochaits (LA 1777), Solanium pimpinellifolum (LA 1579) and cultivated genotypes S. lycopersicum cv. Anit F1, and the susceptible genotype of S. lycopersicum cv. Lice. The seeds were sterilized by soaking in 2% NaClO for a minute and rinsed in sterile warm water (55°C) 3 times, and then were sown in the 48-cell seedling trays. There were four genotypes, four treatments, and three replicates (12 seedlings for each genotype) and irrigated with distilled deionized water from the bottom of the seedling trays. To manage the of heterogeneity of germination and consequently plant growth stage and rate, before starting the main experiment, a group of all genotypes was sown once before the primary trial and the accurate germination date of each of them was recorded. The seeds were then sown on different dates and all germinated in the same week. Three seeds were sown in each cell and two seedlings per cell were removed leaving one seedling per cell. Also, KNO3 of 0.2% was sprayed regularly and evenly on all soil surfaces from the first day of sowing to the day of germination in order to enhance the germination of the seeds. The seedling trays were kept in the growth chamber which had a 16h of lightness and 8 hours of darkness photoperiod at 25± 1ºC. The peat material (Klasmann-Deilmann corporate group) was used. The seedlings at 2 leaves stage were transferred to pots (18 × 15.5 × 14 cm) and then kept at 25± 1ºC for a week with regular cultivation management for environmental acclimatization. The plants were fertilized twice a week with a 20:20:20 NPK fertilizer (1g. / lit.) according to Zucco et al, (2015) and were sufficiently irrigated with tap water every day until the plants began the flowering phase. Then the 3-leave-stage plants in the pots were treated by chilling treatment (at 4 ºC for 4, 6, and 8 h) (Fig. 1). 8 hours after chilling treatment, plants were held at 25°C±1 till the flowering stage. The control plants of each genotype were kept at 25°C±1 to compare with chilling-treated samples.



Figure 1. Plant Growth Stages, 1) Seed cultivation in the seedling trays, 2) Seedling growth, 3) Creation of protective chamber around the seedling trays, 4) Seedling transfer to the pots, 5) pots arrangement 6) flowering stage

3.2. Molecular Analysis

3.2.1. Sampling

3 leaflets were collected (9 leaflets in total) from 3 randomly selected plants at 3 leaves stage in both chilled condition (chilled treated plant) and optimum condition (control plant)) at the 4^{th}, $6^{th,}$ and 8^{th} hours after chilling treatment. The leaflets were washed with distilled water, wrapped in an aluminum foil, and stored at -80ºC immediately using cooling box till used for RNA analysis.

3.2.2. Preparation of Total RNA

3.2.2.1. Lysate Preparation

Total RNA isolation was performed using the Plant/Fungi Total RNA Purification Kit (Cat. 25800) of Norgen Biotech Corp. (Fig. 3). The plant leaf tissue was placed in a mortar with enough liquid nitrogen to completely cover the sample. The samples were ground and turned into a fine powder by using a pestle (Fig. 2). A centrifuge tube containing 50 mg of powder was filled with 600 µL of lysis buffer

and intensively vortexed. After that, it was incubated for 5 minutes at 55°C. Additionally, during incubation, the tube was inverted two or three times to mix the lysate. Then, using one of the supplied collecting tubes, a filter column was put assembled. After being pipetted into the filter column, the lysate was spun for two minutes at 20,000 x g (about 14,000 RPM). A pipette was used to transfer the crystal-clear supernatant from the flow-through into an RNAase-free microcentrifuge tube. The lysate obtained above was mixed with an equal volume of 96–100% ethanol (100 µL of ethanol is added to every 100 µL of lysate) via vortexing.

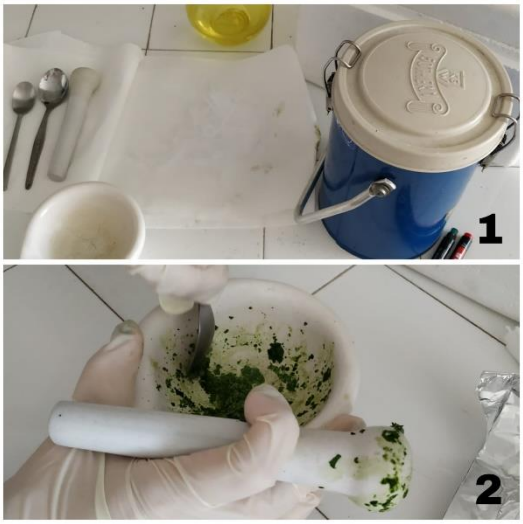


Figure 2. Grinding initial samples 1) Nitrogen container and other equipment required for grinding the initial samples 2) Grinding the tomato leaf with liquid nitrogen by using a mortar and pestle



Figure 3. RNA purification materials, Plant/Fungi Total RNA Purification Kit (Cat. 25800) of Norgen Biotech Corp.

3.2.2.2. Binding to Column

One of the given collecting tubes was used to collect the content of a spin column. Using ethanol as a solvent, 600 µL of the cleared lysate was put into the column and centrifuged for one minute at 3,500 x g (around 6,000 RPM). The spin column and collection tube were put back together after the flow through was discarded.

3.2.2.3. Column Wash

After adding 400 µL of wash solution A to the column, it was centrifuged for one minute at 20,000 x g (about 14,000 RPM). After removing the flowthrough, the spin column and collection tube were put back together. To wash the column a second time, these procedures were repeated. A further 400 µL of wash solution A was added, and the column underwent a third round of washing before being centrifuged for one minute. After removing the flowthrough, the spin column and collection tube were put back together. To completely dry the resin, the column was spun for 2 minutes at 20,000 x g (approximately 14,000 RPM). The collecting tube was thrown away.

3.2.2.4. RNA Elution

The column was placed into a fresh 1.5 mL Elution tube provided with the kit. 50 µL of Elution Solution A was added to the column and centrifuged for 2 minutes at 200 x g (~2,000 RPM), followed by a 2-minute spin at 20,000 x g (~14,000 RPM). Then the extracted RNA was transferred to an elution tube and stored at -80°C (Fig. 4).

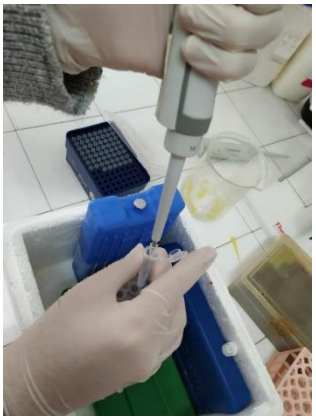


Figure 4. RNA isolation process

3.2.2.4. Measurement of RNA concentration and integrity

Nanodrop 2000c spectrophotometer of Thermofisher scientific corporation was used for RNA concentration measurement (Fig. 5). An agarose gel of 1% was used to evaluate the RNA sample integrity. The gel was run at 100 V until the dye was 80% of the way down the gel. A UV transilluminator was used to visualize the RNA integrity of the samples. The ladder in the first lane was used as a guide to check the size of each band (Fig. 6).



Figure 5. RNA concentration analysis, 1) Sample preparation, 2) Running RNA samples on nanodrop

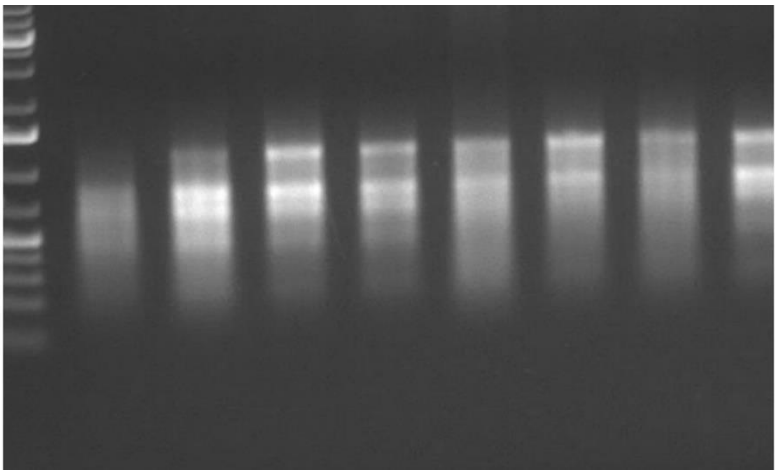


Figure 6. RNA integrity results in the gel electrophoresis

3.2.3. cDNA Synthesis and Quantitative Real-Time PCR:

For the reverse transcription of microRNA from either Total RNA preparations or enriched microRNA preparations, Norgen's microScript microRNA cDNA Synthesis Kit is an all-in-one, ready-to-use product. The 2x Reaction Mix and the microScript microRNA Enzyme Mix are both included in the kit.1 µg of total RNA was used for cDNA synthesis. The First-Strand miRNA cDNA was set up for synthesis reaction in the heat block (Techne Dri-Block – DB3D) (Fig.7). cDNA reaction contained 1 µg RNA, 5 µL 2x Reaction Mix, 0.5 µL microScript miRNA Enzyme Mix, and Rnase-free water was added into the mixture up to 10 µL per sample. First-Strand miRNA cDNA Synthesis reaction was incubated in a heat block manually as described in Table 2.

Table 2. cDNA Synthesis Conditions at different temperatures

Temperature Time	Time
37ºC	30 minutes
50ºC	30 minutes
70ºC	15 minutes
4ºC	Hold





Figure 7. cDNA Synthesis process, 1) cDNA synthesis kit (Norgen corp.) 2) heat block used as a thermocycler.

The cDNA which had been synthesized in the earlier stage can be used as a template in a PCR reaction. The cDNA was diluted 2-fold using nuclease-free water. 1 µL of the diluted cDNA was used in a 20 µL PCR. For quantitative PCR, 10µl of 2x PCR Master, 1 µl miRNA-specific Forward Primer (5 µM) Mix was used. The PCR reaction was set up with the Universal PCR Reverse Primer and a miRNA-specific forward primer, as indicated in Table 3.

Table 3. The PCR Reaction components volume for miRNA

Components	Volume per Reaction
2x PCR Master Mix	10 µL
miRNA-specific Forward Primer (5 µM)	1 µL
Universal PCR Reverse Primer	1 µL
miRNA cDNA	2 µL
Nuclease-Free Water	8 µL
Total Volume	20 µL

The real-time PCR amplification was performed in a Real-time PCR cycler (LightCycler 480 II, Roche) as described in Table 4.

Table 4. Reaction Protocol miRNA Real-time PCR

Temperature	Time	Cycles
94ºC	3 minutes	1 Cycle
94ºC	15 seconds	40 Cycles
60ºC	30 seconds	40 Cycles
72ºC	45 seconds	40 Cycles

The primer sequences used have been mentioned in Table 5. The prepared reaction was run on a 96-well plate of a real-time PCR machine (Fig. 8).

Table 5. Designed primers (Koc et al. 2015).

Name		Sequence
Reference gene (Actin)	**F**	GCCGGGCGTGATCTTACTGA
Reference gene (Actin)	**R**	AGCTACTCCTGGCGGTCTCC
Universal primer		GTGCAGGGTCCGAGGT
miR167	**F**	TCGCGTGAACCTGCCAGCAT
miRI67	**RT**	GTCGTATCCAGTGCAGCGTCCGAGGTATTCGCACTGGATACGACTAGATC
miR169	**F**	GCGGCGGCAGCGAAGGATGACT
miRI69	**RT**	GTCGTATCCAGTGCACGGTCCGAGGTATTCGCACTGGATACGACTCGGCA
miR172	**F**	CGGCGCAGAATCATGATGATG
miRI72	**RT**	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGCAG
miR393	**F**	GCGCGGTCCAAAGCGATCGCA
miR393	**RT**	GTCGTATCGAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGATCAA
miR397	**F**	CGGCGTCATAGAGTGCAGCG
miR397	**RT**	GTCGTATCCAGTGCACGGTCCGAGGTATTCGCACTGGATACGACCATCAA

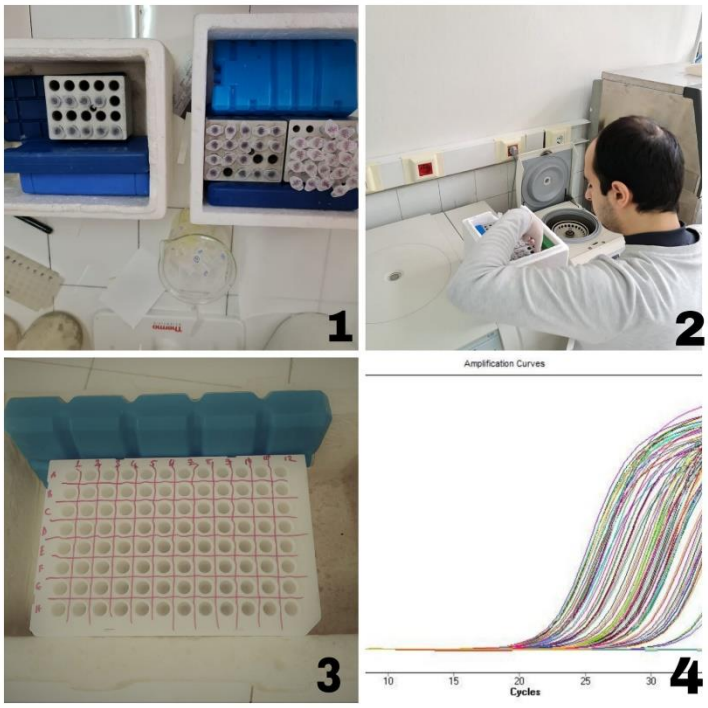


Figure 8. Real-time PCR reaction preparation 1) reaction preparation, 2) Tube centrifuge before running the reactions on the plate, 3) Real-time PCR plate running stage, 4) PCR machine result graph

3.2.4. Real-time Data Analysis:

The double delta Cp analysis and the relative standard curve approach are the two basic methods for analyzing qPCR data (Pfaffl method). The data in this study was calculated by the double delta Cp analysis method. There are five steps in this analysis. First, the Cp values were added from all technical replicates together. The first step was to average the Cp values from each sample's technical replicates. As a result, when performing qPCR in duplicate, these data must first be averaged. Each sample was run twice in the plates. Then the delta Ctp was calculated for each sample. The next step was to calculate delta Cp (ΔCp) for each sample by using the Cp values which was created in the previous step. The formula to calculate delta Cp is presented below.

$$\Delta Cp = Cp \text{ (gene of interest)} - Cp \text{ (housekeeping gene)}$$

After that a reference sample was selected to calculate delta delta Cp. Whichever sample, or group of samples, can be used as the calibrator (reference).

Because this was consistent throughout the analyses and was reported in the results so it is clear. The results produced at the end were relative gene expression values.

Delta delta Cp values were calculated for each sample. Delta delta Cp values were relative to the untreated/control group in this experiment. The formula to calculate delta delta Cp is presented below.

$$\Delta\Delta Cp = \Delta Cp \text{ (Sample)} - \Delta Cp \text{ (Control average)}$$

Finally, to work out the fold gene expression it was needed to do the primer efficiency (which was 1.98 in this case) to the power of negative ΔΔCp. The formula for this can be found below.

$$\text{Fold gene expression} = 1.98^{-(\Delta\Delta Cp)}$$

The significant fold change was ≥1.5 and the q value was 0.001 in this analysis. The analysis was done with two methods. First, the data was calculated manually on excel and then the final fold change was checked using REST software (Pfaffl et al., 2002).

3.3. Physiological Response Analysis:

3.3.1. Chlorophyll Content:

For chlorophyll measurement, a chlorophyll meter (Minolta SPAD-502 meter) was used which is a hand-held tool that is largely employed for the quick, precise, and non-destructive measurement of leaf chlorophyll concentrations. By detecting the absorbance in the 400–500 nm wavelength the SPAD–502 meters may indirectly measure the chlorophyll concentration (Sim et al., 2015).

The SPAD index was measured just after stress treatment and at the beginning of flowering time by using a SPAD. In this experiment each leaf was measured three times, and the average of those measurements served as the sample's SPAD index.

3.3.2. Flowering Day:

Plants can alter their development to cope with stressful situations. Stressed plants may blossom sooner or later in an effort to generate the next generation quickly. This is how plants may protect their species even in harsh conditions (Wada and Takeno, 2010). In the current study the chilling-treated and controls samples continued their growth until the flowering stage. The plants were irrigated with tap water from the bottom of the pots. All four genotypes were grown in the growth chamber conditions with a 16:8 h (light: dark) photoperiod at 25± 1ºC. The date of application of stress and the date of observation of the first flower was recorded separately for each sample to examine the plants in terms of time to enter the reproductive phase.

3.4. Morphological Parameters:

Some physiological parameters such as leaf area, leaf dry weight, leaf fresh weight, root dry weight, root fresh weight, and flowering time were evaluated to assess tomato plants' responses to chilling stress. Physiological parameters were evaluated at the beginning of the flowering stage.

3.4.1. Leaf Fresh and Dry Weights:

3 top fully-grown compound leaves were collected from 3 randomly selected plants under chilling conditions and control at the beginning of the flowering stage. The fresh leaves were washed in distilled water and weighed using a scale with an accuracy of 0.001g. In the next stage, the weighed leaf samples were packed separately and exposed to 65°C for 72 hours in the oven to measure the constant leaf dry weight. The dried leaves were weighed using the same scale (Fig. 9).

3.4.2. Leaf Area Measurement:

The leaf area index of the leaf was laid on the graph sheet with an accuracy of 5 mm and its outline was traced using a pen (Fig. 9). The full squares inside the

leaf trace were counted. Also, the partial squares were numbered inside the leaf trace. The number of full squares was multiplied by 0.25 cm2 and partial squares by 0.125 cm2. Finally, the total surface area was measured using the obtained data.

3.4.3. Specific Leaf Weight:

The Specific Leaf Wight (SLW) was calculated by the following formula:

SLW = LEAF DRY WEIGHT / LEAF AREA

3.4.4. Root Fresh and Dry Weights:

The entire root system per plant was harvested for 3 randomly mature and uniform at the beginning of the flowering stage. The root system of the plant was washed in tap water (Fig 9) and weighed using a scale with an accuracy of 0.001g. In the next stage, the weighed root samples were packed separately and exposed to 65°C for 72 hours in the oven to measure the constant leaf dry weight. The dried roots were weighed using the same scale.



Figure 9. Morphological analysis stages, 1) Root wash, 2) root fresh weight measurement, 3) leaf fresh weight measurement, 4) package arrangement for dry weight analysis, 5) Dry weight measurement, 6) chlorophyll content measurement, 7) leaf area index measurement

Statistical Analysis

All data from physiological response and morphological parameters were analyzed using the data analysis system SPSS 20 software. Significant differences in data between temperatures are indicated: *$p < 0.05$, by One-Way ANOVA. Least Significant Differences (LSD) test were applied to determine the diffences of the treatment.

4. RESULTS

4.1. MiRNA Differential Expression Analysis:

4.1.1. *S. habrochaites* (LA 1777)

The expression patterns of miRNAs in *S. habrochaites* (LA1777) were given in Fig. 10. The expression level of miRNA167 increased 5 times at 4 hours, 4 times at 6 hours and 10 times at 8 hours after chilling treatment. While the miRNA 169 expressed almost 7 folds at 4 hours and nearly 10 folds at 8 hours, the transcript amount of miRNA 169 at 6 hours had a level as that of control. The miRNA 172 was highly expressed over 8 folds at 4 hours and also had a noticeable over 5-fold expression profile at 8 hours. The miRNA 393 did not show any significant differential expression in *S. habrochaites* during chilling treatment. The transcript accumulation of miRNA 397 upsurged 8 folds at 4 hours, then decreased up to the same level of control at 6 hours, and finally, re-increased over 5-folds at 8 hours.

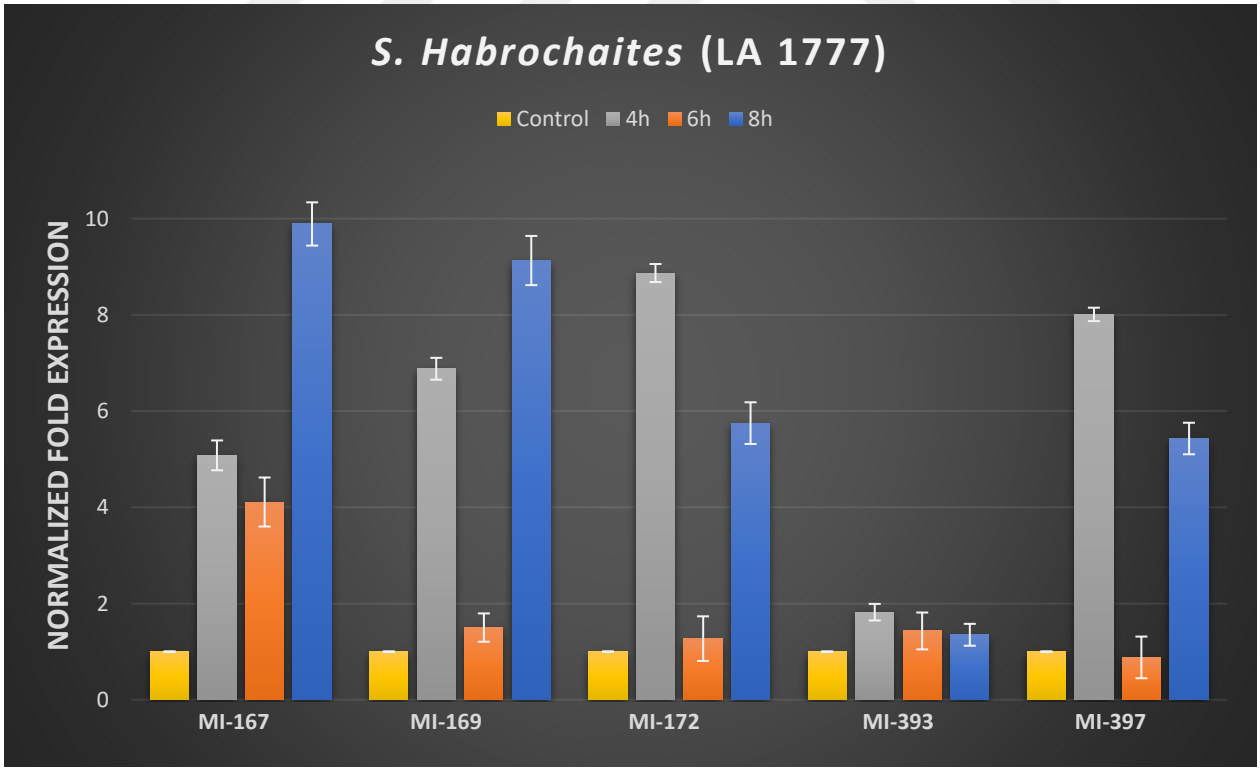


Figure 10. *S. Habrochaites* (LA 1777) miRNA expression profile.

4.1.2. S. pimpinellifolium (LA 1579)

As is shown in the illustration (Fig. 11), the expression of miRNA 167 was induced at 6 hours (7 folds) and at 8 hours (8 folds) after chilling treatment but there was no up-regulation of miRNA 167 at 4 hours. A noticeable differential expression of miRNA 169 was clearly seen at 6 hours and 8 hours after chilling stress, but there was no significant change in expression of miRNA 169 at 4 hours. A significant increase in the expression profile of miRNA 172 was determined at 6 hours (3 folds) and 8 hours (8 folds) after chilling treatment. The transcript abundance of miRNA 393 had a considerable rise up to 6 folds at 6 hours and then decreased at 8 hours (almost 2 folds) after chilling treatment. The expression of miRNA 397 increased at 6 hours of chilling stress by over 7 folds while any upsurged transcript accumulation was not seen at 4 hours and 8 hours and control.

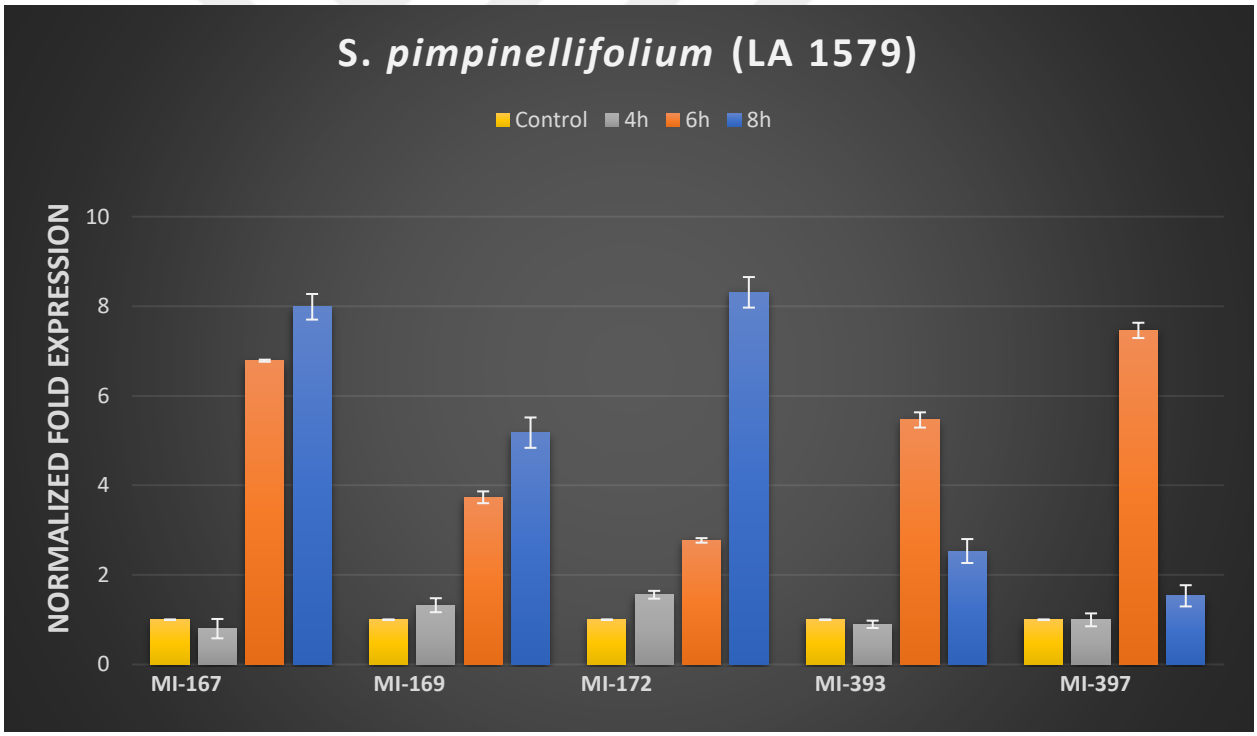


Figure 11. S. *pimpinellifolium* (LA 1579) miRNA expression profile.

4.1.3. S. lycopersicum cv. Anit

The expression pattern of miRNAs in *S. lycopersicum* cv. Anit was given in Fig. 12. The expression level of miRNA167 increased 8 times at 8 hours, and 4

times at 4 hours, and a slight change was seen at 6 hours after chilling treatment. MiRNA 169 expressed 4 folds at 6 hours and over 2 folds at 4 hours. MiRNA 172 was extremely transcribed at 4, 6, and 8 hours by over 5, 6, and 8 folds respectively. The miRNA 393 did not show any significant differential expression in *S. lycopersicum* cv. Anit during chilling treatment. Likewise, the expression level of miRNA 397 at 4 and 8 hours changed slightly. Yet, the transcript abundance of this miRNA at 8 hours soared over 2.5 folds.

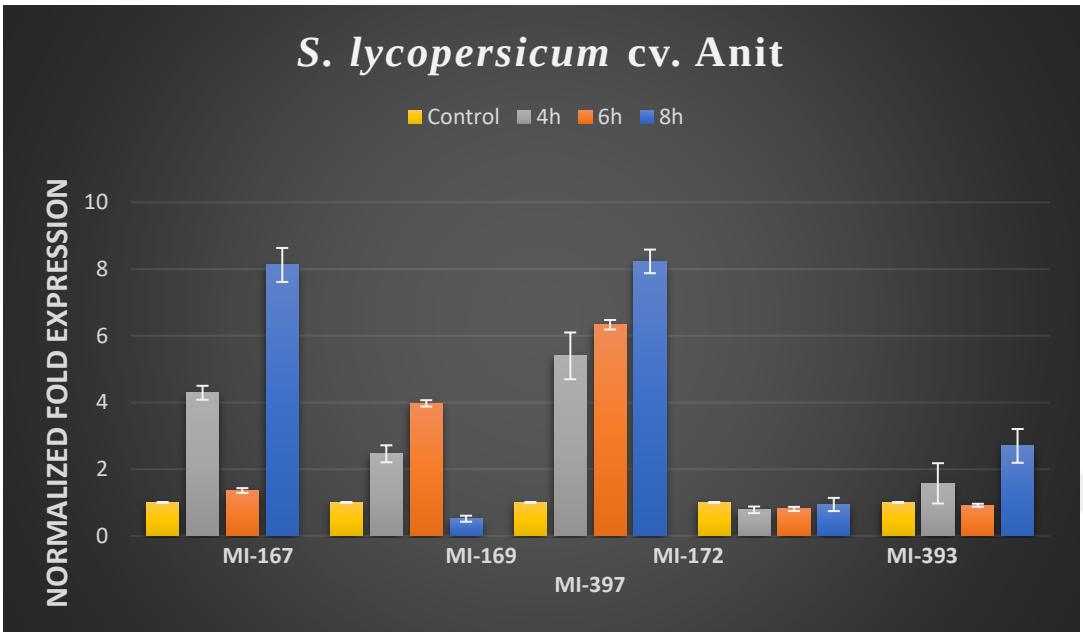


Figure 12. *S. lycopersicum* cv. Anit miRNA expression pr

4.1.4. S. lycopersicum cv. Lice

As is shown in Fig. 13, the expression of miRNA 167 was induced at 4 hours (2 folds) and at 8 hours (1.5 folds) after chilling treatment but there was no up-regulation of miRNA 167 at 6 hours. A noticeable differential expression of miRNA 169 was clearly seen at 8 hours (over 2 folds) after chilling stress, but there was no significant change in expression of miRNA 169 at 4 and 6 hours. A significant increase in the expression profile of miRNA 172 was determined at 4 hours (over 4 folds) and 8 hours (over 2 folds) after chilling treatment. The transcript abundance of miRNA 393 had a considerable increase at 8 hours (over 2 times) but the expression changes at other times were insignificant. The expression of miRNA

397 increased slightly at 8 hours of chilling stress but any upsurged transcript accumulation was not seen at 4 hours and 6 hours.

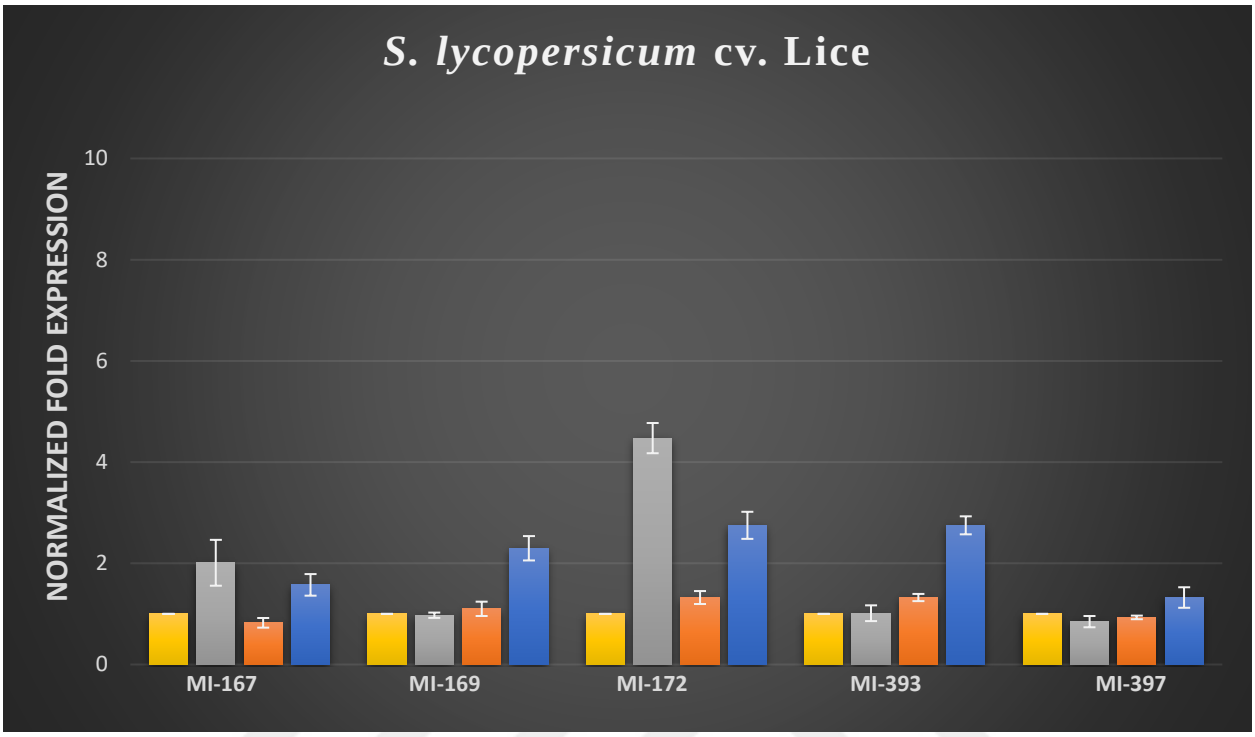


Figure 13. *S. lycopersicum* cv. Lice miRNA expression profile

4.2. Physiological Analysis

4.2.1. Chlorophyll Content

The chlorophyll content of S. *habrochaites* was minimally altered just after being exposed to chilling stress and also at the beginning of the flowering stage. Furthermore, the chlorophyll content of *S. pimpinellifolium* was only a little altered during this study in the mentioned stages. It was also seen that, while there was no significant difference between 4h and 6h of stress, there was a significant difference in all other stress levels between treated and untreated samples just after imposing stress by measuring the leaf chlorophyll content of *S. lycopersicum* cv. Anit F_1. While there was no significant change in this parameter at the beginning of the flowering phase of Anit. Finally, it was discovered that the chlorophyll content of the plant had considerable variations at all levels after the stress by evaluating the

amount of leaf chlorophyll content of *S. lycopersicum* cv. Lice. Also, a significant difference was seen in this index at the beginning of the generative phase when the stress-treated samples were compared to the control samples (Fig. 14).

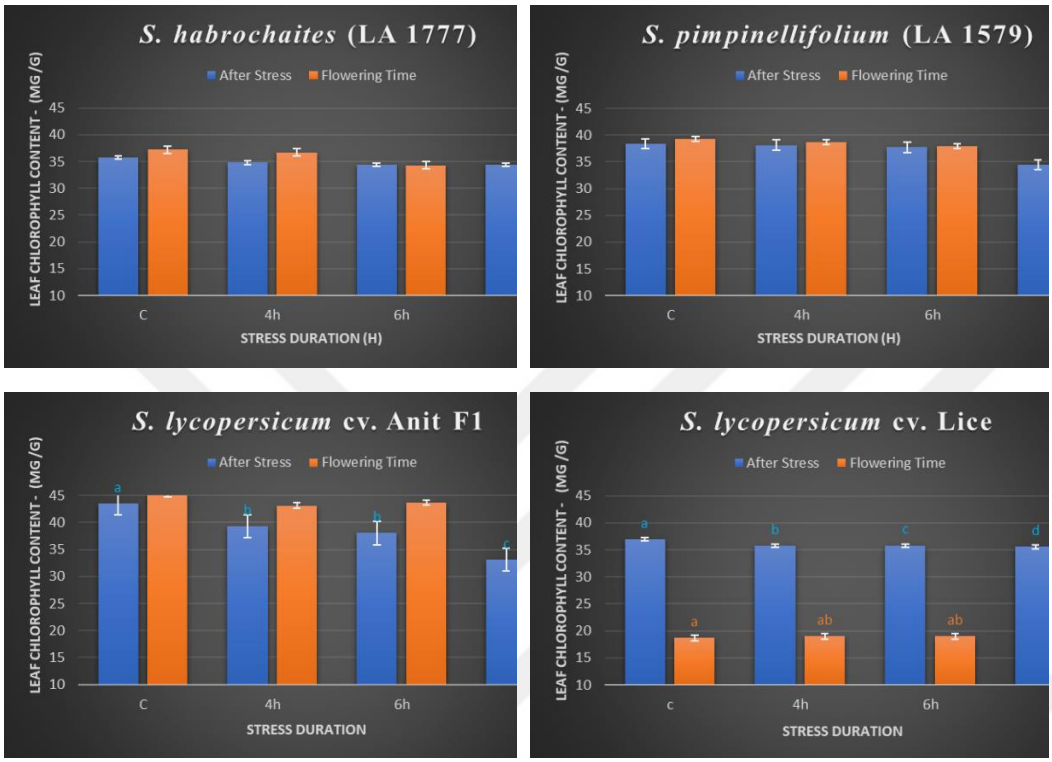


Figure 14. Leaf chlorophyll content results of tomato genotypes

4.2.2. Time to flowering after chilling treatment:

Another physiological parameter in the present study was the time (days) to first flower observation after stress treatment. The genotypes of *S. habrochaites* (LA 1777) and *S. pimpinellifolium* (LA 1579) and *S. lycopersicum* cv. Anit F1 did not show any statistically significant difference compared to the control samples. However, a significant change was seen in *S. lycopersicum* cv. Lice at 8 hours. As the chilling stress duration increased in Lice, flowering time was delayed. (Table 6).

Table 6. Flowering time results. Flowering time between tolerant and susceptible genotypes of tomato was recorded after subjecting different levels of chilling stress (4°). Means with common letters are not significantly different at the $p<0.05$ level. the letters which were different demonstrate significantly different at $p<0.05$ based on LSD test

	Days to flowering time			
Stress level (h)	**LA 1777**	**LA 1579**	**ANIT**	**LICE**
Control	20.33	21.33	17	18.67^{a}
4 hours	20.33	21.33	17	19^{ab}
6 hours	20.33	21.67	17.67	19^{ab}
8 hours	20.67	21.67	18.33	21^{b}

4.3. Morphological Analysis:

4.3.1. Leaf Fresh Weight

Significant morphological changes were seen in several tomato genotypes at varying levels of chilling stress, as shown in Fig. 15. The fresh leaf weight of S. *habrochaites* did not alter considerably. Any significant physiological changes were not seen in S. *pimpinellifolium* at various levels of chilling stress. S. *pimpinellifolium's* fresh leaf weight did not alter considerably, as demonstrated. After 8 hours of chilling treatment, substantial changes were not seen in *S. lycopersicum* cv. Anit F_1. At varying levels of chilling stress, considerable changes were seen in *S. lycopersicum* cv. Lice. The fresh leaf weight of Lice has changed significantly with various levels of stress.

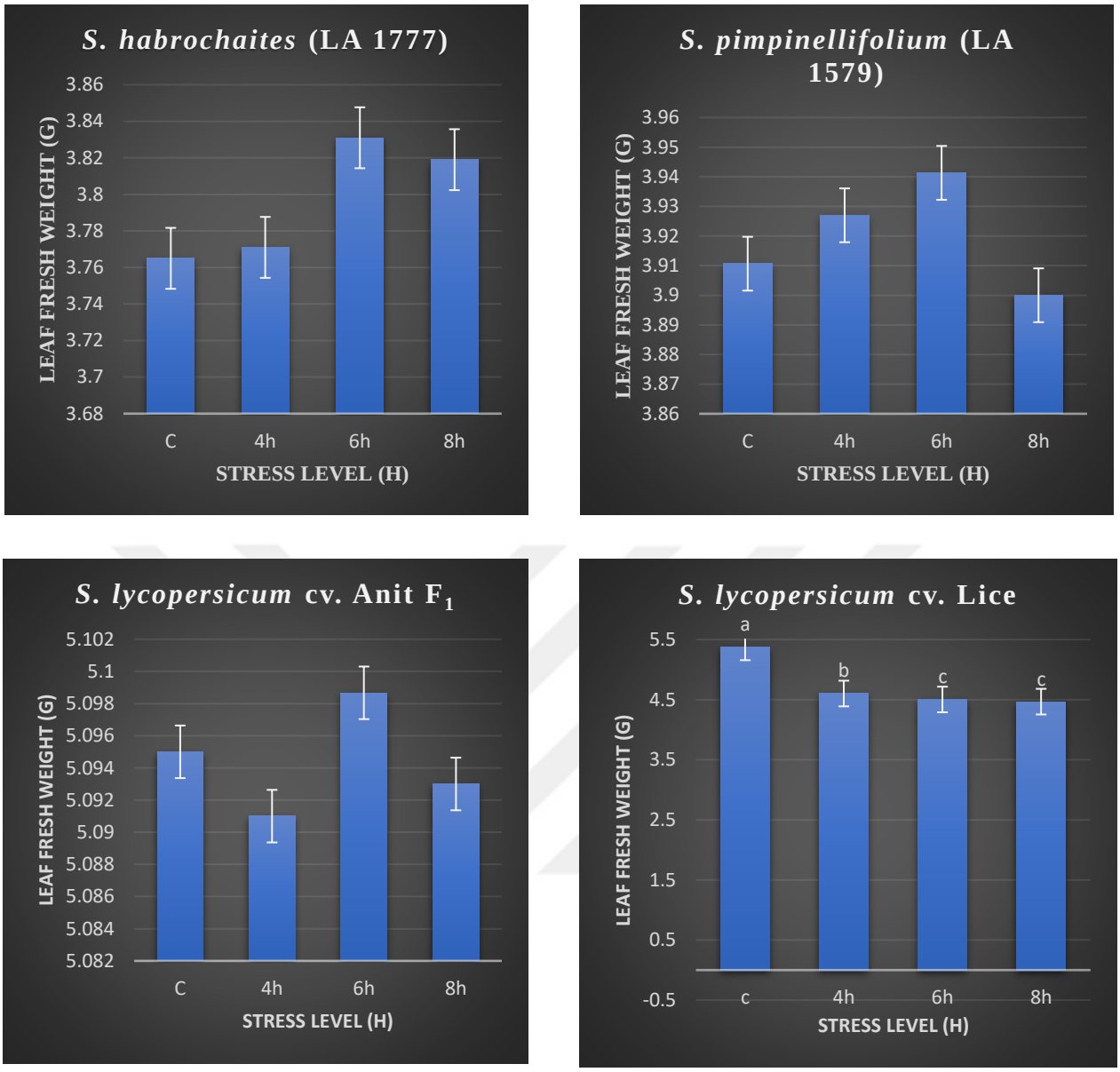


Figure 15. Leaf fresh weight result of tomato genotypes after chilling stress.

4.3.2. Leaf Dry Weight

The leaf dry weight of S. *habrochaites* did not vary significantly, as shown in Fig. 16. In addition, the dry weight of S. *pimpinellifolium* leaves did not alter significantly. Furthermore, *S. lycopersicum* cv. Anit leaf dry weight had a considerable change after 8 hours of stress while the leaf dry weight at different levels of stress remained relatively constant. Lice's leaf dry weight, on the other hand, changed dramatically in almost of levels of chilling stress.

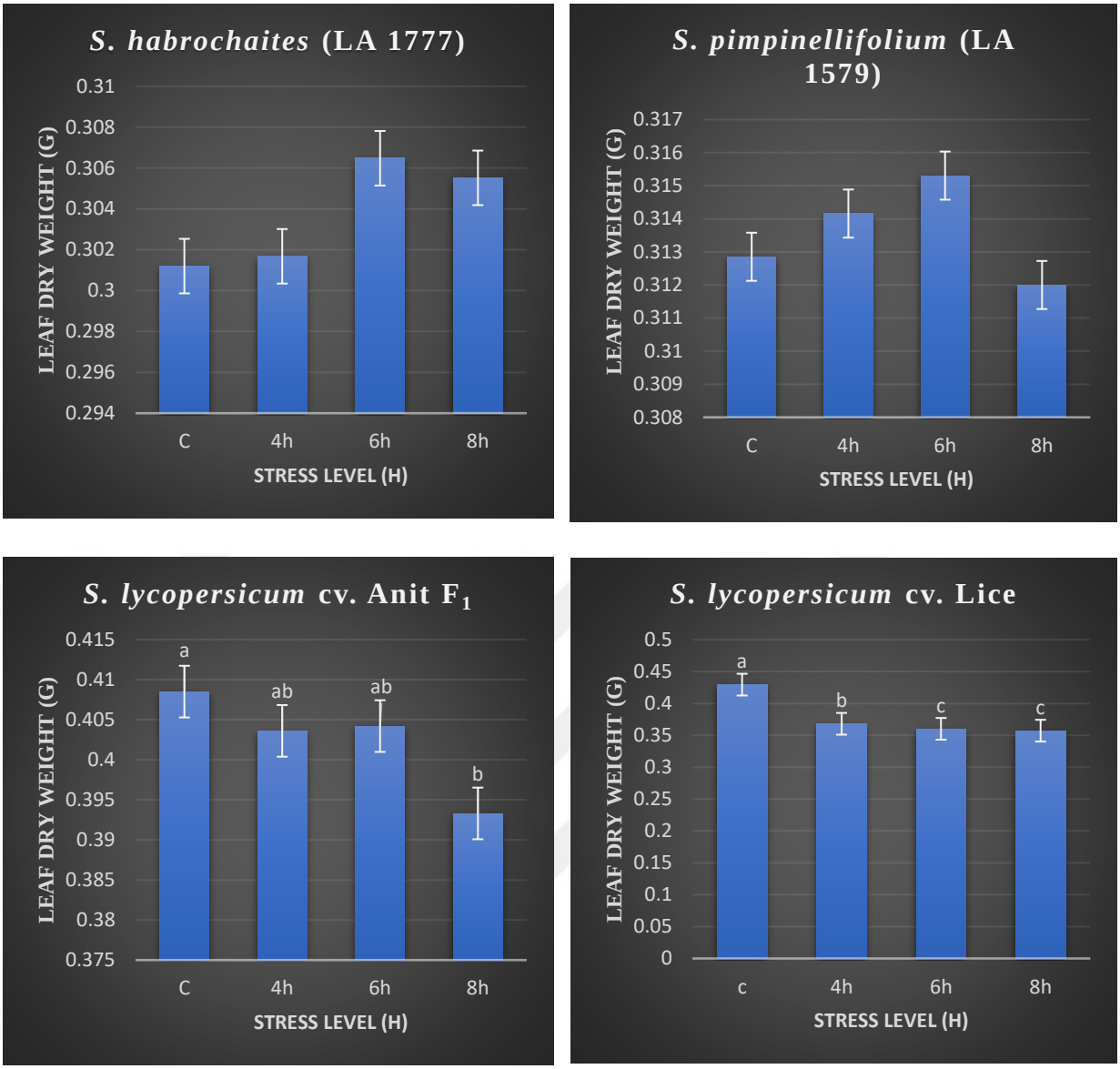


Figure 16. Leaf dry weight result of tomato genotypes after chilling stress

4.3.3. Leaf Area Index

After the calculation of the leaf area index (LAI) of S. *habrochaites* and S. *pimpinellifolium* samples, no significant variation between different degrees of stress was found (Fig. 17). There was no discernible difference between different degrees of stress when the LAI was calculated in *S. lycopersicum* cv. Anit as well. However, the LAI of *S. lycopersicum* cv. Lice indicated a substantial variation between different levels of stress.

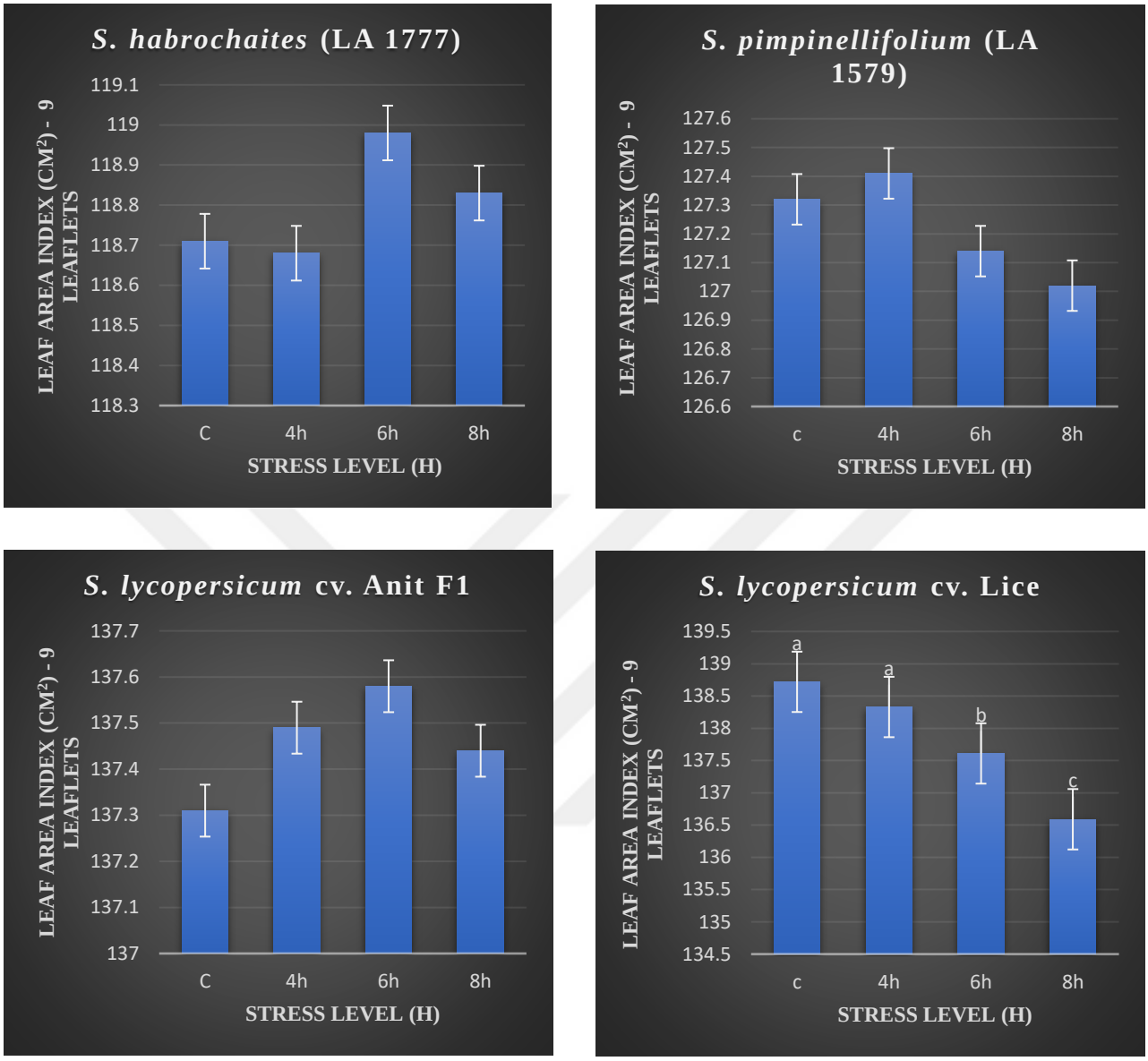


Figure 17. Leaf area index result of tomato genotypes after chilling stress.

4.3.4. Specific Leaf Weight (SLW):

After calculation of the specific leaf weight (SLW) of S. *habrochaites* (LA 1777), S. *pimpinellifolium* (LA 1759), and *S. lycopersicum* cv. Anit, insignificant changes were seen between the chilling-treated samples and control samples. However, a significant decrease in the amount of SLW parameter was observed in *S. lycopersicum* cv. Lice (Fig. 18).

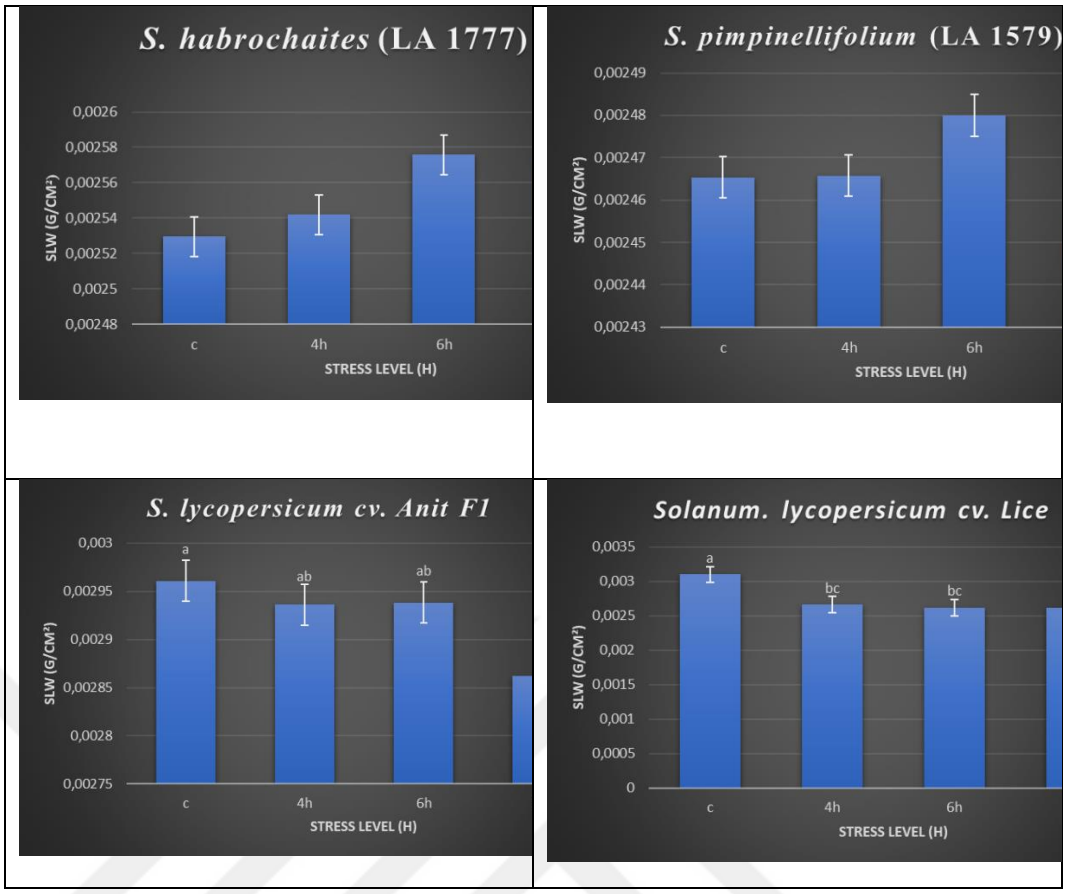


Figure 18. The specific leaf weight (SLW) of tomato genotypes after chilling stress

4.3.5. Root Fresh and Dry Weights

It was found that S. *habrochaites* prevented a notable change in root-related parameters, same as leaf-related parameters. Also, as it is seen in Fig. 19, S. *pimpinellifolium* also prevented significant changes in root-related characteristics after the assessment of the fresh root weight. Additionally, Anit F1's tolerance to chilling stress resulted in a minor change root fresh and dry weights. Eventually, the lack of chilling tolerance in *S. lycopersicum* cv. Lice led to a considerable change in these morphological factors.

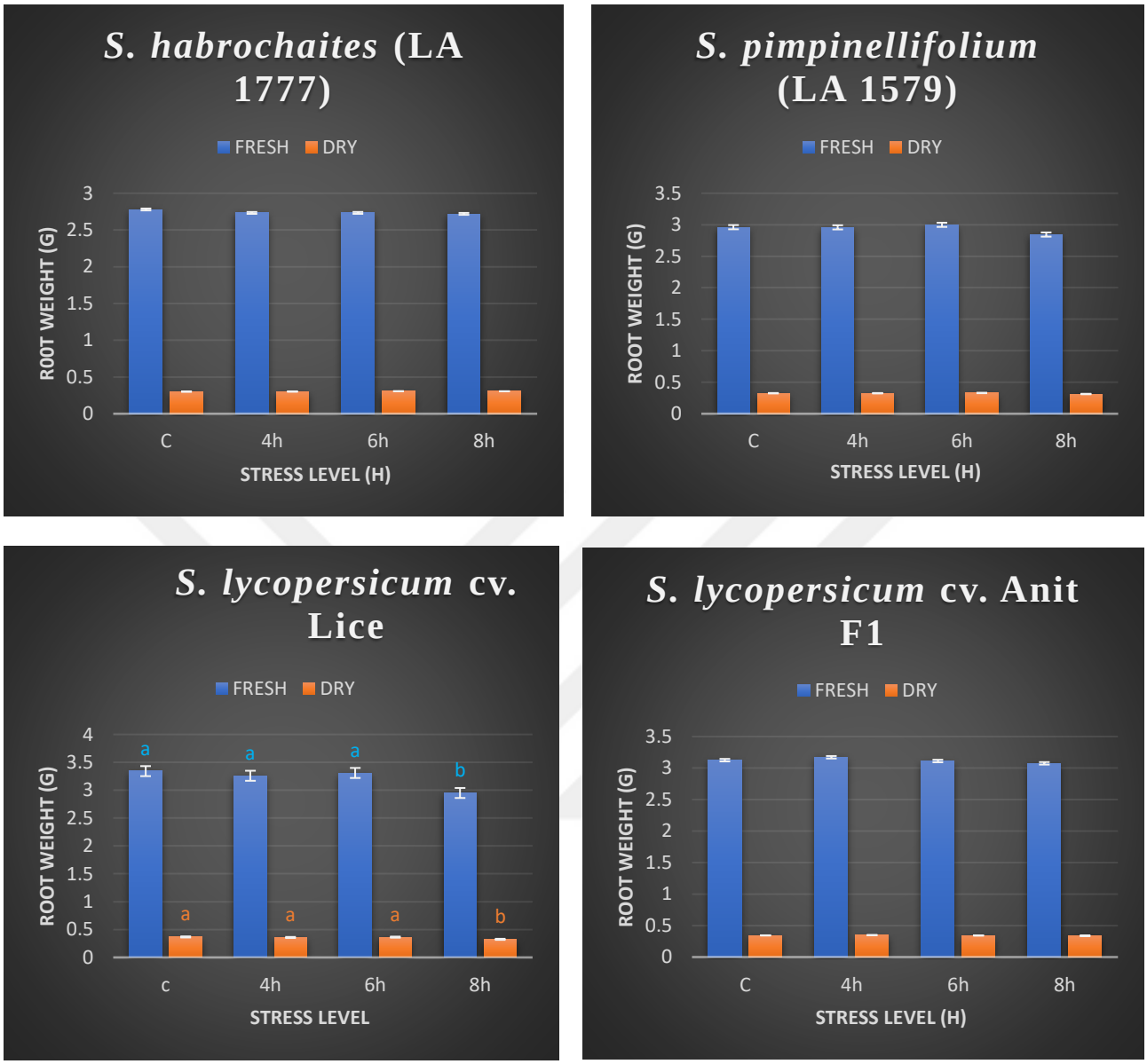


Figure 19. Root fresh and dry weight results of tomato genotypes after chilling stress

5. DISCUSSION

Chilling stress is one of the key environmental constraints in the occurrence of molecular, physiological, biochemical, and morphological changes (Khan et al., 2019; Mesa et al., 2021). Numerous tomato varieties have been shown to be susceptible to chilling stress and heating stress in previous investigations (Liu et al., 2012). Future environmental stresses are anticipated to increase in severity and frequency (Hatfield and Prueger, 2015), therefore, it's important to comprehend how they could impact plant improvement and the molecular and physiological defenses that plants employ. The main objective of the present study was to investigate the role of a group of miRNAs which determined to be related to abiotic stress in former literature, under chilling stress in the wild (S. habrochaites LA 17777 and S. pimpinellifolium LA1579) and cultivated tomato (S. lycopersicum cv. Anit and cv. Lice) genotypes. Therefore, the expression profile of the miRNA 167, miRNA 169, miRNA172, miRNA393, and miRNA 397 were determined in the leaves of tomato genotypes under chilling stress of 4 ° C at 3 different stress levels (4h, 6h, and 8h).

In LA7777 (S. habrocraites), which is cold tolerant (Chen et al., 2015), the expression of miRNA 167, 169, 172 and 397 upregulated up to 9 folds as duration of the chilling stress increased, but miRNA393 down regulated. In LA1579 (S. pimpinellifolium), while the expression of mi RNA 167, 169 and 172 started to upregulate at 6 hours and then continuous to upregulate up to 8 folds, the expression pattern of miRNA397 and 393 showed an increase at 6 hours after chilling treatment then decrease. The expression pattern of miRNA 167 and 172 in Anıt F1, determined as cold tolerant (Tepe and Kabaş, 2019) started to increase from 4 hours after chilling treatment. In Lice, the mi RNA 167, 169 and 172 slightly upregulated but not like that of LA777. The expression level of the miRNA397 had no change during chilling stress in cultivated tomato, cv Lice and Anıt F1. While the expression of miRNA 393 in Anıt F1 had similar level as control plants, its expression level in Lice upregulated at 8hours after chilling stress. The increase the expression of mi RNA 167, 169, 172 and 397 showed that, these miRNAs could be related to chilling stress tolerance by upregulation their expression (Koc et al., 2015). In contrast to this, the upregulation of the expression of miRNA393

determined only in cultivated tomato genotypes Lice, which is non tolerant to chilling stress. Therefore, it can be stated that miRNA393 may not be considerably related to chilling stress at this stress and growth stages in the studied cultivated tomato. This conclusion was also made in another study reported by Koc et al, (2015) in which miRNA 393 did not show a high level of expression at early stages of growth under short period of chilling stress in tomato (S. lycopersicum). However, the expression of miRNA393 increased at 0°C for 24 h in leaf, stem and root of Arabidopsis (Sunkar et al, 2004) and at 4°C for 24 hours in the leaf of Brachypodium (Zhang et al, 2009).

No significant change in expression was seen at the five-leaf stage in LA1777 (S. habrochaites) in miRNA 167 and 169 the study done by Cao et al., (2014) under chilling condition (at 1 hour, 4 hours, 8 hours, 12 hours, 24 hours and 48 hours after subjecting stress), suggesting that these miRNAs may be specific to species or the growth stages. Additionally, the level of expression in miRNA 397 increased in the mentioned study that indicating the importance of this miRNA in three and five-leaf stages of growth under short chilling conditions. MiRNA 167 and other conserved miRNAs had been down-regulated under short periods of chilling stress (2 hours, 6 hours, 12 hours, and 24 hours after treatment) in a study in another member of the Solanaceae family (S. aculeatissimum) at the 3-true-leaf stage in study conducted by Yang et al., (2017). The findings of that study and what is seen in the expression level of miRNA 167 in the current study revealed that conserved miRNAs may have comparable roles across plant species by concentrating on homologous genes involved in the chilly stress response.

According to the results of the present study miRNA 172 was overexpressed noticeably in S. pimpinellifolium (LA 1579) at all levels of low-temperature stress. S. pimpinellifolium is a wild relative of the cultivated tomato and it has a lot of breeding potential for desirable features including abiotic stress tolerance according to a study done by Razali et al., (2018). According to a study carried out by Zhao et al., (2017) miRNA 172 was upregulated in S. pimpinellifolium LA1375 under high-temperature stress. Therefore, this miRNA is a prominent factor in the

transcription regulation in defense mechanisms of this species against temperature stresses.

The subsidiary objective of this study was the evaluation of morpho-physiological parameters including the dry and fresh weight of leaf and root and the calculation of specific leaf weight under a short period of chilling stress. Also, the chlorophyll content of these 4 genotypes was assessed at different levels of chilling stress. It was aimed to see if there was a connection between these morpho-physiological parameters and the exposure to 4h, 6h, and 8h of stress in tomato plants. Abiotic stresses have a significant impact on chlorophyll fluorescence, stem, leaf, and root growth, and the yield and fruit quality in different species of tomato plants including S. lycopersicum as was seen in the previous studies (Hajer et al. 2006). These significant effects were seen in the present study as well. A considerable change in the level of chlorophyll content as one of the most important tomato growth parameters was seen at the stress level (4°C) in the cultivated genotype (S. lycopersicum cv. Lice) just after stress and also at the beginning of flowering stages. These results are completely consistent with a significant decrease in the status of chlorophyll content in another species (Vitis vinifera L.) at early stages of growth under a short period of chilling stress (Aazami et al., 2021). Moreover, the decrease seen in the present study in the amount of chlorophyll content was also seen in two other members of the Solanaceae family (Solanum melongena L. and Solanum tuberosum L.). the amount of chlorophyll content decreased in the eggplant (Solanum melongena L.) under 4°C of stress (Shi et al., 2019) and Tang et al., (2018) reported that some of the morpho-physiological traits including the leaf size and chlorophyll content were affected and their amount decreased considerably in potato (Solanum tuberosum L.) under temperature stress (at 0, 24, 60, and 96 h after stress treatment).

In another study by Rao et al., (2013), it was seen that S. pimpinellifolium demonstrated tolerance to temperature stresses as well and the dry and fresh weights of their leaves and roots did not change excessively. The results of mentioned study have been approved by the data obtained from S. pimpinellifolium (LA 1579) in this thesis. Furthermore, in the mentioned study the chlorophyll content represented a stabilized figure in this study.

Considering another case study carried out by Tepe et al., (2019) S. habrochaites (LA 1777) and S. lycopersicum cv. Anit F1 indicated significant tolerance to chilling stress morphologically. Also, there was not a considerable change in leaf dry weight, leaf fresh weight, root dry weight, and root fresh weight in S habrochaites (LA1777) under a short period of chilling stress (4°C). In addition to this, other wild genotype in present study (S. pimpinellifolium LA 1579) did not showed significant morphological changes under short term chilling stressa thus it can be said that LA1579 could be also tolerant to chilling stress. In contrarily, cultivated tomato, cv Lice had significant morphological changes and could be considered as susceptible to chilling stress.

In relation to flowering time under chilling stress in tomato, the number of prior studies is very limited and there was no previous research about the effect of chilling stress in the vegetive phase on the generative phase. As a result, the result of the present study can be compared to the result of the studies conducted by Jung et al., (2012) and Seo et al., 2009 in which a short period of chilling stress delayed the process of flowering and a long period of chilling stress in the vegetative stage induced early flowering. This is what was slightly seen in, the S. lycopersicum cv. Anit, S. habrochaites LA 1777, and S. pimpinellifolium LA 1579 in this thesis. Also, this mechanism was seen considerably in S. lycopersicum cv. Lice and caused a delay in the flowering phase.

6. CONCLUSION & RECOMMENDATION

This study analyzed some miRNAs (miR167, miR169, miR172, miR393, and miR397) associated with chilling stress during young seedling stage in wild and cultivated tomato genotypes. The outcome revealed that this variation between the expression levels may be connected to tomato genotypes' ability to tolerate chill. Among the miRNAs evaluated, miRNA 167, 169, 172 and 397 were upregulated in the genotypes (S. habrochaites LA 1777 and S. lycopersicum cv. Anit) which already determined tolerant to low temperatures in previous literatures (Venema et al., 1999; Foolad and Lin, 2000; Venema et al., 2005; Tepe et al., 2019). Interestingly, miRNA 167 showed the highest transcript accumulation at 6 hours of chilling exposure in S. pimpinellifolium, 4 and 8 hours in S. lycopersicum cv. Anit, and in all levels of chilling stress in S. habrochaites. It is also worth noticing that miRNA 169 was up-regulated the most at 6 hours of chilling application in S. lycopersicum cv. Anit compared to the control samples. Another important finding in this study is that miRNA 172 can be over-expressed regardless of stress level and tolerability or susceptibility of the plant under chilling stress. It seems that mentioned miRNA is one of the integral parts of tomato plants' defense mechanism against chilling stress.

The physiological responses evaluated in this study showed that chilling stress at these levels and this growth stage could have a significant effect on chlorophyll content, the dry and fresh weights of leaves and roots, and the specific leaf weight of the susceptible genotype (S. lycopersicum cv. Lice). While it could be barely seen a significant difference in these parameters in tolerant genotypes. By comparing the data obtained from S. habrochaites (LA 1777), and S. lycopersicum cv. Anit the tolerance of S. pimpinellifolium (LA1579) and the susceptibility of S. lycopersicum cv. Lice to this level of chilling stress can be concluded morphologically and physiologically. The leaflet area index of the tolerant and sensitive genotypes, however, is unaffected by these stress levels treated during the vegetative phase. The vulnerable cultivar's flowering timing may be significantly delayed by 8 hours of chilling stress. To sum up the findings of this study

demonstrated that chilling-susceptible tomato species can be adversely affected by short-term exposure to chilling stress during the early stages of growth. The physiological, morphological, and molecular responses of the tomato plant to stress treatment at this growth stage seem to be trustable markers for the evaluation process of plant breeding schemes of newly bred lines. In conclusion, the high level of expression in miRNA 167, 169, 172, and 397 could be related to chilling tolerance in tomato genotypes.

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8. THANKS

I would like to express my deepest appreciation to my supervisor Prof. Dr. Hülya İlbi who always supported me to finalize this research project. I would also like to extend my deepest gratitude to Dr. Birsen Çakır, Dr. Aylin Kabaş, and all our knowledgeable professors at the seed science and technology department who covered me to conduct the experiments. I would like to thank my spouse, Yasaman Naziri, for being so supportive while I was working on my thesis.

9. RESUME

I am currently finishing my master's degree in Plant Breeding as a scholarship student under the supervision of Prof. Dr. Hülya İlbi with a GPA of 3.96 / 4.00 at Ege University. I have been first among the students in the class. The master's medium of instruction is entirely in English and I got an overall 7.0 band in Academic IELTS. I am also a certified English tutor with two teaching diplomas from London Teacher Training college. I graduated with a bachelor's degree in Agronomy and Plant Breeding, ranked first among the graduating class with a GPA of 3.55 / 4.00 from the University of Tehran, the most prestigious university in Iran (Ranked 56th in agriculture science), and I have been praised in various festivals with different awards. My bachelor's thesis was about the role of epigenetic modifications in plants exposed to stresses under the supervision of Prof. Dr.Reza Maali Amiri. I have studied three defensive enzymes of chickpea under chilling stress using two cell damage indices and the best wheat cultivar to produce the best hybrid triticale by comparing gene sequences using bioinformatics tools and databases. These two studies have been presented in the 4th international students' science congress held in Izmir in September 2020 and also published in the abstract book and proceedings book of this congress.