T.C.
NİĞDE ÖMER HALİSDEMİR UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF AGRICULTURAL GENETIC ENGINEERING

INVESTIGATION OF POTENTIAL OF MIR160 IN SUSTAINABLE AGRICULTURE THROUGH OVEREXPRESSION IN POTATO CULTIVARS

BEYAZIT ABDURRAHMAN ŞANLI

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Master Thesis

Supervisor

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JULY 2020

Beyazıt Abdurrahman ŞANLI tarafından **Doç. Dr. Zahide Neslihan ÖZTÜRK GÖKÇE** danışmanlığında hazırlanan "**INVESTIGATION OF POTENTIAL OF MIR160 IN SUSTAINABLE AGRICULTURE THROUGH OVEREXPRESSION IN POTATO CULTIVARS**" adlı bu çalışma jürimiz tarafından Niğde Ömer Halisdemir Üniversitesi Fen Bilimleri Enstitüsü **Tarımsal Genetik Mühendisliği** Ana Bilim Dalı'nda Yüksek Lisans tezi olarak kabul edilmiştir.

Başkan : Doç. Dr. Zahide Neslihan ÖZTÜRK GÖKÇE – Niğde Ömer Halisdemir Üniversitesi

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Doç. Dr. Murat BARUT
MÜDÜR

THESIS CERTIFICATION

I hereby acknowledge that this thesis was written by me. All information mentioned each part of this thesis is based upon a scientific foundation and in line with the academic rules. Any knowledge I have utilized during the preparation of the thesis and resources benefitted from the literature have been declared in this thesis.

Beyazıt Abdurrahman ŞANLI

SUMMARY

INVESTIGATION OF POTENTIAL OF MIR160 IN SUSTAINABLE AGRICULTURE THROUGH OVEREXPRESSION IN POTATO CULTIVARS

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July 2020, 115 Pages

Potato *(Solanum tuberosum* L.*)* is one of the major tuber crops which is widely grown in the world following corn, rice and wheat. Potato growth and development as well as yield are negatively affected by heat and drought stress. Here, the function of miR160 was investigated in tolerant (Unica) and sensitive (Russet Burbank) potato genotypes under drought, heat, and their combination by using overexpression approach. In this study, transgenic plants and wild-type plants were compared on the basis of morphological (leaf numbers, biomass, leaf expansion, stem length, and tuber size) and physiological differences (photosynthesis rate, stomatal conductance, transpiration rate, leaf temperature, relative water content, chlorophyll content and proline amount). This study concluded that miR160 interferes directly or indirectly with abscisic acid and auxin pathways and expression of heat shock proteins which thus enables transgenic plants to tolerance abiotic stress conditions. After validation of this study in the field, it will help to develop drought and heat tolerant potato genotypes.

Keywords: Potato (*Solanum tuberosum* L.), abiotic stress, miRNA, drought, heat

ÖZET

PATATES ÇEŞİTLERINDE ANLATIMINI ARTTIRARAK MIR160'IN SÜRDÜRÜLEBİLİR TARIMDA KULLANILABİLME POTANSİYELİNİN DEĞERLENDİRİLMESİ

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Temmuz 2020, 115 Sayfa

Patates (*Solanum tuberosum* L.) dünyada mısır, pirinç ve buğdaydan sonra yaygın olarak yetiştirilen yumru bitkilerinden biridir. Yüksek sıcaklık ve kuraklık, patateste büyüme, gelişme sürecini ve verimi olumsuz etkiler. Bu çalışmada miR160'ın fonksiyonu, yüksek ifade yaklaşımıyla, kuraklık, yüksek sıcaklık ve bunların kombinasyonları altında toleranslı (Unica) ve hassas (Russet Burbank) patates genotiplerinde araştırılmıştır. Bu çalışmada, transgenik bitkiler ve yabani tip bitkiler morfolojik özellikler (yaprak sayıları, biyokütle, yaprak genişlemesi, gövde uzunluğu ve yumru büyüklüğü) ve fizyolojik özellikler (fotosentez oranı, stoma iletkenlik, terleme oranı, yaprak sıcaklığı, bağıl su içeriği, klorofil ve prolin miktarı) bakımından karşılaştırılmıştır. Bu çalışma miR160'ın absisik asit ve oksin yollarına ve ısı şoku proteinlerinin ekspresyonuna doğrudan veya dolaylı olarak müdahale ettiği ve böylece transgenik bitkilerin abiyotik stres koşullarına tolerans göstermesine imkân sağladığı sonucuna varmıştır. Bu çalışma saha denemesinde gerçekleştirildikten sonra, kuraklığa ve ısıya dayanıklı sürdürülebilir patates genotiplerin geliştirilmesine yardımcı olacaktır.

Anahtar Sözcükler: Patates (*Solanum tuberosum* L.), abiyotik stres, miRNA, kuraklık, yüksek sıcaklık

ACKNOWLEDGEMENT

First of all, I would like to thank my supervisor Assoc. Prof. Dr. Zahide Neslihan ÖZTÜRK GÖKÇE as she behaves very patient to me while completing my master degree and have time for me whenever I need to ask anything related to thesis.

I would like to thank to jury members Asst. Prof. Dr. Allah BAKHSH and Asst. Prof. Dr. Fatih HANCI for their positive critics and suggestions.

I would like to thank Prof. Dr. Mehmet Emin ÇALIŞKAN, Assoc. Prof. Dr. Ufuk DEMİREL and Asst. Prof. Dr. Emre AKSOY for their kind assistance during my master period.

I would like to thank my group members Melis YALÇIN and Arslan ASİM for their helps to complete my thesis.

This work was also supported by TUBITAK grant (115-O-405).

The last but not the least, I would like to thank all my family members for providing everlasting motivation to complete this study.

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SYMBOLS AND ABBREVIATION

Symbols	Description
%	Percentage
µ	Micro
µL	Microliter
M	Molar
mg	Milligram
ng	Nanogram
^{o}C	Degree Celsius
α	Alpha
mg/L	Milligrams per liter
g	Gram
L	Liter
mL	Millilitre
g/L	Gram per liter
h	Hour
µmol m^{-2}/sec	Micromole per square meter per second
µmol	Micromole
sec	Second
µg	Microgram
rpm	Revolutions per minute
ng/µL	Nanogram per microliter
min	Minute
bp	Base pair
µM	Micromolar

Abbreviation	Description
ABA	Abscisic acid
AD	Activation Domain
AMIM160	Mutant miR160 gene

A. mongolicu	*Ammopiptanthus mongolicu*
APX1	Cytosolic Ascorbate Peroxidase 1
ARF10	Auxin Response Factor10
ARF16	Auxin Response Factor16
ARF17	Auxin Response Factor17
ARR15	Arabidopsis Response Regulator15
A. thaliana	*Arabidopsis thaliana*
AtTTP	*Arabidopsis thaliana* Zinc Finger Family Gene
A. tumefaciens	*Agrobacterium tumefaciens*
Aux/IAA	Auxin/Indole-3-Acetic Acid
B1	Thiamine
B3	Niacin
B6	Pyridoxin
BAP	6-Benzylaminopurine
(C)	Control
Ca^{+2}	Calcium
CalS5	Callose Synthase 5 Gene
CcCDR	Cold and Drought Regulatory Gene
cDNA	Complementary DNA
CIP	International Potato Centre
DBD	B3-type DNA Binding Domain
DNA	Deoxyribonucleic Acid
DREB	Dehydration Responsive Element Binding Region
EF	Elongation Factor
FAOSTAT	FAO Corporate Statistical Database
g	G-force
GA_3	Gibberellin
GAST	GA-Stimulated Transcript Family
GH3	Grim Domain
GUS	β-glucuronidase
H	Heat
HD	Heat+drought
HD-ZIPIII	Leucine Zipper Transcription Factor
HSP17	Heat Shock Protein17

HSP21	Heat Shock Protein21
HSP70	Heat Shock Protein70
HSP101	Heat Shock Protein101
HvSNAC1	*Hordeum vulgare* NAC Superfamily Transcription Factor
LEA	Late Embryogenesis Abundant Proteins
LEC2	Leafy Cotyledon2
mARF10	Mutated ARF10 gene
MIM160	Repressed miR160 Gene
miRNA	micro RNA
mRNA	messenger RNA
MS	Murashige and Skoog
MS-0	Murashige and Skoog-zero
MT- sHSP	Mitochondrial Small Heat-Shock Gene
M. truncatula	*Medicago truncatula*
mtr-miR160a	*Medicago truncatula* miR160a
MYB	Myeloblastosis Family
NAA	A-Naphtalene acetic acid
O. sativa	*Oryza sativa*
OsmiR160	*Oryza sativa* miR160
OsmiR160a	*Oryza sativa* miR160a
OsmiR160b	*Oryza sativa* miR160b
OsARF18	*Oryza sativa* Auxin Response Factor18
PCR	Polymerase Chain Reaction
Pre-miRNA	Precursor-microRNA
RBB	Russet Burbank
RD	Repression Domain
RNA	Ribonucleic Acid
rRNAs	Ribosomal RNAs
ROS	Reactive Oxygen Species
RT-PCR	Real-time Polymerase Chain Reaction
RWC	Relative Water Content
SAUR	Small Auxin up RNA
SCFTIR1	Auxin Receptor E3 Ubiquitin Ligase
SlARF10	*Solanum lycopersicum* Auxin Response Factor10

S. lycopersicum	*Solanum lycopersicum*
SPAD	Chlorophyll index
S. tuberosum	*Solanum tuberosum*
T. aestivum	*Triticum aestivum*
T-DNA	Transfer-DNA
TaERF3	*Triticum aestivum* Ethylene-responsive Element Binding Factor
TaGASR1	*Triticum aestivum* GA-Stimulated Transcript Family
TaPEPKR	*Triticum aestivum* Phosphoenolpyruvate Carboxylase Kinase
TIR1	Transport Inhibitor Response 1
TMMB	Türk Mühendis ve Mimar Odaları Birliği
UV	Ultra-violet
qRT-PCR	Quantitative Real-time PCR

CHAPTER I

INTRODUCTION

As the world population rapidly increases, it will pose a challenge for feeding humanity. Until quite recently, several approaches have been performed in order to overcome this problem including conversion of forests into arable lands, use of available sources efficiently, reduction and recycling of more waste. Although, the first approach initially seems to be good strategy, it has devastating impact on the environment such as extinction of biodiversity and disturbing natural balance. Today, attempts have been carried out toward increase the yield without losing diversity and consuming fewer sources (water and soil) instead of land expansion. Sustainable agriculture is involved in strategies and applications to develop the production of enough and high-quality foods in a cost-efficient way for the humankind, and methods to protect both environment and available natural agricultural resources (Beddington et al., 2012)

The water sources in the world are decreasing in the course of time as a result of global warming. This phenomenon profoundly affects plant growth and development stages which ultimately cause loss of yield in the agriculture in the worldwide. To cope with these unfavorable effects, attempts have been made to improve better tolerance plants when exposed to abiotic stresses including drought, high/low temperature and salinity. In accordance with this purpose, traditional breeding strategies have demonstrated limited progress because of the complex nature of stress tolerance mechanisms and loss of genetic diversity for most crops. For example, some of crops including wheat, rice, and maize have fewer gene pools than respective wild types regarding genetic variation. Hence, modern approaches have been tailored coupled with conventional methods to improve stress tolerance plants (Onaga and Wydra, 2016)

Potato (*Solanum tuberosum* L.) is one of the important foods with high nutrient content in tubers. In terms of the production amount in the world, it is placed 4th rank with 388 million tons after corn, rice, and wheat. According to FAOSTAT data (2019), China has the greatest amount of production with 99,205,600 tonnes in 2017 among the countries across the world (Figure 1.1). Turkey is placed at 14th rank with 4,800,000 tonnes in the same year. Most of the production in Turkey is provided by Central Anatolian Region

including Niğde, Konya, Sivas, Aksaray and Nevşehir as well as Adana province (Table 1.1). Potato is known as a cool season plant and optimum temperature for the growth is in the range of 15 °C to 18 °C. The ideal soil pH is between 5.5 and 6.0. Composition of potato tubers is composed of carbohydrates (starch), protein, lipids, vitamins (C and B_6), antioxidants (lutein and zeaxanthin) anthocyanins and some minerals including zinc, magnesium, potassium and phosphorus (Burgos et al., 2020). Potato is known as a susceptible plant to abiotic stresses since considerable amount of tuber yield is lost when it faces one or combined adverse environmental conditions. One of the potato cultivars that has been used in this thesis is Unica, developed by CIP in Peru, has red tuber skin and less time required for tuber production, resistance to late blight and viruses, and resistance to heat and drought as well (Muthoni and Kabira, 2015). Other cultivar is Russet Burbank that has brown and large tuber which can be cylindrical shape or slightly flat structure (Bethke et al., 2014), has long-term storage and resistance to black leg, while it is sensitive to, late blight, tuber net necrosis, fusarium dry rot, PVX and PVY (Plant Health and Biosecurity, 2001).

Rank	Country	Potato Production in 2017 in tonnes
1	China	99,205,600
2	India	48,605,000
3	Russian Federation	29,590,000
4	Ukraine	22,208,200
5	United States	20,017,400
6	Germany	11,720,000
7	Bangladesh	10,216,000
8	Poland	9,171,730
9	Netherlands	7,391,880
10	France	7,342,200
11	Belarus	6,414,760
12	United Kingdom	6,218,000
13	Iran	5,102,340
14	Turkey	4,800,000
15	Peru	4,776,290
16	Algeria	4,606,400
17	Belgium	4,416,660
18	Canada	4,410,830
19	Egypt	4,325,480
20	Pakistan	4,142,400
21	Brazil	3,656,850
22	Kazakhstan	3,551,110
23	Romania	3,116,910
24	Colombia	2,819,030
25	Uzbekistan	2,793,690

Figure 1.1. Top 25 countries in potato production were given across the world in 2017 (FAOSTAT, 2019)

Table 1.1. List of top 14 potato production provinces was given in Turkey (TMMOB, 2019)

İl	2014	2015	2016	2017	2018	2018 %
Niğde	618.853	674.773	892.297	835.200	732.188	**16,09**
Konya	509.188	493.748	549.802	567.076	611.957	**13,45**
Afyonkarahisar	301.579	434.929	476.900	473.016	455.352	**10,01**
Kayseri	285.770	287.835	305.470	351.270	385.913	**8,48**
İzmir	391.347	407.745	367.706	396.130	330.143	**7,26**
Nevşehir	218.952	301.039	255.773	249.626	269.620	**5,93**
Adana	206.120	219.221	221.397	241.196	219.076	**4,81**
Aksaray	239.728	242.302	210.959	207.810	202.371	**4,45**
Sivas	171.663	263.167	202.524	182.149	169.737	**3,73**
Bolu	280.735	249.603	226.919	164.778	150.327	**3,30**
Bitlis	132.504	212.490	163.992	154.696	150.043	**3,30**
Erzurum	83.490	78.516	72.173	75.708	85.729	**1,88**
Hatay	51.802	70.231	109.961	148.858	71.145	**1,56**
Tokat	69.815	70.764	67.902	72.542	61.385	**1,35**
Diğer	604.454	753.637	626.225	679.945	655.014	**14,40**
Toplam	**4.166.000**	**4.760.000**	**4.750.000**	**4.800.000**	**4.550.000**	**100,00**

MiRNAs are believed to play important roles in responding to abiotic stress conditions as well as growth, development, signal transduction and hormone metabolism (Khraiwesh et al., 2012). Hence, miRNA-mediated gene regulation can be used in order to develop stress tolerant plants against different stresses such as drought, heat or salt. In parallel with this purpose, identification, characterization and functional analysis of several miRNAs have been studied upon stress treatments in plants including *Arabidopsis thaliana*, soybean, tomato and rice (Song et al., 2019). However, there has been a gap associated with function of miRNAs in potato *S. tuberosum* under high temperature. Until recently, most of the attempts have investigated identification and finding out possible function of miRNAs on drought tolerance mechanisms in potato. In addition, there have been limited number studies involved in function of miRNAs in potato under the combination of environmental stresses. The aim of this study was to investigate role of miR160 in response to drought, heat, drought and heat combined stresses with the help of transgenic approach by overexpressing miR160 in contrasting potato cultivars those are Unica (tolerant) and Russet Burbank (sensitive).

CHAPTER II

LITERATURE REVIEW

2.1 Abiotic Stress

Abiotic stress is defined as any unfavorable condition caused by non-living factors in a given environment. It has negative effects on plant growth, developmental stages and productivity when plants are exposed to one stress alone or combined. The most common types of abiotic stresses that plants encounter throughout their life cycles are drought, high/low temperature, salinity, UV, and heavy metals. Plants, as sessile organisms, have various complex mechanisms and strategies in order to survive and maintain their lifecycles (Tuteja and Gill, 2016).

2.1.1 Drought stress

Drought stress occurs while the amount of available water in the soil becomes less and water consumption is greater compared to retention due to transpiration and evaporation processes. Drought stress inflicts considerable damages to plants and causes several morphological, physiological and biochemical changes based on the stress period, type, age and growth stages of plants. The effects of drought stress have been studied in several organisms including rice, barley, soybean and wheat. Common findings from studies are the increase in root length over the shoot length to use water efficiently, the less reduction in biomass amount in the root than shoot, reduction in leaf biomass and transpiration rate as well (Tuteja and Gill, 2016). Plant organisms have several strategies in response to drought such as drought avoidance, tolerance and escape. Drought avoidance involves changes in morphological features and those are less stomatal conductance, decreased leaf surface area and efficient root structures to absorb water in the soil. Drought tolerance is generally accomplished by tailoring both physiological and molecular networks such as enhanced amount of secondary molecules, antioxidants and scavengers in the cell. Finally, drought escape is a mechanism that includes plant development in advance to complete life cycle of plants in case of possible drought stress. There have been major responses along with minor

responses in plants against drought stress which are shown at physiological, biochemical and molecular level (Onaga and Wydra, 2016) (Figure 2.1).

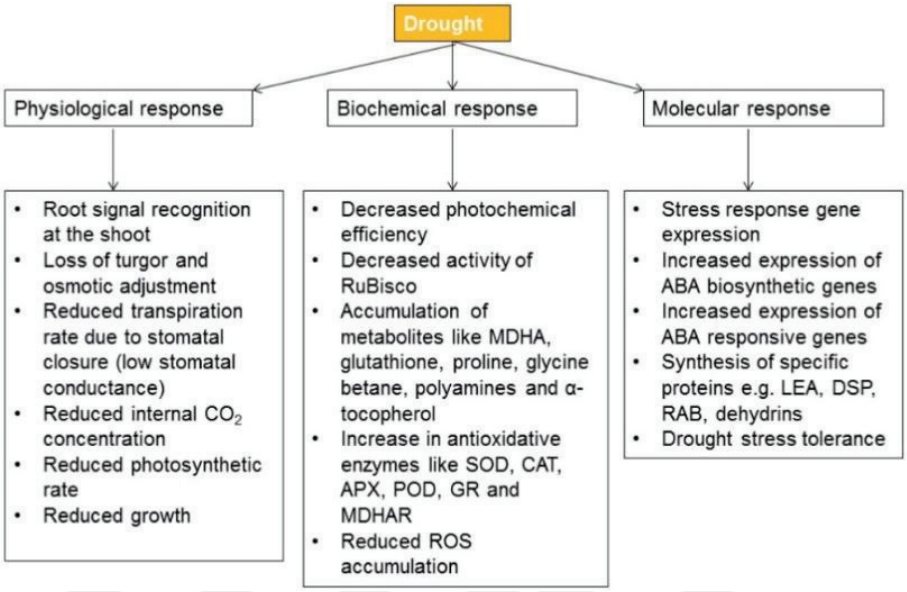


Figure 2.1. Physiological, biochemical and molecular responses were given in plants under drought stress (Onaga and Wydra, 2016)

2.1.1.1 Studies on drought tolerance

With the advent of molecular and genomic approaches, identification of drought-responsive genes has been accelerated in plants. Next, the role of genes associated with either functional or regulatory mechanisms have been investigated using transgenic approach and then tailored to improve plants with enhanced tolerance against drought. Alternatively, several important transcription factors have been utilized to produce enhanced tolerance in plants (Shanker and Maheswari, 2017). Rong et al. (2014) have shown enhanced tolerance in seedlings of wheat lines having overexpressed ethylene-responsive element binding factor gene, *TaERF3*, when exposed to drought stress compared to control group. It was also observed that proline and chlorophyll content was considerably induced, while H_2O_2 level and stomatal conductance was markedly decreased in leaves of transgenic wheats. Al Abdallat et al. (2014) have stated that overexpressed *HvSNAC1*, a stress-related NAC superfamily transcription factor gene, barley displayed increased tolerance upon drought treatment than wild-type barley. They have also revealed that those transgenic plants showed increased water status,

higher level of photosynthesis as well as less amount of water loss compared to wild-type barley. Similarly, Tamirisa et al. (2014) have found that overexpressed Cc*CDR*, cold and drought regulatory protein encoding gene, in transgenic *A. thaliana* plants illustrated increased tolerance under drought conditions. It was proved that those plants had increased relative water content, enhanced amount of proline and reduced sugars, enhanced root length, increased amount of chlorophyll and biomass as well as more stable membrane structure than control plants. In addition, miRNAs can be considered potent regulator in order to develop enhanced tolerant plants, because they have adopted various expression patterns under drought stress, leading to notable changes in the expression of target genes encoding important enzymes function in the committed step of assimilation pathway or transcription factors (Ding et al., 2013) (Figure 2.2).

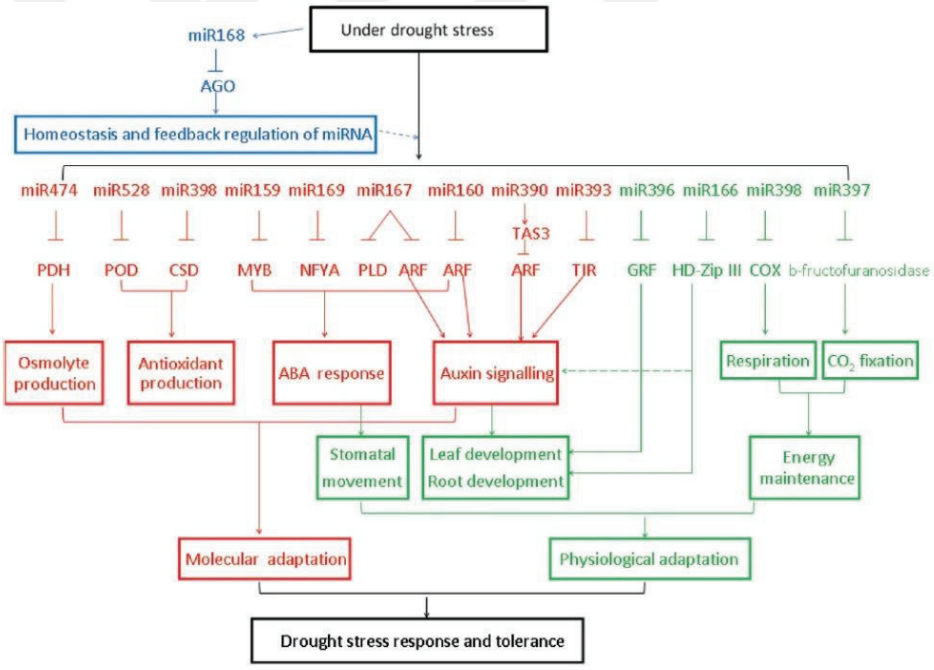


Figure 2.2. MiRNAs and corresponding targets were given under drought stress (Ding et al., 2013)

2.1.2 Heat stress

Heat stress occurs when the optimal temperatures exceed by around 5 °C or more (Guy, 1999). Heat stress negatively influences overall plant growth and development processes, although, the most susceptible parts of plants are seeds against heat fluctuation in a given environment. High temperature stress is mainly categorized in two

groups; those are exposed to sudden change in the temperature for about 1 hour and second group includes gradual increase in the temperature which is so-called priming. The common destructive effects on plants are changed in flowering period, fluidity of membrane structures, protein stabilities, activity of enzymes, inhibition of germination, sugar production, reduction in photosynthesis rate and increased amount of ROS molecules in the cell. When the heat stress signal is perceived by receptors, it triggers release of Ca^{+2} ions and activation of calcium-dependent protein kinase and mitogen-activated protein kinases. Those proteins help induction of some molecules related to tolerance such as antioxidant molecules and osmolytes. In contrast, acclimation causes upregulation of heat shock proteins, leading to enhanced expression at transcriptional and translation levels for dehydrins and late embryogenesis abundant proteins (LEAs), protective compounds and molecules including proline, sugars and sugar derivatives (sugar alcohols), ammonium/sulfonium compounds along with abscisic acid (ABA) hormone. A series of events have been suggested including perception, responses in nucleus and cytoplasm under heat stress in plants (Wahid et al., 2007) (Figure 2.3). Until recently, many studies have been endeavoured to find out possible mechanisms in response to heat stress in different plants including wheat, rice, maize, tomato and grape. For potato production, heat adversely affects tuber initiation, abnormal shapes, and necrosis which ultimately damages yield and quality of potato (Levy and Veilleux, 2007).

2.1.2.1 Studies on heat tolerance

Progress in genetic engineering techniques, bioinformatics along with conventional breeding methods have enabled researchers to understand mechanisms underlying heat tolerance in plants. Queitsch et al. (2000) have studied function of heat shock protein 101 (HSP101) in *A. thaliana.* It was shown that transgenic plants with lower expression of HSP101 due to antisense inhibition displayed similar growth rate that of control group. However, their capacities to get heat tolerance was considerably reduced upon mild pre-treatment. It was revealed that transgenic plants overexpressing HSP101 demonstrated tolerance to higher temperature compared to control plants. In a similar study, effect of mitochondrial small heat-shock protein on thermotolerance has been investigated in tobacco (*Nicotiana tabacum*) (Sanmiya et al., 2004). Researchers have transferred tomato mitochondrial small heat-shock gene (MT- sHSP) into tobacco

plants under 35S promoter and it was observed that seedlings overexpressing MT-sHSP showed better tolerance to sudden heat stress than antisense tobacco lines displaying no expression of MT- sHSP. Growth rate and morphology of transgenic plants were found similar to control plants. One-month-old transgenic seedlings showed thermotolerance under abrupt heat stress, while those showing no expression of MT-sHSP gene displayed sensitivity. In another study, Zhang et al. (2017) have examined heat-responsive genes in wheat (*T. aestivum*). Isolation and characterization of a GA-stimulated transcript (GAST) family gene namely, TaGASR1, was successfully achieved using heat-tolerant variety. It was found that expression of TaGASR1 was substantially stimulated by heat along with drought, salt and ABA. Transgenic wheat lines with overexpressing TaGASR, a heat responsive gene, displayed enhanced tolerance upon heat treatment as compared to control group. It was also observed that ectopic expression of this gene increased thermotolerance and decreased ROS accumulation in *A. thaliana* upon heat treatment. Recently, function of a member of transcription factor belonging to dehydration-responsive element-binding (DREB) family has been investigated in *A. thaliana* under heat stress (Yin et al., 2018). It was implied that AmDREB2 gene was successfully isolated from *Ammopiptanthus mongolicu* plant with enhanced tolerance to abiotic stresses. The expression of that gene in the seedling of *A. mongolicu* was triggered by heat along with drought. Transgenic *A. thaliana* plants overexpressing AmDREB2C showed increased tolerance to heat and drought. It was also reported that this gene induced more production of linoleic acid both in seeds and leaf tissues of transgenic *A. thaliana* plants. Zang et al. (2018) have stated that transformation of *TaPEPKR* gene, phosphoenolpyruvate carboxylase kinase-related kinase in wheat (*T. aestivum*), into *A. thaliana* and another wheat cultivar, namely, Liaochun10, ensured increased tolerance under heat and drought stress. It was said that the transformation into *A. thaliana* plant was done to compare possible effects of that gene between monocotyledonous and dicotyledonous plants. It was proposed that *TaPEPKR* gene might be used in transgenic studies because it has significant function to gain tolerance to heat as well as dehydration.

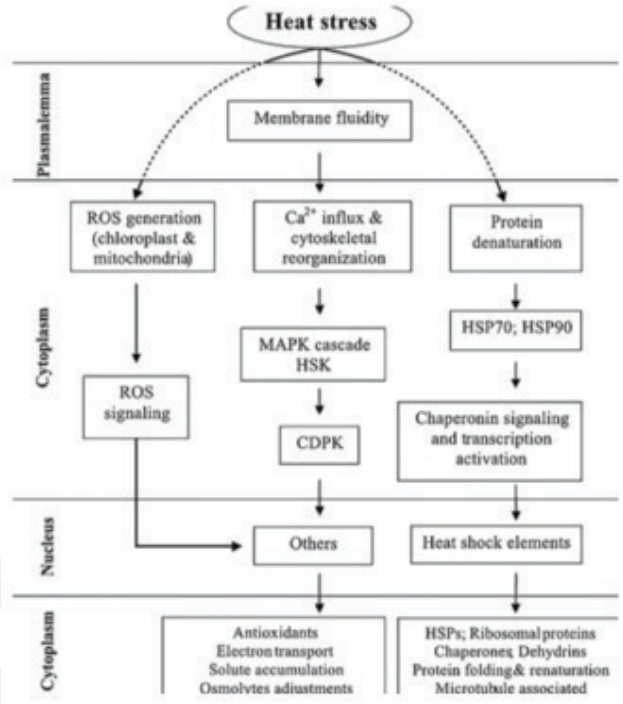


Figure 2.3. An array of events and changes were given upon heat treatment in the cell (Wahid et al., 2007)

2.1.3 Combination of drought and heat stress

Plants simultaneously deal with several environmental constraints under field conditions. Those conditions involve several stresses such as drought combined with heat, drought combined with salt, heat combined with salt, or any of those abiotic stress and biotic stress simultaneously, unlike controlled environments such as greenhouses or laboratories (Suzuki et al., 2014) (Figure 2.4). In contrast to effect of these environmental limitations individually, a combination of drought and heat stress has not been investigated in detail in plants (Rizhsky et al., 2004). Combined stress causes either positive or negative interaction in plants. The effect of combined stress on growth, development, and yield have been studied in plants such as maize, sorghum and barley (Mittler, 2006).

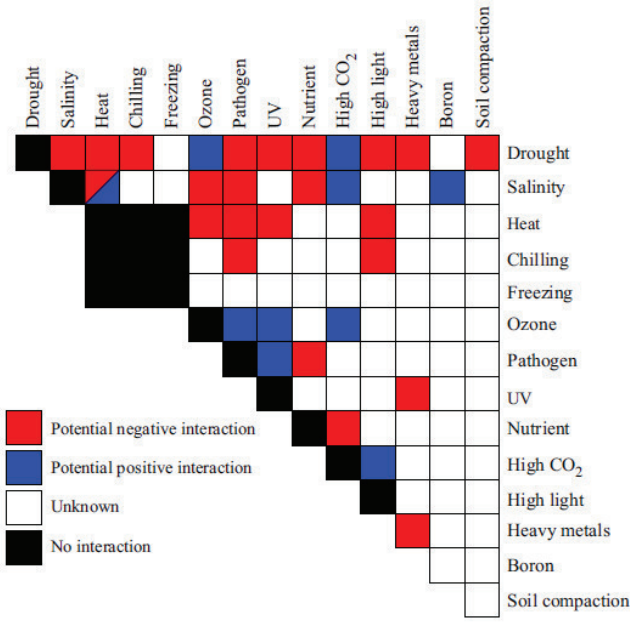


Figure 2.4. Potential interactions for abiotic and biotic stresses that plants face in the nature (Suzuki et al., 2014)

2.1.3.1 Studies on combined effect of drought and heat stress

Rizhsky et al. (2004) have studied response mechanism under drought combined with heat conditions in *A. thaliana*. It was found that the response of plants against a combination of drought and heat stress was different from plants exposed to either drought or heat stress. Accumulation of sucrose and maltose in plants was observed greater compared to plants subjected to drought or heat individually. However, accumulation of proline was not seen in those plants. In exchange for proline, sucrose functioned as osmoprotectant in *A. thaliana* plants. It was also shown that expression of 454 transcripts specific to combined stress condition. Koussevitzky et al., (2008) have revealed specifical accumulation of 45 proteins under a combination of drought and heat stress in *A. thaliana*. They function in several important processes in the cell including scavenging of reactive oxygen species, Calvin cycle as well as malate metabolism. It was found that malic enzyme accumulated much upon combined stress treatment, leading to reduction in malic acid and oxaloacetate amount. Hence, it was proposed that malate might be a potent regulator in response to combined stress in *A. thaliana.* Besides, it was shown that accumulation of cytosolic ascorbate peroxidase 1 (APX1) protein and corresponding mRNA occurred under drought combined with heat. APX1-deficient plants displayed higher sensitivity to combined conditions and greater

accumulation of hydrogen peroxide compared to wild-type plants. However, mutant plants whose activity of either stromal or mitochondrial ascorbate peroxidase 1 (APX1) was repressed did not show higher sensitivity as compared to APX1-deficient or wild type plants. Similar study was done to find out potential effects of drought, heat and combined stress on growth and physiology in *A. thaliana* (Vile et al., 2012). Plants under a combination of drought and heat developed less than those treated with drought or heat alone. It was suggested that drought combined with heat effect was mostly additive such as plant mass based on multiple and single trait analysis, yet, some traits specific to individual stress such as increase in biomass distribution in roots under drought stress. Prasad et al. (2011) have endeavor effect of drought and heat stress alone as well as combined stress in grain filling stage on yield in wheat (*T. aestivum).* Photosynthesis activity was found the lowest in plants in response to a combination of drought and heat compared to plants subjected to drought or heat. The additive interaction of drought and heat stress was prominently shown in those traits including total dry weight and spikelet fertility in wheat (Prasad et al. 2011).

2.2 MicroRNAs (MiRNAs)

MiRNAs are one group of small RNAs and play important roles in gene regulation at transcriptional and post-transcriptional level by repressing or inhibiting protein expression of corresponding target. They are mostly between 20-24 nucleotides in length and conserved in most of species along with species-specific ones. They have linked several important processes in plants including flowering period, growth, hormone regulation, signal pathways, homeostasis, response to biotic and abiotic stresses (Ding et al., 2013) (Figure 2.5). MiRNAs have been studied in order to identify, and then, to unravel their possible functions with related mechanisms under abiotic stress conditions in different organisms. They have displayed different expression patterns regarding stress type and organisms. For example, expression of miR156, target of Squamosa promoter binding protein-like, has upregulated in *T. aestivum* under drought condition while, the expression has downregulated under heat stress in the same organism. In addition, expression of miR167, target of Auxin response factor, has stimulated under drought stress *Oryza sativa* although miR168, target of Argonaute1, has repressed in rice. (Mangrauthia et al., 2013)

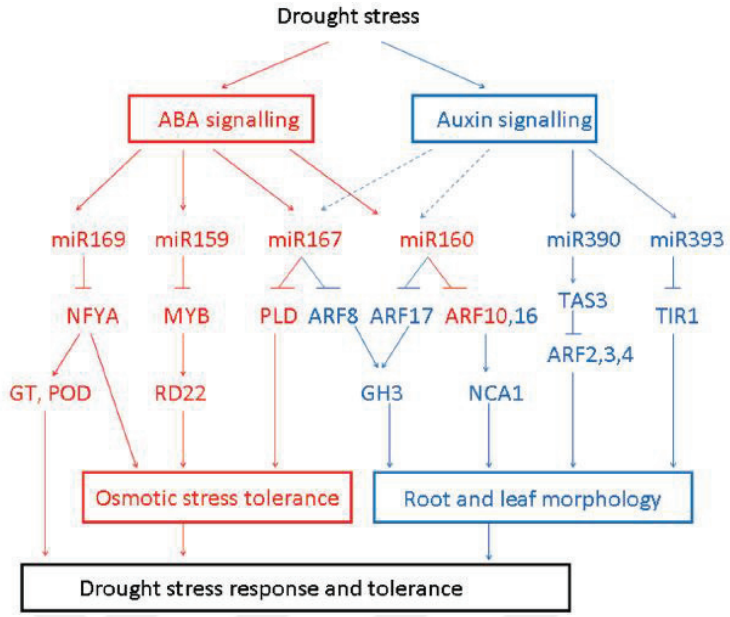


Figure 2.5. Regulation of ABA signalling and auxin signalling were shown by modulation of several miRNAs under drought stress (Ding et al., 2013)

2.2.1 MiRNA studies in potato

Drought, the most common abiotic stress, adversely influences potato yield by restricting amount of water plants absorbed from the soil and enhancing salt ions in the soil, causing movement of water from plant to environment. First study to identify miRNAs on potato (*S. tuberosum)* growth and development have been made by Zhang et al. (2009) and 48 potential miRNAs have been found by using *in silico* approach. They have also revealed 186 potent target genes that are involved in flower, leaf, root and stem development, signal transduction, metabolism pathways and stress response. In order to validate function of miRNAs identified by bioinformatics tools, 12 miRNAs have been chosen to perform RT-PCR analysis. They concluded that some miRNAs have expressed in all parts of plants with varying levels of expression based on tissue type. Moreover, Yang et al. (2010) have found 71 possible miRNAs belonging to 48 families in potato. They have stated that 65 out of 71 miRNAs have initially been identified and 7 miRNAs have been further selected to verify their functions in potato. Consequently, different expressions of those miRNAs in potato have been proved using real time PCR regarding tissue type. Researchers suggested that expression of each miRNA with varied levels in different tissues might be associated with the functions of miRNAs in regulating the organ or vegetative development stages in potato. Xie et al.

(2011) argued that number of miRNAs in potato has yet been found less compared to other organisms. Therefore, they have studied identification of miRNAs via bioinformatics approach along with newly modified comparative genome strategy. As a result, 202 potential miRNAs belonging to 78 families have been shown and 54 out of 78 have been stated as novel family. Following identification step, expression levels of 12 miRNAs have been investigated in several tissues including young leaf, immature flower and mature flower tissues. They conclude that miRNAs analyzed have been expressed for all tissue types apart from the one which has not shown any expression in young leaf. Besides, researchers have revealed 1094 target genes involved in encoding transcriptional factors, in response to stress, regulation of metabolic pathways and signal transduction in accordance with miRNAs identified in potato. They have analyzed only the expressions of the 12 miRNAs selected in young leaf, immature flower and mature flower tissues, and as a result they observed that 11 of the miRNAs analysed were expressed in all three tissues and one was not expressed in young leaf tissue. Zhang et al. (2013 and 2014) have identified 259 miRNAs that belong to the 159-miRNA family with the help of next generation sequencing approach and the potato genome sequence. They have shown that only 28 families of these miRNA families are conserved in all plants, while others have been suggested as potato-specific miRNAs. Potential targets of those miRNAs identified are involved in several processes including kinase and ion balance, defense mechanism, flowering and tuber formation (Zhang et al., 2013). In a similar study aimed at identification and characterization of miRNAs with samples from three different tissues and four different stages of tuber development, 89 conserved miRNAs, 147 potato-specific miRNAs and 112 potato-specific potential miRNAs have been identified in potato (Lakhotia et al., 2014). In the lights of expression analysis results, researchers have proved that some of miRNAs have shown tissue-specific expression although a few have only expressed in tuber formation stage. Ou et al. (2014) have examined genome-wide investigation of miRNAs and their respective target genes in cold-stored potato tubers. They have identified 53 known and 60 novel miRNAs along with 70 target genes. It has been demonstrated that miRNAs play important roles in regulation of the gene expression in post-harvest tubers. Additionally, varied expression patterns of 11 miRNAs and respective 34 target genes have been confirmed in two different potato cultivars showing different response to storage (Ou et al., 2014). Similarly, Din et al. (2014) have identified 120 novel miRNAs belonging to 110 families with a comparative genomic approach. Later, they

have validated the expression of randomly chosen 10 miRNAs using quantitative RT-PCR method. Researchers have also indicated 433 potential target genes involved in metabolism, transcription factors, growth and development, other physiological events in accordance with identified 120 novel miRNAs (Din et al., 2014).

To date, attempts have largely been conducted to identify miRNAs that are only involved in the regulation of tolerance mechanism under droughts condition among abiotic stresses. For the first time, the findings of these studies were published in 2011. Until recently, five studies were published with three of those by the same group (Hwang et al. 2011a; 2011b; 2011c). They have identified stu-miR396, stu-miR156a and stu-miR157a with a changing expression under drought stress. The expression of stu-miR396 has gradually increased from the 1st hour to the 6th hour after the drought treatment. The expression of stu-miR159a and stu-miR157a, however, was repressed in the 1st and 3rd hour and then induced in the 6th hour. In another study, Hwang et al. (2011b) have examined the expression pattern of miR171 family and indicated change in the expressions of miR171a, miR171b and miR171c under drought conditions which has been accomplished by both air seasoning and treated with 15% PEG 6000. In air seasoning treatment, the expressions of the selected miRNAs have decreased in the 1st hour of stress application and then triggered up to the 6th hour. Similarly, the expression of miR171a has repressed after 1 hour, and has induced after 3rd hour and remained steady at this level for 48 hours in the latter treatment. The expression of miR171b has reduced in the 1st hour, and then has reached expression of control group in the 3rd hour in air seasoning treatment followed by surpassing in the control group in 6th hour. For the PEG application, there has been slight reduction in the expression of miR171b until the 6th hour. However, the expression of miR171b has been observed greater as compared to the control group from the 12th hours. It has been found that expression pattern for miR171c was the same as that of miR171a in air seasoning treatment. Expression of miR171c has diminished after 1 hour, and later increased slightly followed by reaching control group after 48 hours in PEG application. Hwang et al. (2011c) have displayed change in the expressions of stu-miR172c, stu-miR172d, and stu-miR172e miRNAs under drought conditions. A total of 11 miRNAs belonging to 6 miRNAs families and respective targets involved in regulation of proline have been identified in response to drought (Yang et al., 2013). Based on findings from qRT-PCR analysis, 10 out of 11 miRNAs have successfully been verified and expression of those

have upregulated upon drought apart from the one having reduced expression. In consideration of expression and functional analysis results miR172, miR396a, miR396c and miR4233 might be involved in regulating expression of the pyrroline-5-carboxylate synthase gene, controlling proline biosynthesis pathway. Additionally, researchers have shown regulation of pyrroline-5-carboxylate synthase gene and proline dehydrogenase genes that play important roles in proline synthesis might be controlled by miR2673 and miR6461, respectively. Recently, Zhang et al. (2014) have identified 458 known and 674 novel miRNAs in control group, and 471 known and 566 novel miRNAs in drought treated group. In this comprehensive study, conserved miRNAs with two times greater expression have been chosen under drought stress. There has been an increase in the expression of 100 miRNAs while, expression of 99 miRNAs have decreased upon drought. For novel miRNAs, it has been observed that increase in the expression of 119 miRNAs was unlike 151 miRNAs showing reduction pattern in the expression. Furthermore, researchers have predicted a number of 246 genes targeted by known miRNAs along with 241 genes targeted by novel miRNAs. As a result of the expression analysis on miRNA and respective target genes miR814, miR835 and miR4398 have been involved in the regulation of drought-related genes. MYB transcription factor, hydroxyproline-rich glycoprotein, aquaporin and WRKY transcription factor have been targeted by those miRNAs, respectively.

2.3 Auxin Response Factors in Plants (ARFs)

Auxins are essential groups of plant hormones that regulate fundamental processes in plants including growth and developmental stages, division, elongation and differentiation of cells, response against abiotic stress and tropisms. Recently, many studies have identified mechanisms underlying the auxin perception, response and function of possible genes targeted by auxins in *A. thaliana* together with other organisms (Rosado et al., 2012; Guilfoyle, 2015). Auxin response factors (ARFs) are a group of DNA-binding proteins regulating auxin-mediated transcription following releasing from complex, namely, auxin receptor E3 ubiquitin ligase SCFTIR1 (Mallory et al., 2005). ARF proteins generally have three domains which are B3-type DNA binding domain (DBD), activation/repression domain (AD/RD) and a carboxy-terminal dimerization domain. B3-type DBD is known as conserved domain and contains 100 - 120 residues. The content of middle region, AD or RD, have important role in deciding

role of ARF proteins. Glycine residue rich region act as activator, while serine residue rich region function as repressor. Dimerization domain mediates protein interactions with Aux/IAA proteins in response to auxin along with other ARFs. Upon increase in auxin level, it triggers degradation of Aux/IAA proteins, leading to homodimerization of ARF proteins. Following activation, ARF proteins regulate expression of early auxin-response genes. ARF10 and ARF16 demonstrate similarity regarding sequence, meaning that they have DNA-binding domain, C-terminal domain along with middle region (AD/RD) while, ARF17 lacks C-terminal domain (Guilfoyle, 2015). Expression of auxin responsive genes including Auxin/Indole-3-Acetic Acid (Aux/IAA), Small auxin up RNA (SAUR) as well as Grim domain (GH3) have been either stimulated or repressed by binding of member ARF family to specific AuxRE sequence in their promoter regions (Li et al., 2016). It has been proposed that auxin response factors family has a crucial role in the regulation of expression level for auxin response genes. For the first time, Ulmasov et al. (1997) have cloned ARF1 protein in *A. thaliana*. Later, Guilfoyle and Hagen (2007) have identified 22 ARF genes plus one transgene in *A. thaliana.* Moreover, 22 ARF genes have been identified in tomato, 25 genes in rice (Wang et al., 2007), 24 genes in *Medicago truncatula* (Shen et al., 2015a), and 47 genes in banana (Hu et al., 2015). Recently, attempts have been made associated with ARF gene family to understand possible mechanism using bioinformatics approach and molecular studies in *M. truncatula* and *Gossypium raimondii* (Li et al., 2016). They have suggested a novel understanding underlying mechanism regulating both ARF gene expression and protein activity in those organisms.

2.4 MiR160 Family

MiR160 family is a kind of conserved miRNA family and has different numbers of members in plants. Two members have been identified in *Dimocarpus longan*, three members in *A. thaliana* (miR160a, miR160b, miR160c) six members in *O. sativa* and *Zea mays,* with the highest number of seven members in *Populus trichocarpa.* Each member of this family plays an important role in seed germination or vegetative phase of plants. Rhoades et al. (2002) have found 23 auxin response factor (ARF) genes and three of which ARF10, ARF16, and ARF17 are targeted by miRNA160 in *A. thaliana*. This study has proposed three genes including *MIR160a, MIR160b* and *MIR160c* that encodes miR160. The function of miR160a in *A. thaliana* has been investigated and it

has been found in controlling embryo development during very early stage of embryogenesis (Liu et al., 2010b). Moreover, miR160c controls development of root cap structure in *A. thaliana* (Lin. et al., 2015). Mallory et al. (2005) have shown that enhanced ARF17mRNA level and varied accumulation of auxin conjugating proteins jointly induced developmental anomalies in leaf, stem, root, reduction in petal size and aberrant stamen structure. In agreement with an earlier study, roles of miR160 coupled with miR166 and miR393 have studied in *A. thaliana* and it has been found that those regulate morphological changes as well as hormone balance via controlling transcript level of ARF10, leucine zipper transcription factors (HD-ZIPIII) and transport inhibitor response 1 (TIR1) (Khraiwesh et al., 2012). This study proved the post-transcriptional control of ARF17 which was dictated by miR160. In another study, Sorin et al. (2005) have found importance of ARF17 regulation directed by miR160 associated with auxin homeostasis and generation of lateral roots in *A. thaliana*. The transgenic plants overexpressing ARF17 had lower numbers of adventitious roots compared to control group together with decreased expression in GH3 genes. It was observed that negative regulation caused by ARF17 on adventitious root production was achieved by suppressing GH3 in *ago1* mutants with constant adventitious root production, leading to disruption in auxin homeostasis depending on light effect. Wang et al. (2005) have shown requirement of ARF10 and ARF16 during cap formation process. Independent relation between auxin and miR160 on regulation of root cap production and lateral root formation has unambiguously been observed in root structure, unlike aerial parts of plants including leaf structures that did not show any certain pattern. Moreover, the expression of miR160 was observed less in young leaves as compared with the old ones showing that target gene expression was gradually reduced throughout leaf development process. Similar study in *M. truncatula* has revealed role of miR160 on orchestrating the root structure and nodule development (Pilar et al., 2013). They have identified two different miR160 variant including *M. truncatula* miR160abde (mtr-miR160abde) and miR160c (mtr-miR160c) along with 17 possible ARF genes using bioinformatics approach. It was observed that four potential targets in the roots was cleaved by miR160. Expression of mtr-miR160d and mtr-miR160c was overlapped with showing different pattern throughout development of root and nodule. Transgenic plants overexpressing mtr-miR160a with two different promoters including p35S:miR160a and pCsVMV:miR160a were demonstrated defects depending on promoter type. First group illustrated reduction in root length, while second group displayed less number of

nodules as well as deficiency in gravitropism. Zhang et al. (2012) have stated that miR160 along with other miRNAs including miR156, miR159, miR160, miR166, miR167, and miR390 may take an active role during development of cotyledonary embryo in *Larix leptolepis.* They have shown that two targets of miR160 and expression of miR160 was detected maximum level at sixth stage of somatic embryogenesis. In the other study, down-regulation of ARF10 targeted by miR160 on both seed germination and post-germination processes has been demonstrated in *A. thaliana* (Liu et al., 2007). Transgenic lines with silent mutations in ARF10 gene, called as mARF10, were displayed some developmental deficiencies in leaves, stems and flowers. They have asserted that both seeds and plants developed from those transgenic lines were hypersensitive against ABA hormone while, plants containing 35S: miR160 constructs showed decreased sensitivity against ABA throughout germination process. Besides, expressions of ABA-responsive genes in germinating mARF10 seeds were enhanced depending upon the comparison of all transcripts for ARF10 and mARF10 seeds, respectively. Yang et al. (2013) have proposed that miR160-mediated ARF17 factor have contributed both pollen wall and primexine production in *A. thaliana.* ARF17 mutants showed male sterility during vegetative growth phase and was deprived of primexine structure, causing deformation in the pollen wall as evidenced by transmission electron microscopy. Moreover, it was demonstrated that ARF17 binds promoter region of callose synthase 5 (CalS5) gene, function in callose synthesis, stating that callose accumulation was considerably decreased in those mutants compare to wild type plants. Shi et al. (2015) have proposed that expression of CalS5 gene was regulated by ARF17 which was targeted by miR160. It was found that expression of CalS5 was considerably decreased and callose wall was observed quite thinner in AtTTP-OE transgenic plants overexpressing *AtTTP* gene, belonging to zinc finger family. They proved that the expression of miR160 was reduced in those plants by using quantitative RT-PCR and Northern blotting. Those plants also showed decreased male fertility. Altogether, it was suggested that miR160 together with AtTTP played an important role in pollen wall production as well as callose biosynthesis. Liu et al. (2016) have shown that miR160 acted as negative regulator during callus formation process by affecting relation between two important phytohormones namely, auxin and cytokinin. Callus induction displayed in a faster and more fertile pattern in miR160-resistant form of ARF10 (mARF10) plants, while transgenic ones overexpressing miR160c, arf10, as well as arf10 arf16 mutants showed slower and less fertile pattern as compared with

wild type plants. Moreover, it was found that Arabidopsis response regulator15 (ARR15) expression was suppressed by ARF10 directly binding to the promoter region of that gene. When the *ARR15* gene was knock-out in transgenic plants containing pro35S:miR160c constructs, callus induction was increased and effect of overexpression on phenotype was mitigated in those plants. MiR160 was believed to have function in somatic embryogenesis stimulation via affecting auxin-related mechanisms and downregulation of ARF10/ARF16/ARF17 targeted by miR160 was found in Arabidopsis embryogenic culture (Wójcik et al., 2017). It was found that both ARF10 and ARF16 expressions were enhanced in miR160 mutants. ARF16 was accumulated in mutants that have the miRNA resistant of ARF16. They were suggested that variation in accumulation amount of ARF10 and ARF16 at different times might be resulted from redundancy in miR160 family. Consequently, it has been proposed that miR160 together with miR166/miR165 provided LEC2 (Leafy Cotyledon2)-based embryogenic transition by organizing pathways associated with auxin biosynthesis. Leafy Cotyledon2 is known as an important transcription factor throughout somatic embryogenesis process. Likewise, ARF10/ARF16/ARF17 repression targeted by miR160 have coordinated embryogenic response in the *D. longan* (Lin et al., 2015). Recently, function of miR160 in tomato (*Solanum lycopersicum)* was investigated by knock down miR160 with the help of short tandem target mimic approach (Damodharan et al., 2016). Those transgenic plants showed aberrant floral abscission and production of floral organs with reduced blade. They have displayed that miR160 was found in a greater amount in developing ovaries in the light of findings Northern blot analysis. It has been suggested that downregulation of miR160 resulted in deformation in ovary formation due to more elongation at proximal ends as well as induction to getting thin placenta structure. Upon fertilization, elongated tomato fruits were produced because of observed morphological defects. Liu et al. (2016) have studied function of miR160 on the regulation of water balance in *S. lycopersicum*. Transgenic plants overexpressing miR160-resistant form SlARF10 (mSlARF10) showed much greater in size and less in amount along with limited leaflet blade. The water loss was found much than wild type plants. Hydraulic conductance in those transgenic lines was higher than control group due to the fact that they had larger stoma and increased aquaporin activity. It was also found that SlARF10 stimulated the ABA signalling by adjusting stomata structure to relieve water loss and aquaporin activity to regulate water movement, ensuring that water loss in leaf tissues is regulated by SlARF10 in tomato. It was suggested that

miR160 participates in controlling water level in leaf tissue. Huang et al. (2016a) have investigated role of miR160 on growth and development in *O. sativa.* They have identified two miR160a and miR160b that contribute formation of mature miR160 (OsmiR160). Transgenic plants showing OsmiR160-resistant form of OsARF18 displayed morphological anomalies including small sized seeds, short stature and wrapped leaves. Starch amount accumulated in leaves was decreased in those plants. The cleavage site in OsARF18 transcript was detected using gene-specific 5′ RACE method. Expressions of both OsmiR160a and OsmiR160b were found to be significantly higher in different tissues including leaf and stem based on real-time analysis. Altogether, it was suggested that downregulation of OsARF18 targeted by OsmiR160 play an important role in growth coupled with development by changing auxin response (Huang et al., 2016a).

2.4.1 Role of miR160 under stress conditions

Xin et al. (2010) have identified heat-responsive and powdery mildew-responsive miRNAs including miR160 in wheat (*T. aestivum*). Based on findings from comparison analysis in heat tolerant line (TAM107), it was shown that expression of miR160 was induced upon heat treatment. It was also suggested that miR160 expression was considerably decreased in the stem structure of wheat upon fungi infection by *C. quercuum* f.sp. *fusiforme.* Barrera-Figueroa et al. (2011) have identified drought-responsive miRNAs including miR160 family in two contrasting cowpea cultivars. It was found that miR160a and miR160b expression were considerably regulated in drought-tolerant cultivar (IT93K503-1), while those expressions were not controlled in drought-sensitive cultivar (CB46). The target of both miR160a and miR160b was found to be ARF10. Together with 44 drought-responsive miRNAs identified, miR160 might be used to improve cowpea plants with enhanced tolerance under drought stress. Ballén-Taborda et al. (2013) have identified 60 conserved miRNAs along with 821 cassava-specific miRNAs by bioinformatics approach along with high-throughput sequencing in *Sorghum bicolor* showing tolerance against drought as well as other stresses. They proved that expression of 5 possible miRNAs showed the condition-specific pattern including miR160 compared to control group. MiR160 expression in root structure was downregulated and target expressions, ARF10 and ARF16, were upregulated in response to drought stress. Bakhshi et al. (2014) have characterized three miRNAs

including miR160 and investigated function of those miRNAs on root structure in response to drought in *O. sativa.* The expression of miR160 repressed 3-fold in drought-treated roots than control group. It was proposed that downregulation of miR160 may attribute to the needs of plants with enhanced root length together with more lateral roots in response to drought stress. For the first time, Ebrahimi Khaksefidi et al. (2015) have proposed the role of miR160 in *Helianthus annuus* under various stress conditions such as heat, salt, drought and cadmium. They identified some of conserved miRNAs including miR160 and then examined expression of those miRNAs in leaves and roots of sunflower upon stress treatments. Considering protein sequence results of new possible target of miR160, it has three different domains such as kinase, phosphorylase and ARF, respectively. The expression of that gene was reduced at 12 hours with 2-fold and at 24 hours with 8-fold, while it was induced at 48 hours for leaves and root samples under drought stress. Upon heat treatment, miR160 expression was triggered at 1.5 hours and reduced after 1.5 hours in leaves. It was upregulated under severe heat conditions but yet, less than control group. MiR160 expression in roots reduced at 3rd hours with 10-fold than control group and this trend followed at 6 hours. By contrast, target gene expression was considerably increased at 3rd hours stating that there was a negative relation between that new gene identified and miR160. The expression pattern in leaves showing reduction trend was opposite to that of roots under salt stress conditions. Likewise, the role of miR160 has been investigated both root and leaf tissues upon drought and salt stresses in *S. lycopersicum* (Bouzroud et al., 2018). Expression of miR160 in leaves was induced with 2-fold upon salt stress after 24 hours and the target gene expression was repressed at that time. Expression of miR160 was stimulated at 1st hour, while miR160 remained constant in response to salt stress. In leaf tissues, miR160 expression was dramatically reduced following 48 hour drought condition. SlARF10A*, S. lycopersicum* Auxin Response Factor10, expression was triggered in 5-day-old roots exposed to drought stress whereas miR160 expression was observed similar to that of control group. It was proposed that miR160 may be involved in the post-transcriptional regulation of SlARF10A. Cui et al. (2018) have studied function of miR160 coupled with miR164 in two contrasting beets (*Beta vulgaris*) in response to salt stress. They have predicted targets of miR160, ARF17 and ARF18, with the help of bioinformatics tools and degradome sequencing. Expression of miR160/miR164 and respective targets in root/leaf samples were tested using quantitative real-time PCR. MiR160 expression at four-leaf stage was found less in

"O"68 variety, showing higher tolerance against salt stress, than Shuang 6 variety, sensitive to salt stress seedlings. By contrast, expression of miR160 at six-leaf stage was observed higher in "O"68 compared to Shuang 6. It was shown that miR160 expression was less in root tissue as compared to leaf tissue in response to salt stress after 72 h. It was suggested that negative regulation of miR160 in leaf and root tissues in line with up-regulation of respective targets, ARF17/ARF18, participates in the adaptation of the beet against high salt stress. Lin et al. (2018) have studied relation between miR160 and heat shock proteins under heat stress in *A. thaliana.* It was created different mutant plants including those overexpressing miR160 precursor and plants showing that expression of miR160 (MIM160) was repressed. Germination of seeds together with seedling survival enhanced in transgenic lines overexpressing miR160 in response to heat stress. Hypocotyl elongation as well as rachis were observed greater in those plants compared to wild-type group upon heat treatment. Adaptation of transgenic plants showing no expression of miR160, AMIM160, was found worse against heat stress. It was also found that expressions of HSP17, HSP21 and HSP70 were controlled via heat stress in those transgenic plants. Accordingly, it was proposed that miR160 enable plant organisms to survive under heat condition by affecting heat shock protein expressions (Figure 2.6).

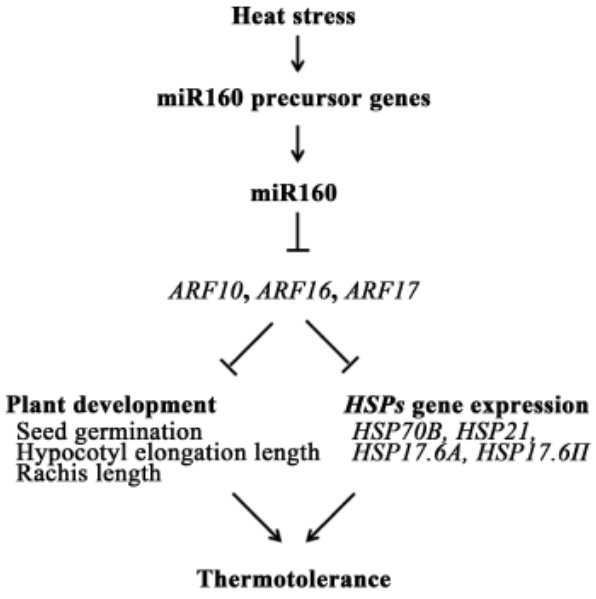


Figure 2.6. MiR160-directed regulation in response to heat stress (Lin J-S. *et al*., 2018)

Ding Y. et al. (2017) have studied effect of miRNAs including miR160 on male sterility under heat stress in *Gossypium hirsutum*. They used two contrasting cultivars as insensitive line (84021) and sensitive line (H05). MiR160 expression was repressed in insensitive lines, while it was induced in sensitive lines. It was also shown that transgenic plants overexpressing miR160 induced sensitivity against heat stress by repressing expression of ARF10 and ARF17, which ultimately results in anther dehiscence.

2.5 Aims and Objectives

The aim of this study is to investigate the function of miR160 in Unica and Russet Burbank cultivars, showing contrasting abiotic stress tolerance, exposed to drought, high temperature and a combination of heat and drought stress using transgenic approach by overexpression of pre-miRNA of miR160.

CHAPTER III

MATERIALS AND METHODS

In this study, role of miR160 was examined under drought, high temperature and a combination of drought and heat conditions using Unica and Russet Burbank potato cultivars via producing transgenic potato plants overexpressing pre-miR160. Pre-miR160 sequence identified in potato was taken from the previous study (Kaplan, 2017).

3.1 Synthesis of aPre-miRNA cDNA and Cloning Into Transformation Vector

In order to achieve cDNA of pre-miRNA, total RNA isolations using Trizol (Invitrogen, Catalog number: 155926) were made for both potato cultivars (Figure 3.1). Manufacturer's protocol was adopted except for a few changes in some steps. Firstly, 200 mg of leaf which was already fragmentized in the mortar containing liquid nitrogen was mixed with 1.5 mL of Trizol. Next, this mixture was vortexed for a while and waited at room temperature for 10 minutes. Following centrifuge step through maximum rpm at 4 °C for 10 minutes, 450 µL of upper phase was taken into a DNase/RNAase free tube and mixed with 400 µL chloroform. Subsequently, the solution was centrifuged at 14,000 rpm at 4 °C for 15 minutes followed by 5 minutes waiting period at room temperature. 500 µL of cold isopropanol was added on the tube including upper phase after centrifuge step. This mixture was once centrifuged with 11,000 rpm at 4 °C for 10 minutes after 10 minutes waiting period at room temperature. Supernatant phase was removed and tube was placed downward position to ensure to dry the pellet for around 10 minutes. Before dissolving in DEPC water, 1 mL of 75% ethanol was added on the resultant pellet. This protocol was performed as three replicates to avoid errors. The value of RNA concentration was found by taking the average of two different measurements via nanodrop machine. Finally, 1 µg of total RNA was used in order to test RNA quality as well as vigour in 1% TBE agarose gel. After visualization of RNA sample, it was kept at -20 °C. Restriction sites of two endonucleases, *NcoI* and *BstEII*, were attached ends of cDNA sequences of miR160 (Table 3.1). Next, attachment of those sites to the cDNA was verified by PCR method and then visualized via agarose gel electrophoresis. Bands on the gel including desired

fragments were extracted and purified with the help of GeneJET Gel Extraction kit (Thermo Scientific) and DNA Cleanup Micro Kit (Thermo Scientific). This fragment was ligated into pCAMBIA1301 vector in exchange for GUS second exon cut by same restriction enzymes. The ligation reaction was made with the help of T4 ligase at the rate of 1:6 cDNA and vector, respectively. PPB primers were used for validation of ligation products (Table 3.2). The stages of PCR condition were set up as specified; 94˚C x 3 min., 34x {94˚C x 15 sec., 55˚C x 15 sec., 72˚C x 15 sec.}, 72˚C x 7 min.

Figure 3.1. The figure shows pre-miR160 structure

Table 3.1. Mature miRNA and pre-miRNA sequence were given along with corresponding target mRNA sequence

	miR160
Mature miRNA Sequence	UGCCUGGCUCCCUGUAUGCCA
Pre-miRNA Sequence	UGCCUGGCUCCCUGUAUGCCAUUUGCAAAGCUCACCGUAA UAUAUCGAUGGGCCUUGUUGAAUGGCGUAUGAGGAGCCAA GCAUA
Target Sequence *PGSC0003DMT400045323, auxin response factor ARF16 Solanum tuberosum auxin response factor 18-like (LOC102599257), mRNA*	ATGAGAGTTTCTTCTTCTGGATTTAATCCTCAACAAGAAAAG CTGGAGAAAAGAAGTTCTTAATTCTGAACTTTGGCATGCTTG TGCTGGACCTCTTGTTTCTCTTCCTCCTGTTGGATCTAGAGTT GTTTATTTTCCTCAAGGACATTCTGAACAAGTTGCTGCTTCT ACTAATAAGGAAGTTGATGCTCATATTCCTAATTATCCTGGA CTTCCTCCTCAACTTATTTGTCAACTTCATAATCTTACTATGC ATGCTGATGTTGAAACTGATGAAGTTTATGCTCAAATGACTC TTCAACCTCTTTCTCCTCAAGAACAAAAGGATGTTTGTCTTC TTCCTGCTGAACTTGGAATTCCTTCTAAGCAACCTACTAATT ATTTTTGTAAGACTCTTACTGCTTCTGATACTTCTACTCATGG AGGATTTTCTGTTCCTAGAAGAGCTGCTGAAAAGGTTTTTCC TCCTCTTGATTATTCTCAACAACCTCCTTGTCAAGAACTTATT GCTAAGGATCTTCATGGAAATGAATGGAAGTTTAGACATAT TTTTAGAGGACAACCTAAGAGACATCTTCTTACTACTGGATG GTCTGTTTTTGTTTCTGCTAAGAGACTTGTTGCTGGAGATGC TGTTATTTTTATTTGGAATGAAAATAATCAACTTCTTCTTGG AATTAGAAGAGCTAATAGACCTCAAACTGTTATGCCTTCTTC TGTTCTTTCTTCTGATTCTATGCATATTGGACTTCTTGCTGCT GCTGCTCATGCTGCTGCTACTAATTCTAGATTTACTATTTTTT ATAATCCTAGAGCTTCTCCTTCTGAATTTGTTATTCCTCTTGC TAAGTATGCTAAGGCTGTTTATCATACTAGAATTTCTGTTGG AATGAGATTTAGAATGCTTTTTGAAACTGAAGAATCTTCTGT TAGAAGATATATGGGAACTATTACTGGAATTTCTGATCTTGA TCCTGTTAGATGGCCTAATTCTCATTGGAGATCTGTTAAGGT TGGATGGGATGAATCTACTGCTGGAGAAAGACAACCTAGAG TTTCTCTTTGGGAAATTGAACCTCTTACTACTTTTCCTATGTA TCCTTCTCCTTTTTCTCTTAGACTTAAGAGACCTTGGCCTTCT GGACTTCCTTCTCTTCCTGGATTTCCTAATGGAGATATGACT ATGAATTCTCCTCTTTCTTGGCTTAGAGGAGATATGGGAGAT CAAGGAATGCAATCTCTTAATTTTCAAGGATTTGGAGTTACT CCTTTTATGCAACCTAGAATGGATGCTTCTATGCTTGGACTT CAACCTGATATTCTTCAAACTATGGCTGCTCTTGATCCTTCT AAGCTTGCTAATCAATCTCTTATGCAATTTCAACATTCTATT CCTAATTCTTCTGCTCCTCTTTCTCAATCTCAAATGCTTCAAC CTTCTCATTCTCAACAAAATCTTATTCAAGGATTTTCTGAAA ATCATCTTATTTCTCAAGCTCAAATGCTTCAACAACAACTTC AAAGAAGACAAAATTTTAATGATCAACAACAACTTCTTCAA CCTCAACTTCAAAGACATCAAGAAGTTAATTCTCAATTTCAA CATCAACAACAAACTAAGACTATTTCTGGACTTTCTCAAATG GCTTCTGCTACTCATCCTCATCTTTCTCATCTTCAAGTTCTTT

	CTTCTACTGGATCTCCTCAAACTTTTTCTGATATTCTTGGAAA TCATGTTAATGCTTCTTCTAATTCTAATATGCAATCTCTTCTT TCTTCTTTTTCTTGTGATGGAGCTTCTACTGTTCTTAATGTTC ATGAAACTCATCCTCTTGTTTCTTCTTCTTCTTCTTCTAAGAG AATTGCTCTTGAATCTCAACTTCCTTCTAGAGTTACTCCTTTT GTTGTTTCTCAACCTGAAGATGTTATTGCTCATAATACTAAG GTTTCTGATCTTTCTTCTCTTCTTCCTCCTTTTCCTTCTAGAGA ATCTTTTTCTGATTATAGAGGAGTTGAAGATTCTCAATCTAA TGCTCTTTATGGATTTACTGATTCTCTTAATATTCTTCAAACT GGAATGTCTAATATGAAGGGATCTTCTGGAGATAATGGATC TCTTTCTATTCCTTATGCTACTTCTACTTTTACTTCTACTGTTG GAAATGAATATCCTCTTAATTCTGATATGACTGCTTCTTCTT GTGTTGATGAATCTGGATTTCTTCAATCTTCTGAAAATGGAG ATCAAGCTAATCCTACTAATAGAATTTTTGTTAAGGTTCAAA AGTCTGGATCTTTTGGAAGATCTCTTGATATTTCTAAGTTTTC TTCTTATCATGAACTTAGATCTGAACTTGCTAGAATGTTTGG ACTTGAAGGACTTCTTGAAGATCCTGAAAGATCTGGATGGC AACTTGTTATTGTTGATAGAGAAAATGATGTTCTTCTTCTTG GAGATGATCCTTGGCAAGAATTTGTTAATAATGTTTGGTATA TTAAGATTCTTTCTCCTCATGAAGTTCAACAAATGGGAAAGG AAGGACTTGATCTTCCTAATGGAGTTCAAGCTCAAACTCAA ACTCTTCCTGGAAATGTTAATGGATGTGATGATTATATGAAT CAAAAGGGATCTAGAAATACTATGAATGGAATTCCTCTTGG ATCTCTTGATTAT

Table 3.2. Sequence and length of PBB forward and reverse primers sequences were given along with their lengths

Primer	Primer Sequence	Length
PBB-Forward	GAGAACACGGGGGACTCTTG	20
PBB-Reverse	TAATCATCGCAAGACCGGCA	20

3.2 Transfer of The Positive pCAMBIA1301 Vectors to *Agrobacterium tumefaciens*

Transfer of positive recombinant vectors into *A. tumefaciens* LBA4404 strain achieved by electroporation technique. For this, Agrobacterium strain was extracted from stock at -80 °C and waited around 10 minutes in the box full of ice. Next, addition of 4 µL ligation reaction was made on bacteria cells and mixed for 2-3 minutes. Those cells and ligation products were transferred into a new tube which was already kept at cold condition. They were later exposed to electroporation. Cells containing constructs were switched into a new tube and mixed with 400 µL LB media. Following incubation at 220 rpm at 28 °C around 4 hours, 200 µL sample was inoculated in the media with 25 mg / L kanamycin using spread plate technique and kept at 28 °C for one day. Colonies

were classified as positive and negative depending on findings from colony PCR technique. The stages of PCR condition were adjusted as specified; (94˚C x 2 min, 34x {94˚C x 15 sec, 58.4 x 15 sec, 72˚C x 20 sec) 72˚C x 10 min). Later, positive clones were kept at -80 °C.

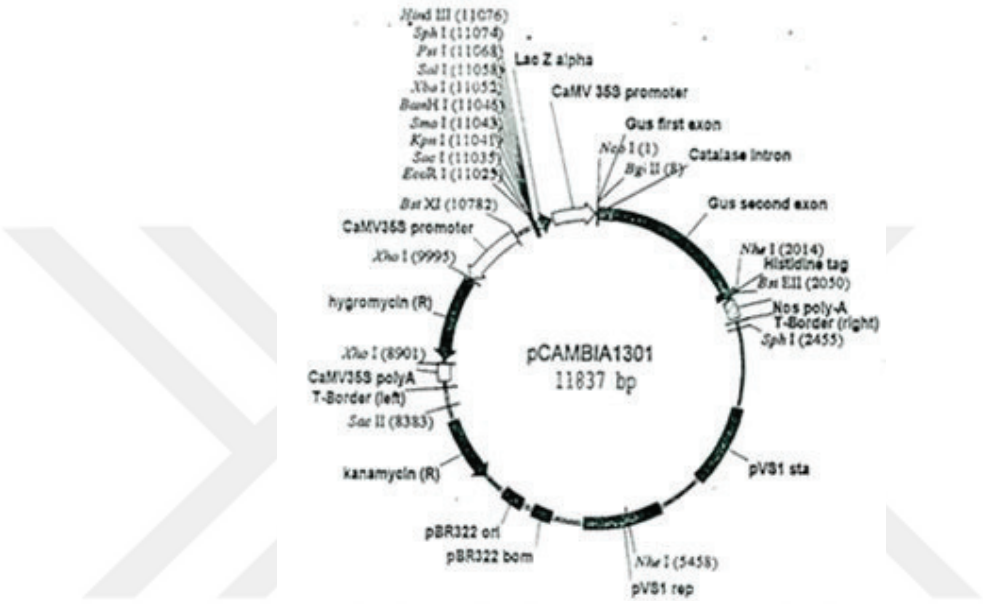


Figure 3.2 The pCAMBIA1301 vector was shown containing restriction sites, antibiotic-coding sites and other regions

3.3 Sterilization of Potato Cultivars and Transformation of *Agrobacterium tumefaciens* With Positive Constructs to Potato

3.3.1 Sterilization of potato cultivars

In this study, two types of potato cultivars were utilized, Unica, tolerant to abiotic stress (Cabello et al., 2012), and Russet Burbank susceptible to abiotic stress (Stark et al., 2013). Both potato tubers were sterilized before transformation. For this aim, Tween-20 chemical was used for washing sprouts, and then, they were treated with 70% ethanol for 5 minutes. Subsequently, Mancozeb antifungal solution (2 mg/L) was added on sprouts around 15 minutes. After treating with 70% ethanol, they were washed several times using distilled water. Next, they were dipped into solution containing 1:20 diluted Sulcid antibiotic for approximately 10 minutes. Before washing with distilled water, they were treated with 1% hydrogen peroxide for 1 minute. Lastly, sterilized sprouts

were placed in Murashige and Skoog (MS) media. After sterilization step, several nodes derived from were regularly cut and used further for the multiplication of potato plants in a media containing 4 g MS salts, 7-7.5 g agar, and 30 g sucrose for 1 L with adjusted pH 5.7.

Concurrently, bacterial cultures were taken from stock and grown at 28 °C along with 240 rpm in the liquid medium containing 1000 µL/L kanamycin. Subsequently, those cells were inoculated into internodes of four-week-old plants after growing in at 28 °C 10 ml LB liquid media for one day.

3.3.2 Transformation of *Agrobacterium tumefaciens* to potato and transfer of potential transgenic plants to greenhouse

Internodes of potato plants, used as explant, were regularly cut from Unica and Russet Burbank cultivars. 500 µL bacterial culture was inoculated on internodes in the petri plate including an amount of 15-25 ml water for nearly 3 minutes. Explants were gently mixed with bacterial culture for 3 minutes in the petri plates including MS-0 medium plus 1 mg/L acetosyringone. After inoculation step, plates were placed in growth chamber around 2 days. Following 2 days, inoculated internodes were transferred another media callus-induction media containing 4.4 g MS salts and vitamins (Duchefa Biochemie Product no: M0231.0050), 100 mg/L ascorbic acid, 7.8 g/L agar, 0.1 mg/L NAA (A-Naphthalene acetic acid) (Duchefa Biochemie Product no: N0903.0025 Lot.No. 013138.03), 1 mg/L BAP (6-Benzylaminopurine) (Duchefa Biochemie Product no: B0904.0025 Lot.No. 012974.04), 2 mg/L Zeatin riboside (Duchefa Biochemie Product no:Z0937.0050 Lot.No. 011832.02), 30 g/L sucrose, 0.1mg/L GA3 (Gibberellin) (Sigma-Aldrich Lot.7165164.633 Cas-no:77-06-5), 0.1 mg/L NAA, 4 mg/L hygromycin, (Alfa Aesar Lot. R09D029) as well as 500 mg/L Sulcid (1g ampicillin + 0.5 g sulbactam). Those were placed in the growth chamber providing approximately 16 hours photoperiod at a constant temperature of 27±1 °C. When the calli formation was observed in the plates, it was transferred to other media namely, shoot induction medium consisting of 4.4 g MS salts and vitamins, 30 g/L sucrose, 7.8 g/L agar, 100 mg/L GA_3, 0.1 mg/L NAA, 1 mg/L BAP, 2 mg/L hygromycin as well as 300 mg/L Sulcid. After transgenic sprouts developed enough to transfer MS-0 media lack of antibiotics, they were switched into growth chamber ensuring 16 hours of

photoperiod plus 25±2 °C constant temperature. While plantlets reach enough maturity to adapt acclimation in the greenhouse, they were transferred to soil containing peat moss and perlite with 2:1 ratio.

3.3.3 Validation of potential transgenic potato cultivars

When the plantlets were developed enough maturity, leaf samples were taken to verify whether potential transgenic plants were produced by PCR technique. DNA isolation was done with the help of GeneJet Plant Genomic DNA Purification kit (Thermo Fischer Scientific) and then, genomic DNAs were tested by gel electrophoresis followed by purity and quality measurements via nanodrop machine. Two different groups of primers including Hygromycine Forward-Reverse primers and 35S Forward and NOS Poly-A Reverse primers were utilized to prove possible transgenic plants for PCR reactions (Table 3.3) (Table 3.4). Moreover, those transgenic plants were investigated to detect any contamination caused by *A. tumefaciens* (Table 3.5) (Table 3.6).

Table 3.3. Primers including which were used to verify possible transgenic plants

Primer	Sequence
35S Forward	5'-ACGCACAATCCCACTATCCTTC-3'
NOS Poly-A tail Reverse P	5'-AACCCGGGCCCATCTAGTAACATA-3'
Hygromycine Forward	5'-TTGGGAATCCCCGAACATCG-3'
Hygromycine Reverse	5'-CTCTCGGAGGGCGAAGAATC--3'

Table 3.4. Stages and amounts of chemicals used for PCR were shown as a table

Chemicals	Amount (µL)
DNA	2
35S Forward	2
NOS Poly-A tail Reverse	2
Master-mix	6
dH_2O	8
Total	20

Primer	Temperature(C)	Time (h:m:s)	
35S Forward and NOS Poly-A tail Reverse	94 °C	0:02:00	
	94 °C	0:00:15	34 cycles
	58.4 °C	0:00:15	
	72 °C	0:00:20	
	72 °C	0:10:00	

Table 3.5. Primers which were used to detect the contamination caused by *A. tumefaciens* in possible transgenic plants

Primer	Sequence
ChvA gene Forward	5′-CGAAACGCTGTTCGGCCTGTGG-3′
ChvA gene Reverse	5′-GTTCAGCAGGCCGGCATCCTGG-3′

Table 3.6. Stages and amount of chemicals used for PCR

Primer	Temperature(C)	Time (h:m:s)	
ChvA gene Forward and *ChvA* gene Reverse	94 °C	0:02:00	
	94 °C	0:00:15	34 cycle
	64 °C	0:00:15	
	72 °C	0:00:20	
	72 °C	0:10:00	

Chemicals	Amount (µL)
DNA	2
ChvA gene Forward	2
ChvA gene Reverse	2
Master-mix	6
dH_2O	8
Total	**20**

3.3.4 Development of validated transgenic plants and stress application

After plants were stayed for 4 weeks in the growth chamber, they were switched to pots consisting of peat moss and perlite in the greenhouse as well growth chamber. Before starting stress treatment, irrigation was done in a soil-field capacity manner in a controlled condition with 24 °C for 16 hours light and 16 °C for 8 hours night in a day. Insecticides coupled with pesticides application were used at regular intervals.

Stress treatments were started at tuber development stage. Four different groups were classified such as control, drought, high temperature (heat), a combination of drought and high temperature stress for both growth chamber and greenhouse conditions (Table 3.7). For heat stress treatment, wild-type plants, control group and transgenic plants were placed in the growth chamber, while control group and transgenic plants were situated in greenhouse for drought stress application. All control group was regularly watered during stress period. Plants belonging to drought treatment were not irrigated unlike plants belonging to heat stress were irrigated regularly with an increase in the temperature (Table 3.8). In addition, not only plants were not watered but also

temperature was increased in accordance with the schedule prepared under drought combined with heat stress. When stress application was completed for plants in greenhouse and growth chamber, a few of leaves including control group and stress treatment group were sampled and then placed immediately into liquid nitrogen tank. Later, all samples were kept at -86 °C.

Table 3.7. Table shows experimental plan for development of transgenic plants

Cultivar	Greenhouse		Growth Chamber		
	Control Group	Drought Stress	Control Group	Heat Stress	Heat+Drought Stress
Unica cultivar	each pot has one plant	each pot has one plant	each pot has one plant	each pot has one plant	each pot has one plant
Russet Burbank cultivar	each pot has one plant	each pot has one plant	each pot has one plant	each pot has one plant	each pot has one plant

Total pot numbers were decided as 4 for each box.

Table 3.8. The table includes experimental plan under heat stress plus a combination of drought and heat stress

Temperature duration	11-9-2019	12-9-2019	13-9-2019	14-9-2019	15-9-2019	16-9-2019	17-9-2019	18-9-2019	19-9-2019	20-9-2019	21-9-2019	22-9-2019	23-4-2019 Harvesting
Daylight Temperature (°C) for 14h	24	25	26	27	29	31	33	35	37	38	39	39	39
Night Temperature (°C) for 10h	18	19	20	21	22	23	24	25	26	26	27	27	27
Average Temperature	21.5	22.5	23.5	24.5	26.1	27.7	29.3	30.8	32.4	33	34	34	34

3.4 Measurements of Physiological Parameters

Physiological parameters that were measured during stress treatment including, photosynthesis and transpiration rate, stomatal conductance, leaf temperature,

chlorophyll index, relative water content (RWC) and proline content, and then, results of transgenic plants were compared with control plants.

3.4.1 Photosynthesis rate/stomatal conductance/transpiration rate

Starting from the initial day of stress treatment, those parameters were measured for all control plants and plants exposed to stress at midday under conditions with 1000 μmol/m^2/sec light intensity, 400 moly CO_2 along with 500 μmol/s air flow by LICOR-6400 machine in 0, 2^{nd}, 7^{th}, 9^{th}, 12^{th}, 13^{th}, 15^{th}, 18^{th} and 20^{th} days. Generally, third or fourth of potato leaves which was considered to reach maturity, were chosen to achieve correct measurements. Those measurements were replicated to prevent possible errors.

3.4.2 Chlorophyll index

It was measured for all control plants and stress treatment plants at midday using Konica Minolta SPAD-502 Plus Chlorophyll meter machine. Third or fourth of potato leaves which were mostly considered to reach maturity, were chosen to achieve correct measurements in given days including in 0, 2^{nd}, 7^{th}, 9^{th}, 12^{th}, 13^{th}, 15^{th}, 18^{th} and 20^{th} days.

3.4.3 Leaf temperature

It was measured both in control and stress treatment plants at mid-day hours using IRT instrument (MASTECH BM380 Laser Temperature Measuring Instrument). In order to achieve correct measurement, fourth of potato leaves was selected during the measurement. Third or fourth of potato leaves which were mostly considered to reach maturity, were chosen to achieve correct measurements in given days including in 0, 2^{nd}, 7^{th}, 9^{th}, 12^{th}, 13^{th}, 15^{th}, 18^{th} and 20^{th} days.

3.4.4 Relative water content (RWC)

It was examined in both control plants and stress treatment plants. Third or fourth of potato leaves which were mostly considered to reach maturity, were chosen to achieve correct measurements in given days including in 0, 2^{nd}, 7^{th}, 9^{th}, 12^{th}, 13^{th}, 15^{th}, 18^{th} and

20th days. Those measurements were replicated to avoid possible errors. First of all, plant leaves were harvested, and their fresh weights were measured using a sensitive scale. Afterwards, leaves were exposed to pure water in order to trigger the turgor around 18-24 hour and their resultant weights were measured once more. Next, the leaves were placed into microwave for 10 minutes, and then, kept at 95 °C around 3 hours to achieve complete dryness. In last step leaves, which was already dried, were weighed and RWC was calculated using the formula written below.

RWC= [(Fresh Weight-Dry Weight) / (Turgor Weight-Dry Weight)] × 100. (3.1)

3.4.5 Proline content

Proline measurement in control and stress treatment plants were made depending on recovered protocol which was originally developed by Bates et al. (1973). Initially, 100 mg of leaf sample was added in 2 mL of 3% sulfosalicylic acid solution and mixed by using vortex. In the second step, mixture was precipitated by centrifugation in 10,000xg at 4 °C for 10 minutes. Subsequently, 200 µl of supernatant was transferred to a new tube and the same amount of ninhydrin solution was added on upper phase in the tube. Following mixing around 15 seconds, the mixture was incubated at 90 °C for 1 hour. After 1 hour, reaction was ended in the ice and addition of 1 ml of toluene was done in the tube. It was incubated at room temperature in dark condition for 20 minutes followed by mixing around 15 seconds. Next, collection of toluene phase was achieved and absorbance value was measured at 520 nm using the spectrophotometer. Lastly, proline amount was calculated by using the given formula:

(µg proline in extract/115.5)/g sample = µmol/g fwt (3.2)

3.5 Molecular Studies on Transgenic Plants

Molecular studies were performed in three stages including total RNA isolation, cDNA synthesis and qRT-PCR. Initially, total RNA isolation was made by the protocol mentioned in 3.1. Secondly, cDNA synthesis of miRNA was achieved by Omniscript Reverse Transciption Kit (Omniscript RT Kit, Catalog No: 201511). Although cDNA synthesis of respective target was accomplished by Oligo dT primer, cDNA synthesis of

miRNA was done by miR160-SL-RT (Table 3.9). Three replications were made to avoid possible errors. For the cDNA synthesis, mRNA samples were incubated at 65 °C for around 3-5 minutes. Required chemicals were mixed together with both target gene and miRNA (Table 3.10.). Next, RNA was added the on mixture prepared and incubation was done at 37 °C for about 1 hour. Subsequently, inactivation was achieved at 70 °C for 15 minutes.

Table 3.9. Primer sequence for cDNA synthesis

Primer Name	Sequence (5'-3')
miR160-SL-RT	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACTGGCAT

Table 3.10. Type and amount of chemicals required for cDNA synthesis

Chemical	Concentration (Mm)	Amount (µL)
Oligo dT Primer	10 µM	2
miR160-SL-RT Primer	10 µM	2
RT Buffer	10X	2
dNTP Mix	5 µM	2
RNAse Inhibitor	40 µM	0.25
OmniScript RT		1
miRNA (RNA sample)		5
dH_2O	100 ng/µL	7.75
Total		20

The relation between gene expression of miR160 and corresponding target was tested using stem-loop qRT-PCR. Elongation factor 1-α (Ef1α) was used as the reference gene showing no change in the gene expression upon stress treatment in plants. The amount and content of PCR components were set up as stated below (Table 3.11) (Table 3.12). Moreover, sequences of primers including ef1α (elongation factor 1-α), miR160-Tg (Target gene) miR160 and miRNA-R-Universal were given in Table 3.13.

Table 3.11. Amount of chemicals used in qRT-PCR analysis

Chemical	Amount (µL)
cDNA	2.5
Forward Primer (2 µM)	0.4
Reverse Primer (2 µM)	0.4
Total Mix (QIAGEN)	5.0
dH2O	1.7
Total Volume	20

Table 3.12. Each stages and time of qRT-PCR condition were shown below the table

Temperature (°C)	Time (h:m:s)	
95	0:05:00	
95	0:00:45	30 Cycles
58	0:01:00	
72	0:00:45	
4	∞	

Table 3.13. Primer sequences used for qRT-PCR analysis

	Forward Primer (5'-3')	Reverse Primer (5'-3')
ef1α (elongation factor 1-α)	GGACCCAACTGGTGCCAAAG	CTCGCCACCGCCTATCAAGT
miR160-Tg (Target gene)	TTCTTAGGCCCAACAACACC	TGGCTTGGGAATCATGGTAT
miR160	TGC CTG GCT CCC TGT ATG C	
miRNA-R-Universal		CCAGTGCAGGGTCCGAGGTA

CHAPTER IV

RESULTS

4.1 Synthesis of Pre-miRNA cDNA and Cloning Into Transformation Vector

Modified form of pCAMBIA1301 vector was used for cloning. *Pfu* polymerase was used for the synthesis of cDNA and several amplification temperatures were tested for gradient PCR (Figure 4.1.). Later, optimization of PCR was achieved by changing several factors such as amount of cDNA and amplification temperature (Figure 4.2.).

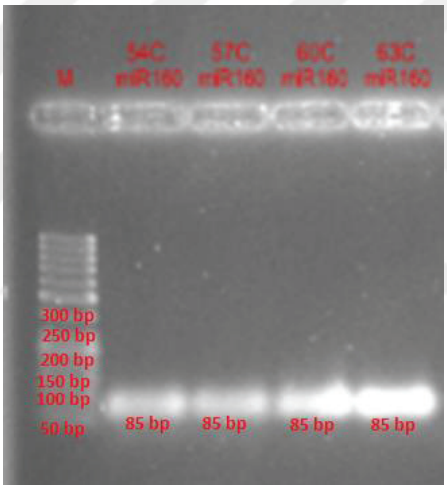


Figure 4.1. Gel image of pre-miR160 was taken from gradient PCR

Figure 4.2. Gel image of pre-miR160 was taken after the optimization process

The pre-miRNA band on Figure 4.2 was purified using the Thermo Scientific GeneJET Gel Extraction and DNA Cleanup Micro Kit to clone into the vector followed by cutting from the gel. Next, pre-miRNA structure was cut with *NcoI* and *BstEII* restriction enzymes before cloning into the vector (Figure 4.3.).

Figure 4.3. Gel image of pre-miRNA was shown after gel extraction and cutting with restriction enzyme

Additionally, modification of pCAMBIA1301 vector was done in a manner in which region of *GUS* gene was extracted from plasmid. The pCAMBIA1301 plasmid was then isolated and cut with *NcoI* and *BstEII* restriction enzymes. The bands shown in the red boxes belonging to pCAMBIA vector which was digested and lacked of GUS gene were purified by extraction from the gel (Figure 4.4.).

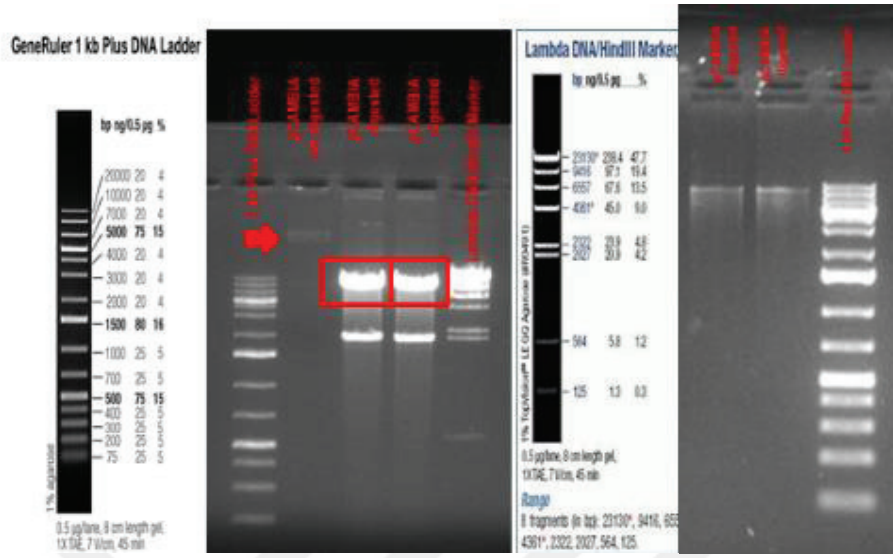


Figure 4.4. Modification of pCAMBIA vector was shown in the red-labelled boxes

Modified pCAMBIA vector and pre-miRNA amplicon was ligated and then, it was tested with the help of PBB primers by using PCR (Figure 4.5.).

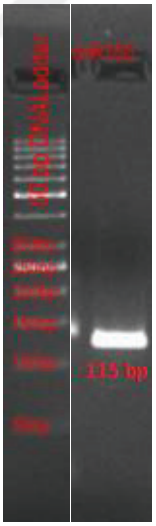


Figure 4.5. Ligation was tested with PBB primers

After validation of PCR, colonies containing pCAMBIA1301+miR160 ligation indicated in the red boxes were grown in a solid media and selected for colony PCR (Figure 4.6.). Gel image of colony PCR using miR160 specific primers were demonstrated for the conformation (Figure 4.7.).

Figure 4.6. Positive colonies containing pCAMBIA1301+miR160 construct were chosen for colony PCR

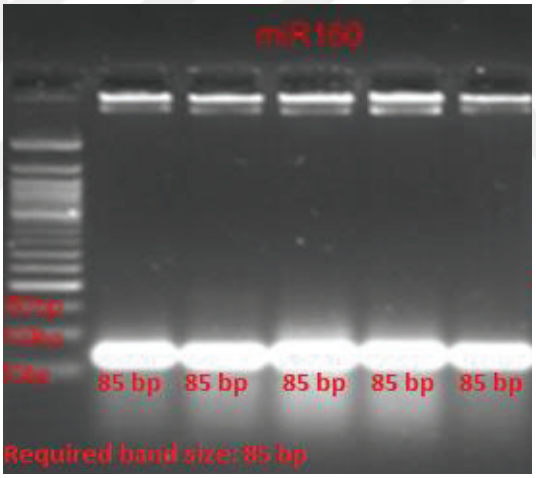


Figure 4.7. Gel image of colony PCR for the selected colonies using miR160 specific primers. 50 bp marker was used for the gel image

Based on those results, chosen *Agrobacterium* colonies were used for production of transgenic potato. Besides, those colonies were sub-cultured regularly in order to maintain the culture.

4.2 Sterilization of Potato Cultivars and Transformation of *Agrobacterium tumefaciens* With Positive Constructs to Potato

After sterilization which was already done depending upon part 3.3.1, nodes derived from both Unica and Russet Burbank cultivars were planted in a MS-0 media in glass tubes (Figure 4.8.).

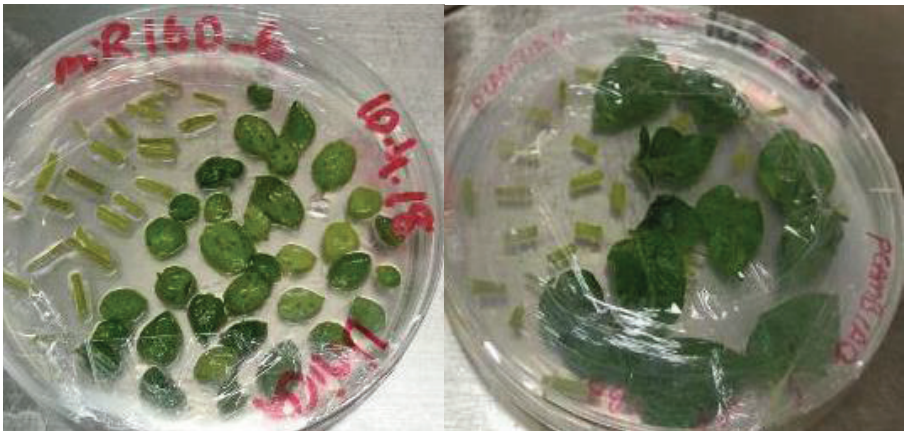


Figure 4.8. Nodes and leaves cutting from Unica and Russet Burbank cultivars

Colonies including pCAMBIA1301+miR160 construct was grown in the liquid media for transformation. Explants were transferred from co-cultivation media to regeneration selection (callus-induction media) media until callus formation was observed in the growth chamber (Figure 4.9.). Callus formation took place around 3 or 4 weeks in potato.

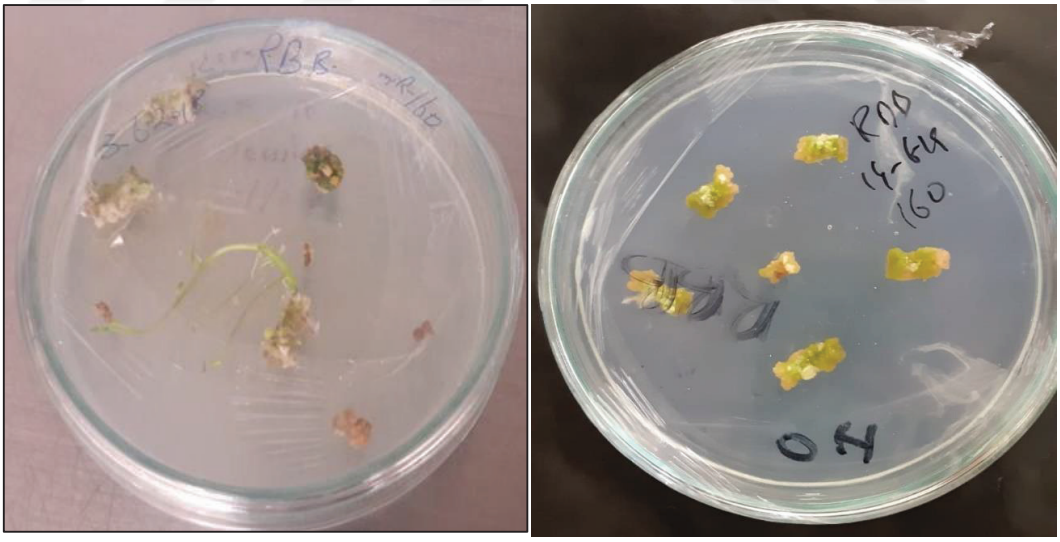


Figure 4.9. Callus formation derived from Russet Burbank cultivar

Subsequently, those shoots were transferred to MS-0 media deficient in antibiotics and grown in growth chamber (Figure 4.10.). Potential transgenic plants were switched into soil and grown for about 3 to 4 weeks in the growth chamber. Those development of plants were controlled regularly as well as irrigated as need. Four-week-old plants were switched into the greenhouse.

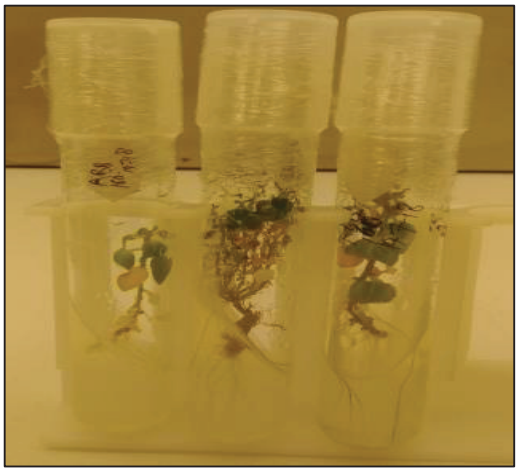


Figure 4.10. Potential transgenic plants for Russet Burbank grown in MS-0 media

4.2.1 Validation of potential transgenic potato cultivars

Validation of possible transgenic Unica and Russet Burbank cultivars were done by 35S and NOS primers (Figure 4.11.).

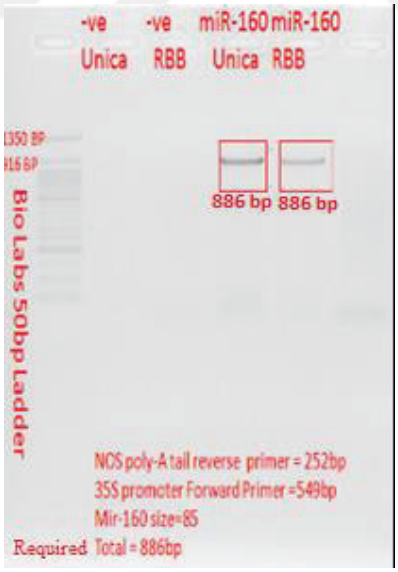


Figure 4.11. Validation of genomic DNA of transgenic Russet Burbank and Unica cultivars was shown using 35S and NOS primers

Conformation of potential transgenic Unica and Russet Burbank cultivars were also done by hygromycin primers (Figure 4.12.).

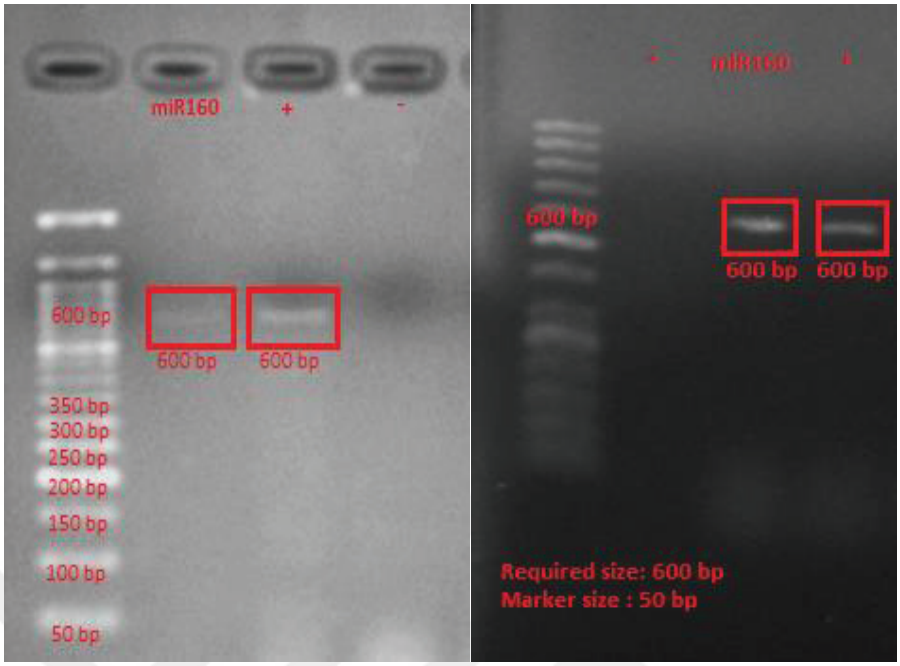


Figure 4.12. Validation of genomic DNA of transgenic Russet Burbank (left) and Unica (right) cultivars was shown using hygromycin primers

Following conformation stage with primers, transgenic plants were transferred to the greenhouse to start stress treatment (Figure 4.13) (Figure 4.14). Leaf number and area in transgenic Russet Burbank plants were slightly greater than transgenic Unica plants, whereas stem structure in transgenic Unica plants were a little taller than transgenic Russet Burbank plants.

Figure 4.13. T0 generation of transgenic Russet Burbank potato plants overexpressing miR160 which were used for stress treatments

Figure 4.14. T0 generation of transgenic Unica potato plants overexpressing miR160 which were used for stress treatments

4.3 Images and Physiological Parameters in Wild-type and Transgenic Plants

4.3.1 Images of wild-type and transgenic plants before stress treatments

Before starting stress treatments, images of wild-type and transgenic plants were taken to see morphological changes in Unica and Russet Burbank cultivars (Figure 4.15) (Figure 4.16).

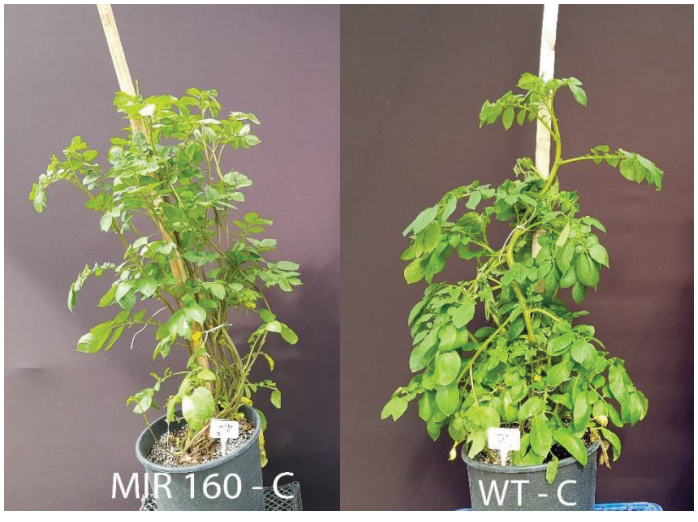


Figure 4.15. T0 generation of transgenic Unica control plant (left) and wild-type Unica plant (right) before stress treatment were shown

Figure 4.16. T0 generation of transgenic Russet Burbank control plant (left) and wild-type Unica plant (right) before stress treatment were shown

Some of morphological changes including leaf structure, biomass and stem length were observed between transgenic and wild-type plants. In Unica cultivar, the number of leaves and biomass in transgenic plants were less than wild-type plants. Less expansion in the leaf structure of transgenic plants was detected. By contrast, biomass in Russet Burbank cultivar was greater in favour of transgenic plants. Besides, stem structure in wild-type Russet Burbank plants was observed to be taller than transgenic plants. Leaf size in wild-type Russet Burbank was found to be a little greater as compared with transgenic ones. Those results were just obtained from the first observations unlike taking regular measurements.

4.3.2 Images and physiological parameters in wild-type plants after stress period

Stress treatment which was already mentioned in detail in the method part of the study was applied for Unica and Russet Burbank cultivars. Upon drought treatment, images of control group and drought-treated plant were shown for both cultivars (Figure 4.17) (Figure 4.18). Moreover, images of plants which was subjected to heat and heat combined with drought stress was illustrated for Unica and Russet Burbank cultivars (Figure 4.19) (Figure 4.20).

Figure 4.17. Wild-type plants including control group (left) and drought-treated (right) for Unica cultivar after 20-day drought treatment were shown

Figure 4.18. Wild type plants including control group (left) and drought-treated (right) for Russet Burbank cultivar after 20-day drought treatment were shown

Figure 4.19. Wild-type Unica cultivar including control group (left), heat-treated (middle) and a combination of drought and heat (right) after 12-day high temperature and combined stress were shown

Figure 4.20. Wild-type Russet Burbank cultivar including control group (left), heat-treated (middle) and a combination of drought and heat (right) after 12-day high temperature and combined stress were shown

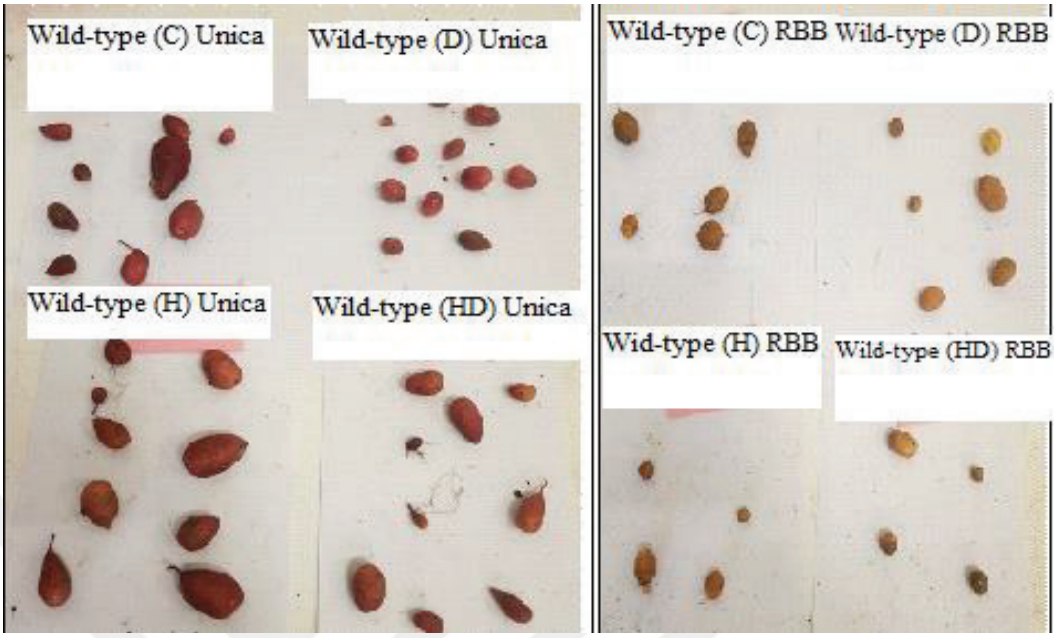
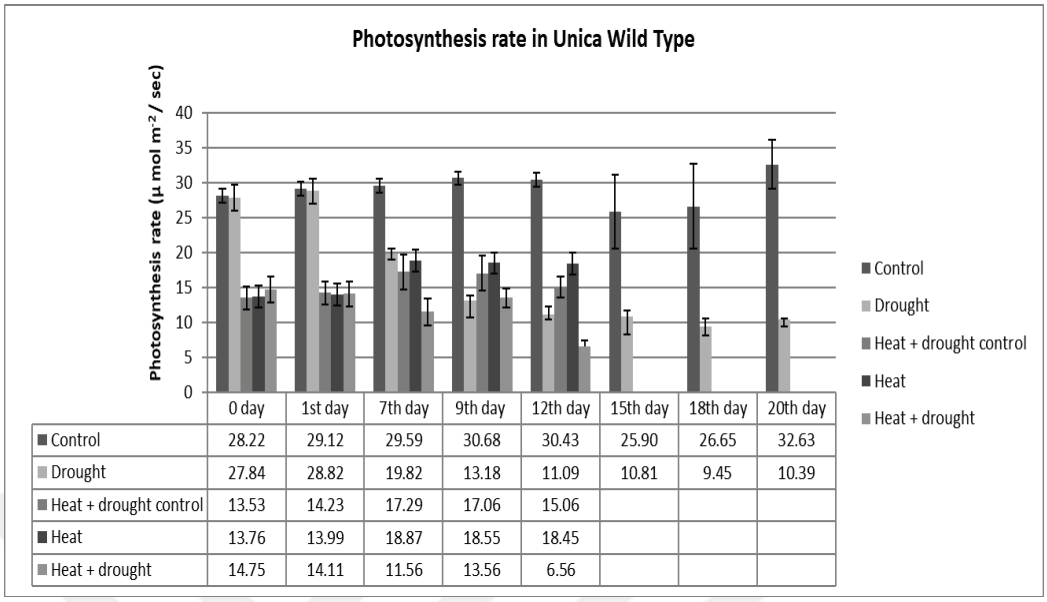


Figure 4.21 Wild-type Unica and Russet Burbank tubers cultivars after 20-day drought and 12-day heat and combined treatment were shown

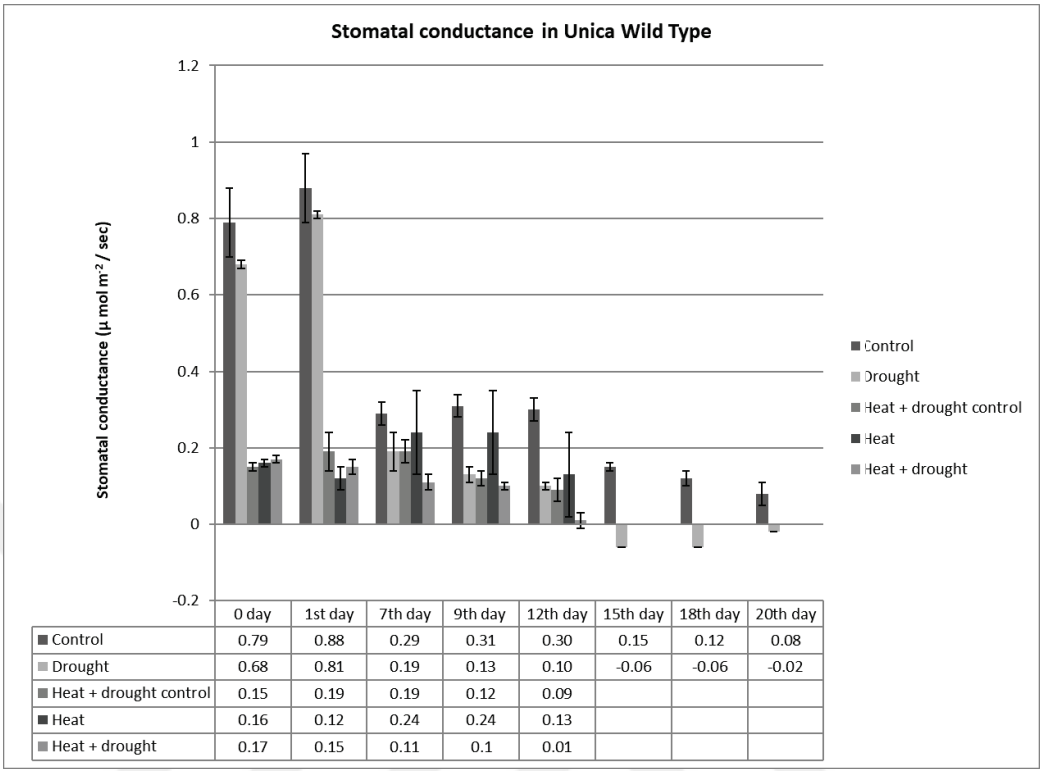
Physiological traits including photosynthesis rate, stomatal conductance, transpiration rate, SPAD measurement, leaf temperature, relative water content and proline accumulation during stress treatments period were taken for both wild-type cultivars. Photosynthesis rate, stomatal conductance and transpiration rate for Unica cultivar classified as control group, drought-treated plants, control for combined stress-treated plants, heat-treated plants and combined stress-treated plants were shown (Figure 4.22) (Figure 4.23) (Figure 4.24). Those parameters also measured for Russet Burbank cultivar categorized as control group, drought-treated plants, control for combined stress-treated plants, heat-treated plants (Figure 4.25) (Figure 4.26) (Figure 4.27). SPAD measurements in both cultivars were taken for drought, heat and a combination of heat and drought stresses (Figure 4.28) (Figure 4.29) (Figure 4.30) (Figure 4.31). Leaf temperatures of plants were measured for Unica and Russet Burbank cultivars under stress conditions (Figure 4.32) (Figure 4.33) (Figure 4.34) (Figure 4.35). Relative water content was taken for both cultivars for drought, heat and a combination of heat and drought stress (Figure 4.36) (Figure 4.37) (Figure 4.38) (Figure 4.39). Proline accumulation in Unica and Russet Burbank was measured for the plants classified as control group, drought-treated plants, control for combined stress-treated plants, heat-treated plants and combined stress-treated plants (Figure 4.40) (Figure 4.41).



	0 day	1st day	7th day	9th day	12th day	15th day	18th day	20th day
Control	28.22	29.12	29.59	30.68	30.43	25.90	26.65	32.63
Drought	27.84	28.82	19.82	13.18	11.09	10.81	9.45	10.39
Heat + drought control	13.53	14.23	17.29	17.06	15.06			
Heat	13.76	13.99	18.87	18.55	18.45			
Heat + drought	14.75	14.11	11.56	13.56	6.56			

Figure 4.22. Change in the photosynthesis rate in wild-type Unica cultivar was shown for control group, drought-treated plants, control for combined stress-treated plants, heat-treated plants and combined stress-treated plants

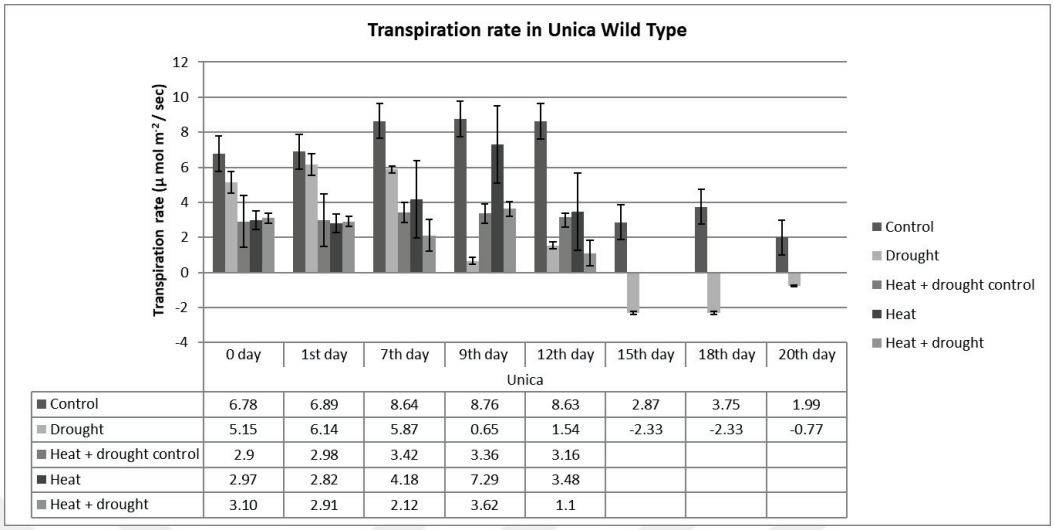
As seen in Figure 4.22, photosynthesis activity in plants categorized as control group and drought-treated plants were measured on 1st, 7th, 9th, 12th, 15th, 18th and 20th day, while it was measured on 1st, 7th, 9th and 12th day under heat and combined stress. Photosynthesis rate in control group showed slight fluctuation for 20 days although, it showed mostly reduction in drought-treated plants from initial day to the end of stress period. For heat-treated plants, photosynthesis rate initially increased until 7th day and then less reduction was observed for the last two measurements. When heat + drought control was compared with heat+drought treated plants, photosynthesis activity in control group plants fluctuated between 15-17 µmol m^{-2}/sec upon stress treatment and in combined stress-treated plants reduced notably with the lowest value of 6.56 µmol m^{-2}/sec. The greatest difference was observed in the last day of stress treatment.



	0 day	1st day	7th day	9th day	12th day	15th day	18th day	20th day
Control	0.79	0.88	0.29	0.31	0.30	0.15	0.12	0.08
Drought	0.68	0.81	0.19	0.13	0.10	-0.06	-0.06	-0.02
Heat + drought control	0.15	0.19	0.19	0.12	0.09			
Heat	0.16	0.12	0.24	0.24	0.13			
Heat + drought	0.17	0.15	0.11	0.1	0.01			

Figure 4.23. Change in the stomata conductance in wild-type Unica cultivar was shown for control group, drought-treated plants, control for combined stress-treated plants, heat-treated plants and combined stress-treated plants

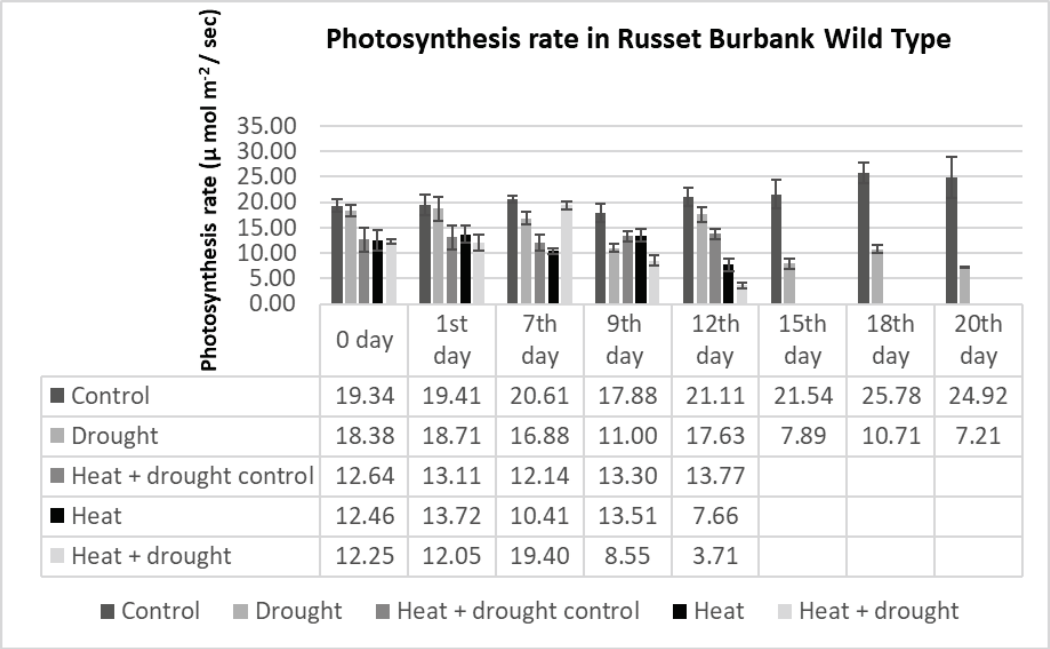
As seen in Figure 4.23, stomatal conductance in plants categorized as control group and drought-treated plants were measured on 1st, 7th, 9th, 12th, 15th, 18th and 20th day, while it was measured on 1st, 7th, 9th and 12th day under heat and combined stress. Stomatal conductance in control group showed considerable reduction for 20 days, with the greatest value of 0,88 µmol m^{-2}/sec. Stomatal conductance in drought-treated plants showed significant reduction at 7th day. For heat-treated plants, stomatal conductance on 7th day doubled compared to 1st day and then reached to value which was close to the first day measurement. When heat + drought control was compared with heat + drought treated plants, stomatal conductance in control group plants fluctuated between 0.09-0.15 µmol m^{-2}/sec under stress treatment and in combined stress-treated plants decreased gradually with the lowest value of 0,01 µmol m^{-2}/sec. The greatest difference was observed in 9th day of stress treatment.



	0 day	1st day	7th day	9th day	12th day	15th day	18th day	20th day
	Unica							
Control	6.78	6.89	8.64	8.76	8.63	2.87	3.75	1.99
Drought	5.15	6.14	5.87	0.65	1.54	-2.33	-2.33	-0.77
Heat + drought control	2.9	2.98	3.42	3.36	3.16			
Heat	2.97	2.82	4.18	7.29	3.48			
Heat + drought	3.10	2.91	2.12	3.62	1.1			

Figure 4.24. Change in the transpiration rate in wild-type Unica cultivar was shown for control group, drought-treated plants, control for combined stress-treated plants, heat-treated plants and combined stress-treated plants

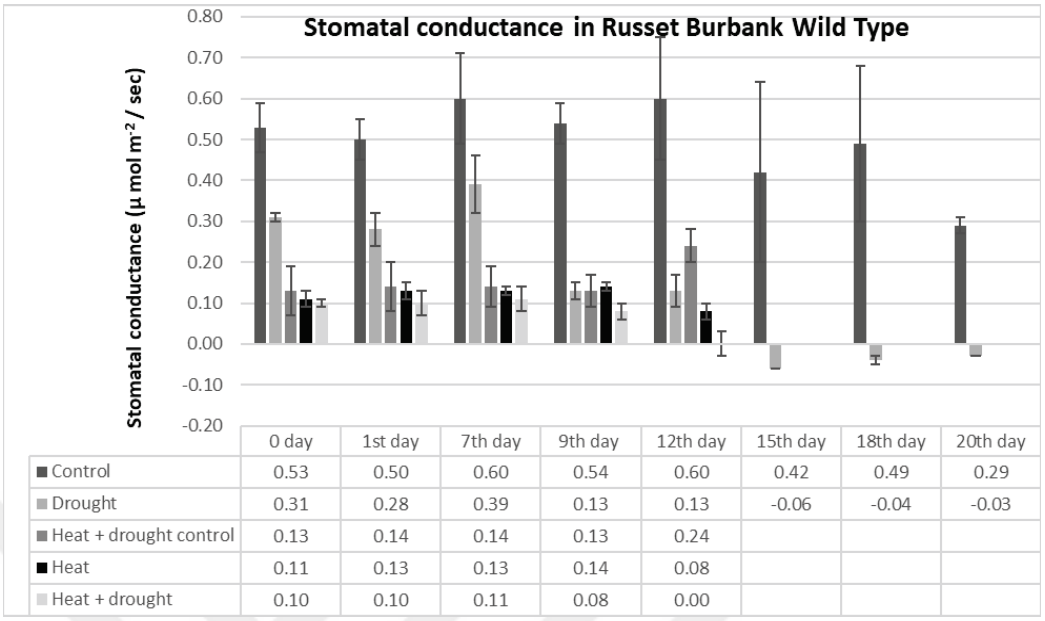
As seen in Figure 4.24, transpiration rate in plants categorized as control group and drought-treated plants were measured at 1st, 7th, 9th, 12th, 15th, 18th and 20th day, while it was measured at 1st, 7th, 9th and 12th day under heat and combined stress. Transpiration rate in control group showed considerable reduction at 15th day with the value of 2.87 µmol m^{-2}/sec. Transpiration rate in drought-treated plants showed significant reduction on the 9th day. For heat-treated plants, transpiration rate increased until 9th day and it decreased nearly by half at the last measurement. When heat + drought control was compared with heat + drought treated plants, transpiration rate in the control group enhanced slightly until 7th day and in combined stress-treated plants decreased mostly with the lowest value of 1.1 µmol m^{-2}/sec. The greatest difference was observed at 12th day of stress treatment.



	0 day	1st day	7th day	9th day	12th day	15th day	18th day	20th day
Control	19.34	19.41	20.61	17.88	21.11	21.54	25.78	24.92
Drought	18.38	18.71	16.88	11.00	17.63	7.89	10.71	7.21
Heat + drought control	12.64	13.11	12.14	13.30	13.77			
Heat	12.46	13.72	10.41	13.51	7.66			
Heat + drought	12.25	12.05	19.40	8.55	3.71			

Figure 4.25. Change in the photosynthesis rate in wild-type Russet Burbank cultivar was shown for control group, drought-treated plants, control for combined stress-treated plants, heat-treated plants and combined stress-treated plants

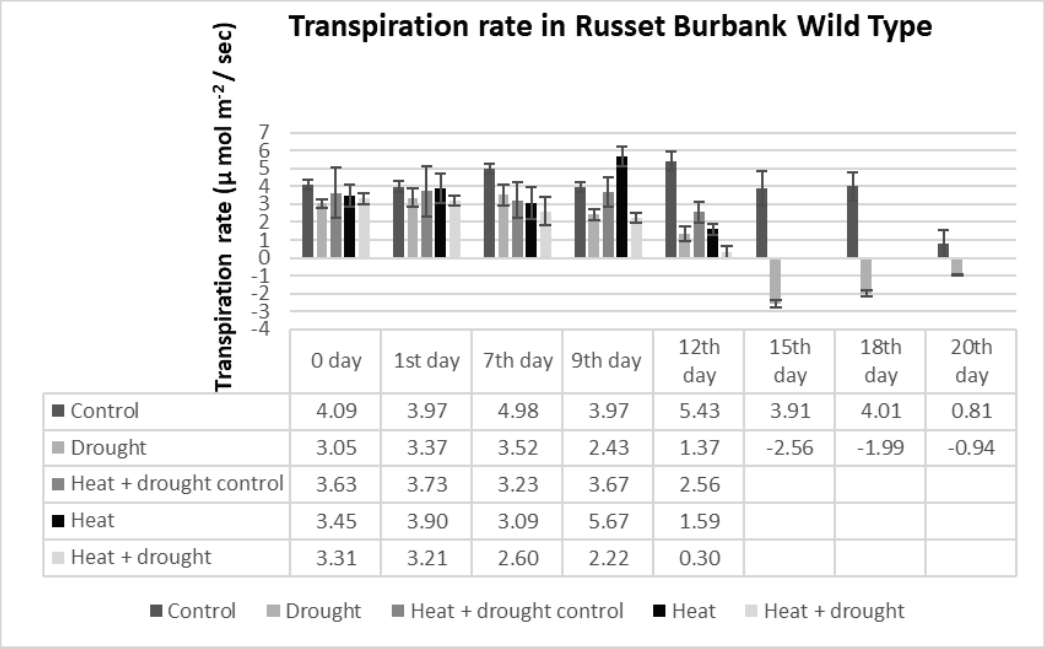
As seen in Figure 4.25, photosynthesis activity in plants categorized as control group and drought-treated plants were measured at 1st, 7th, 9th, 12th, 15th, 18th and 20th day, while it was measured at 1st, 7th, 9th and 12th day under heat and combined stress. Photosynthesis rate in the control group showed slight fluctuation during 20 days with the greatest value of 25.78 µmol m^{-2}/sec, whereas it followed mostly reduction pattern in drought-treated plants from initial day to the end of stress period. For heat-treated plants, photosynthesis rate fluctuated around 11.0 µmol m^{-2}/sec during the stress period. When heat + drought control was compared with heat + drought treated plants, photosynthesis rate in control group remained nearly steady and in combined stress-treated plants reduced greater than 2-fold on 9th day. The greatest difference between those groups was observed on 12th day of stress treatment.



	0 day	1st day	7th day	9th day	12th day	15th day	18th day	20th day
Control	0.53	0.50	0.60	0.54	0.60	0.42	0.49	0.29
Drought	0.31	0.28	0.39	0.13	0.13	-0.06	-0.04	-0.03
Heat + drought control	0.13	0.14	0.14	0.13	0.24			
Heat	0.11	0.13	0.13	0.14	0.08			
Heat + drought	0.10	0.10	0.11	0.08	0.00			

Figure 4.26. Change in the stomatal conductance in wild-type Russet Burbank cultivar was shown for control group, drought-treated plants, control for combined stress-treated plants, heat-treated plants and combined stress-treated plants

As seen in Figure 4.26, stomatal conductance in plants categorized as control group and drought-treated plants were measured at 1st, 7th, 9th, 12th, 15th, 18th and 20th day, while it was measured at 1st, 7th, 9th and 12th day under heat and combined stress. Stomatal conductance in control group showed little fluctuation with the greatest value 0.6 µmol m^{-2}/sec. Stomatal conductance in drought-treated plants showed significant reduction on the 9th day. For heat-treated plants, stomatal conductance did not change significantly in 12-day stress period. When heat + drought control was compared with heat + drought treated plants, stomatal conductance in control group fluctuated around 0.45 µmol m^{-2}/sec and in combined stress-treated plants peaked on the 7th day with a value of 0.11 µmol m^{-2}/sec. The largest difference was taken at the last measurement of stress treatment.



	0 day	1st day	7th day	9th day	12th day	15th day	18th day	20th day
Control	4.09	3.97	4.98	3.97	5.43	3.91	4.01	0.81
Drought	3.05	3.37	3.52	2.43	1.37	-2.56	-1.99	-0.94
Heat + drought control	3.63	3.73	3.23	3.67	2.56			
Heat	3.45	3.90	3.09	5.67	1.59			
Heat + drought	3.31	3.21	2.60	2.22	0.30			

Figure 4.27. Change in the transpiration rate in wild-type Russet Burbank cultivar was shown for control group, drought-treated plants, control for combined stress-treated plants, heat-treated plants and combined stress-treated plants

As seen in Figure 4.27, transpiration rate in plants categorized as control group and drought-treated plants were measured at 1st, 7th, 9th, 12th, 15th, 18th and 20th day, while it was measured at 1st, 7th, 9th, and 12th day under heat and combined stress. Transpiration rate in control group showed considerable reduction at 20th day with the value of 0.81 µmol m^{-2}/sec. Transpiration rate in drought-treated plants showed significant reduction on the 12th day. For heat-treated plants, transpiration rate increased until 9th day and it decreased nearly 3-fold on the 12th day than previous measurement. When the control group for heat + drought control was compared with heat + drought treated plants, transpiration rate in the control group showed fluctuation, while it decreased overwhelmingly on the 9th day in combined stress-treated plants. The greatest difference was observed at the 12th day of stress treatment, similar to Unica cultivar.

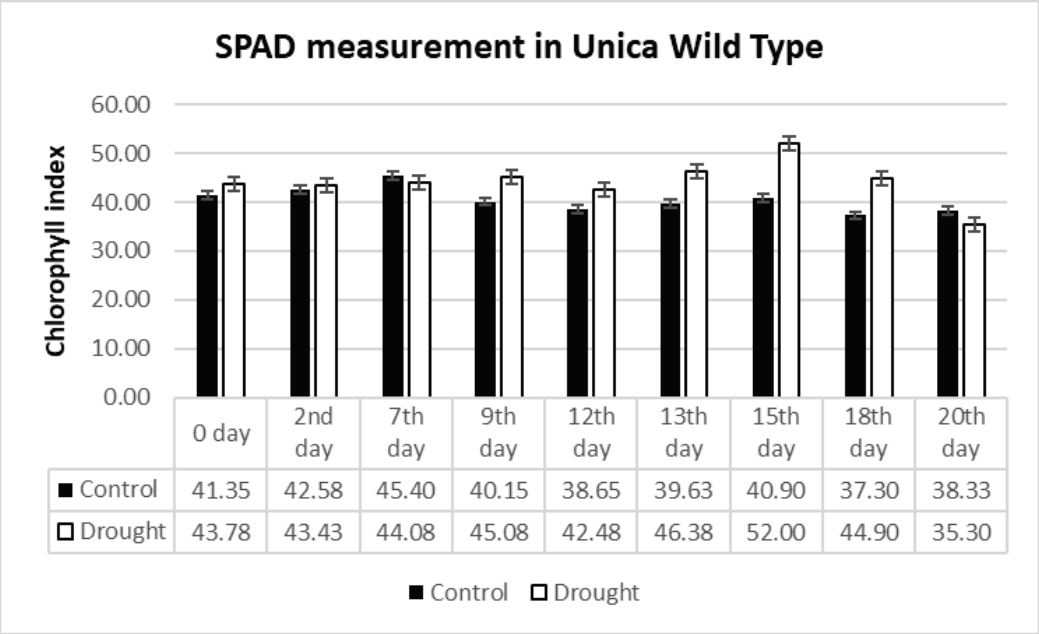


Figure 4.28. Change in the SPAD values in wild-type Unica cultivar was shown for control group and drought-treated plants after 20-day drought treatment

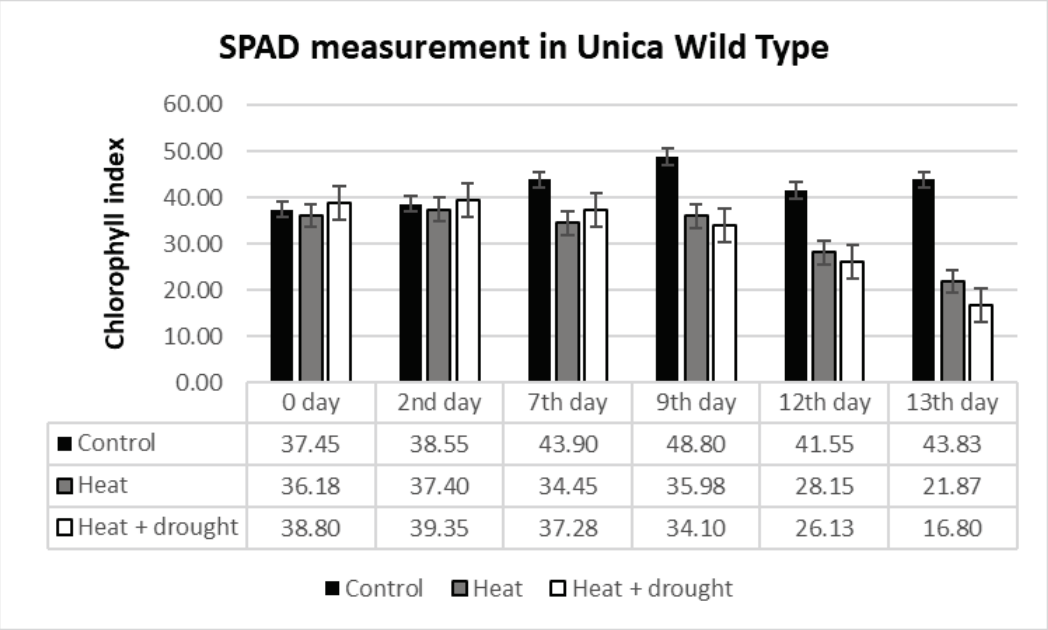
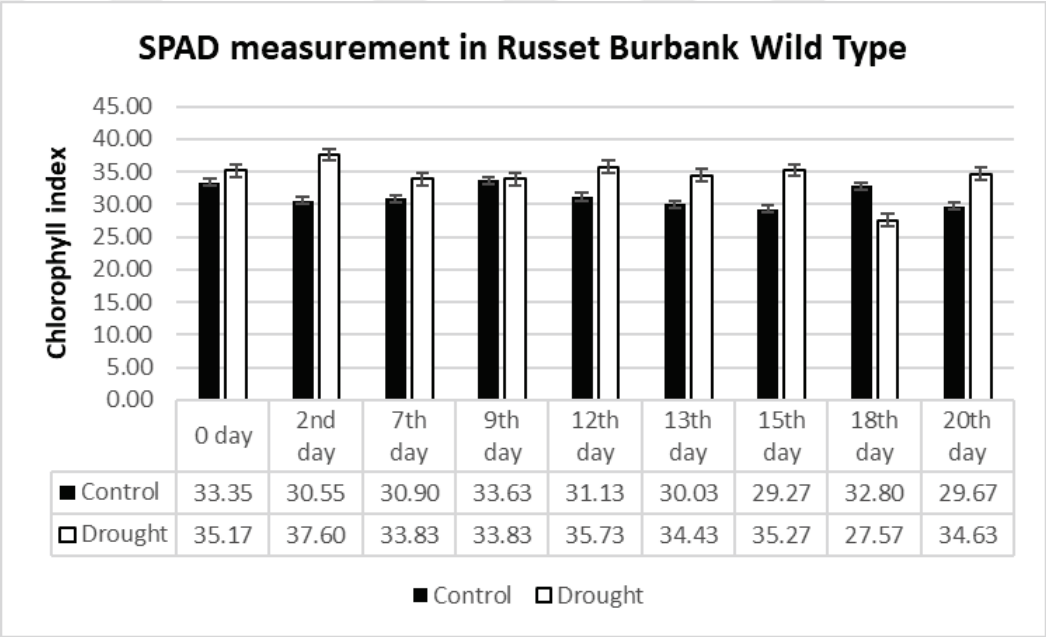


Figure 4.29. Change in the SPAD values in wild-type Unica cultivar was shown for control group, heat-treated plants and combined stress-treated plants after 12-day heat and combined treatment

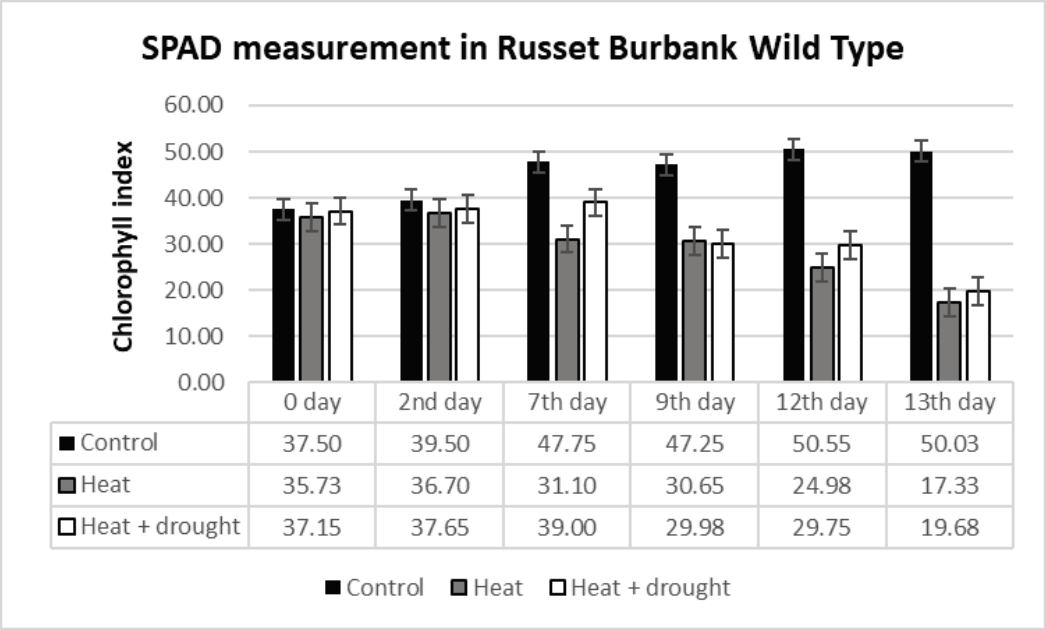
As seen in Figure 4.28 and Figure 4.29, chlorophyll index in Unica plants categorized as control group and drought-treated plants were measured at 0^{th}, 2^{nd}, 7^{th}, 9^{th}, 12^{th}, 13^{th},

15th, 18th and 20th day, while it was measured at 0th, 2nd, 7th, 9th, 12th and 13th day under heat and combined stress. Chlorophyll index in control group showed slight fluctuation with the greatest value of 45.4 in the 7th day. Chlorophyll index in drought-treated plants peaked on 15th day and reached minimum level on the 20th day. For heat-treated plants, chlorophyll index decreased severely on 12th day and it reached nearly half of control group at the end of 12-day stress treatment. Similarly, when heat + drought control was compared with heat + drought treated plants, chlorophyll index in the combined stress-treated plants displayed sharp reduction on the 13th day and the greatest difference between those groups were observed at the last measurement.



	0 day	2nd day	7th day	9th day	12th day	13th day	15th day	18th day	20th day
Control	33.35	30.55	30.90	33.63	31.13	30.03	29.27	32.80	29.67
Drought	35.17	37.60	33.83	33.83	35.73	34.43	35.27	27.57	34.63

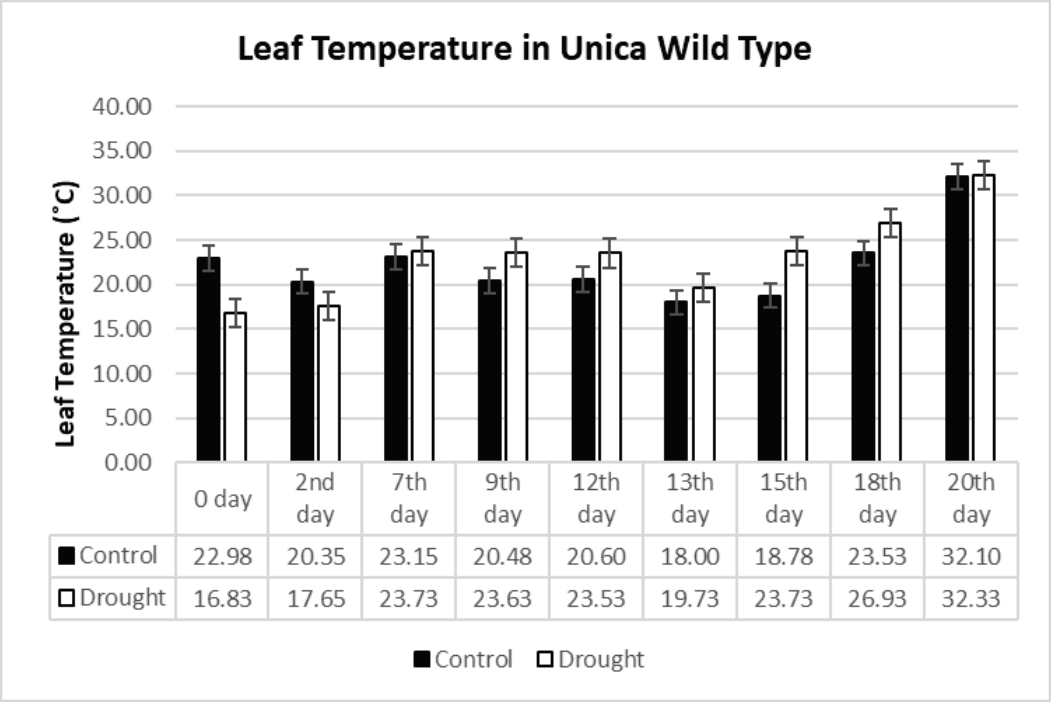
Figure 4.30. Change in the SPAD values in wild-type Russet Burbank cultivar was shown for control group and drought-treated plants after 20-day drought treatment



	0 day	2nd day	7th day	9th day	12th day	13th day
Control	37.50	39.50	47.75	47.25	50.55	50.03
Heat	35.73	36.70	31.10	30.65	24.98	17.33
Heat + drought	37.15	37.65	39.00	29.98	29.75	19.68

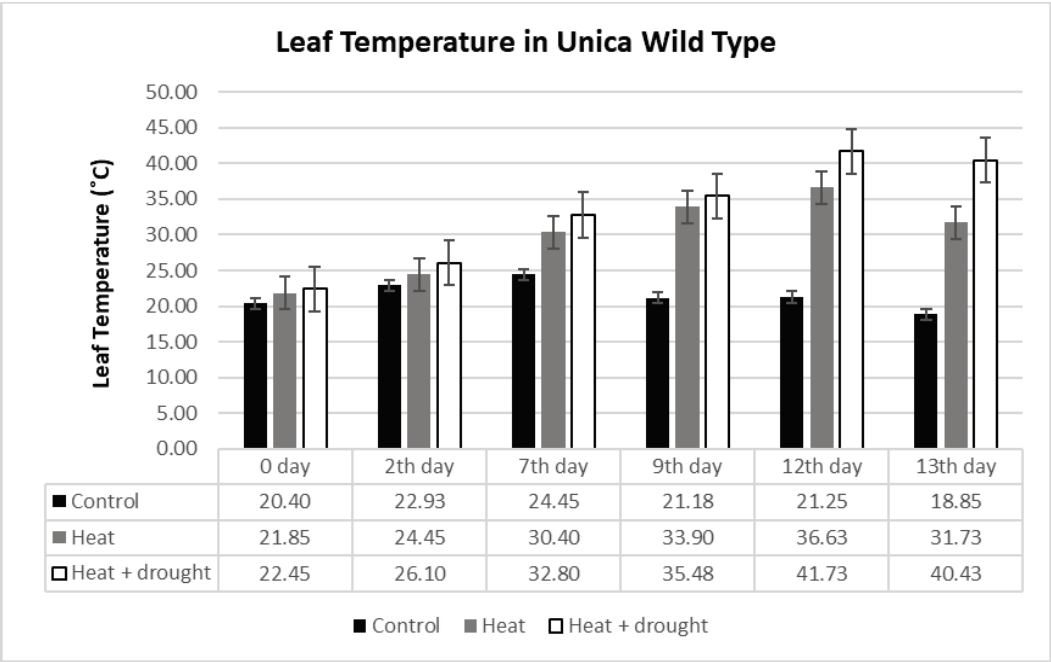
Figure 4.31. Change in the SPAD values in wild-type Russet Burbank cultivar was shown for control group, heat-treated plants and combined stress-treated plants after 12-day heat and combined treatment

As seen in Figure 4.30 and Figure 4.31, chlorophyll index in Russet Burbank plants categorized as control group and drought-treated plants were measured at 0^{th}, 2^{nd}, 7^{th}, 9^{th}, 12^{th}, 13^{th}, 15^{th}, 18^{th} and 20^{th} day, while it was measured at 0^{th}, 2^{nd}, 7^{th}, 9^{th}, 12^{th} and 13^{th} day under heat and combined stress. Chlorophyll index in control group showed little fluctuation with the greatest value of 33.63 on the 9^{th} day. Similar to control group, chlorophyll index in drought-treated plants fluctuated slightly during whole stress period with the lowest value of 27.57 on the 18^{th} day. For heat-treated plants, chlorophyll index decreased intensely in the 12^{th} day and it reached approximately one third of control group at the end of 12-day stress treatment. When heat + drought control was compared with heat + drought treated plants, chlorophyll index in the combined stress-treated plants showed significant decrease in the 13^{th} day and the largest difference between those groups were observed at the last measurement, similar to Unica cultivar.



	0 day	2nd day	7th day	9th day	12th day	13th day	15th day	18th day	20th day
Control	22.98	20.35	23.15	20.48	20.60	18.00	18.78	23.53	32.10
Drought	16.83	17.65	23.73	23.63	23.53	19.73	23.73	26.93	32.33

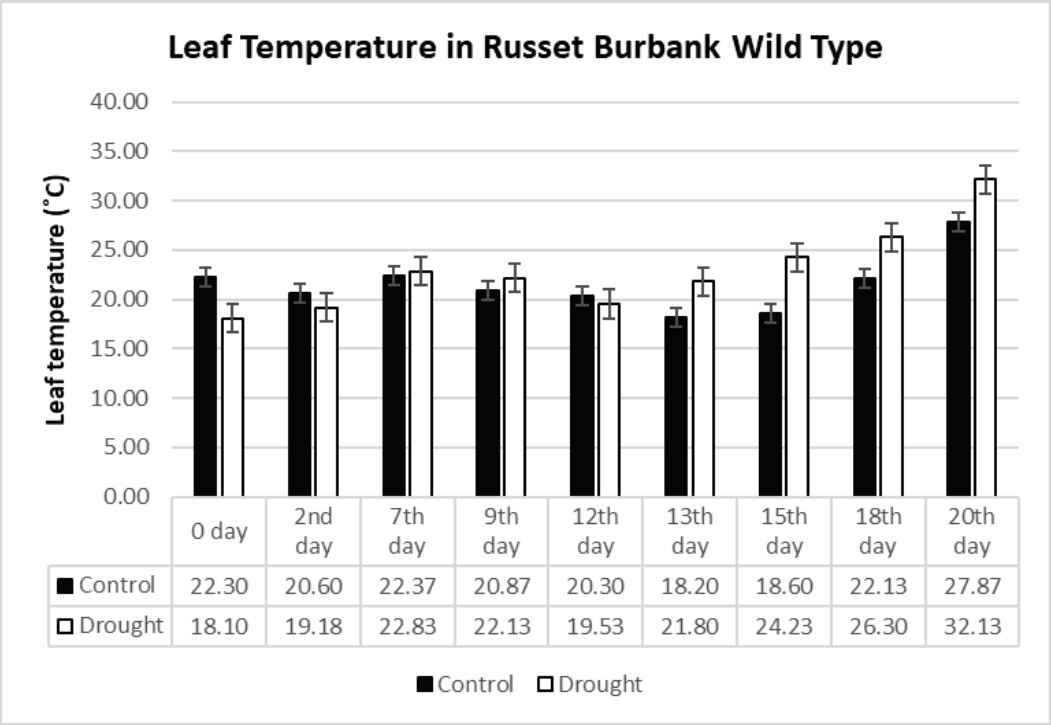
Figure 4.32. Change in the leaf temperature (˚C) in wild-type Unica cultivar was shown for control group and drought-treated plants after 20-day drought treatment



	0 day	2th day	7th day	9th day	12th day	13th day
Control	20.40	22.93	24.45	21.18	21.25	18.85
Heat	21.85	24.45	30.40	33.90	36.63	31.73
Heat + drought	22.45	26.10	32.80	35.48	41.73	40.43

Figure 4.33. Change in the leaf temperature (˚C) in wild-type Unica cultivar was shown for control group, heat-treated plants and combined stress-treated plants after 12-day heat and combined treatment

As seen in Figure 4.32 and Figure 4.33, leaf temperature in Unica plants categorized as control group and drought-treated plants were measured at 0^{th}, 2^{nd}, 7^{th}, 9^{th}, 12^{th}, 13^{th}, 15^{th}, 18^{th} and 20^{th} day, while it was measured at 0^{th}, 2^{nd}, 7^{th}, 9^{th}, 12^{th} and 13^{th} day under heat and combined stress. Leaf temperature in control group showed slight fluctuation with the greatest value of 32.10 ˚C on the 20^{th} day. Leaf temperature in drought-treated plants peaked on the 20^{th} day and reached minimum value on the 2^{nd} day. For heat-treated plants, leaf temperature increased notably on the 7^{th} day and it reached 2-fold of control group at the last measurement. Similarly, when the control group for heat + drought control was compared with heat + drought treated plants, leaf temperature in the combined stress-treated plants increased until 12^{th} day and it was always found greater than control group during stress period.



	0 day	2nd day	7th day	9th day	12th day	13th day	15th day	18th day	20th day
Control	22.30	20.60	22.37	20.87	20.30	18.20	18.60	22.13	27.87
Drought	18.10	19.18	22.83	22.13	19.53	21.80	24.23	26.30	32.13

Figure 4.34. Change in the leaf temperature (˚C) in wild-type Russet Burbank cultivar was shown for control group and drought-treated plants after 20-day drought treatment

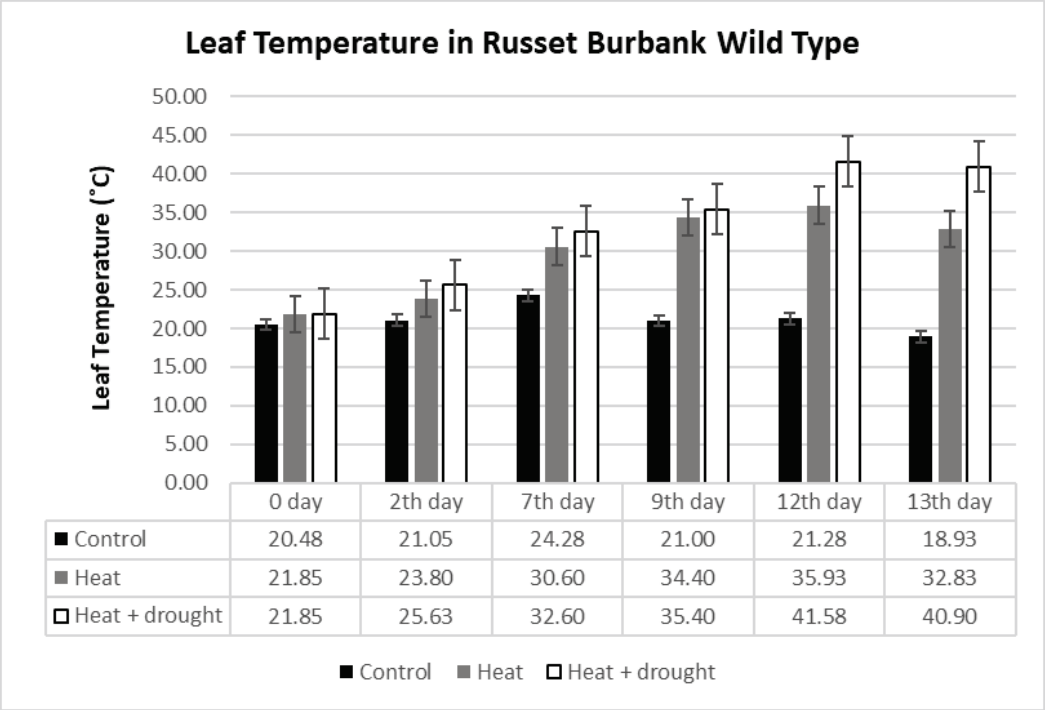


Figure 4.35. Change in the leaf temperature (˚C) in wild-type Russet Burbank cultivar was shown for control group, heat-treated plants and combined stress-treated plants after 12-day heat and combined treatment

As seen in Figure 4.34 and Figure 4.35, leaf temperature in Russet Burbank plants categorized as control group and drought-treated plants were measured at 0^{th}, 2^{nd}, 7^{th}, 9^{th}, 12^{th}, 13^{th}, 15^{th}, 18^{th} and 20^{th} day, while it was measured at 0^{th}, 2^{nd}, 7^{th}, 9^{th}, 12^{th} and 13^{th} day under heat and combined stress. Leaf temperature in control group showed slight fluctuation with the greatest value of 27.87 ˚C on the 20^{th} day. Leaf temperature in drought-treated plants peaked on the 20^{th} day. For heat-treated plants, leaf temperature increased notably on the 12^{th} day and it reached nearly 2-fold of control group at the last measurement. Similarly, when the control group for heat + drought control was compared with heat + drought treated plants, leaf temperature in the combined stress-treated plants increased until 12^{th} day with the greatest value of 41.58 ˚C and it reached greater than 2-fold of control group on the 13^{th} day.

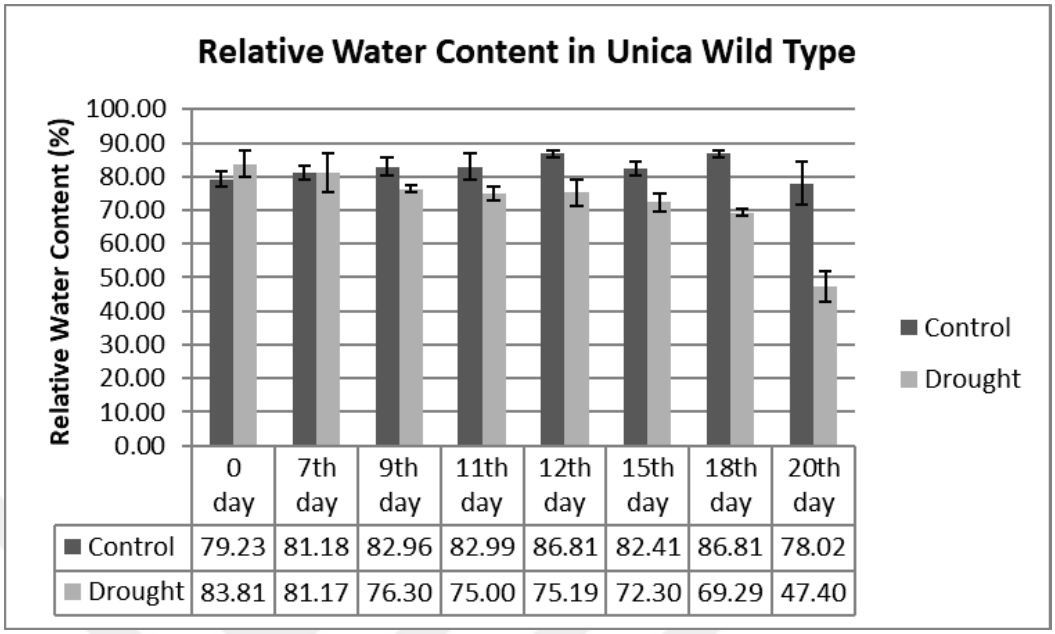


Figure 4.36. Change in the relative water content (%) in wild-type Unica cultivar was shown for control group and drought-treated plants after 20-day drought treatment

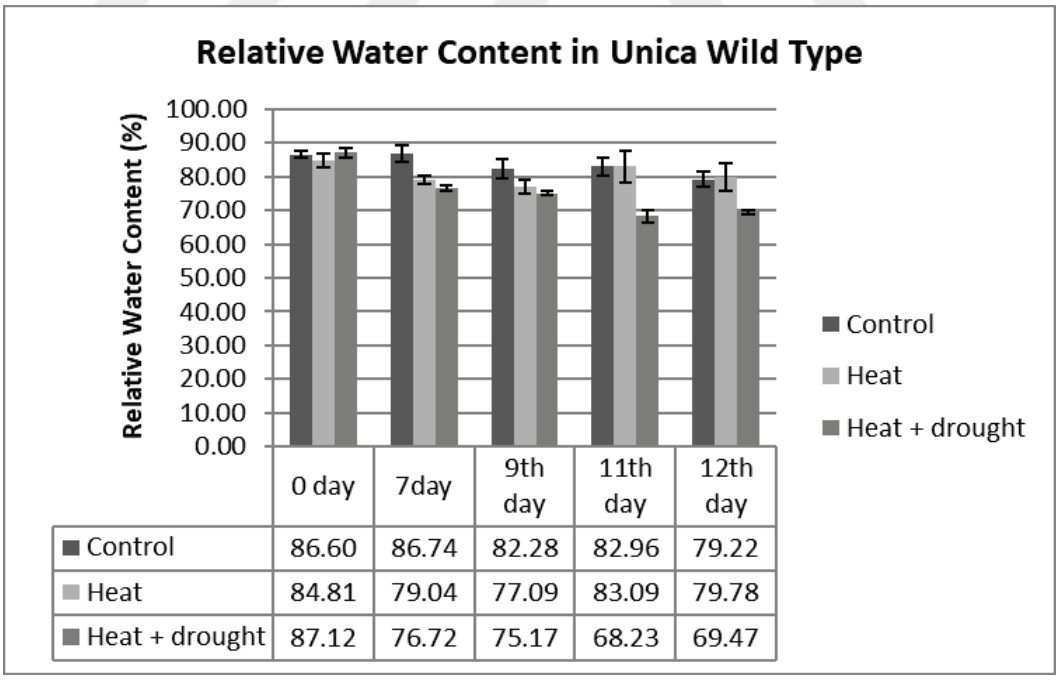
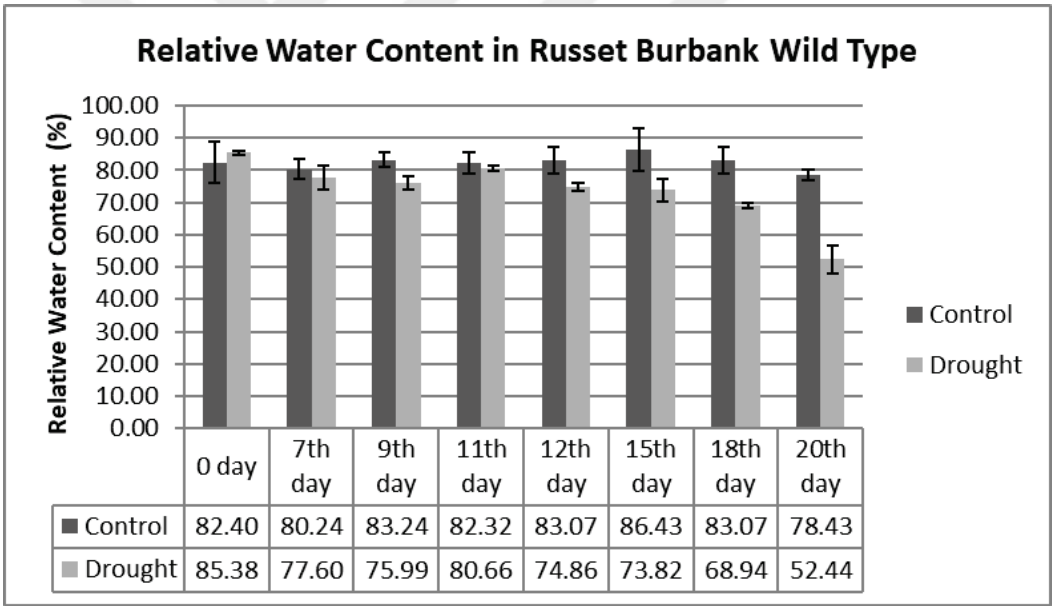


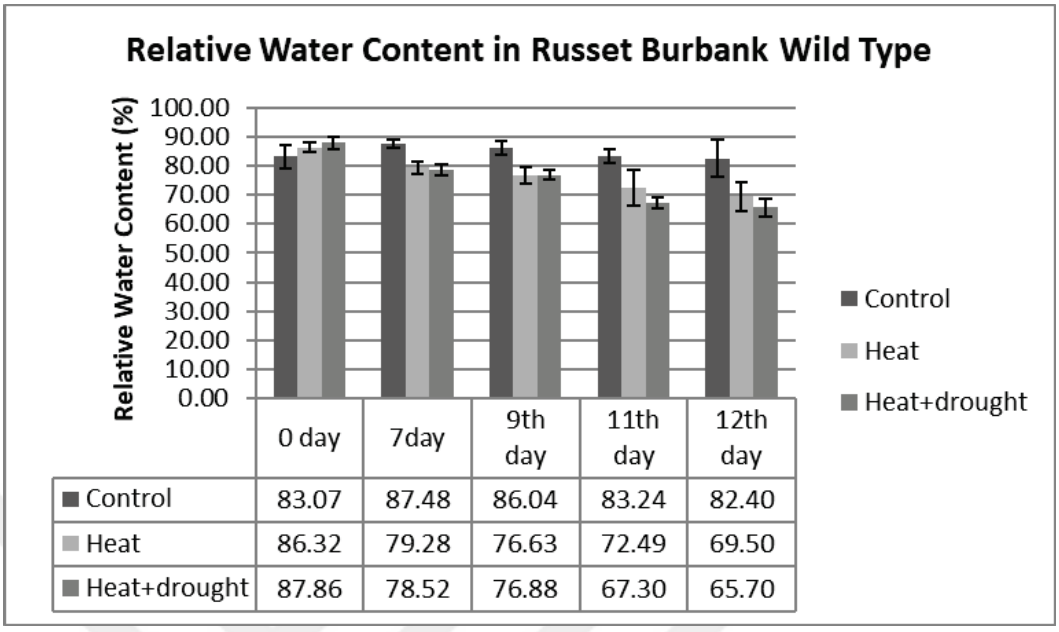
Figure 4.37. Change in the relative water content (%) in wild-type Unica cultivar was shown for control group, heat-treated plants and combined stress-treated plants after 12-day heat and combined treatment

As seen in Figure 4.36 and Figure 4.37, relative water content in Unica plants categorized as control group and drought-treated plants were measured at 0^{th}, 7^{th}, 9^{th}, 11^{th},12^{th}, 15^{th}, 18^{th} and 20^{th} day, while it was measured at 0^{th}, 7^{th}, 9^{th}, 11^{th} and 12^{th} day under heat and combined stress. Relative water content in control group showed slight fluctuation with the greatest value of 86.81% on the 20^{th} day. Relative water content in drought-treated plants decreased gradually apart from 12^{th} day and reached minimum value on the 20^{th} day. For heat-treated plants, relative water content fluctuated under heat stress and it reached similar value of control group at the last measurement. When heat + drought control was compared with heat + drought treated plants, relative water content in the combined stress-treated plants reduced gradually until 11^{th} day and it was mostly less compared to control group during stress period.



	0 day	7th day	9th day	11th day	12th day	15th day	18th day	20th day
Control	82.40	80.24	83.24	82.32	83.07	86.43	83.07	78.43
Drought	85.38	77.60	75.99	80.66	74.86	73.82	68.94	52.44

Figure 4.38. Change in the relative water content (%) in wild-type Russet Burbank cultivar was shown control group and drought-treated plants after 20-day drought treatment



	0 day	7day	9th day	11th day	12th day
Control	83.07	87.48	86.04	83.24	82.40
Heat	86.32	79.28	76.63	72.49	69.50
Heat+drought	87.86	78.52	76.88	67.30	65.70

Figure 4.39. Change in the relative water content (%) in wild-type Russet Burbank cultivar was shown for control group, heat-treated plants and combined stress-treated plants after 12-day heat and combined treatment

As seen in Figure 4.38 and Figure 4.39, relative water content in Russet Burbank plants categorized as control group drought-treated plants were measured at 0^{th}, 7^{th}, 9^{th}, 11^{th},12^{th}, 15^{th}, 18^{th} and 20^{th} day, while it was measured at 0^{th}, 7^{th}, 9^{th}, 11^{th} and 12^{th} day under heat and combined stress. Relative water content in control group showed slight fluctuation with the greatest value of 86,43% on 15^{th} day. Relative water content in drought-treated plants decreased gradually except on 11^{th} day and reached minimum value on 20^{th} day. For heat-treated plants, relative water content followed reduction pattern under heat stress, and it was less compared to control group at the last measurement. When heat + drought control was compared with heat + drought treated plants, relative water content in the combined stress treated plants reduced gradually until the end of stress period and in control group remained steady around 83%. Moreover, it was quite less compared to that of control group on the 12^{th} day.

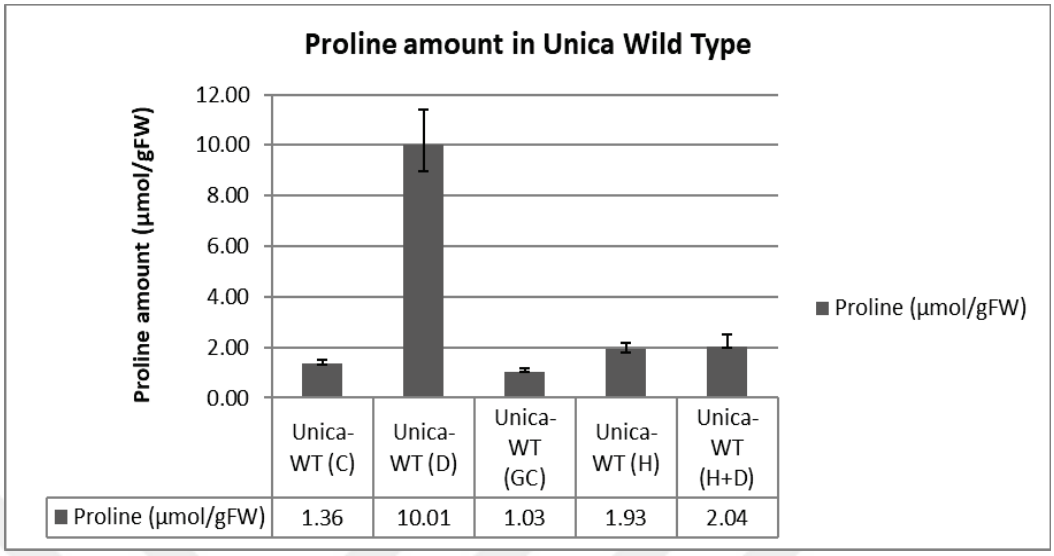


Figure 4.40. Proline accumulation in Unica wild-type cultivar was shown for control group, drought-treated plants, control for combined stress-treated plants, heat-treated plants and combined stress-treated plants

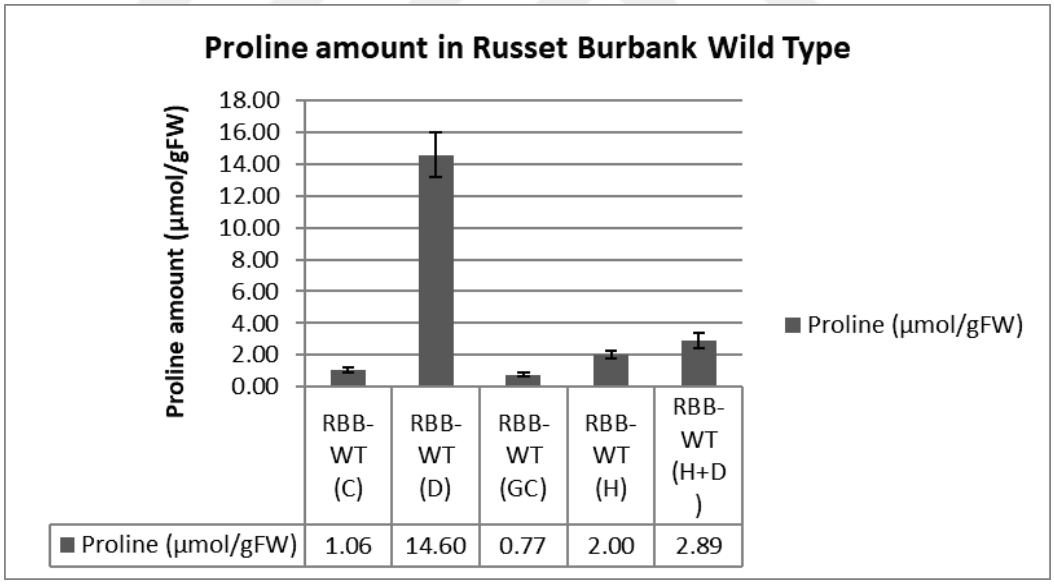


Figure 4.41. Proline accumulation in Russet Burbank wild-type cultivar was shown for control group, drought-treated plants, control for combined stress-treated plants, heat-treated plants and combined stress-treated plants

Proline amount in Unica wild-type and Russet Burbank cultivars were measured under drought, heat and a combination of heat and drought stresses. In Unica cultivar, proline accumulation in drought-treated plants was found the highest level than others with around 7-fold greater than control group. In Russet Burbank cultivar, proline

accumulation in drought-treated plants was observed greater than others with around 12-fold greater than control group. Correspondingly, proline accumulation in drought-treated plants was observed greater compared to other wild-type plants subjected to heat or heat + drought stresses.

4.3.3 Images and physiological parameters in transgenic cultivars after stress period

After drought treatment, images of wild-type control group, drought-treated wild-type plants, T0 generation of transgenic control plants and drought-treated plants were shown for both cultivars (Figure 4.42) (Figure 4.43). Images of plants which were subjected to heat and heat combined with drought stress were illustrated for Unica and Russet Burbank cultivars (Figure 4.44) (Figure 4.45). Besides, tubers of T0 transgenic plants for Unica and Russet Burbank cultivar after stress treatments were shown in Figure 4.46.

Figure 4.42. Wild-type control group, T0 transgenic control plants, drought-treated wild-type plants and drought-treated T0 transgenic plants for Unica cultivar after 20-day drought treatment (C, control; D, drought) were shown

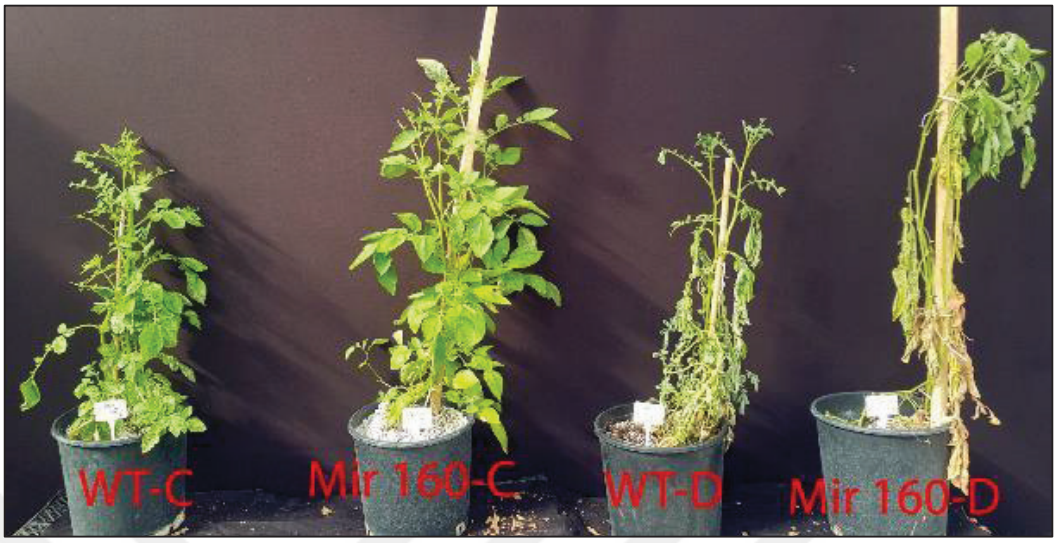


Figure 4.43. Wild-type control group, T0 transgenic control plants, drought-treated wild-type plants and drought-treated T0 transgenic plants for Russet Burbank cultivar after 20-day drought treatment (C, control; D, drought) were shown

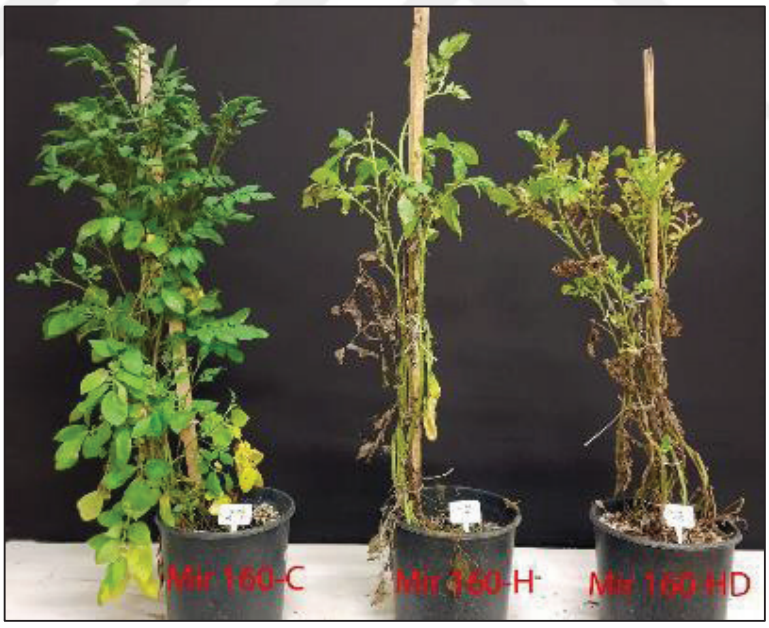


Figure 4.44. T0 generation of transgenic control group, heat-treated and combined-treated transgenic plants for Unica cultivar after 12-day heat and combined treatment (C, control; H, heat; HD, heat plus drought) were shown

Figure 4.45. T0 generation of transgenic control group, heat-treated and combined stress-treated transgenic plants for Russet Burbank cultivar were shown after 12-day heat and combined treatment (C, control; H, heat; HD, heat plus drought)

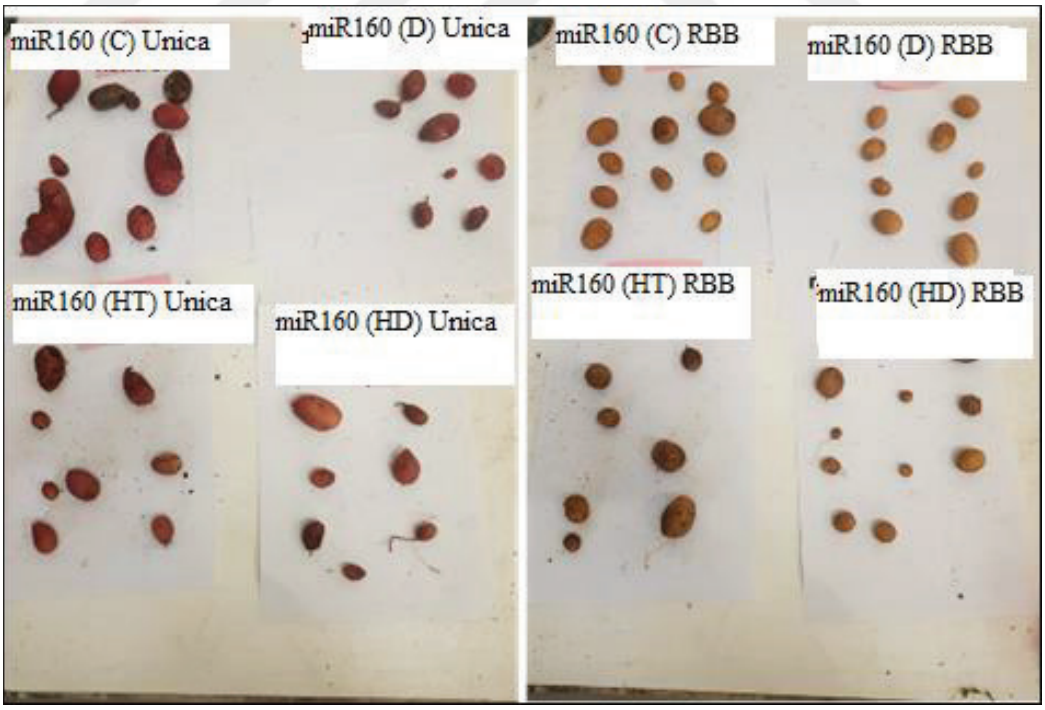
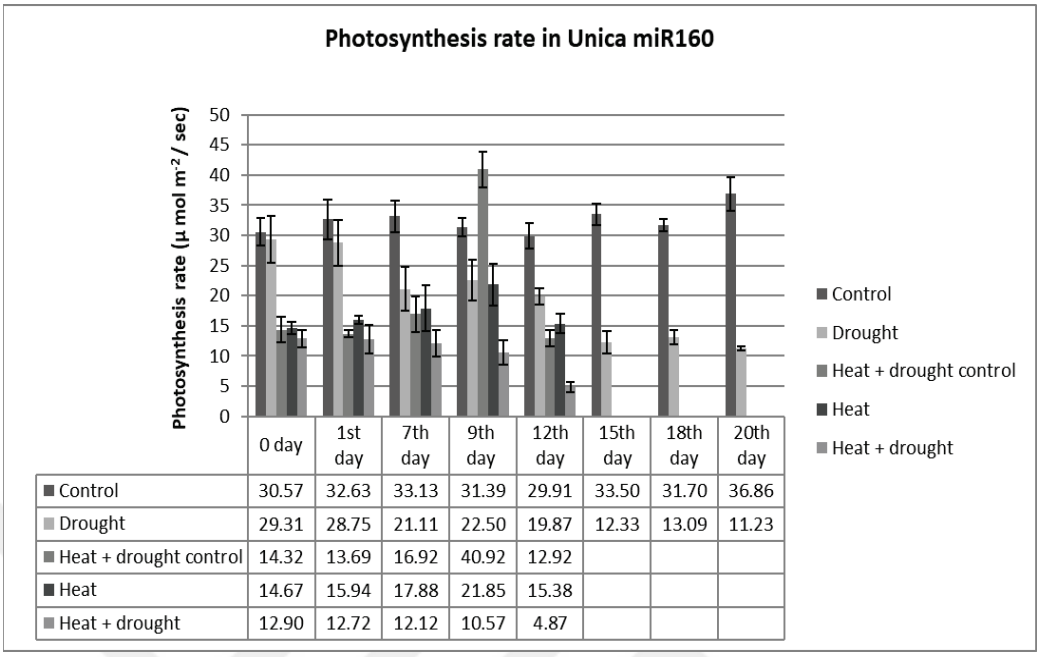


Figure 4.46. T0 generation of transgenic tubers in Unica and Russet Burbank cultivars were shown after 20-day drought and 12-day heat and combined treatment

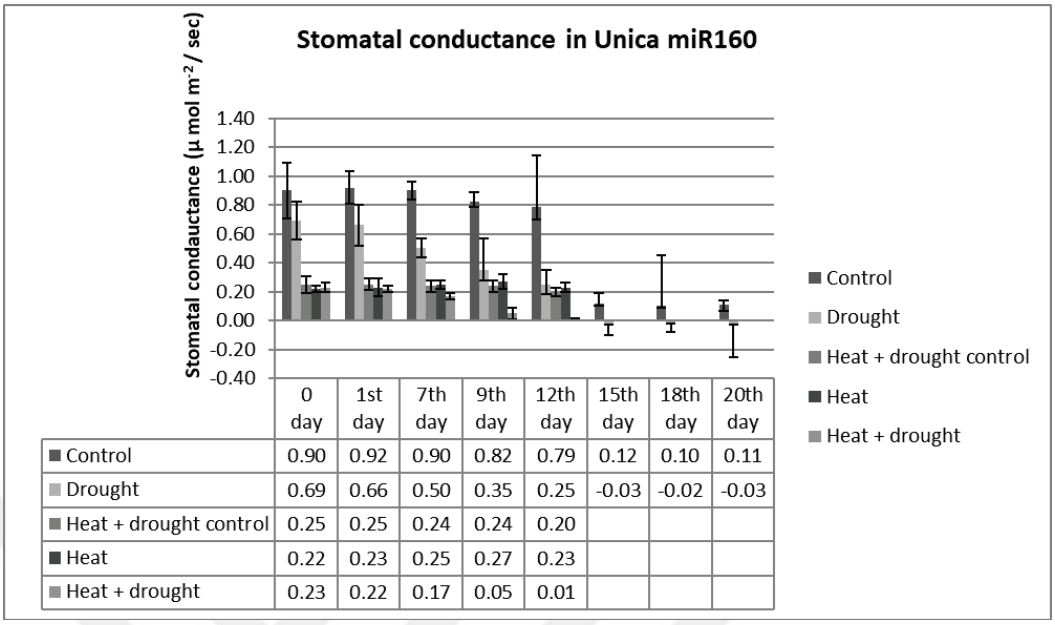
Similar to measurements taken in wild-type plants, physiological measurements such as photosynthesis rate, stomatal conductance, transpiration rate, SPAD measurement, leaf temperature, relative water content and proline accumulation during stress treatments was done for transgenic cultivars overexpressing miR160. Photosynthesis rate, stomatal conductance and transpiration rate in transgenic Unica cultivar classified as control group, drought-treated plants, control for combined stress-treated plants, heat-treated plants and combined stress-treated plants were given in Figure 4.47 Figure 4.48 and Figure 4.49. Those parameters were also measured for Russet Burbank cultivar categorized as control group, drought-treated plants, control for combined stress-treated plants, heat-treated and combined stress-treated plants (Figure 4.50) (Figure 4.51) (Figure 4.52). SPAD measurements in both cultivars were taken for drought, heat and a combination of heat and drought stresses (Figure 4.53) (Figure 4.54) (Figure 4.55) (Figure 4.56). Leaf temperatures of plants were measured for transgenic Unica and Russet Burbank cultivars under stress conditions (Figure 4.57) (Figure 4.58) (Figure 4.59) (Figure 4.60). Relative water content was taken in both cultivars under drought, heat and a combination of heat and drought stresses (Figure 4.61) (Figure 4.62) (Figure 4.63). Lastly, proline content in transgenic Unica and Russet Burbank plants classified as control group, drought-treated plants, control for combined stress-treated plants, heat-treated plants and combined stress-treated plants were measured under stress conditions (Figure 4.64) (Figure 4.65).



	0 day	1st day	7th day	9th day	12th day	15th day	18th day	20th day
Control	30.57	32.63	33.13	31.39	29.91	33.50	31.70	36.86
Drought	29.31	28.75	21.11	22.50	19.87	12.33	13.09	11.23
Heat + drought control	14.32	13.69	16.92	40.92	12.92			
Heat	14.67	15.94	17.88	21.85	15.38			
Heat + drought	12.90	12.72	12.12	10.57	4.87			

Figure 4.47. Change in the photosynthesis rate in transgenic Unica cultivar was shown for control group, drought-treated transgenic plants, control group for combined stress-treated transgenic heat-treated transgenic plants and combined stress-treated transgenic plants

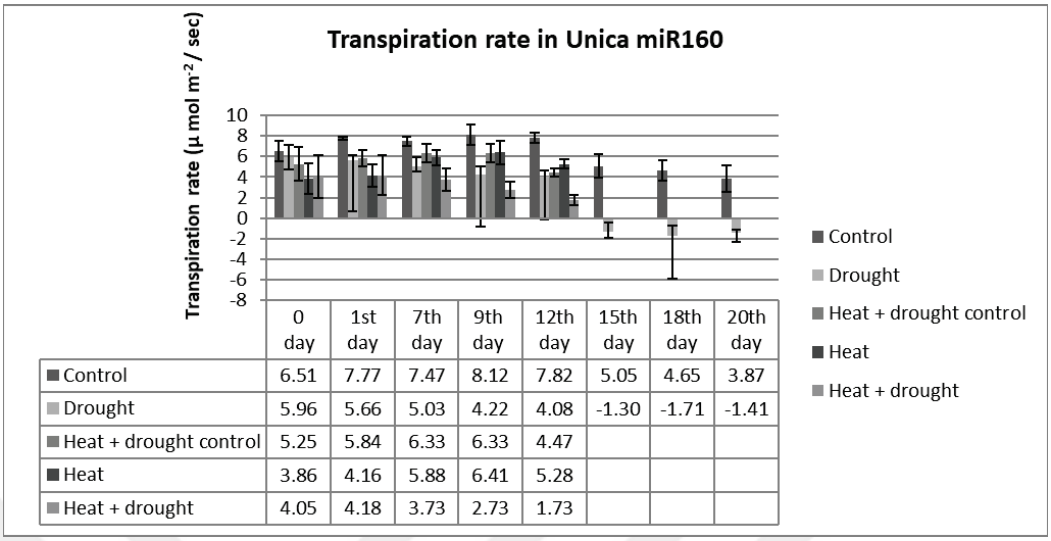
As seen in Figure 4.47, photosynthesis activity in plants categorized as control group and drought-treated plants were measured at 1st, 7th, 9th, 12th, 15th, 18th and 20th day, while it was measured at 1st, 7th, 9th and 12th day under heat and combined stress. Photosynthesis rate in the control group showed slight fluctuation for 20 days, although it decreased mostly in drought-treated plants with the lowest value of 11.23 µmol m^{-2}/sec at the last measurement from initial day to the end of stress period. For heat-treated plants, photosynthesis rate increased until the 9th day with the greatest value of 21.85 µmol m^{-2}/sec and then slight reduction was observed on the 12th day. When heat+drought control was compared with heat+drought treated plants, photosynthesis rate in control group plants generally remained steady around 15 µmol m^{-2}/sec except the significant increase on the 9th day and combined stress-treated transgenic plants showed decrease in the photosynthesis activity.



	0 day	1st day	7th day	9th day	12th day	15th day	18th day	20th day
Control	0.90	0.92	0.90	0.82	0.79	0.12	0.10	0.11
Drought	0.69	0.66	0.50	0.35	0.25	-0.03	-0.02	-0.03
Heat + drought control	0.25	0.25	0.24	0.24	0.20			
Heat	0.22	0.23	0.25	0.27	0.23			
Heat + drought	0.23	0.22	0.17	0.05	0.01			

Figure 4.48. Change in the stomatal conductance in transgenic Unica cultivar was shown for control group, drought-treated transgenic plants, control group for combined-treated transgenic plants heat-treated transgenic plants and combined stress-treated transgenic plants

As seen in Figure 4.48, stomatal conductance in plants categorized as control group and drought-treated plants were measured at 1st, 7th, 9th, 12th, 15th, 18th and 20th day, while it was measured at 1st, 7th, 9th and 12th day under heat and combined stress. Stomatal conductance in control group showed considerable reduction for 20 days, with the greatest value of 0.82 µmol m^{-2}/sec. Stomatal conductance in drought-treated transgenic plants decreased greatly under stress condition. For heat-treated plants, stomatal conductance remained steady around 0.25 µmol m^{-2}/sec during stress treatment. When heat + drought control was compared with heat + drought treated plants, stomatal conductance in control group did not change considerably and in combined stress-treated transgenic plants showed remarkable decrease with the lowest value of 0.01 µmol m^{-2}/sec.



	0 day	1st day	7th day	9th day	12th day	15th day	18th day	20th day
Control	6.51	7.77	7.47	8.12	7.82	5.05	4.65	3.87
Drought	5.96	5.66	5.03	4.22	4.08	-1.30	-1.71	-1.41
Heat + drought control	5.25	5.84	6.33	6.33	4.47			
Heat	3.86	4.16	5.88	6.41	5.28			
Heat + drought	4.05	4.18	3.73	2.73	1.73			

Figure 4.49. Change in the transpiration rate in transgenic Unica cultivar were shown for control group, drought-treated transgenic plants, control group for combined stress-treated transgenic heat-treated transgenic plants and combined stress-treated transgenic plants

As seen in Figure 4.49, transpiration rate in plants categorized as control group and drought-treated plants were measured at 1st, 7th, 9th, 12th, 15th, 18th and 20th day, while it was measured at 1st, 7th, 9th and 12th day under heat and combined stress. Transpiration rate in control group showed considerable reduction on the 15th day and reached minimum value of 3.87 µmol m^{-2}/sec at the last measurement. Transpiration rate in drought-treated plants reduced remarkably during the stress period. For heat-treated plants, transpiration rate enhanced until 9th day and then reached 5,28 µmol m^{-2}/sec on 12th day. When heat + drought control was compared with heat + drought treated plants, transpiration rate in the control group enhanced slightly apart from the 12th day and in combined stress-treated transgenic plants decreased greatly from beginning of the stress treatment to end. The greatest difference was found on the 12th day of stress treatment.

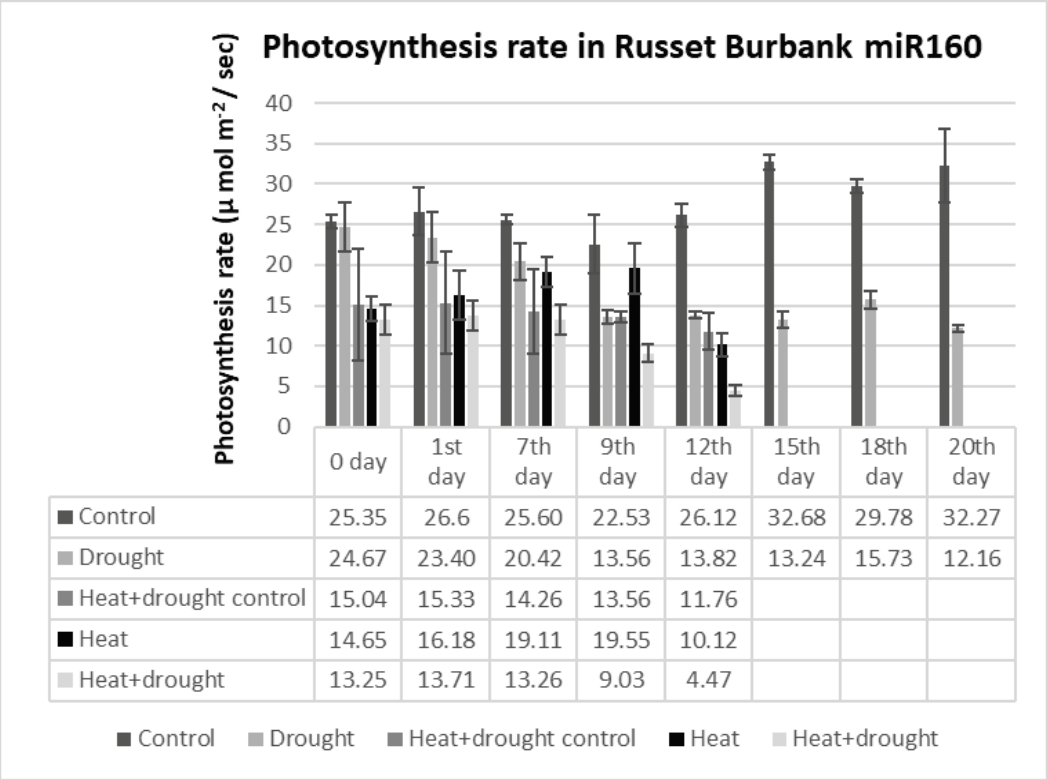
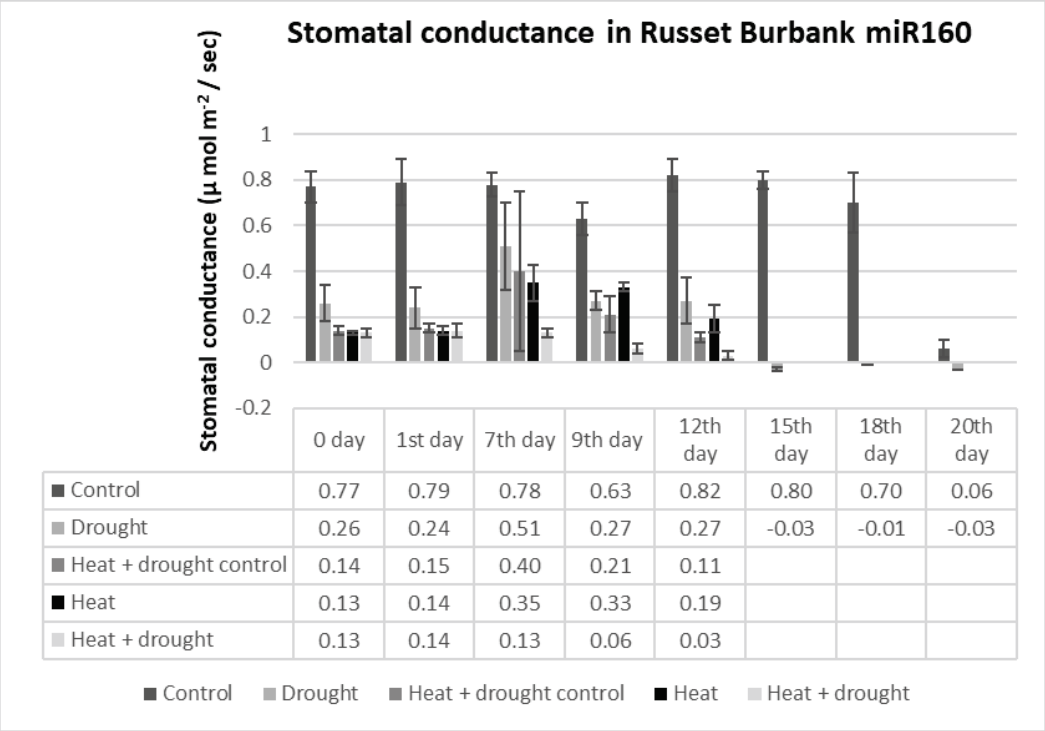


Figure 4.50. Change in the photosynthesis rate in transgenic Russet Burbank cultivar was shown for control group, drought-treated transgenic plants, control group for combined stress-treated transgenic plants heat-treated transgenic plants and combined stress-treated transgenic plants

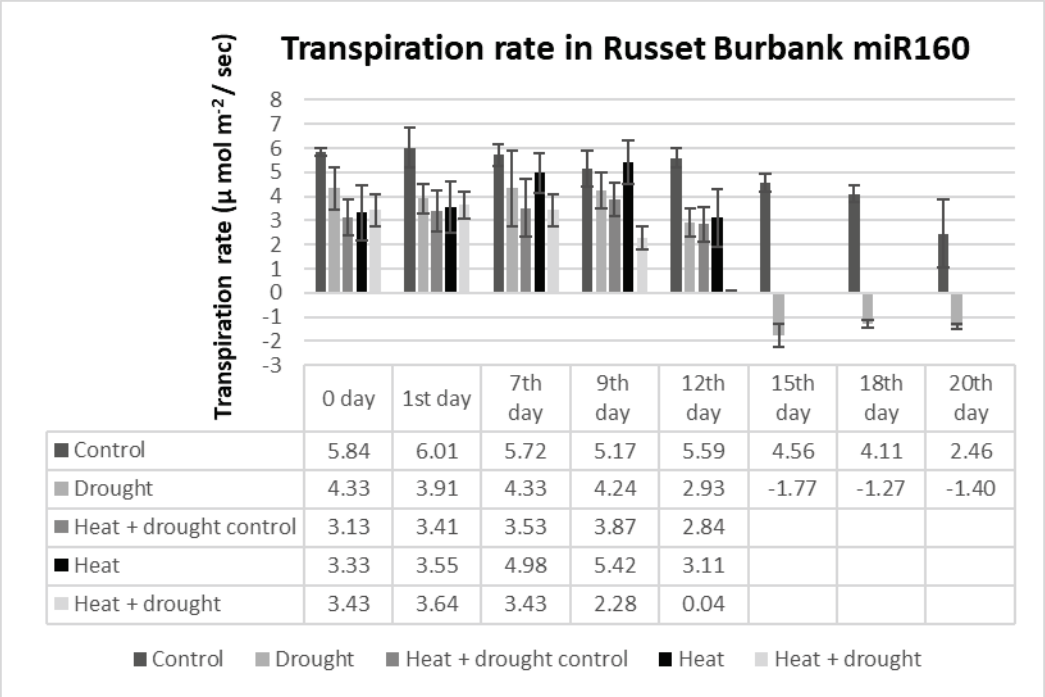
As seen in Figure 4.50, photosynthesis activity in plants categorized as control group and drought-treated plants were measured at 1st, 7th, 9th, 12th, 15th, 18th and 20th day, while it was measured at 1st, 7th, 9th and 12th day under heat and combined stress. Photosynthesis rate in control group increased in 20 days with the greatest value of 32.68 µmol m^{-2}/sec in the 15th day whereas, it reduced mostly in drought-treated plants with the lowest value of 12.16 µmol m^{-2}/sec at the last measurement from initial day to the end of stress period. For heat-treated plants, photosynthesis rate increased until 9th day with the greatest value of 19.55 µmol m^{-2}/sec and then slight reduction was found on the 12th day. When heat + drought control was compared with heat + drought treated plants, photosynthesis rate in control group plants decreased gradually apart from the slight increase on the 1st day and in combined stress-treated transgenic plants reduced significantly on the 12th day. The greatest difference was found at the last day of stress treatment.



	0 day	1st day	7th day	9th day	12th day	15th day	18th day	20th day
Control	0.77	0.79	0.78	0.63	0.82	0.80	0.70	0.06
Drought	0.26	0.24	0.51	0.27	0.27	-0.03	-0.01	-0.03
Heat + drought control	0.14	0.15	0.40	0.21	0.11			
Heat	0.13	0.14	0.35	0.33	0.19			
Heat + drought	0.13	0.14	0.13	0.06	0.03			

Figure 4.51. Change in the stomatal conductance in transgenic Russet Burbank cultivar was shown for control group, drought-treated transgenic plants, control group for combined stress-treated transgenic plants heat-treated transgenic plants and combined stress-treated transgenic plants

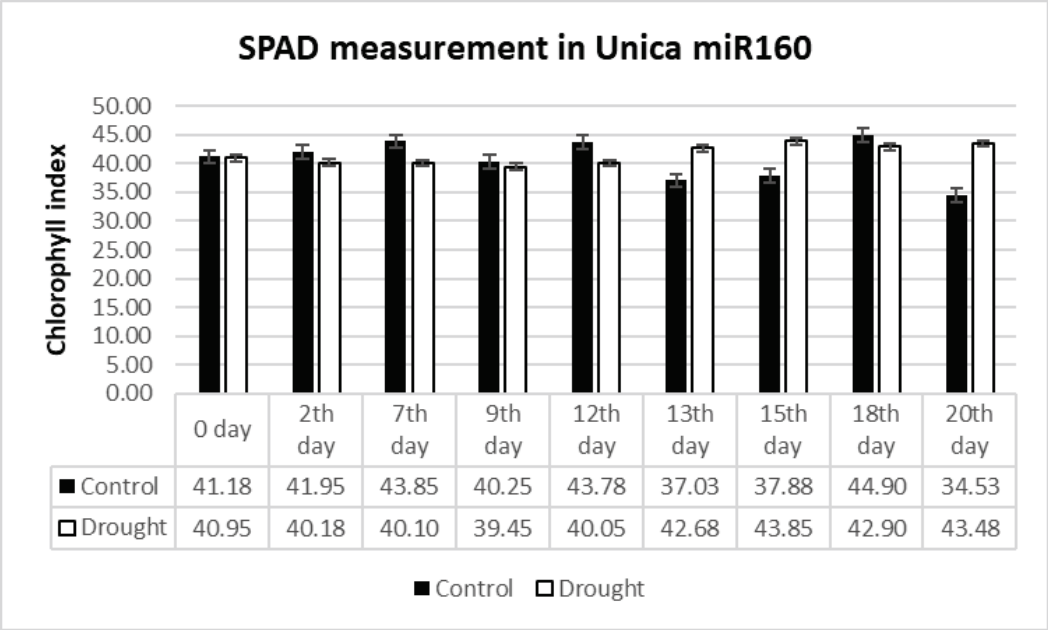
As seen in Figure 4.51, stomatal conductance in plants categorized as control group and drought-treated plants were measured at 1st, 7th, 9th, 12th, 15th, 18th and 20th day, while it was measured at 1st, 7th, 9th and 12th day under heat and combined stress. Stomatal conductance in control group displayed notable reduction for 20 days, with the greatest value 0.82 µmol m^{-2}/sec. Stomatal conductance in drought-treated transgenic plants reduced greatly during stress period. For heat-treated plants, stomatal conductance went up by the 7th day and then went down slowly by 0.19 µmol m^{-2}/sec upon stress treatment. When heat + drought control was compared with heat + drought treated plants, stomatal conductance in control group did not change considerably and in combined stress-treated transgenic plants showed significant decrease with the lowest value of 0.03 µmol m^{-2}/sec on the 12th day.



	0 day	1st day	7th day	9th day	12th day	15th day	18th day	20th day
Control	5.84	6.01	5.72	5.17	5.59	4.56	4.11	2.46
Drought	4.33	3.91	4.33	4.24	2.93	-1.77	-1.27	-1.40
Heat + drought control	3.13	3.41	3.53	3.87	2.84			
Heat	3.33	3.55	4.98	5.42	3.11			
Heat + drought	3.43	3.64	3.43	2.28	0.04			

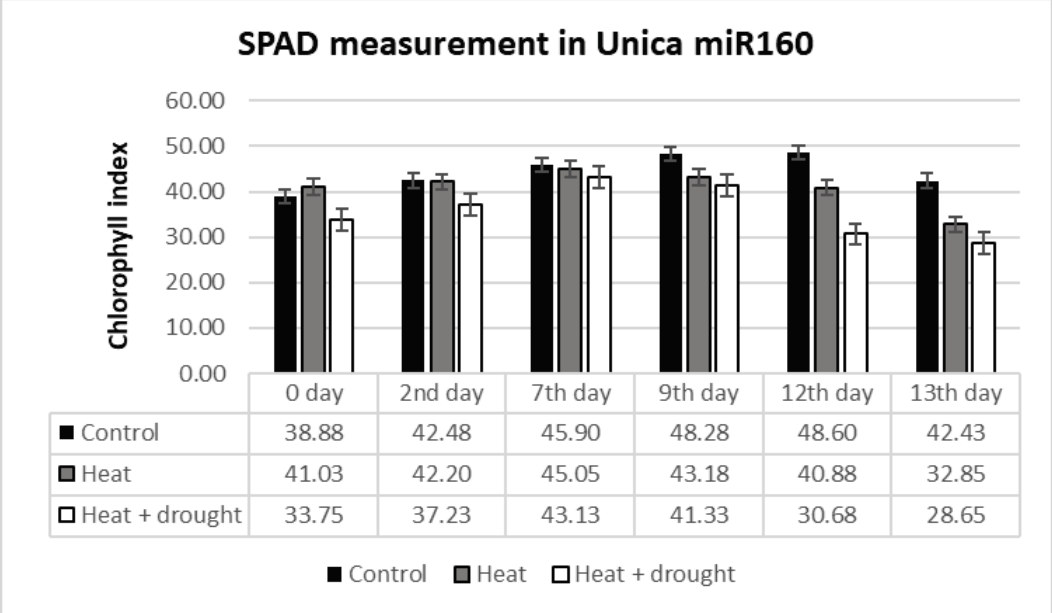
Figure 4.52. Change in the transpiration rate in transgenic Russet Burbank cultivar were shown for control group, drought-treated transgenic plants, control group for combined stress-treated transgenic plants heat-treated transgenic plants and combined stress-treated transgenic plants

As seen in Figure 4.52, transpiration rate in plants categorized as control group and drought-treated plants were measured at 1st, 7th, 9th, 12th, 15th, 18th and 20th day, while it was measured at 1st, 7th, 9th and 12th day under heat and combined stress. Transpiration rate in control group displayed a noteworthy reduction on the 20th day and reached minimum value of 3.87 µmol m^{-2}/sec at the last measurement. Transpiration rate in drought-treated plants reduced remarkably during the stress period. For heat-treated plants, transpiration rate peaked on the 9th day with the value of 5.42 µmol m^{-2}/sec and then reached 3.11 µmol m^{-2}/sec on the 12th day. When heat + drought control was compared with heat + drought treated plants, transpiration rate in the control group enhanced slightly apart from 12th day and in combined stress-treated transgenic plants initially increased slightly and then decreased gradually until last day with the lowest value of 0.04 µmol m^{-2}/sec on the 12th day. The greatest difference was observed on the 9th day of stress treatment.



	0 day	2th day	7th day	9th day	12th day	13th day	15th day	18th day	20th day
Control	41.18	41.95	43.85	40.25	43.78	37.03	37.88	44.90	34.53
Drought	40.95	40.18	40.10	39.45	40.05	42.68	43.85	42.90	43.48

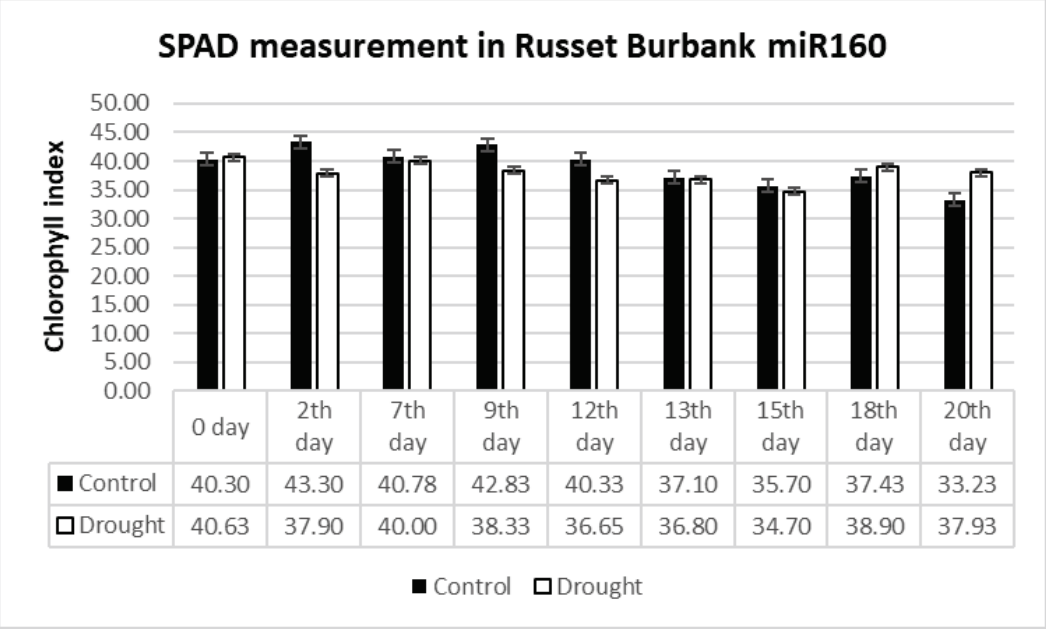
Figure 4.53. Change in the SPAD values in transgenic Unica cultivar was shown for control group and drought-treated plants after 20-day drought treatment



	0 day	2nd day	7th day	9th day	12th day	13th day
Control	38.88	42.48	45.90	48.28	48.60	42.43
Heat	41.03	42.20	45.05	43.18	40.88	32.85
Heat + drought	33.75	37.23	43.13	41.33	30.68	28.65

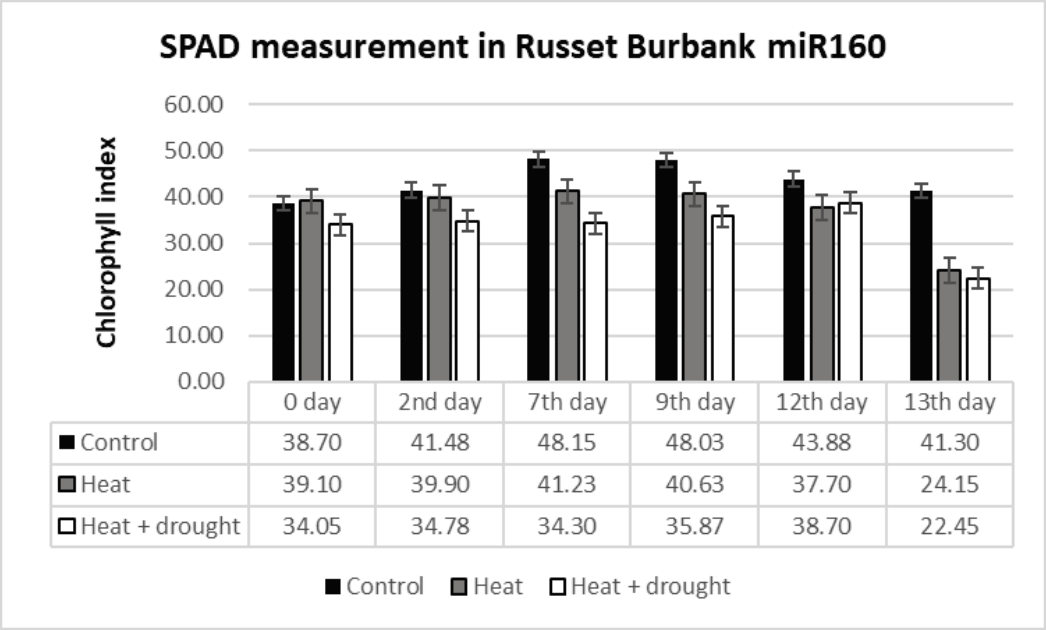
Figure 4.54. Change in the SPAD values in transgenic Unica cultivar was shown for control group, heat-treated plants and combined stress-treated plants after 12-day heat and combined treatment

As seen in Figure 4.53 and Figure 4.54, chlorophyll index in transgenic Unica plants categorized as control group and drought-treated plants, were measured at 0^{th}, 2^{nd}, 7^{th}, 9^{th}, 12^{th}, 13^{th}, 15^{th}, 18^{th} and 20^{th} day, while it was measured at 0^{th}, 2^{nd}, 7^{th}, 9^{th}, 12^{th} and 13^{th} day under heat and combined stress. Chlorophyll index in control group increased gradually until the 12^{th} day with the greatest value of 43.85 in the 7^{th} day. Chlorophyll index in drought-treated plants peaked on the 15^{th} day and reached minimum level on 9^{th} day. For heat-treated plants, chlorophyll index reduced remarkably on the 13^{th} day and it was found less than control group at the end of 12-day stress treatment. Similarly, when heat + drought control was compared with heat + drought treated plants, chlorophyll index in the combined stress-treated plants displayed steep reduction on the 12^{th} day and in control group increased gradually until the 12^{th} day with the greatest value of 48.6. The greatest difference between those groups was observed at the last measurement of stress period.



	0 day	2th day	7th day	9th day	12th day	13th day	15th day	18th day	20th day
Control	40.30	43.30	40.78	42.83	40.33	37.10	35.70	37.43	33.23
Drought	40.63	37.90	40.00	38.33	36.65	36.80	34.70	38.90	37.93

Figure 4.55. Change in the SPAD values in transgenic Russet Burbank cultivar were shown for control group and drought-treated plants after 20-day drought treatment



	0 day	2nd day	7th day	9th day	12th day	13th day
Control	38.70	41.48	48.15	48.03	43.88	41.30
Heat	39.10	39.90	41.23	40.63	37.70	24.15
Heat + drought	34.05	34.78	34.30	35.87	38.70	22.45

Figure 4.56. Change in the SPAD values in transgenic Russet Burbank cultivar was shown for control group, heat-treated plants and combined stress-treated plants after 12-day heat and combined treatment

As seen in Figure 4.55 and Figure 4.56, chlorophyll index in transgenic Russet Burbank plants categorized as control group and drought-treated plants were measured at 0^{th}, 2^{nd}, 7^{th}, 9^{th}, 12^{th}, 13^{th}, 15^{th}, 18^{th} and 20^{th} day, while it was measured at 0^{th}, 2^{nd}, 7^{th}, 9^{th}, 12^{th} and 13^{th} day under heat and combined stress. Chlorophyll index in control group fluctuated during stress period with the greatest value of 43.3 on the 2^{nd} day. Similarly, chlorophyll index in drought-treated plants fluctuated and reached minimum level on the 15^{th} day under drought stress. For heat-treated plants, chlorophyll index reduced remarkably on the 13^{th} day and it was found considerably less than control group at the end of 12-day stress treatment. Similarly, when heat + drought control was compared with heat + drought treated plants, chlorophyll index in the combined stress-treated plants showed rapid reduction at the 12^{th} day and in control group went up gradually until the 7^{th} day with the greatest value of 48.15. The greatest difference between those groups was found on the 13^{th} day.

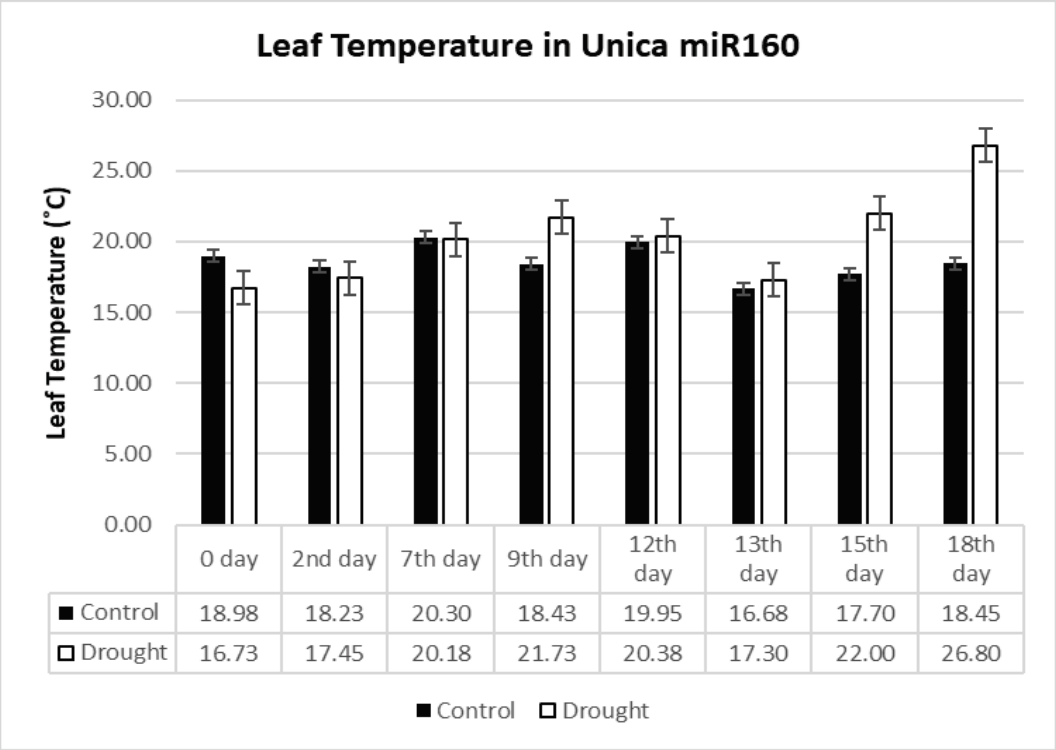


Figure 4.57. Change in the leaf temperature (˚C) in transgenic Unica cultivar were shown for control group and drought-treated plants after 20-day drought treatment

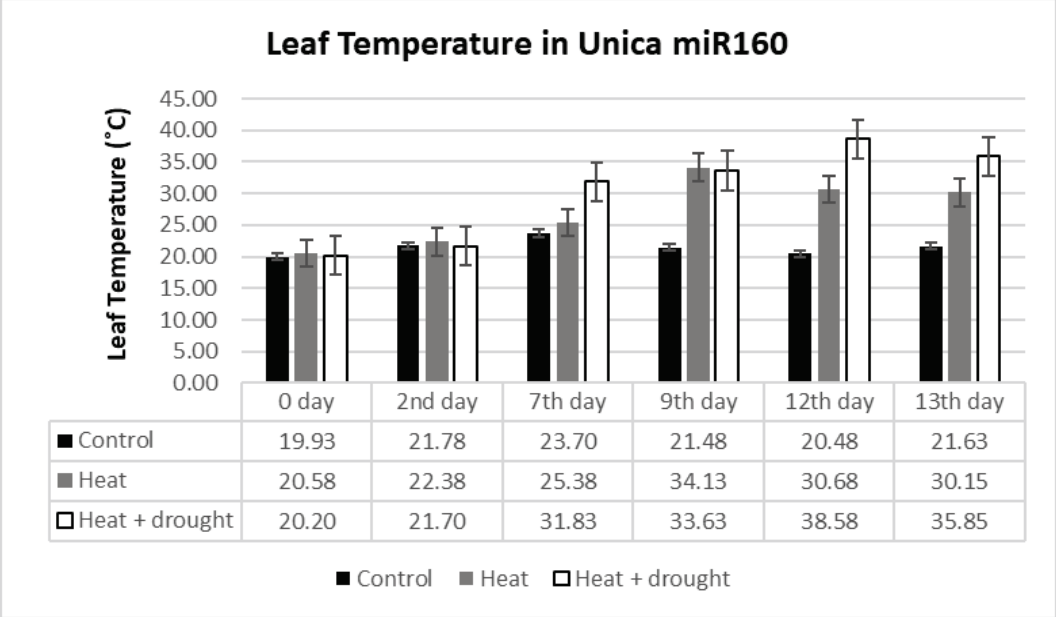
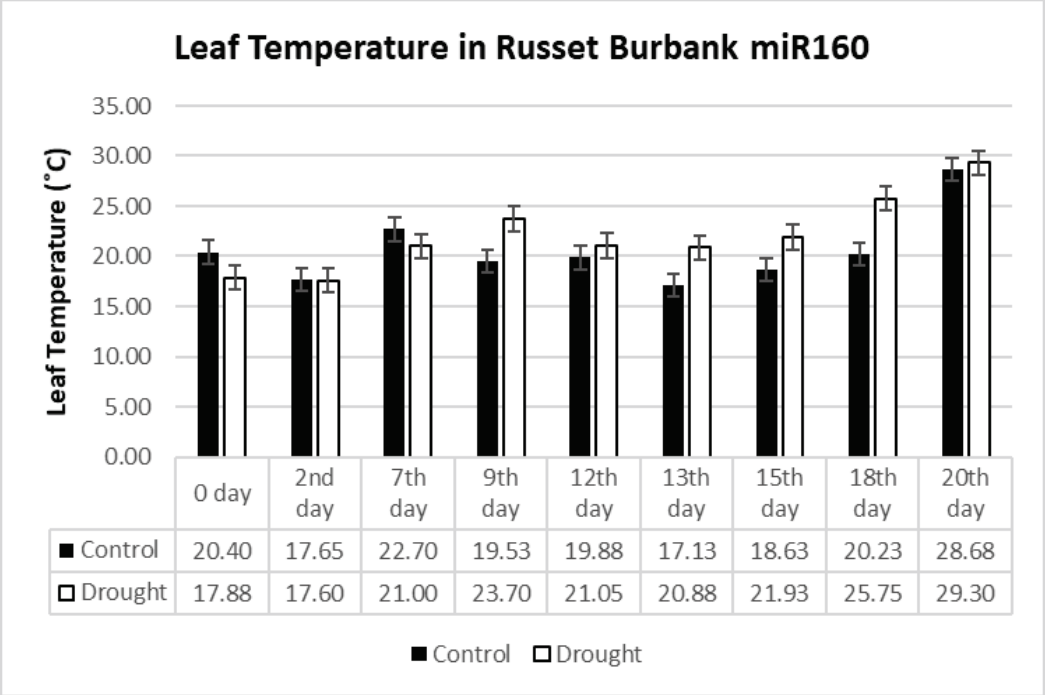


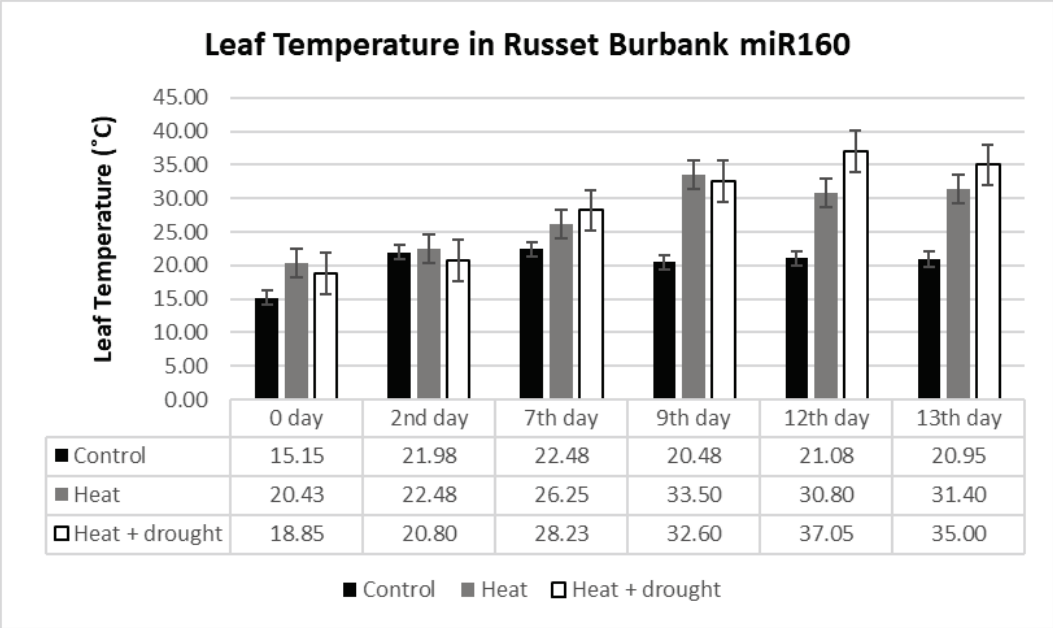
Figure 4.58. Change in the leaf temperature (˚C) in transgenic Unica cultivar was shown for control group, heat-treated plants and combined stress-treated plants after 12-day heat and combined treatment

As seen in Figure 4.57 and Figure 4.58, leaf temperature in transgenic Unica plants categorized as control group and drought-treated plants were measured at 0th, 2nd, 7th, 9th, 12th, 13th, 15th, 18th and 20th day, while it was measured at 0th, 2nd, 7th, 9th, 12th and 13th day under heat and combined stress. Leaf temperature in control group showed slight fluctuation with the greatest value of 32.10 ˚C on the 20th day. Leaf temperature in drought-treated plants peaked on the 20th day as well fluctuation during stress period. For heat-treated plants, leaf temperature augmented significantly in the 9th day and was greater than control group at the last measurement. Similarly, when heat + drought control was compared with heat + drought treated plants, leaf temperature in the combined stress-treated plants increased until 12th day with the maximum value of 38.57 ˚C and in control group fluctuated around 21 ˚C. The biggest difference between those groups were found on the 12th day during stress period.



	0 day	2nd day	7th day	9th day	12th day	13th day	15th day	18th day	20th day
Control	20.40	17.65	22.70	19.53	19.88	17.13	18.63	20.23	28.68
Drought	17.88	17.60	21.00	23.70	21.05	20.88	21.93	25.75	29.30

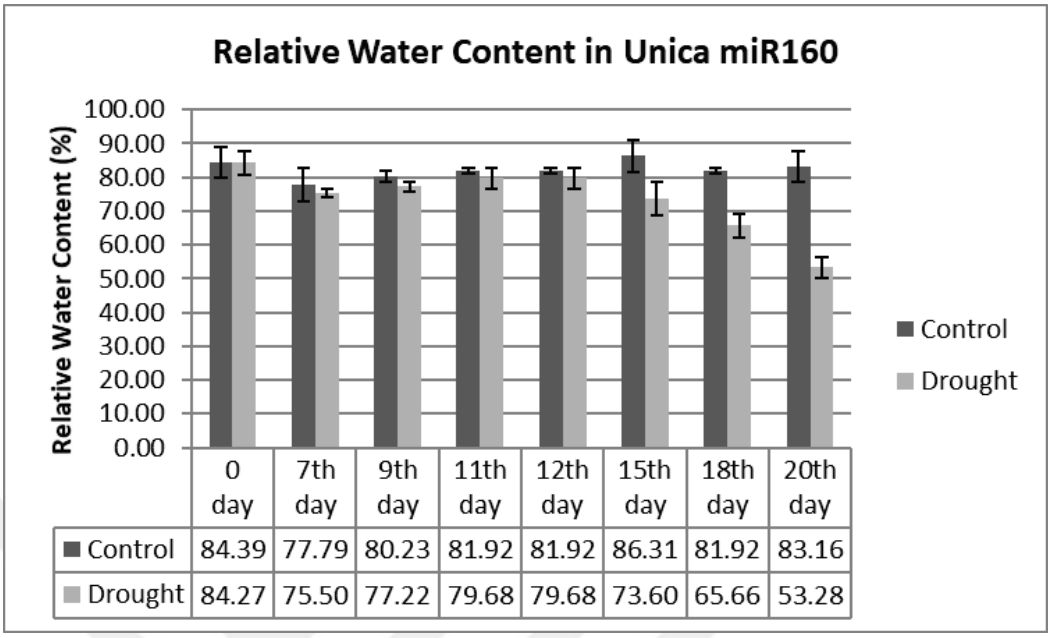
Figure 4.59. Change in the leaf temperature (˚C) in transgenic Russet Burbank cultivar was shown for control group and drought-treated plants after 20-day drought treatment



	0 day	2nd day	7th day	9th day	12th day	13th day
Control	15.15	21.98	22.48	20.48	21.08	20.95
Heat	20.43	22.48	26.25	33.50	30.80	31.40
Heat + drought	18.85	20.80	28.23	32.60	37.05	35.00

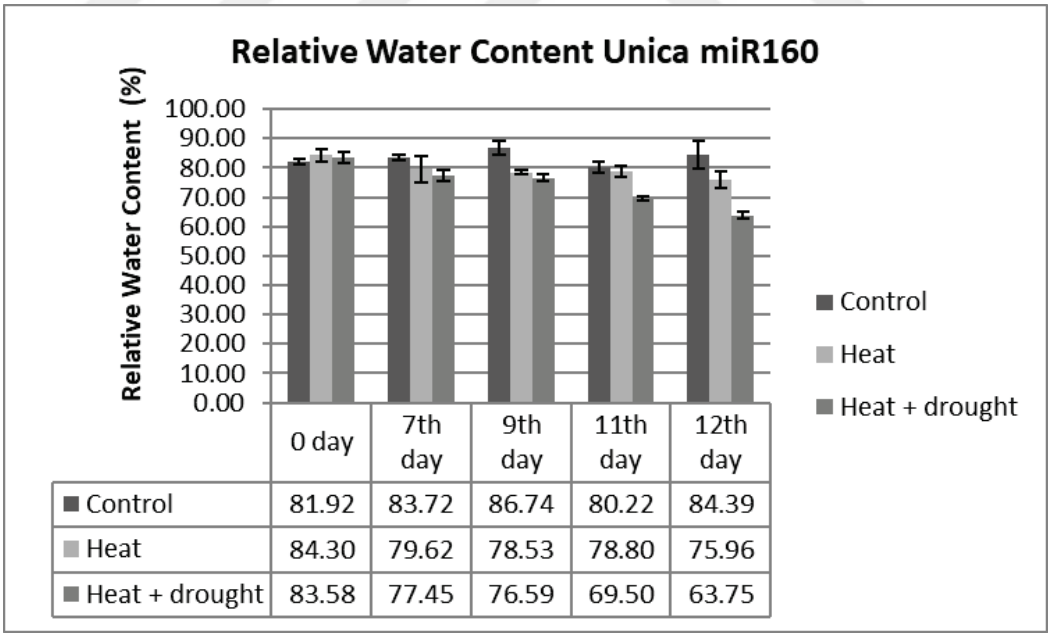
Figure 4.60. Change in the leaf temperature (˚C) in transgenic Russet Burbank cultivar was shown for control group, heat-treated plants and combined stress-treated plants after 12-day heat and combined treatment

As seen in Figure 4.59 and Figure 4.60, leaf temperature in transgenic Russet Burbank plants categorized as control group and drought-treated plants, were measured at 0^{th}, 2^{nd}, 7^{th}, 9^{th}, 12^{th}, 13^{th}, 15^{th}, 18^{th} and 20^{th} day, while it was measured at 0^{th}, 2^{nd}, 7^{th}, 9^{th}, 12^{th} and 13^{th} day under heat and combined stress. Leaf temperature in control group showed slight fluctuation with the greatest value of 28.67 ˚C on the 20^{th} day. Leaf temperature in drought-treated plants peaked at the 20^{th} day as well as fluctuation under stress condition. For heat-treated plants, leaf temperature increased considerably in the 9^{th} day and was greater compared to that of control group at the last measurement. Similarly, when heat + drought control was compared with heat + drought treated plants, leaf temperature in combined stress treated plants increased until the 12^{th} day with the maximum value of 37.05 ˚C and in control group fluctuated at around 20˚C. The largest difference between those groups were found on the 12^{th} day during stress period, similar to Unica.



	0 day	7th day	9th day	11th day	12th day	15th day	18th day	20th day
Control	84.39	77.79	80.23	81.92	81.92	86.31	81.92	83.16
Drought	84.27	75.50	77.22	79.68	79.68	73.60	65.66	53.28

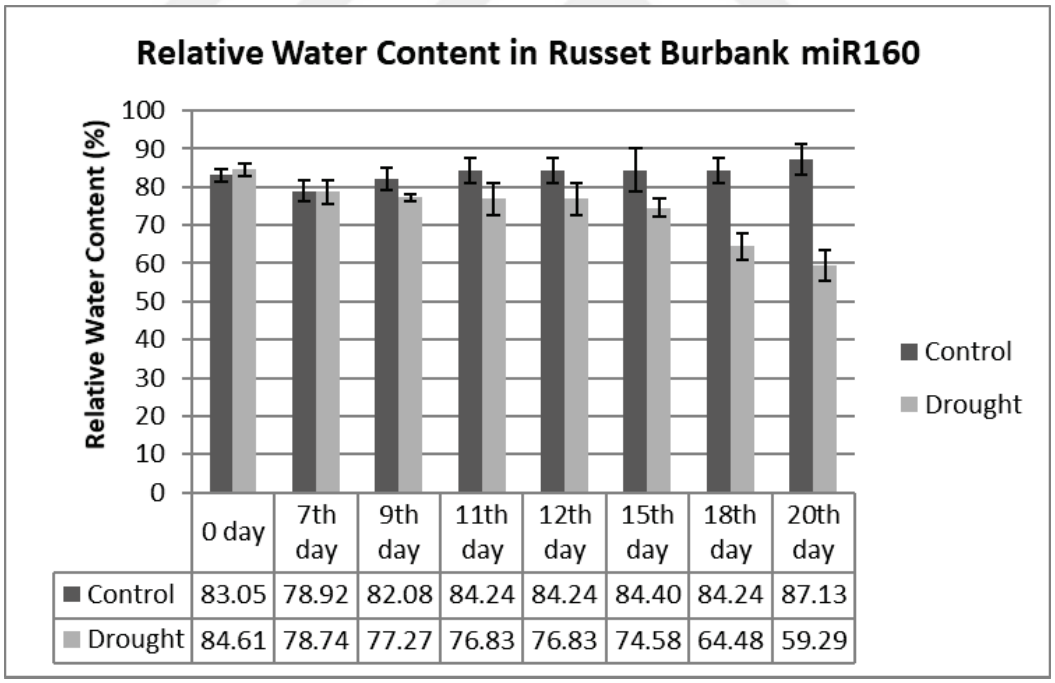
Figure 4.61. Change in the relative water content (%) in transgenic Unica cultivar for control group and drought-treated plants after 20-day drought treatment was shown



	0 day	7th day	9th day	11th day	12th day
Control	81.92	83.72	86.74	80.22	84.39
Heat	84.30	79.62	78.53	78.80	75.96
Heat + drought	83.58	77.45	76.59	69.50	63.75

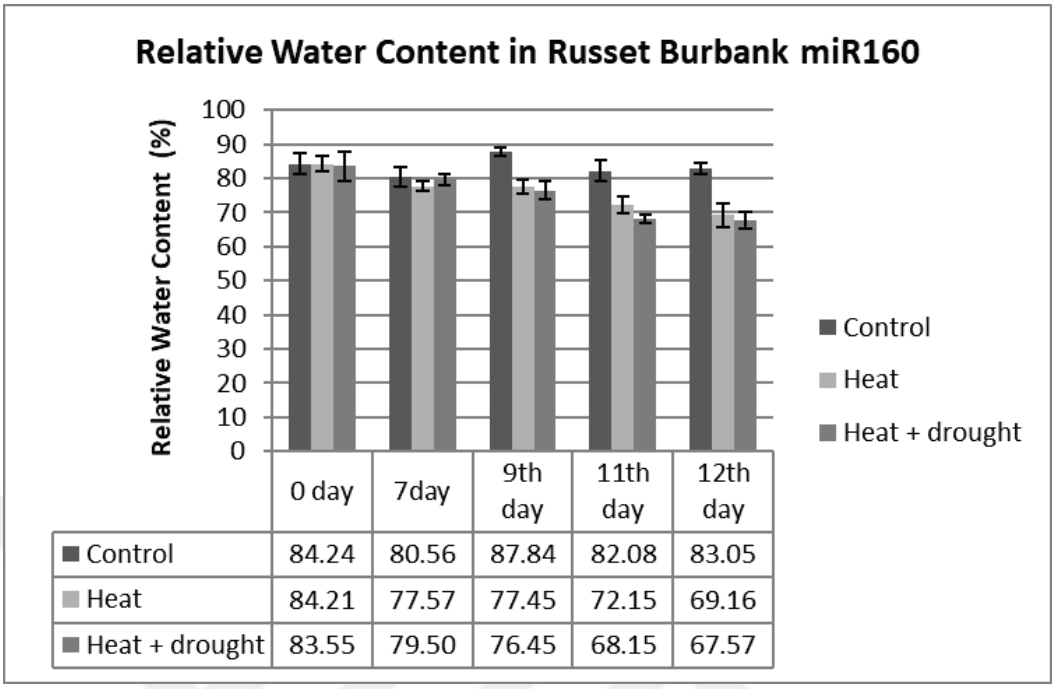
Figure 4.62. Change in the relative water content (%) in transgenic Unica cultivar for control group, heat-treated plants and combined stress-treated plants after 12-day heat and combined treatment was shown

As seen in Figure 4.61 and Figure 4.62, relative water content in transgenic Unica plants categorized as control group, drought-treated plants, were measured at 0^{th}, 7^{th}, 9^{th}, 11^{th},12^{th}, 15^{th}, 18^{th} and 20^{th} day while, it was measured at 0^{th}, 7^{th}, 9^{th}, 11^{th} and 12^{th} day under heat and combined stress. Relative water content in control group showed slight fluctuation with the greatest value of 86.31% in the 15^{th} day. Relative water content in drought-treated plants initially increased slightly until 11^{th} day and then started to decrease gradually by 20^{th} day with the lowest value of 53.28%. For heat-treated plants, relative water content reduced significantly on the 7^{th} day under heat stress and it reached the smallest value of at the last measurement. When heat + drought control was compared with heat + drought treated plants, relative water content in combined stress treated plants decreased gradually by 63.75 % and in control group displayed less fluctuation with the value of around 82% during stress period. The largest difference between those groups was observed on the 12^{th} day upon stress treatment.



	0 day	7th day	9th day	11th day	12th day	15th day	18th day	20th day
Control	83.05	78.92	82.08	84.24	84.24	84.40	84.24	87.13
Drought	84.61	78.74	77.27	76.83	76.83	74.58	64.48	59.29

Figure 4.63. Change in the relative water content (%) in transgenic Russet Burbank cultivar was shown for control group and drought-treated plants after 20-day drought treatment



	0 day	7day	9th day	11th day	12th day
Control	84.24	80.56	87.84	82.08	83.05
Heat	84.21	77.57	77.45	72.15	69.16
Heat + drought	83.55	79.50	76.45	68.15	67.57

Figure 4.64. Change in the relative water content (%) in transgenic RBB cultivar was shown for control group, heat-treated plants and combined stress-treated plants after 12-day heat and combined treatment

As seen in Figure 4.63 and Figure 4.64, relative water content in transgenic Russet Burbank plants categorized as control group and drought-treated plants were measured at 0^{th}, 7^{th}, 9^{th}, 11^{th},12^{th}, 15^{th}, 18^{th} and 20^{th} day, while it was measured at 0^{th}, 7^{th}, 9^{th}, 11^{th} and 12^{th} day under heat and combined stress. Relative water content in control group showed slight fluctuation between 78.92-87.13%. Relative water content in drought-treated plants went down gradually by the 20^{th} day with the lowest value of 59.29% at last measurement. Similarly, relative water content in heat-treated plants reduced considerably on the 7^{th} day under heat stress and it reached the smallest value at the last measurement. When the heat + drought control was compared with heat + drought treated plants, relative water content in combined stress treated plants decreased gradually by 67.57% until 12^{th} day and in control group showed less fluctuation with the value of around 83% during stress period. The largest difference between those groups was observed on the 12^{th} day with the value of 15.48% upon stress treatment.

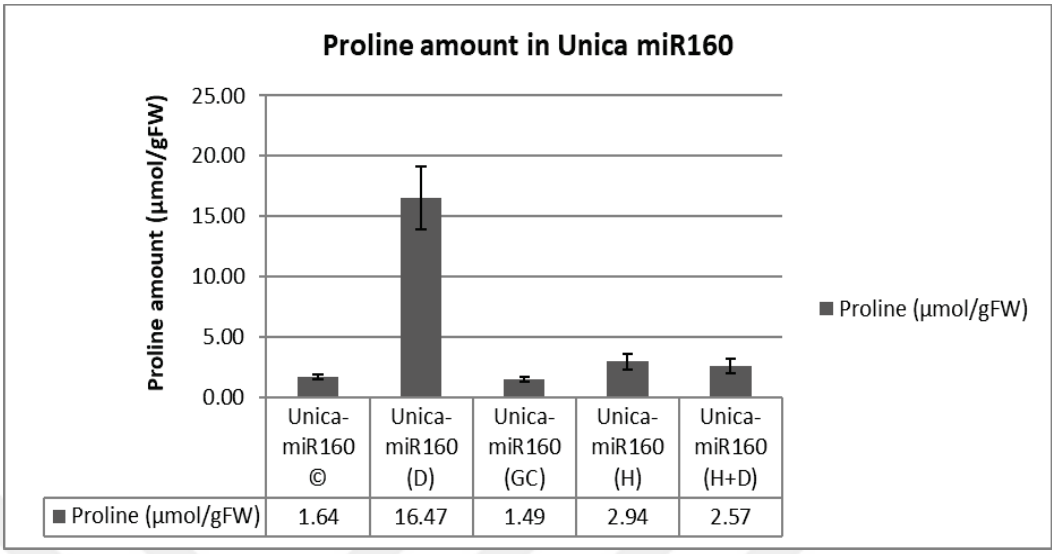


Figure 4.65. Proline accumulation in transgenic Unica plants were shown for control group, drought-treated plants, control for combined stress-treated plants, heat-treated plants and combined stress-treated plants

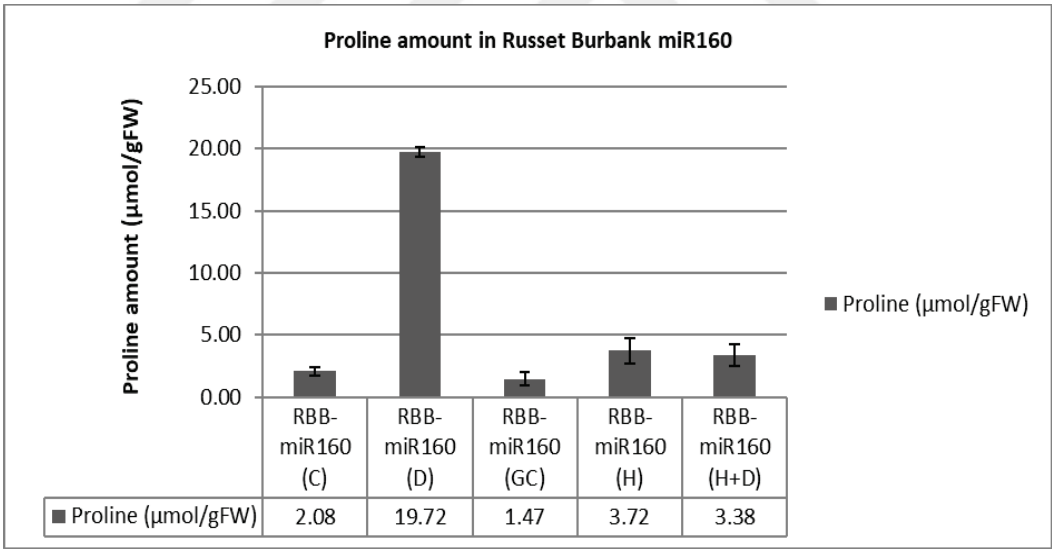


Figure 4.66. Proline accumulation in transgenic Russet Burbank plants were shown for control group, drought-treated plants, control for combined stress-treated plants, heat-treated plants and combined stress-treated plants

Proline amount in transgenic Unica and Russet Burbank plants were measured under drought, heat and a combination of heat and drought stresses. In Unica cultivar, proline accumulation in drought-treated plants was found the highest level than others with around 9-fold greater than control group. In Russet Burbank cultivar, proline content in drought-treated plants was observed greater than others with around 8-fold greater than

control group. Besides, proline accumulation in drought-treated plants was observed greater compared to other wild-type plants subjected to heat or heat + drought stress.

4.3.5 Comparison of physiological parameters in wild-type and transgenic plants

4.3.4.1 Photosynthesis rate

On 20th day, photosynthesis rate decreased by 68.16 % in wild-type Unica plants under drought stress whereas, photosynthesis rate in transgenic Unica plants reduced by 69.13 %. On the12th day, photosynthesis activity increased by 15.99% in transgenic Unica plants upon heat stress and 22.51% in wild-type Unica plants. However, heat + drought stress caused the greatest reduction in photosynthesis rate by 56.44 % on the 12th day in wild-type Unica plants and significant reduction in photosynthesis rate by 74.17% on the 9th day in transgenic plants. Besides, photosynthesis rate reduced by 71.07 % in wild-type Russet Burbank plants under drought stress, while it decreased by 62.32% in transgenic Russet Burbank plants. Upon heat treatment, photosynthesis activity in wild-type Russet Burbank plants decreased by 44.37 % on the 12th day and in transgenic Russet Burbank plants reduced by 13.5% on the 12th day. After heat combined with drought stress period, the rate of 73.5% reduction was observed in wild-type Russet Burbank plants 61.09% decrease was found in transgenic Russet Burbank plants on the 12th day.

4.3.4.2 Stomatal conductance

On the 12th day, stomatal conductance reduced by 66.67% and 68.35% under drought stress in wild-type Unica and transgenic Unica plants, respectively. After combined stress, stomatal conductance decreased considerably 88.89% and 95.00% on the 12th day in wild-type Unica and transgenic Unica plants, respectively. Upon heat treatment, at the rate of 66.67% reduction in stomatal conductance was discovered in wild-type Russet Burbank plants, whereas 72.73% increase in transgenic Russet Burbank plants were found on the 12th day.

4.3.4.3 Transpiration rate

On the 12th day, transpiration rate restricted by 74.77% and 47.58% upon drought treatment in wild-type Russet Burbank and transgenic Russet Burbank plants, respectively. It was reduced notably by 88.29% and 98.59% in wild-type Russet Burbank and transgenic Russet Burbank plants on the 12th day under a combination of heat and drought condition, respectively. Additionally, a reduction of 65.19% in transpiration rate in wild-type Unica plants was found on the same day, while a reduction of 61.30% was observed in transgenic Unica plants.

4.3.5.4 Chlorophyll index

On the 13th day, chlorophyll index decreased by 7.89% and 25.92% in drought-treated wild-type Unica and transgenic Unica plants, respectively. By contrast, chlorophyll index reduced by 50.10% and 22.57% after heat stress in wild-type Unica and transgenic Unica plants, respectively. Upon combined stress, a decrease of 61.67% was observed on the 13th day in wild-type Unica and of 33.42% in transgenic Unica plants. On the other hand, chlorophyll index decreased by 60.67% and 45.64% on the 13th day stress in wild-type Unica and transgenic Unica plants under a combination of drought and heat stress, respectively. A decrease of 60.67% in chlorophyll amount was discovered in heat-treated wild-type Russet Burbank and of 45.64% was found on the 13th day in heat-treated transgenic plants.

4.3.5.5 Leaf temperature

The greatest changes in the leaf temperatures were observed in different days in wild-type and transgenic plants. On the 15th day, leaf temperature increased by 26.36% in drought-treated wild-type Unica although, it raised by 45.26% in transgenic Unica plants under drought stress. Upon heat treatment, leaf temperature enhanced by 72.35% on the 12th day and 58.91% on the 9th day in wild-type and transgenic Unica plants, respectively. On the 15th day, leaf temperature increased by 30.29% in drought-treated wild-type Russet Burbank although, it enhanced by 27.32% in transgenic Russet Burbank plants under drought stress. Upon heat treatment, leaf temperature enhanced by 73.49% on the 13th day and 63.61% on the 9th day in wild-type and transgenic Russet

Burbank plants, respectively. Increase in the leaf temperature in both cultivars caused by heat + drought was observed more severe than individual effect.

4.3.5.6 Relative water content

On the 20th day, relative water content limited by 39.25% and 35.93% in drought-treated wild-type Unica and transgenic Unica plants, respectively. It was also reduced by 33.14% in wild-type Russet Burbank plants and 31.95% in transgenic Russet Burbank plants. A reduction of 15.66% in relative water content was discovered on the 12th day in heat-treated wild-type Russet Burbank plants, while a decrease of 16.72% in relative water content was detected on the 12th day in heat-treated transgenic Russet Burbank plants. It fell by 20.27% on the 12th day in wild-type Russet Burbank plants and 18.64% in transgenic Russet Burbank plants under heat combined with drought stress.

4.3.5.7 Proline content

Proline content in wild-type and transgenic plants increased considerably under drought stress compared to heat or combined stresses. In Unica cultivar, change in the proline content in drought-treated wild-type plants was found 7-fold greater than control, while it was observed 9-fold higher in drought-treated transgenic plants as compared to respective control plants. In Russet Burbank cultivar, proline content in the drought-treated wild-type plants was 12-fold greater than control group, whereas it increased by 8-fold in drought-treated transgenic plants compared to control plants. Upon heat treatment, proline content in transgenic Unica plants slightly increased compared to wild-type Unica plants, whereas proline content in wild-type Russet Burbank plants augmented a little more than transgenic Russet Burbank plants. By contrast, proline amount in wild-type Unica plants was measured slightly higher compared to transgenic Unica plants, although, it was found more than 2-fold in wild-type Russet Burbank plants than transgenic Russet Burbank plants.

4.4 Molecular Studies on Transgenic Plants

Before starting qRT-PCR, total RNA measurement was done for samples including control group for drought and drought-treated plants, heat-treated plants, combined stress-treated plants and control group for combined stress-treated plants by using spectrophotometer (Table 4.1). Besides, gel electrophoresis results were shown followed by dilution of samples (Figure 4.67).

Table 4.1. RNA concentration (ng/ µl) of samples for qRT-PCR

	Concentration ng/µl	**260/280**	**260/230**
Drought Control			
Unica Wild Type	2121.12	1.990	1.174
Unica miR160	771.92	2.040	1.065
Russet Burbank Wild Type	1047.72	2.026	0.79
Russet Burbank miR160	926.60	2.007	0.859
Drought Stress			
Unica Wild Type	796.20	2.064	1.727
Unica miR160	7233.68	2.081	0.905
Russet Burbank Wild Type	1246.16	2.087	1.397
Russet Burbank miR160	726.08	1.994	1.024
Heat Stress			
Unica Wild Type	1530.92	2.088	1.177
Unica miR160	1588.80	2.062	1.104
Russet Burbank Wild Type	1059.68	2.081	1.143
Russet Burbank miR160	867.60	2.023	0.713
Heat + Drought Stress			
Unica Wild Type	1695.50	2.080	1.636
Unica miR160	1463.84	2.064	1.038
Russet Burbank Wild Type	1327.16	2.113	1.450
Russet Burbank miR160	1274.80	2.083	1.250
Heat + Drought Control			
Unica Wild Type	964.28	2.046	0.908
Unica miR160	1466.60	2.062	0.928
Russet Burbank Wild Type	721.64	2.047	0.702
Russet Burbank miR160	1066.80	2.027	0.583

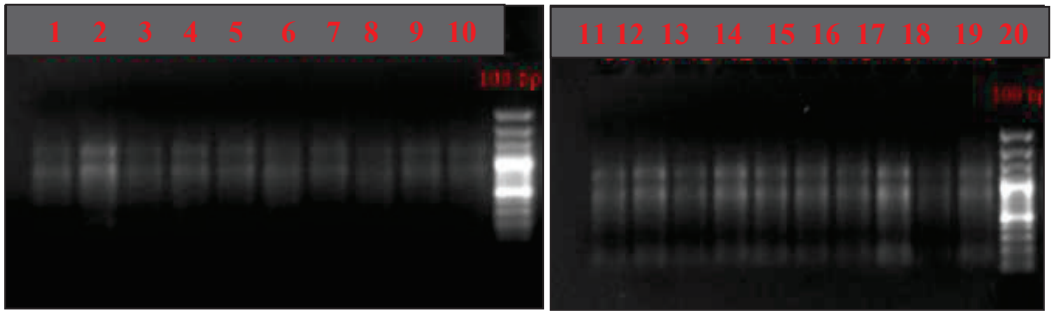


Figure 4.67. Agarose gel image was given after dilution step using Thermo Scientific 100 bp marker. (1) Drought control Unica wild type, (2) Drought control Unica miR160, (3) Drought control RBB wild type, (4) Drought control RBB miR160, (5) Drought Unica wild type, (6) Drought Unica miR160, (7) Drought RBB wild type, (8) Drought RBB miR160, (9) Heat Unica wild type (10) Heat Unica MiR160, (11) Heat RBB wild type, (12) Heat RBB miR160, (13) Heat + drought Unica wild type (14) Heat + drought Unica miR160, (15) Heat + drought RBB wild type, (16) Heat + drought RBB miR160, (17) Heat + drought control Unica wild type ,(18) Heat + drought control Unica miR160, (19) Heat + drought control RBB wild type, (20) Heat + drought control RBB miR160

Expression of EF-1α was tested before starting measurement of miR160 expression and corresponding target gene in transgenic plants (Figure 4.68). Subsequently, expression of miR160 and relative target gene were tested in transgenic Unica and Russet Burbank lines using qRT-PCR (Figure 4.69) (Figure 4.70) (Figure 4.71) (Figure 4.72). Melting curve related to miR160 and target gene were also taken from qRT-PCR (Figure 4.73).

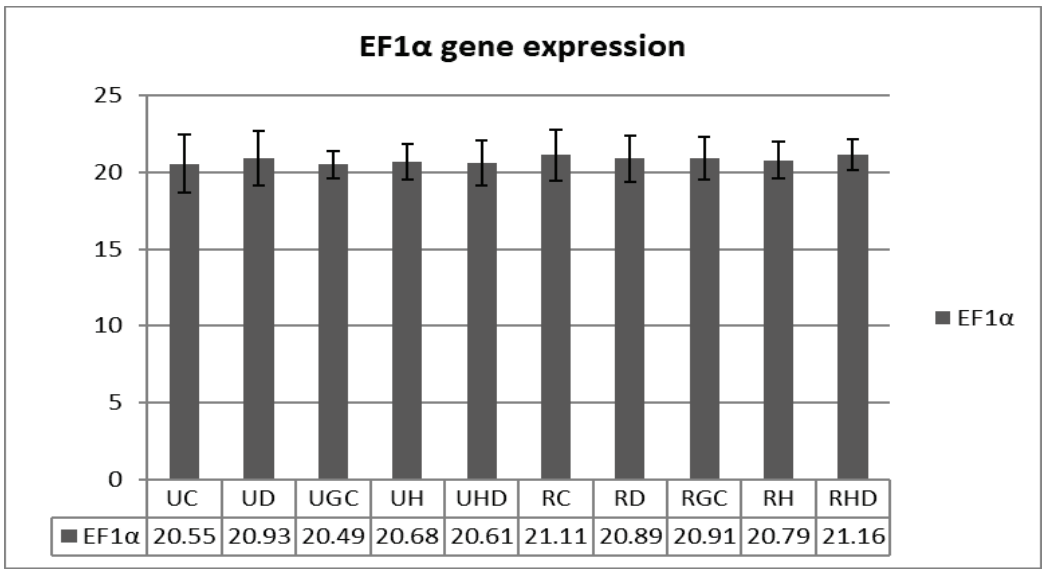


Figure 4.68. Expression of EF-1α gene was shown in T0 transgenic Unica and Russet Burbank lines

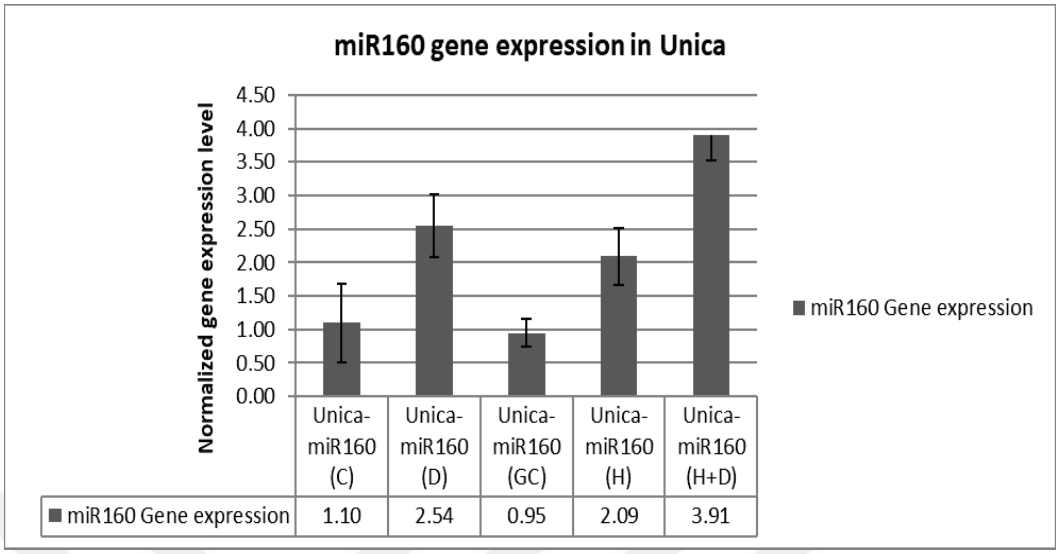


Figure 4.69. Expression of miR160 was given in T0 transgenic Unica line

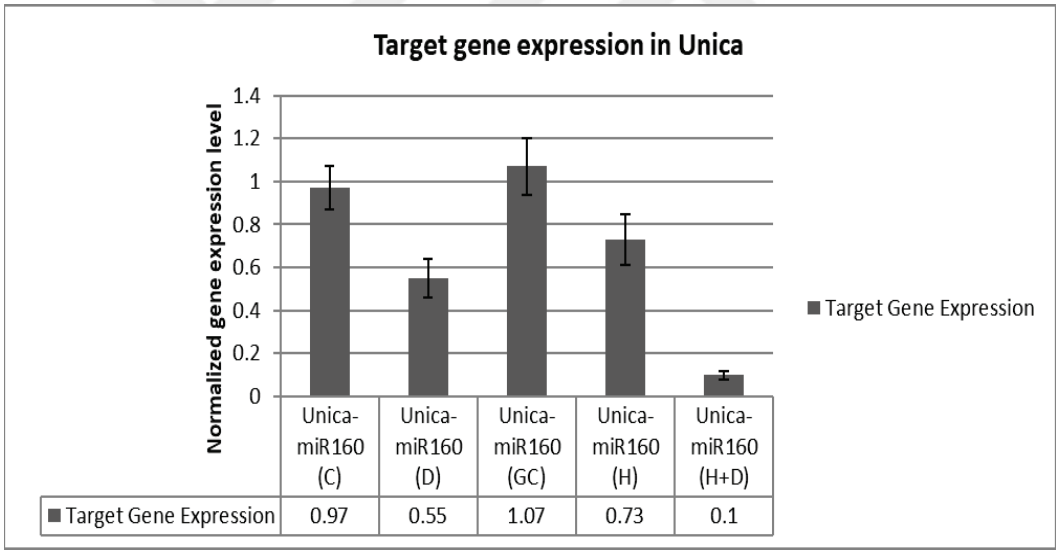


Figure 4.70. Expression of target gene was given in T0 transgenic Unica line

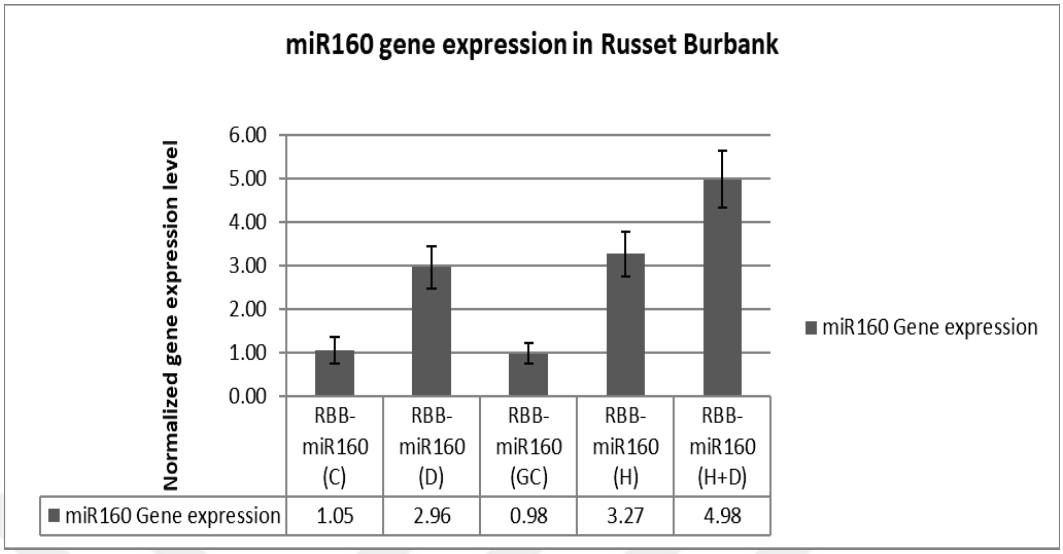


Figure 4.71. Expression of miR160 was displayed in T0 transgenic Russet Burbank line

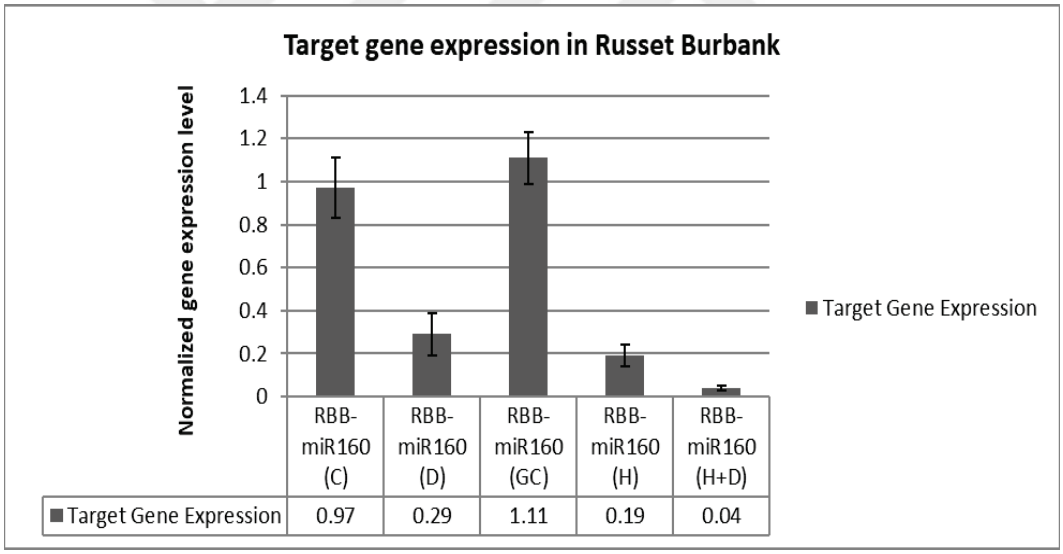


Figure 4.72. Expression of target gene was given in T0 transgenic Russet Burbank line

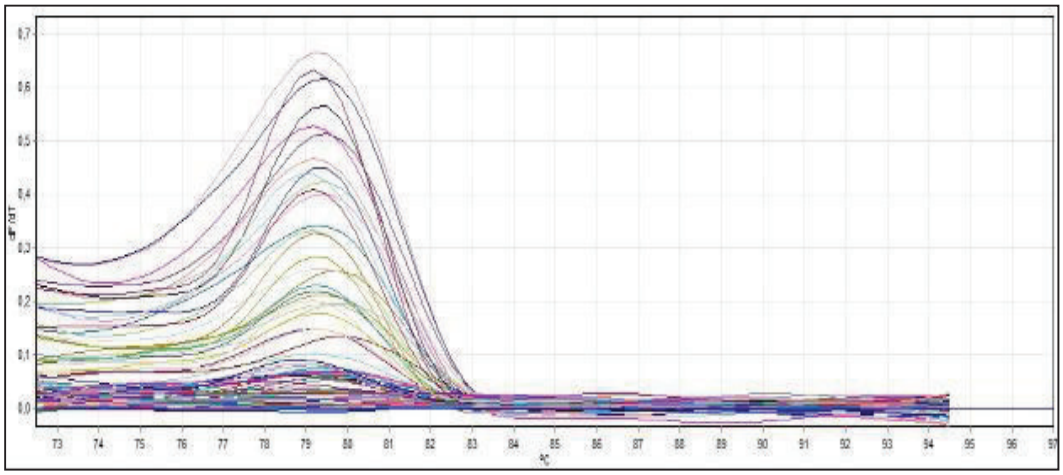


Figure 4.73. Melting curve for specific to miR160 and target gene

As seen in Figure 4.73, amplification of miR160 and target gene were achieved since two different peaks were observed.

CHAPTER V

DISCUSSION

In this thesis, function of miR160 in contrasting potato cultivars including Unica and Russet Burbank was studied under drought, heat and a combination of drought and heat stresses. For this purpose, this study examined responses of wild-type and transgenic plants under stress conditions as regards of morphological differences, physiological traits including photosynthesis rate, stomatal conductance, transpiration rate, chlorophyll index, leaf temperature, relative water content and proline amount, as well as molecular proof.

5.1 Comparison of Wild-type and Transgenic Plants Before Stress Treatment

5.1.1 Morphological traits

Overexpression of miR160 caused some morphological differences such as leaf number, leaf surface and stem length in transgenic plants as compared to wild-type plants. As specified in Figure 4.15, it was observed that decreased foliage in transgenic Unica plants were found compared to wild-type plants indicating that photosynthesis, stomatal conductance and transpiration rate in transgenic plants could be expected less than wild-type plants. Damodharan et al. (2016) have studied role of miR160 in tomato (*Solanum lycopersicum)* by knock down of miR160 using short tandem target mimic approach. They have found that sly-miR160a triggers blade outgrowth, leaf and leaflet production along with floral organs by quantitatively affecting corresponding target SlARF10A. Moreover, stem length was shorter in transgenic Russet Burbank plants than wild-type plants. Biomass in transgenic Russet Burbank plants were greater than wild-type suggesting that photosynthesis and transpiration rate along with stomatal conductance in transgenic plants could be greater than wild-type plants as they have more leaves, providing larger surface area and stomata for CO_2 assimilation. Liu et al. (2016) have examined the role of miR160 on the controlling of water balance in *S. lycopersicum*. They have discovered that transgenic plants overexpressing miR160-resistant form SlARF10 (mSlARF10) displayed greater size of leaves but yet less, than wild-type plants as well as limited leaflet blade. It was also shown that SlARF10 triggered the

ABA signalling via adjusting stomata structure to overcome water loss and aquaporin activity to regulate water movement, providing that water loss in leaf tissues is regulated by SlARF10 in tomato. Hence, miR160 contributes controlling water amount in leaf tissue.

5.2 Comparison of Wild-type and Transgenic Plants After Stress Treatments

5.2.1 Morphological traits

In Unica cultivar, leaf numbers in drought-treated transgenic plants were found a little greater than wild-type plants. In Russet Burbank cultivar, leaf sizes and stem structure in drought-treated transgenic plants were observed higher compared to wild-type plants. It was expected that leaf sizes would be less in order to avoid water loss by transpiration in response to drought stress. Biomass in wild-type Russet Burbank plants were observed slightly more than transgenic plants suggesting that overexpression could delay leaf senescence under drought stress. Altogether, it can be said that wild-type and transgenic plants were partially affected under stress conditions when they were compared before stress period.

The effect of heat and heat + drought stresses on transgenic plants was observed different from each other. Leaf senescence was triggered earlier in combined-stress treated plants than heat stress alone indicating that combined stress could impair severely than single stress. Vile et al. (2012) have revealed additive effect of combined stress on plant mass in *A. thaliana*, although some traits were known to be specific to plant organism including increase in root structure upon drought treatment.

The effects of stresses on tuber structure were observed different from each other. The most important finding was that tuber size in transgenic Russet Burbank plants were bigger than wild-type plants suggesting that overexpression of miR160 along with downregulation of target gene in Russet Burbank cultivar provide enhanced tolerance under drought and heat stress alone. By contrast, tuber size in heat-treated Unica transgenic plants were observed smaller than wild-type plants. Dahal et al. (2019) have proposed that heat impairs severely both tuber quality and yield in potato by reducing

dry matter content in the tuber and sucrose movement to stolon. Overall, the effect of stress on tuber structure could differ with respect to cultivar type.

5.2.2 Physiological traits

5.2.2.1 Photosynthesis rate, stomatal conductance and transpiration rate

In the present study, photosynthetic activities of wild-type and transgenic plants was impaired in Unica and Russet Burbank cultivars under drought stress. It has been shown that water limitation causes decrease in photosynthesis rate in potato plants (Monneveux et al., 2013).

Dahal et al. (2019) have proposed that drought triggers the downregulation of regulatory enzymes in photosynthesis and enzymes playing important roles in sucrose synthesis as well as upregulation of enzymes serving in degradation of sucrose molecule in potato.

As seen in Figure 4.49 and Figure 4.50, it was found that photosynthesis activity in transgenic plants was close to wild-type plants for both cultivars upon drought stress. By contrast, it was observed that photosynthesis rate impaired considerably in transgenic Russet Burbank plants than transgenic Unica plants suggesting that cultivar type could be an important factor on photosynthesis. In parallel to this result, it was proven that photosynthesis activity in sensitive potato cultivars severely affected as compared to tolerant ones upon 12 days of drought stress (Demirel et al., 2020). Moreover, photosynthesis rate in potato has been affected by several traits including light intensity, conductance of mesophyll cells in leaves and relative water content (Romero et al., 2017).

As seen in Figure 4.22 and Figure 4.47, it was observed that photosynthesis activity in transgenic Unica plants and wild-type Unica plants increased under heat stress. However, it was shown that heat prevents PSII activity in potato and subsequently causes reduction in photosynthesis (Havaux, 1993). Nevertheless, Hancock et al. (2014) have discovered that heat increased photosynthesis activity and reduced the yield in potato when the temperature was increased moderately during stress period. In Russet Burbank cultivar, photosynthesis activity in transgenic plants was found greater than

wild-type plants suggesting that overexpression of miR160 could be the cause of that change in auxin signalling in the leaves. Similarly, Lin et al. (2018) have proposed that overexpression of miR160 in *A. thaliana* confers enhanced tolerance to heat by modulating some of heat shock-proteins such as HSP17, HSP21 and HSP70B.

As seen in Figure 4.22, Figure 4.25, Figure 4.47 and Figure 4.50, it was found that photosynthesis activity in transgenic and wild-type plants were decreased for both cultivars under heat combined with drought stress. It was also observed that effect of combined stress on photosynthesis was quite severe than heat alone suggesting that a combination of heat and drought causes detrimental effects on photosynthesis than single stress irrespective of cultivar type. Recently, Demirel et al. (2020) have proved that combined stress impaired photosynthesis in both tolerant and sensitive potato plants following 12 days of stress period.

As seen in Figure 4.24 and Figure 4.49, it was found that transpiration rate in wild-type and transgenic plants decreased under drought stress. However, transpiration rate in transgenic plants was higher than wild-type plants indicating that overexpression of miR160 could contribute mechanisms associated with auxin metabolism and enables transgenic plants to have a greater level of transpiration. Transpiration rate in potato plants has been reduced under drought condition to mediate their water status by closing stomatal apertures (Dahal et al., 2019). This closed form of stomatal structures causes less amount of CO_2 to be utilized in Calvin cycle because it inhibits CO_2 entry in leaves. It also brings imbalance between Calvin cycle and electron transport chain followed by ATP and NADPH accumulation. As a result, oxidative stress resulting from energy instability in chloroplasts causes considerable damage on cell constituents (Dahal et al., 2019).

As seen in Figure 4.24 and Figure 4.49, it was explored that transpiration rate in wild-type and transgenic plants enhanced under heat condition suggesting that increase in the transpiration could be considered as an adaptation strategy upon heat treatment in potato plants (Dahal et al., 2019). Nevertheless, transpiration rate in wild-type and transgenic plants decreased significantly under combined stress than heat stress alone suggesting that several mechanisms could be triggered and precede to some extent the effect of heat. As seen in Figure 4.27 and Figure 4.52, it was explored that transpiration rate in

transgenic plants were found greater than wild-type plants under combined stress indicating that overexpression of miR160 could modulate some mechanisms in transgenic plants via affecting auxin pathway.

As seen in Figure 4.26 and Figure 4.48, stomatal conductance in wild-type and transgenic plants reduced in potato plants in order to prevent water loss under drought condition. It has been shown that stomatal conductance is closely related to abscisic acid in response to mild water stress (Liu et al., 2005). As it was found less difference on stomatal conductance for Unica and Russet Burbank cultivars suggesting that both cultivars affected negatively regardless of cultivar type.

As seen in Figure 4.48 and Figure 4.51, stomatal conductance in transgenic Unica and Russet Burbank plants were found greater than wild-type plants suggesting that overexpression of miR160 could contribute enhanced tolerance to heat stress. It has been proposed that stomatal conductance in tomato was decreased under moderate heat condition as it blocks activation state of rubisco enzyme (Morales et al., 2003).

As seen in Figure 4.26 and Figure 4.51, stomatal conductance in transgenic Russet Burbank plants was detected better than wild-type Russet Burbank plants upon combined stress suggesting that overexpression of miR160 along with downregulation of target gene could change abscisic acid mechanisms in potato, which may enable stomata apertures to be open for a longer time. Besides, drought effect could be more predominant than heat effect in potato plant. In Unica cultivar, effect of combined stress on wild-type and transgenic plants was not pronounced as Russet Burbank cultivar. Rizhsky et al. (2004) have studied that heat combined with drought stress causes considerable reduction in stomatal conductance in *A. thaliana* and blocks a mechanism which normally happens upon heat treatment in order to help cooling in leaves.

5.2.2.2 Chlorophyll index

Drought reduces downregulation of genes encoding chlorophyll a and chlorophyll b binding proteins in potato plants (Dahal et al., 2019). Hence, it causes reduction in chlorophyll content in potato. It was also reported that devastating damage resulting

from reactive oxygen species on chloroplast organelles contributes reduction in chlorophyll content upon drought treatment (Mafakheri et al., 2010).

As seen in Figure 4.28, Figure 4.29, Figure 4.53 and Figure 4.54, chlorophyll content in transgenic plants was greater than wild-type plants for both cultivars under drought stress suggesting that overexpression of miR160 along with repression of target gene could positively affect chlorophyll amount in the leaves by restoring function of chlorophyll binding proteins. Besides this, reduction in the leaf area have accumulated chlorophyll content in potato plants upon drought treatment (Rolando et al., 2015).

On 18^{th} day, control plants showed larger value than transgenic plants under drought stress stating that variation in leaf samples used for measurement on that day could be a possible factor in this difference. Similarly, Shahriari and Karimi (2001) have proposed that chlorophyll content increased in tolerant wheat cultivars in response to drought. They have also reported that leaves in resistant cultivars found a little darker than sensitive ones.

As seen in Figure 4.28, Figure 4.29. Figure 4.53 and Figure 4.54., chlorophyll content in transgenic plants was greater than wild-type plants under heat stress. However, it was found that chlorophyll amount in transgenic and wild-type plants decreased partially upon heat treatment. Reynolds et al. (1990) have shown that high temperature causes reduction in chlorophyll amount as well as CO_2 fixation in potato plants.

As seen in Figure 4.29 and Figure 4.53, chlorophyll content in transgenic Unica plants was larger than wild-type Unica plants under combined stress although, chlorophyll content in transgenic Russet Burbank plants was a little greater compared to wild-type plants suggesting that overexpression could confer enhanced chlorophyll amount in transgenic plants. Similarly, Handayani and Watanabe (2020) have stated that chlorophyll amount increased in potato plants under a combination of heat and drought stresses. However, they have not found any relation between high chlorophyll content and photosystem II quantum yield. Besides, there were not a significant difference on chlorophyll content in transgenic plants in terms of cultivar type suggesting that 12-days stress period was not enough to see possible change between the cultivars in this study. Demirel et al. (2020) have proposed that chlorophyll amount in potato plants decreased

considerably under 12 days of stress periods irrespective of being tolerant or sensitive species.

5.2.2.3 Leaf temperature

In this study, leaf temperature of transgenic plants was found slightly larger than wild-type plants under drought stress suggesting that timing of measurement or uncontrolled factors including varied ambient temperature during measurements could be the reason of this small difference. Drought stress leads to increase in leaf temperature by causing less dissipation to environment resulting from transpiration via closing stomatal apertures (Gimenez et al., 2013). It has been stated that genotypes showing cooler leaf temperatures in irrigated conditions were more sensitive to drought stress compared to those showing warmer leaf temperatures in potato (Stark et al., 1991).

As seen in Figure 4.32. and Figure 4.57, leaf temperature in wild-type plants was found a little greater than transgenic plants under heat stress suggesting that leaf samples used for measurements may differ from each other. Moreover, overexpression and downregulation of target gene protein can change auxin and abscisic acid mechanisms in the leaves, causing less canopy temperature in transgenic plants.

As seen in Figure 4.32. and Figure 4.34., leaf temperature in wild-type plants was found a little higher than transgenic plants under combined stress indicating that leaf samples used for measurements may differ from each other. Another reason could be overexpression affects some mechanisms including opening and/or closing of stomatal apertures related to auxin signalling in the leaves, giving rise to less canopy temperature in transgenic plants. Demirel et al. (2020) have found that canopy temperature in resistant potato genotypes was greater under a combination of drought and heat stress than heat stress alone. However, they have stated that canopy temperature in sensitive cultivars were less under combined stress than heat stress alone.

5.2.2.4 Relative water content

As seen in Figure 4.36 and Figure 4.61., relative water content decreased in both wild-type and transgenic plants under drought stress. Similarly, Liu et al. (2005) have found

that relative water content in potato leaves reduced under severe water deficient conditions. It was observed slight difference between transgenic and wild-type plants upon drought treatment.

As seen in Figure 4.39 and Figure 4.63., relative water content decreased in both wild-type and transgenic plants under heat and combined stress. However, relative water content in transgenic plants was higher under heat stress compared to a combination of drought and heat stress suggesting that combined stress could impair severely for both cultivars. Demirel et al. (2020) have found that relative water content in sensitive potato cultivars was a little higher under 12 days of heat stress than 12 days of combination of drought and heat stresses, while relative water content in resistant potato cultivars was significantly greater under heat stress compared to combined stress. Handayani and Watanabe (2020) have also reported relative water content in five potato varieties decreased under 21 days of drought, heat and combined stress. Moreover, a small difference was found in transgenic and wild-type plants upon heat or combined stress treatment.

5.2.2.5 Proline content

Proline plays an important role in the protection of plant cells by regulating osmotic adjustment and conservation of cell membrane as well as protein molecules (Kavi-Kishor et al., 2005). As seen in Figure 4.40 and Figure 4.45., proline content in transgenic Unica plants increased slightly than wild-type Unica plants under drought stress whereas, proline content in transgenic Russet Burbank plants enhanced less than wild-type Russet Burbank plants suggesting that accumulation of proline content could depend on cultivar type. It was found that proline content in transgenic Russet Burbank plants were higher than transgenic Unica plants upon drought treatment. Proline content in drought sensitive potato varieties found greater than drought tolerant potato cultivar (Jerez et al., 1993). Similarly, proline amount in sensitive potato cultivars triggered earlier compared to tolerant cultivars during 42-days drought stress period (Schafleitner et al., 2007).

As seen in Figure 4.40., Figure 4.41, Figure 4.65 and Figure 4.66., proline content was observed to be larger in transgenic plants than wild-type plants under heat stress

suggesting that overexpression may contribute to an increase in proline accumulation in transgenic plants by indirectly changing the expression of genes encoding proline synthesis upon combined stress treatment. However, Demirel et al. (2020) have stated that proline accumulation was not observed in tolerant and sensitive potato plants under heat stress. Yet, Naz et al. (2018) have proposed that amount of proline and soluble sugar molecules was increased under 25 days of heat stress in potato plants. Hence, they have concluded that proline accumulation can enable plants to be less affected caused by reactive oxygen species.

5.3 Comparison of Findings From qRT-PCR in Transgenic Plants

As seen in Figure 4.69. and Figure 4.70., miR160 expression was found at the highest level and corresponding target decreased significantly under combined stress condition than other stresses for both cultivars suggesting that combined stress affected more severely compared to single stress treatments. It was also found that effect of combined stress on Russet Burbank plants were greater than Unica plants indicating that cultivar type being sensitive or tolerant could be important under abiotic stress conditions.

CHAPTER IV

CONCLUSION

In this thesis, function of miR160 was analysed in Unica (tolerant) and Russet Burbank (sensitive) cultivars under single stress including drought and heat as well combined stress condition by using overexpression transgenic approach.

For this aim, wild-type and transgenic plants were compared to find out morphological and physiological differences. Drought stress was carried out in the greenhouse, while heat and combined stress were performed in the growth chamber. Some of morphological traits including biomass, leaf numbers, leaf expansion and stem length as well as tuber size differed between wild-type and transgenic plants. Besides, findings from physiological parameters showed pronounced difference between wild-type and transgenic plants under abiotic stress conditions such as transpiration rate, stomatal conductance and chlorophyll index. Moreover, effect of combined stress was generally found more severe than single stress treatment.

Altogether, overexpression of miR160 could alter some mechanisms either directly or indirectly related abscisic acid or auxin pathways or heat-shock protein expressions, enabling transgenic plants to have an enhanced tolerance to abiotic stress conditions. Based on findings, transgenic Unica and Russet Burbank plants showed enhanced tolerance to drought and heat stress as compared to wild-type plants. This study can help improvement of plants showing improved tolerance to abiotic stresses in line with the sustainable agriculture. However, these findings11 can be just considered as preliminary results which need to be further validated in field studies.

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