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NİĞDE ÖMER HALİSDEMİR UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

T.R.
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Doctor of Philosophy Thesis

Supervisor
Assoc. Prof. Dr. Ufuk DEMİREL

December 2021

The study titled **"The Response of Some miRNAs in Potato to Combined Abiotic Stress"** is presented by **Arslan ASIM** under the supervision of Assoc. Prof. Dr. Ufuk DEMIREL and accepted as a Doctoral thesis by the jury at the Department of Agricultural Genetic Engineering of the Niğde Ömer Halisdemir University, Graduate School of Natural and Applied Sciences.

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This thesis has been found appropriate at the date of ..../..../2021 by the jury mentioned above who have been designated by Board of Directors of Graduate School of Natural and Applied Sciences and has been confirmed with the resolution of Board of Directors dated …./…./2021 and numbered ……

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THESIS CERTIFICATION

I certify that the thesis has been written by me and that, to the best of my knowledge and belief. All information presented as part of this thesis is scientific and in accordance with the academic rules. Any help I have received in preparing the thesis, and all sources used, have been acknowledged in the thesis.

ARSLAN ASIM

SUMMARY

THE RESPONSE OF SOME miRNAs IN POTATO TO COMBINED ABIOTIC STRESS

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Potato (*Solanum tuberosum* L.) is a significant food source with high nutritional value and rich content. Abiotic stress conditions, mainly drought and heat are main factors affecting its growth. Combined stress factors cause adverse effects as compared to an individual stress factor. MicroRNAs (miRNAs) are considered as major players to defensively deal against stress conditions and to increase crop production. Overexpression of miRNA is an effective tool to investigate the role of miRNA during stress conditions. Current study aims to understand functions of stress responsive miRNAs (miRNA novel 8, miR156d-3p, and miR172b-3p) and their target genes (photosystem II core complex proteins, phospholipid-transporting ATPase, and ERTF RAP2-7-like) under drought, heat, and drought+heat by overexpressing the miRNAs in a stress-resistant potato cultivar, Unica. Along with physio-biochemical analyses, molecular analyses were performed by RT-qPCR to analyze the expression level of the miRNAs and their target genes. All transgenic lines showed differential response against different stress conditions with the overexpression of miRNAs and down regulation of their target genes. In conclusion, current study revealed the changes in potato due to overexpression of stress responsive miRNAs.

Keywords: Potato, abiotic stress, drought, heat, combined stress, miRNA

ÖZET

PATATESDEKİ BAZI miRNA'LARIN KOMBİNE ABİYOTİK STRESE TEPKİSİ

ASIM, Arslan
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Patates (*Solanum tuberosum* L.), yüksek besin değeri ve zengin içeriği ile önemli bir besin kaynağıdır. Abiyotik stres koşulları, özellikle kuraklık ve ısı, büyümesini etkileyen ana faktörlerdir. Kombine stres faktörleri, bireysel stres faktörlerine kıyasla daha olumsuz etkilere neden olur. MikroRNA'lar (miRNA'lar) stres koşullarına karşı savunmacı bir şekilde başa çıkmak ve mahsul üretimini artırmak için ana oyuncular olarak kabul edilir. MiRNA'nın aşırı ekspresyonu, stres koşulları sırasında miRNA'nın rolünü araştırmak için etkili bir araçtır. Mevcut çalışma, kuraklık altında strese duyarlı miRNA'ların (miRNA novel 8, miR156d-3p ve miR172b-3p) ve hedef genlerinin (fotosistem II çekirdek kompleks proteinleri, fosfolipid taşıyan ATPaz ve ERTF RAP2-7 benzeri) işlevlerini anlamayı amaçlamaktadır. Strese dayanıklı bir patates çeşidi olan Unica'da miRNA'ları aşırı ifade ederek ısı ve kuraklık + ısı. Fizyo-biyokimyasal analizle birlikte, miRNA'ların ve hedef genlerinin ekspresyon seviyesini analiz etmek için RT-qPCR ile moleküler analiz yapıldı. Tüm transgenik çizgiler, miRNA'ların aşırı ekspresyonu ve hedef genlerinin aşağı regülasyonu ile farklı stres koşullarına karşı farklı tepki gösterdi. Sonuç olarak, mevcut çalışma, strese duyarlı miRNA'ların aşırı ekspresyonu nedeniyle patatesteki değişiklikleri ortaya çıkardı.

Anahtar Kelimeler: Patates, Abiyotik stres, kuraklık, ısı, birleşik stres, miRNA

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SYMBOLS AND ABBREVIATION

Symbols	Description
bp	Base pair
%	Percentage
µL	Microliter
µ	Micro
M	Molar
µ	Micro
mg	Milligram
ng	Nanogram
^{o}C	Degree celsius
mg/L	Milligrams per liter
µmol	Micromol
W	Watt
ng/µL	Nanogram per microliter
sec	Second
rpm	Revolutions per minute
min	Minute
µL/L	Microliter per liter

Abbreviation	Description
AGO1	ARGONAUTE1
APX	Ascorbate Peroxidase
A.tumefaciens	*Agrobacterium tumefaciens*
B1	Thiamine
B3	Niacin
B6	Pyridoxin
BAP	6-Benzylaminopurine
BLAST	Basic Local Alignment Search Tool
C	Control

AA	Ascorbic Acid
CAT	Catalase
cDNA	Complementary DNA
CIP	International Potato Center
CS	Chinese Spring
CSD	Copper/zinc Superoxide Dismutase
CPL1	C-Terminal Domain Phosphatase-Like1
Cu	Copper
DCL1	DICER-LIKE1
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
E	Transpiration Rate
EST	Expressed Sequence Tag
EF	Elongation Factor
FAOSTAT	FAO Corporate Statistical Database
Fe	Iron
GA_3	Giberellin
GM	Genetically Modified
GO	Gene Ontology
GRN	Gene Regulatory Network
GSS	Genome Survey Sequence
GUS	β-glucuronidase
(H)	Heat
(HD)	Heat + drought
HECT-type	Homology to E6-Associated Carboxyl-Terminus
HEN	HUA ENHANCER1
HSF	Heat Shock Factor
HSP	HEAT SHOCK PROTEIN90
HST	HASTY
HYL1	HYPONASTIC LEAVES1
K mineral	Potassium
LB	Lysogeny Broth
MFE	Minimum Free Energy
MFEI	Minimum Free Energy Index

Mg	Magnesium
Mn	Manganese
miRNA	micro RNA
miRNA*	Antisense Sequence of miRNA
mRNA	messenger RNA
MS	Murashige and Skoog
MS-0	Murashige and Skoog-zero
NAA	A-Naphtalene Acetic Acid
NCBI	National Center for Biotechnology Information
NGS	Next-generation Sequencing
P	Phosphorus
P5CS	Pyrroline-5-Carboxylate Synthetase
P5CR	Pyrroline-5-Carboxylate Reductase
PCR	Polymerase Chain Reaction
Pn	Photosynthesis
POD	Peroxidase
Pre-miRNA	Precursor-microRNA
Pri-miRNA	Primary-microRNA
ProDH	Proline Dehydrogenase
RING-type	Really Interesting New Gene
RISC	RNA-induced Silencing Complex
RNA	Ribonucleic Acid
RNAi	RNA Interference
rRNAs	Ribosomal RNAs
RWC	Relative Water Content
SE	SERRATE
siRNAs	Small Interfering RNAs
smRNAs	Small Modular RNAs
SOD	Superoxide Dismutase
SPAD	Chlorophyll index
sRNA	Small RNA
stRNAs	Small Temporal RNAs
SVM	Support-vector Machine
SQN	SQUINT

T-DNA	Transfer-DNA
Ti	Tumour-inducing
tncRNAs	Tiny Non-coding RNAs
tRNAs	Transfer RNAs
UPS	Ubiquitin 26S Proteasome System
Ub	Ubiquitin
YEP	Yeast extract peptide
qRT-PCR	Quantitative Real-time PCR
Xg	G-force

CHAPTER I

INTRODUCTION

Potato (*Solanum tuberosum* L.) is a main crop harboring rich food contents and high nutritional value. It is ranked as the 3rd after wheat and rice according to human consumption with the production of 368 million tons from 17.5 ha in the world (Tang et al., 2021). The literature gives an idea that all around the world potato have over 4,000 edible varieties and is declared as food of more than a billion poor people (Vincent et al., 2013). China, India, Ukraine, Russia, Poland, USA, Belarus, France and Netherlands are among the major potato producer countries. Seventy percent of the total potato production is being shared by these countries where Turkey is ranked as 13th in terms of potato production (Karan et al., 2021). Three percent 3% of the national economy of Turkey is being contributed by potato in terms of area, production, and trade among tuber crops. Being raw material provider for agricultural industry in Turkey, it is a highly significant crop. In Turkey an area of 140766 hectares is used for potato cultivation resulting in 4979824 tons of production with an average yield of 324,475 hectogram per hectare (FAOSTAT, 2017). Central Anatolia including Niğde participate more than 61% potato production in this regard (Dangol et al., 2018).

Potato is a temperate-cool climate plant with an optimum temperature requirement of 17-24 °C for better plant growth and yield (Demirel et al., 2020). Potato is propagated vegetatively, which is adversely affected by numerous biotic and abiotic factors resulting in 40 % crop losses during field production and storage (Oerke, 2006). Generally, abiotic stress factors affect tuber yield and quality in an adverse way. Potato is sensitive to abiotic stress factors that includes fluctuation in air temperatures (high/low), drought and salinity. Efforts to increase tolerance of agricultural plants against these adverse environmental conditions have gained momentum because of global warming and a decrease in irrigation water resources (Mittler, 2006). In changing climates, temperature deviations are about to increase (IPCC, 2014). Several studies have reported the response of plants to single and combined stress conditions (Rensink et al., 2005). Earlier reported studies showed that combined stress conditions caused specific responses of plants that cannot be observed in case of single stress condition. However, studies on understanding biological basis of potato response to adverse

environmental conditions are still limited compared to other important agricultural crops, especially wheat. In present conditions, the need of the era is to unravel the responses against single and combined stress conditions. The understandings of responses are important to unveil molecular mechanisms that are involved in crop maintenance to ensure yield and quality.

Micro RNAs (miRNAs) belong to small RNAs that play regulatory roles in post transcriptional level. Plant miRNAs are known to regulate organ development, root development, morphogenesis of leaf, and floral identity. The discovery of miRNAs has altered our basic knowledge of regulation of gene expression. MiRNAs have a great potential to understand abiotic stress tolerance mechanisms to develop stress resilient plants. Additionally, it allows new opportunities to plant breeders for creating new varieties. There are studies proving the important regulatory roles of miRNAs due to different stresses. miRNA population in potato was mostly identified with *in silico* approaches and only a few were functionally proven to own roles in abiotic stress tolerance. Currently, limited information is available about potato miRNAs, transcriptomics, function of miRNAs and their regulation role in individual drought or heat stress and their combination. Most of the literature related to potato miRNAs is on drought stress tolerance, and there are no reports on expressional variations of miRNAs with the exposure to high temperature stress, combination of high temperature and drought in potato.

Although various functions of miRNAs have been investigated in Arabidopsis and some major crops, limited number of studies has been conducted on miRNAs in potato. While most of these are at bioinformatics level, only few studies have been related to find functions of miRNAs and their target genes (mRNA) in potato (Zhang et al., 2009; Yang et al, 2010; Hwang et al., 2011; Xia et al., 2011; Zhang et al., 2013, Lakhotia et al., 2014). Detection of involved miRNAs in regulation of drought and heat tolerance has been mostly studied among abiotic stress factors in potato so far (Hwang et al, 2011 a, b, c; Yang et al., 2013; Zhang et al., 2014). Whereas, according to our knowledge no study has been conducted about combined stress (drought+heat) conditions in transgenic potato plants.

There is also limited study about identification of miRNA functions in single or combined stresses in potato. Transgenic approach is the biggest shortcomings for identification of miRNAs' function in potatoes. We carried out a preliminary study to identify no water and high temperature stress responded miRNAs in potato. To achieve this aim, miRNA profile under stress was determined by using next generation sequencing (NGS) approach. Differentially expressed miRNAs in response to several abiotic stress conditions (control, elevated temperature, drought and high temperature + drought) were analyzed via bioinformatic tools. From our previous study, following miRNAs (Table 1.1) were selected and transferred into a stress tolerant potato variety Unica.

Table 1.1. miRNAs and target genes

miRNA	**Target Gene by psRNATarget (Solanum transcript library)**	
stu-miR156d-3p	PGSC0003DMT400001292	Phospholipid-transporting ATPase *Solanum tuberosum* heterogeneous nuclear ribonucleoprotein 1-like (LOC102604973), transcript variant X2, mRNA
Stu-miR172b-3p	PGSC0003DMT400065313	*Solanum tuberosum* ethylene-responsive transcription factor RAP2-7-like (LOC102606071), transcript variant X2, mRNA
miRNA-novel-8	PGSC0003DMT400018554, PGSC0003DMT400052092	Photosystem II core complex proteins psbY, chloroplast, Mitochondrial transcription termination factor family protein

The aim of this study was to determine the role and function of selected miRNAs in potato by exposing them to drought, heat and drought+heat stress conditions. For this aim, a transgenic approach was used. Selected miRNAs were over-expressed via transferring DNA encoding Pre-miRNAs with a constitutive promoter (35S promoter)

in a drought and heat tolerant variety, Unica. Identification of target genes and miRNAs involved in regulation of drought and heat combination response of potato may help to regulate the pathways leading to drought and heat combination tolerance in the future. miRNAs normally control more than one or many target genes, so their functions and nomenclature are supposed to be confusing and misled by much research (Zhang et al. 2018). Therefore, keeping in account complex networking of many interdependent genes or dual role of some transcription factors (TFs) it was supposed to be a possibility to develop more tolerant or susceptible potato transgenic lines against single/combined abiotic stress conditions by using over expression of miRNA approach.

The aim of proposed thesis was following.

1) Production of improved knowledge about role and importance of miRNAs and their mRNAs (target genes) in drought, heat and heat+drought tolerance of potato.
2) Minimizing the confusions present in miRNA nomenclature.
3) Gaining skills to develop new plant variations by using miRNA approach.
4) Production of transgenic lines influencing the sustainability of potato cultivation under diverse climatic conditions.

CHAPTER II

LITERATURE REVIEW

2.1 Historical Perspective of Potato (*Solanum tuberosum*)

Potato (*Solanum tuberosum*) was originated in Peru where it was domesticated by Inca Indians. Nikolai Vavilov was the first botanist who identified plant diversity centers. He used the term homologous series in order to explain the crop diversity. According to his classification, Central Andes is referred to as the diversity center and origin of potato (Vavilov 1926). Mexican center (Central and North America) and Andean center (South America) have been reported as two diversity centers for wild potatoes (Hijmans et al. 2007). Potato had a single origin as believed by some scientists that *Solanum brevicaule* complex considered as ancestor of common potato originated from Southern Peru (Spooner et al. 2005) but Hawkes (1994) and Huaman and Spooner (2002) suggested multiple origins of cultivated potatoes. Chiloe region (Chile) is considered as the source of introduction of potatoes in Europe (Juzepczuk and Bukasov, 1929). Contrarily, cultivated potatoes were selected/adapted from potato wild species cultivated in Andean region i.e., Bolivia, Northern Argentina, and Peru (Hawkes, 1994). Molecular studies have suggested that in 1700s Andean potato was dominated, before Chilean potato was introduced in Europe (Ames and Spooner, 2008).

Some 8,000 to 10,000 years ago, anchester of cultivated potato occured from diploid (2n=2x=24) wild potato species. It later spread widely throughout the globe and helped in food security (Pearsall, 2008). First domesticated potato was *Solanum stenotomum,* which is the descendant of diploid (2x) wild species and ancestor of cultivated species of potato (Hawkes, 1990). During domestication period, wild potato species of *S. brevicaule* complex undergo selection for traits such as shorter stolons, large tubers, varying in color, shapes, and less bitter taste. Apart from these underground traits, aboveground characteristics showed higher segregation in foliage and flower that possess a high vigor (Spooner et al., 2005). Domestication results in genetic bottleneck effect in potatoes which is the main cause of limited diversity in present day cultivars

(Brush et al. 1995). For instance, with the introduction of potato in different continents such as America, and Europe, signifies a chunk of variation in comparison to extensive diversity found at the origin center.

Potato has somewhat similar botanical appearance as several poisonous plants (Nightshade family) that's why it slowly became a part of new world and European food culture. However, by the passage of time potato got farmers attraction due to its hardiness and resistance against European damp climatic conditions, and due to being a staple food it quickly displaced other crops. At present, it covers a large cultivated area having above 4,500 consumable varieties worldwide and around 100 countries it is being commercially sold (Brown and Henfling, 2014) (Figure 2.1). Especially in Ireland the potato got prosperity, where it was encouraged not only for its hardy nature and ease in cultivation, but also due to its rich nutritional values and economy. The potato tubers are nutritiously enriched with carbohydrates, protein, vitamins and many minerals mainly potassium, calcium and antioxidants that are linked with many benefits of health like reduction in heart disease, cancerous disorders and cataracts risks and improvement in immune system (Brown, 2005).

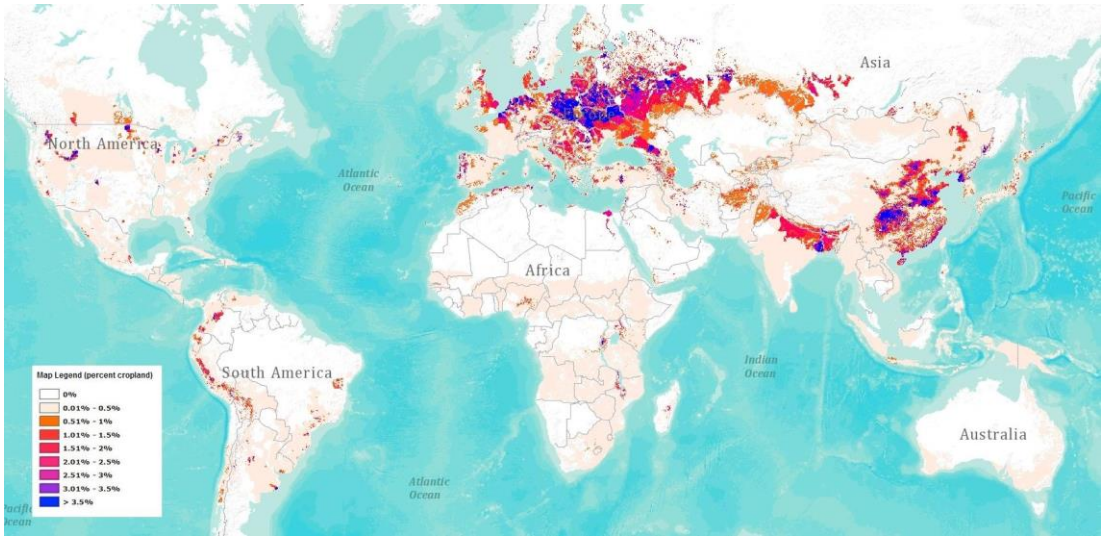


Figure 2.1. Worlwide distribution of potato cultivation (*PotatoPRO, 2019*)

2.2 Importance of Potato

According to an estimate total world potato production is 370,436,581 tons in 2019 (Source: FAOSTAT, 2019). Europe and Asia followed by America covers major percentage of worldwide potato production (Figure 2.2, 2.3) (FAOSTAT, 2019). Potato is considered as important food for humans and animals due to its rich important minerals (Mg, P, Mn, Fe, Cu), carbohydrate content and vitamins. Its nutritional value, potential of high yield and widely consumption rate causes potato to be considered as a major crop to deal against the deficiency of nourishment (Djami-Tchatchou et al., 2017).

In terms of potato production Turkey stands at 13th position in the world. Maximum part of total population of the country depends directly or indirectly on agriculture, so it would not be wrong to say that potato is an important part of Turkey. Potato cultivation is being reported as primary way of income for tens of thousands producer's families and quite important staple food source for them as well in Turkey. About 56% of the total potato production is freshly consumed in Turkey (Kart et al., 2017).

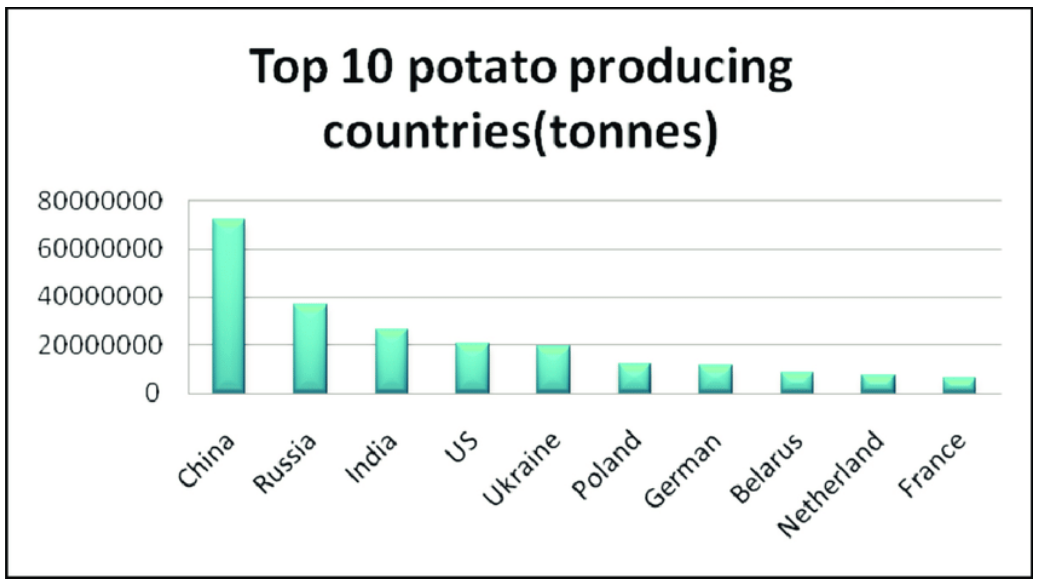


Figure 2.2. Major potato producing countries in the world (FAOSTAT, 2019)

Potatoes have been found since about 150 years in Turkey; and showing growth as a sector by passing through the production, consumption, and marketing. Actually, behind the prosperous position of potato production, one of main reasons is its naturally

favorable agro-ecological resources. In Turkey, more than 70 provinces are growing potatoes including Niğde, which is sharing around more than 14% of total production (TÜİK, 2020). The abiotic and biotic stress factors during the vegetative growth phase causes major decrease in potato production in Turkey similar to other developed countries. The literature reveals that more than 17 mites and 270 insect species are involved in attacking potato fields and even in storage conditions worldwide (Alkan et al., 2017). Major insect pests include Colorado potato beetles (*Leptinotarsa decemlineata*), leaf hoppers (*Empoasca fabae*), potato tuber moth (*Phthorimaea operculella*) and cutworms (*Agrotis ipsilon*) (Vaneva and Dimitrov, 2013). Out of all of these insects, Colorado Potato beetle is appraised as main disastrous pest of potato worldwidely and also in Turkey (Alyokhin, 2009).

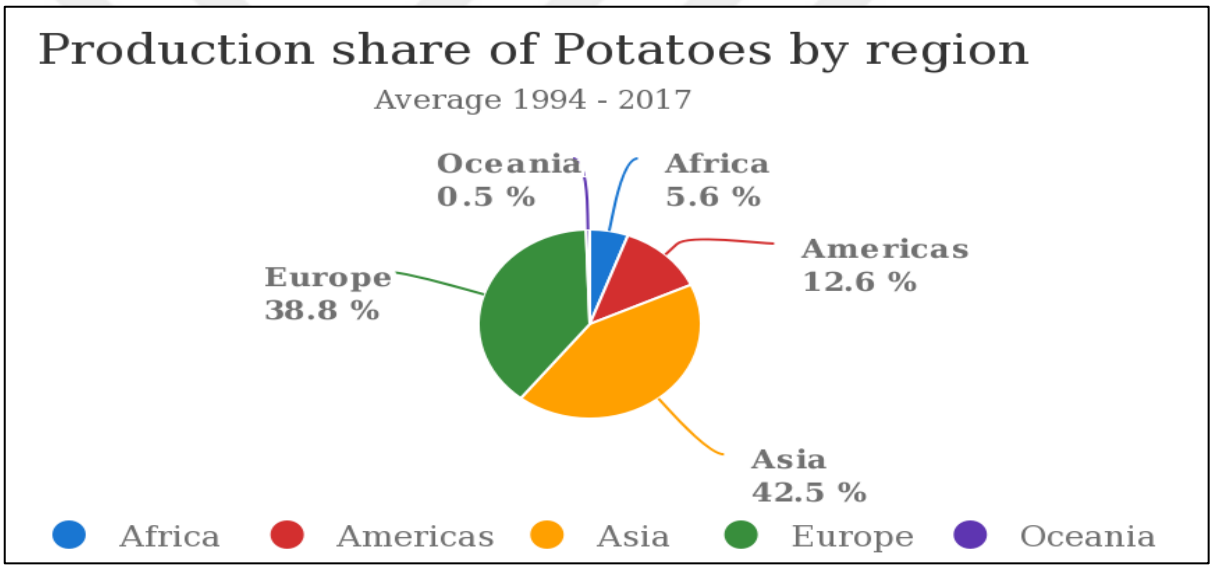


Figure 2.3. Region wise production of potato (FAOSTAT, 2019)

2.3 Abiotic stress in Potato

Abiotic stresses include heat, cold, salinity and drought stresses which are main factors responsible for potato yield loses up to 34% of total production (Oerke, 2006). Shallow root system of potato attribute to capture less water and an inability of photosynthetic machinery to recover during high heat and drought conditions. Stress factors mainly drought and heat causes decrease in potato plant growth, its life cycle and reduction in size and number of tubers (George et al. 2017).

2.3.1 Influence of drought stress on potato

Water deficit condiitons causes morphological alteration in root system of potato (Wishart et al. 2013, 2014). These alterations include expanding lateral root proliferation, increase in thickness, decrease in root elongation and hair production that helps to maintain root-soil linkage which goes down during dry periods (Schmidt et al. 2012; Haling et al. 2013). Moreover, drought tolerance of plants increases by hydraulic resistance of the roots associated with xylem vessels and by decreasing the water extraction rate from soil without rapid reduction of water available. Hydraulic conductivity changes beside the root depending upon the root type and tissue age and can be mediated by aquaporin expression and permeability of cell membrane (Doussan et al. 1998). Water scarcity causes an increase in hydraulic conductivity of root (to maintain water uptake), although continuous water extraction may later cause decrease in hydraulic conductivity of root (to save soil from loss) also these changes also refer to aquaporin regulation and activity (Maurel et al. 2010). For a period, increase in tuberization of root exodermis /endodermis casues further decrease in root hydraulic conductivity (Vandeleur and Mayo 2009). After severe and continuous conditions of water scarcity, due to xylem embolism hydraulic conductivity of root can negatively affected in the plant (Cruiziat et al., 2002). In different genotypes of potato having bigger root systems, specifically the stolon bearing roots, improves the capability to deal with drought stress conditions to ensure higher yield of potato (Puértolas et al. 2014). However, it is being noticed that genotypes having bigger roots systems also show quick closure of their canopy, which ultimately extends their duration and activity of photosynthesis and results in quick water loss due to more evaporation from soil rather than activity of plant transpiration. It has been recommended that breeding aimed for increased root hydraulic resistance and root hair length, greater numbers of seminal roots is possible (Bengough et al. 2011). With the passage of time due to considerable decrease in resources of water in the world, plants having more ability to cope with abiotic stresses may play significant roles in agriculture sector (Çalışkan, 2016). In terms of water availability, potato is declared as drought sensitive, speciallly during tuber formation water is required by potato plant. Environmental conditions can affect some potato cultivars depending upon their susceptibility to abiotic stress (Çalışkan, 2016).

Recently, observations have revealed about the relations of water at the soil-root interface modulates soil physical conditions, soil-root contact and variation in moist or dry soil water uptake (Koebernick et al. 2017).

2.3.2 Impacts of high temperature and its combination with drought stress on potato

Being specie adopted in temperate cool climate, fluctuation in temperature shows different effects on different processes in physiology of potato. Tuber developmental process is more sensitive to high temperature levels because signal of tuberization retards at high temperature (Ewing 1981). Out of all temperature sensitive phenomenon, s one very important is transportation of carbon to sink organs in potato. High temperature causes less carbon incorporation into starch in tubers (Wolf et al. 1991). These results in ultimate sucrose gathering at unloading sites of phloem, thus resulting in less sucrose transport to the tuber and ultimately resulting decrease in sink strength. High temperature negatively affects heat sensitive tuber-bearing and photosynthetic performance. Solanum specie show severe chlorophyll loss and hindrance of CO_2 fixation than heat tolerant specie (Reynolds et al. 1990). Behind many tuber disorders including abnormal shape of tuber, high temperature is also a big cause for formation of secondary tuber (associated with high branching and elongation of stolon) or chain tuberization. Elevated temperature is also reported to be responsible for high adverse impacts on skin quality of potato (Heltoft et al., 2017). During tuber filling stage the stress of high temperature is reported to have large impact on gene regulation involved in the steroidal glykoalkloid and anthocyanin pathways in phellodem (below skin tissue) of red skin potatoes. Less hoarding of anthocyanins is supposed to be responsible for the less collection of anthocyanins which may reduce the nutritive value of tubers (Kumar et al. 2017). Moreover, at maturation stage of tuber, high temperature effects tuber dormancy and can ultimately cause early sprouting. So, different responses of heat stress may negatively impact quality and yield of tuber. Germplasm of potato can be widely categorized based on their heat tolerance ability (Levy and Veilleux 2007). Genotypes having tolerance against heat should also maintain their processing quality, health promoting qualities and nutritional qualities rather than showing maintenance only in yield.

Over past two decades, the capability of plants to tolerate these stresses has been continuously tried to unveil at molecular level. These research efforts have ended up in identifying many genes that activate under high temperature, salt and drought stress aiming that overexpression of stress responsive genes would increase tolerance of plants against stress. Some transgenics showed very less or no improvement in tolerance of stress, because of the lack of capability to understand complex genetic processes underlying plant stress tolerance.

2.4 Unica Potato Cultivar

Unica cultivar is generally known as comparatively tolerant to abiotic stress factors. In 1990s by crossing 'Aphrodite' and CIP No. 387521.3, Unica (Accession number: 392797.22) was developed in Peru (Figure 2.4). Its tuber skin is red and due to having resistance against abiotic stress conditions and potato virus Y which is a limiting factor for yield and quality of potato. Unica is widely cultivated in Uzbekistan, Laos, Vietnam, and Peru. Its name was derived from University of Ica. Flesh of tuber has cream colour and better quality for fresh consumption and French fry (Contreras-Liza et al., 2017). It has been extensively reported in different studies as tolerant cultivar to different abiotic stress factors (Asim et al., 2021; Ramirez et al., 2015; Demirel et al., 2020; Gokçe et al., 2021).

Figure 2.4. Unica potato variety (Rosales and Bonierbale, 2007; Potato Association, 2019)

2.5 RNA Interference

Literature elaborates that in addition to protein level, plants also response to stress conditions at transcriptional and post transcriptional level. Gene regulatory mechanism is suppressed at transcriptional level in RNA silencing by post-transcriptional gene silencing (PTGS) or by RNA interference (RNAi) (Gupta et al., 2014). In early 1990s the first report of RNAi was in petunia plants (Napoli et al., 1990). Transgenic plants were produced to obtain dark purple flowers but unexpectedly due to homologous gene silencing, white and chimeric flowers were shown up. This mechanism was named as 'co-suppression' (Cogoni and Macino, 2000). Small RNAs role as bio-regulators in this silencing process. Depending on biogenesis/genomic loci's structure, they can be categorized as siRNAs (small interfering RNA), miRNAs (micro-RNA), smRNAs (small RNA), tncRNAs (transfer RNA-derived non-coding RNAs), piRNAs (piwi-interacting RNA), and stRNAs (small temporal RNA). Out of all types of small RNA (sRNAs), the miRNAs have been found and reported to play vital role in stress responses (Copper et al., 2019, Bej and Basak, 2014). Movahedi et al. (2018) reported the pathways of gene-silencing and knockdown of genes in eukaryotic organisms related to 20 to 25 nucleotides in length little RNAs, which involve siRNAs and miRNAs. These little RNAs are exploited to suppress upstream or downstream of gene expression in the transcription pathway. The biological gene expression inhibitor is RNAi that outcomes in the destruction of mRNA, resulting in inhibition of protein generation. Moreover, the important role of RNA silencing consists of having a part in the pathways of plant response to the stresses. Contrarily, the proteins that hamper antiviral RNAi activation in plants are called VSRs (Viral Suppressors of RNA silencing), results in repressing the plant RNA-silencing. The prevention of starting immune response of plant antiviral RNAi caused by VSR proteins. This research mainly studies plant sRNAs and their part against abiotic stresses in plant.

2.6 MicroRNAs

2.6.1 General information and biogenesis of miRNAs.

In 1993, miRNAs were firstly discovered in nematodes (Lee et al., 1993), they are about 20-24 nucleotides in length and single strand small RNAs. MiRNAs are endogenous

sRNAs that are negative regulator of their complementary target mRNAs. They are reported to perform important biological functions in plants (Gökçe et al., 2021). Generally, most of the coding genes that synthesizes protein are regulated by miRNAs (Grosswendt and Rajewsky, 2017). miRNAs partially bind with target mRNAs. This binding result in cleavage of phosphodiester bonds in mRNA and mRNA is cleaved or protein synthesis is suppressed. Thus, target mRNA expression is regulated post-transcriptionally. MiRNA's with similar complementary sequences can attach with different mRNAs, they can regulate expression of more than one target mRNA at post transcriptional level (Selbach et al., 2008).

MiRNAs are known to be single-stranded RNA's having DICER enzymes behind their production and they also act on precursors with intra-molecular stem-loop structures. Mature miRNAs bear hydroxyl group at 3′ and phosphate group at 5′ end. These molecules are involved in mediation of post transcriptional silencing (PTS) with the binding at specific sites in the 3′ untranslated region (UTR) of their mRNA, translational inhibition or/and triggering degradation of mRNA.

The biogenesis of plant miRNAs occurs in nucleus (transcription and processing) via RNAi silencing (Figure 2.5). RNA polymerase II encodes pri-miRNAs processing to form miRNAs. The folding of pri-miRNAs intitates to form incomplete hairpin structure, that is cleaved by DICER-LIKE 1 (DCL1) for the formation of short incomplete double-stranded structure (pre-miRNAs). The RNase III enzyme DCL1 is required for miRNA maturation (Cambiagno et al., 2021). The DCL1 is in nucleus and can form both the first pair of cuts made by Drosha and the second pair of cuts made by Dicer. The resulting miRNA/miRNA duplex comes out of the nucleus with the help of by HASTY (HST). Plant ortholog of Exportin 5 assembles with RISC complex in the cytoplasm (Park et al., 2005). The *S*-adenosyl methionine (SAM)-dependent methyltranferase HEN1 adds terminal methyl group and modifies miRNA by HEN1. It protects miRNA from further degradation/modification that can facilitate its assembly RISC complex (Boutet et al., 2003). After that RISC conducts sRNA-directed gene silencing in RNAi and miRNA pathways (Carbonell, 2017). RISC that belongs to Argonaute (Ago) protein family directs RISC pairs to a target mRNA and functions as an endonuclease, for the cleaving of mRNA. Resemblance with the operation of RNAi interaction is key mechanisms to cleave and degrade mRNA. Firstly, the

complementary open reading frame of mRNA and the miRNA results to halt the process of translation of mRNA. Secondly, miRNAs incompletely attach with the 3′UTR region of target mRNA, that inhibits RISC complex translation/degradation of mRNAs.

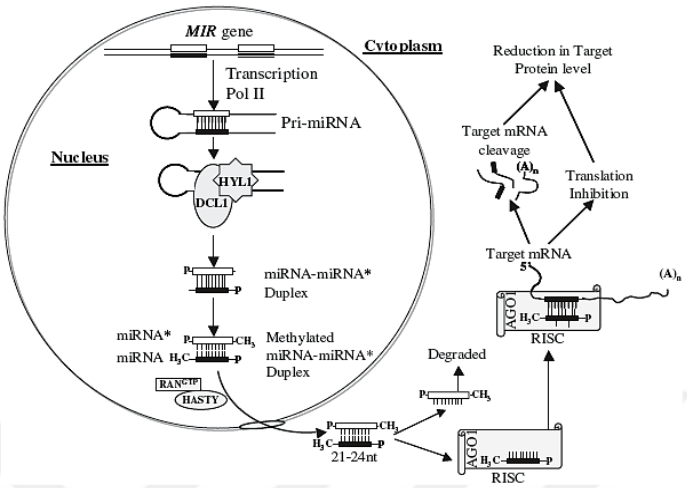


Figure 2.5. Model of Biogenesis of miRNAs and Post-transcriptional Gene Regulation in Plants

2.6.2 MiRNA identification

For identification of miRNA the following characteristics are used:

1. In plants length of miRNA varies between 19-24 nucleotides while in case of animals it is between 20-22 nucleotides.
2. Stem loop structures are formed, and all miRNAs are less conserved in plants which is in contrast to miRNAs in animal (Bonnet et al., 2014).
3. All precursors of miRNA have less free energy (-31–57kcal/mol) as compared to tRNAs (-26–29kcal/mol) or rRNAs (-33kcal/mol).

Four techniques can be used for identification of miRNAs: (a) genetical screening, (b) computational identification, (c) small RNA isolation cloning and (d) mining method with expressed sequence tags. Computational miRNA identification is practiced by data of next-generation sequencing (NGS). To specify DNA sequence by parallel sequencing, small DNA fragments are used in NGS. For the identification of plant miRNAs by NGS, the initial step is isolation of total RNA from plant followed by construction of small RNA library. Generation of cDNA library from small RNA is followed by sequencing with NGS technique. Sequences of sRNA are compared with

miRBase databases to find homologous miRNAs. Then novel and known miRNAs can be categorised and selected. Then for miRNA enrichment, Gene Ontology (GO) analysis is performed to remove RNA of large size or to increase concentration of miRNA (Devi et al., 2018). All new miRNAs lacking homology, can be identified and selected in plants by these criteria. (1) more than 3 nucleotides interior bulge, (2) overhanging of 2 nucleotides at 3´ end over 5´ end, (3) folding to appropriate hairpin structure, (4) there should be less than 6 mismatches in target RNA sequences and mature miRNA sequences, (5) whole structure should not contain more than successive 4 nucleotides mismatches, (6) mature miRNA should stay on hairpin stem region, (7) percentage of uracil and adenine should be in 30-70%, (8) limit of folding energy should not be less than -20 kilocalories/mol. In estimated secondary structure higher minimum free energy index (MFEI) and negative minimum free energy (MFE) by following equation 2.1. It should be >0.75 (Devi et al., 2018; Bakhshi and Ehsan, 2019).

$$\text{MFEI}=\frac{\frac{MFE}{lengthofpre-miRNAseq}*100}{G+C}\% \qquad (2.1)$$

RNAhybrid, psRNATarget, TargetMiner, miRanda, SVMicrO and psRobot are common tools that can be utilized in target prediction of selected novel miRNAs (Devi et al., 2018). Quantitative real-time PCR (qRT-PCR) technique is used to validate target of miRNA. The expressional level of miRNAs and mRNAs are opposite to each other. Overexpression or target gene silencing techniques and phenotypic changes observation are used for identification of miRNA and target gene roles (Koroban et al., 2016).

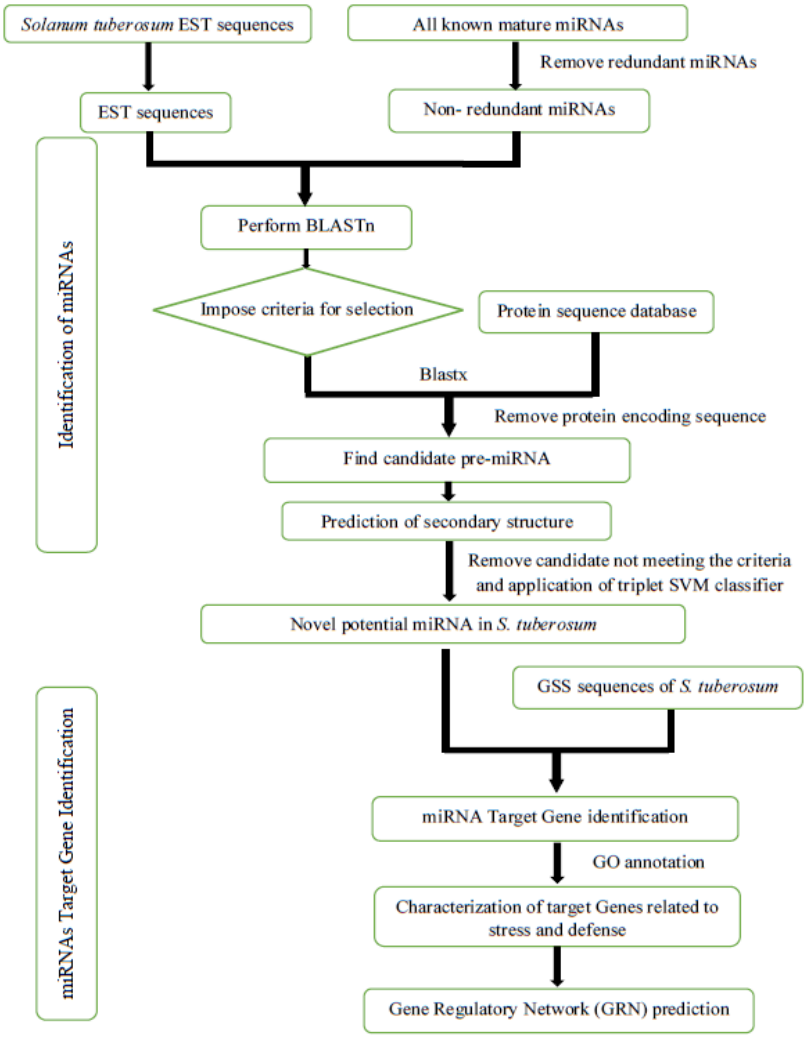


Figure 2.6. Flowchart diagram of miRNAs and target genes prediction in potato (Kumar et al., 2018)

2.6.3 Potential of miRNA against abiotic stresses

miRNAs regulate many events such as initiation and development of root, transmission bundles, transition to generative stage, flower differentiation and development (Chuck et al., 2009). Additionally, miRNA play crucial role in transduction of hormonal signaling and in environmental stress response (Sunkar et al., 2006; Liu and Chen, 2010). The discovery of abovementioned functions of these regulatory miRNAs has promoted an idea of silencing some genes by miRNA or increasing expression of certain genes (Chung et al., 2017). In this way, it will be possible that desired agricultural characteristics directly in the molecular arrangement and in plant breeding.

In summary, molecular regulatory studies of miRNA have potential to confer stress resistance that can improve quality and yield of major crops.

Çelik and Akdaş (2019) worked on *Ricinus communis* L. and reported about its capability of assembling of heavier metals like nickel which are required by plants in small amount, but they act as potential contaminant if their concentration increases. MicroRNAs (miRNAs) are behind detoxification of heavy metals. In this study, expressional levels of heavy metal stress responsive miRNAs and mRNA were evaluated and validated via qRT-PCR. It revealed contrasting regulations of stress-related miRNAs and targets mRNA under stress treatments. This regulation showed that regulatory processes vary between the tissues under nickel stress. Data described that miR838 was the most responsive miRna to the Ni2+ stress. In contrast target gene of miR398 that is Cu-Zn/SOD was up-regulated in root and leaf tissues. Different expression levels of miR159 and miR319 and TCP were significant in leaf tissues.

Chiou (2007) has indicated the new functions of miRNAs to regulate plant stress adaptive responses under nutrient stress. Plant miRNAs and their target mRNAs usually are reversely proportional to each other by affecting post-transcriptional cleavage. In case of phosphate and sulphate deficienct soil conditions miR395 and miR399 are up-regulated, respectively. Due to adjust the expression level of sulphate transporter (AtSULTR2;1) and ATP sulphurylase (APS) miR395 is reported to be a participant in sulphate assimilation and allocation. In the same way up-regulated miR399 ultimately resulted in decreased gene expression of UBC24, which is involved to encode ubiquitin-conjugating E2 enzyme. The plants showing over expression of miR399 are reported to be defective in UBC24 display Pi toxicity due to excessive uptake of Pi, enhancement in translocation of root to shoot and Pi retention in old leaves. It suggested that in Pi homeostasis the regulation of UBC24 is critical which is miR399 mediated. Moreover, among many plant species the conservation and existence of miR399, miR395 and their mRNAs revealed miRNA-mediated nutrient stress responses.

Shukla et al. (2008) described those abiotic stresses are playing a big role behind reduction in crops yield world widely. To minimize the loss caused by stress factors, plants have evolved and adopted reprogramming gene expression at different transcriptional, translational levels. Analysis of such processes will result in developing

novel strategies to improve tolerance against plant stress. The up/down regulation of the small RNAs in response to stress explores that these small RNAs play a role in stress tolerance. Down regulation of the target genes might occur in result of stress-induced small RNAs. In response to stress down regulation of sRNAs, caused the accumulation of mRNAs, that positively contribute for stress adaptation.

Lv et al. (2010) reported cold responsive 18 miRNAs were identified by using microarrays in rice. Most of them were down-regulated. Members of the miR319 and miR167 families exhibited similar profiles. Intriguingly, members of miR171 family showed various expression patterns. Only under cold stress three miRNAs obtained from sequence of transposable element were massed within an intronic region that proved to be co-transcribed within the host gene. The importance of hormones in this miRNA mediated defensive system can be indicated by the presence of hormonal responsive elements in cold responsive miRNAs at upstream region. Under cold stress two miRNA target pairs validated by 5′ RACE showed adverse expression profiles. Finally, in support of our results the predicted stress related targets of these miRNAs provided further authentication. These findings confirm that miRNAs role as universal regulators in rice.

Liu et al. (2008) reported that in Arabidopsis thaliana by affecting three abiotic stresses 14 stress-inducible miRNAs were identified using microarray data. Among them, 10 cold-regulated, four drought and 10 high salinity miRNAs were detected. miR171 miR396 and miR168 responded to all of the stresses. Expression profiling by RT-PCR analysis caused great cross-talk among the cold stress, drought and high-salinity signaling pathways. The presence of stress-related elements in miRNA promoter regions provided further authentication in favor of their results. These findings declared miRNA as universal regulators under stress conditions.

Li et al. (2016) reported that one of the most important plant hormones in strawberry is abscisic acid (ABA) which has critical role in ripening of fruits and adaptive responses to stress conditions. It was indicated by the previous results from the high-throughput sequencing that Fan-miR73, a novel microRNA (miRNA) was target of a ABA-insensitive (ABI)15 transcription factor that plays significant role in the ABA signaling pathway. This research established that exogenous applying of ABA promotes ripening

of fruits by differentially controlling the ABA metabolism transcripts and genes connected to transduction signals, like SnRK2.2, ABI1, NCED1, PYR1. Down-regulated expression of Fan-miR73 was due to the exogenous applying of the ABA treatment in a manner of dose dependence, that consequenced in ABI5 transcript accumulation of fruits that were ripening-extended. Moreover, the results from salt stress and radiation of UV-B showed declined transcript levels of Fan-miR73, while ABI5 expression was inclined. In addition, during the stages of fruit ripening and stress stimuli reaction were observed excessive negative correlations of transcriptional profusion ABI5 and Fan-miR73. Results from the research added new possible regulatory roles of miRNA connected to ABI5 post-transcriptional alterations in ripening of strawberry, as well as reaction to stress.

Ma et al. (2015) reported that the critical role of MicroRNAs (miRNAs) in development and growth of plants was established but wheat dehydration stress response and their role in it is not studied well. In addition, the systems by which miRNAs extend to various levels of tolerance to stress dehydration in various genotypes of wheat still remains unclear.

Omidvar et al. (2015) reported that the male sterile (7B/1tomato) mutant Solanum lycopersicum L. 'Rutgers' has wide abiotic stress tolerance in specific BL (blue-light) manner in comparison to the wild type. This opens the 7B-1 as a prospective candidate for stress controlling and breeding of hybrid seeds. To establish sRNAs (small RNAs) connected to stress response of 7B/1, BL-grown 7B-1 and its two libraries of sRNA and seedlings of WT were concomitantly treated with mannitol and abscisic acid which were sequenced, than profile comparison of small RNAs was done. In two libraries were done identification of 27 putative novel miRNAs and 29 known microRNAs (miRNAs). Upregulation of a novel miRNA denoted miR#C and miR2916, miR5301, miR5300 while on other hand down regulation of miR472, miR166, miR159, miR482, and two novel miRNAs, miR#D and miR#A was reported in stress-exposed 7B/1 seedlings. Fast amplification validation analysis of 5′ DNA complementary ends (5′-RACE) of miRNA targets was done that may have role in regulation of stress. The response of some expressions of miR472, miR166, miR-A, miR159, miR-D and miR482 in cooperation with targets were studied in reaction to mannitol, ABA, treatments of cold and NaCl and the observation of tough negative correlation was done

among the miRNA levels and tarhet expressions. The only miR159 and miR166 samples were responsive to the treatment of cold. Regulation of MiR#A and targets of it was done by mannitol and ABA as possible 0.5 h later the treatments; regulation of other targets and miRNAs was only done 2 h after. This shows a part in miR#A early reaction to stress. Our information guesses that miR482, miR472, miR#A, miR159, miR#D and miR166 might promote intensified BL-determined tolerance of 7B-1 to abiotic stress.

Akdogan et al. (2016) described that the group of little noncoding regulative RNAs represented by microRNAs (miRNAs), that control expression of genes by leading the target mRNA division or inhibition of translation. The crop development and growth often influenced by environmental stress like drought. In addition, it was showed that some miRNAs of plants are participating in reaction to the drought stress. In this research, screening miRNA microarray made possible to investigate similarities of responsive to stress of drought responsive miRNAs roots as well as in the leaves of wheat.

The analysis of miRNA microarray displayed that some miRNAs were differentially expressed among 244 miRNAs (115 up-regulated, 129 down-regulated), and 285 miRNAs (207 up-regulated and 78 down-regulated) in tissues of root and leaves. In the wheat grown under the stress conditions of drought among differentially expressed miRNAs were investigated 23 miRNAs that were exhibited in leafs and some 26 miRNAs in roots. In bread wheat after treatment of drought the strong differentiation in expression of miR482, miR408, miR172, miR477, miR159, miR160, miR169, miR2118, miR166, miR395, miR396, miR472, miR5049 and miR1858 was established. The association network survey established that miR395 has relations with some transcripts of target, and common target genes were found in miR159 and miR319. Some altered expression motifs upon stress of drought were found in wheat cultivars that sensitive and tolerant to drought, in transcript expression levels of studied miRNA and its targets.

Gu et al. (2017) reported that despite the fact of well studying the toxicity in melatonin alleviated Cd (cadmium) in plants and animals, the controlling systems in plants not investigated well. In this work we found stimulative effect of Cd stress in endogenous

processing of melatonin in tissues of root of alfalfa seedlings. The melatonin level was increased in response to the exogenous melatonin pretreatment, were observed reducing the Cd induced seeds growing. Moreover, was observed extended tolerance of Arabidopsis transgenic plants which were melatonin-rich that over expressed alfalfa SNAT (a melatonin synthetic gene), in comparison to the wild type plants of Cd states. Cd levels were declined in tissues of root. In contrast to the only Cd stress, the transporter of ABC and alfalfa seedlings transcripts in PCR2, PDR8 and HMA4 in Arabidopsis, appeared to be melatonin up regulated. Meanwhile, the down-regulated transcripts of Nramp6 were observed. Differences in above mentioned transporters related to the lower assembling of Cd. In addition, melatonin upgraded the imbalance which was triggered by Cd redox. All of them might be provided by differences of the Cu/Zn SOD gene regulated by miR398a and miR398b. The similar results were received from the contents analyses of H2O2, histo-chemical staining, confocal microscope of laser scanning. Taking into consideration, we obviously guessed that melatonin improved Cd tolerance by reducing accumulation of cadmium and modifying the microRNAs-mediated redox homeostasis.

Kaja et al. (2015) reported one of the most important parts in various biological processes are done by MicroRNAs (miRNAs); consequently, characterization and investigation of these little controlling RNAs is a critical stage for understanding the biology. The total number of known microRNAs comes to 200 in apple (Malus domestica), which might be just a part of miRNAome diversity. Therefore, for better annotating miRNAs it is required more effort and their roles in the species of economic importance. We provided 12 small RNA libraries deep sequencing, gained for fire blight-sensitive trees and fire blight-resistant. In results obtained from sequencing, we found 116 novel miRNAs and established most of the already found miRNAs in apple. The selected candidates were then verified experimentally with stem-loop quantitative PCR and RT-PCR and provided contrasting analysis of expression. In the end, we found and indicated supposed targets of all familiar miRNAs of apple. The analysis of gene ontology enrichment guesses important parts of microRNAs in reaction to stress, which includes infection of pathogens. In present research, 116 new and established the expression of 143 already familiar micro RNAs were found. In addition, this information guesses that miRNAs of apple plants may be counted as controllers and markers of hostility to fire blight. The performed analyses made possible to explain the

four miRNAs of apple plants that possibly having part in resistance to fire blight in trees of apple plants: mdm-miR167b-g, mdm/miR160e, mdm/miR169a and mdm/miR168a,b.

For checking if SPs may promote activity of plant miRNAs, artificial SP trans genes having various miRNA binding sites focusing on various families of miRNA in Arabidopsis were induced. Moreover, the results of silencing were examined to the correlating mimic and short tandem target MIMICs transgenes via calculating commonness and acuteness of abnormal phenotypic changes obtained from all transgenes. Although, there is no obvious impact of SPs and binding sites of wild-type miRNAs, the generation of vigorous loss-of-function phenotypes can occur having SPs with micro-RNA binding sites and cmSPs (two central mismatches). Although, their results strongly differed, starting from prompting strong loss of function phenotypes to faulting to build any influence on phenotype.

Lu et al. (2019) reported that cotton producing is overly controlled by drought stress, mainly if experienced in the stage of seedling or initiation of fiber and stage of elongation, but the fundamental systems of drought regulation still unclear. For that reason, functional study and characterization of microRNA-mediated networks defining regulatory system of stress are important to deciphering response of the plant to drought. In this research, three treatments (drought, re watering and CK) were used to obtain 357, 377 and 379 miRNAs with annotations in cotton ZhongH177 variety and it was separated into 73 families of miRNA with differing numbers of to 24. They identified 136 DEGs - differential expressed genes with important changes of expressions, where 33 of DEGs appeared to be up regulated, other 103 DEGs down regulated in stress of drought. Although, the time when plants were re watered most of the DEGs returned beginning expression levels. To sum up, were identified 2657 targets and discovered to be mostly enriched in ways of interaction of plants and pathogens, amino acids biosynthesis and signal transduction of plant hormones. The tolerance to drought remarkably was increased in two Arabidopsis transgenic lines, discovering that micro RNAs had role in response to the drought in cotton plants. The examination of the expression motifs of two miRNA precursors and modification of methylation of two targets supposed that micro RNAs or micro-RNA precursors may have a role in controlling of methylation target levels. Our work shows evidence to the drought transcriptional reactions that is useful for the future studies of drought tolerant systems.

2.7 Studies on miRNAs that Play a Role in Regulating Potato Plant Growth

Recently a total of 7,025 miRNA entries from 86 plant species have been registered in the miRbase (Guo et al., 2020). Numerous miRNA targets have been estimated, some of which have been confirmed and verified experimentally. Efforts to identify miRNAs and target genes are responsible to perform functions and growth in potato plant have begun in 2009 and are still in progress. Zhang et al. (2009) compared potato expressed sequesnce tags (EST), genomic survey sequence (GSS) and nucleotide databases using known miRNAs from other plant species and identified 48 potential miRNAs in potato by *in silico* approach. As a result of the bioinformatics analyses, they reported that these miRNAs could regulate 186 potential target genes in leaf, flower and stem development, metabolic pathways, signal transduction and stress reaction. To confirm presence of potato miRNAs predicted by bioinformatics methods, RT-PCR analyzes were performed for only 12 from randomly selected 48 candidate miRNAs (Zhang et al., 2009). As a result, it was determined that few miRNAs expressed in all plant parts, but expression levels vary depending on the tissues. While another *in silico* approach has been progressing to predict miRNAs, Yang et al. (2010) identified 71 potential miRNAs from 48 families and out of 71, approximately 65 miRNAs were predicted for the first time. Only 7 miRNAs were selected from the estimated 71 miRNAs for verification purposes and expression levels in different tissues of the potato were examined by qRT-PCR. The results showed that all of these 7 miRNAs can be successfully replicated and differ in expression levels in different tissues (Yang et al., 2010). Researchers have reported that expression of miRNA at different levels in separate tissues may be related to function of miRNAs for regulating vegetative development of the potato. Xie et al. (2011) considered that previously identified miRNAs were low compared to the number of miRNAs detected in other plants and they thought that there could be more miRNAs in potato. Then, using newly modified genome comparison strategy with bioinformatics approaches, they identified a total of 202 potential miRNAs from 78 families. They found that 54 of these 78 families were new in potatoes. They analyzed only expressions of selected 12 miRNAs from 202 potential miRNAs in young leaves, immature flowers and mature flower tissues and observed that out of analyzed miRNAs 11 showed expression in all tissues while one of them did not show expression in young leaves. Xie et al. (2011) estimated approximately 1,094 target miRNAs genes, and they reported the function of genes that

encode for transcription factors; some genes are functional in the stress response, signal transduction and other metabolic stages. In another study conducted to predict miRNAs and target genes in different agricultural plants belonging to *Solanaceae* family *in silico* approach, 22 miRNAs and 221 target genes were reported in potato (Kim et al., 2011).

Zhang et al. (2013, 2014) identified 259 miRNAs associated with 159 miRNA families in potato, by NGS approach and potato genome sequencing consortium public data (PGSC, 2011). In these miRNA families, they reported that 28 miRNA families are conserved in other plants and that others are potato specific. Predicted potential target genes include transcription factors (TFs), genes involved in defense mechanisms, genes involved in kinase and ion balance, and genes responsible for flowering and formation of tuber (Zhang et al., 2013). In another study, aiming to identify and characterize potato miRNAs from samples taken from four different stages of tuber growth and three different tissues, 89 conserved miRNAs from 33 members of the family, 112 potato-specific candidate miRNAs and 147 potato-specific miRNAs were identified (Lakhotia et al., 2014). Some of the miRNAs found are miR160, miR164, miR172, and miR171, and the target genes are auxin response factor 16 (*ARF16*), relative to apetala2 1 (*RAP1*), no apical meristem (*NAM*), and hairy fringe meristem (*HAM*). As a result of the expression analyzes performed in this study, it has been shown some miRNAs produced specific expression in particular tissues and some miRNAs are specific for cycle.

Many reports indicated that mRNA turnover and miRNAs are key components underlying PTR genes encoding sugar metabolic enzymes/transporters. The first study to investigate miRNAs and target genes in cold-stored potato tubers was performed by Ou et al., (2014). They reported, 53 known, 60 new miRNAs and 70 target genes. It was also reported that miRNAs were active in regulating gene expression in post-harvest tubers. In addition, there were differences in expression of 11 miRNAs and 34 mRNAs in two potato genotypes having different response to storage conditions (Ou et al., 2015). In another study comparative genomic approach was used by Din et al. (2014) to identify 120 novel miRNAs from 110 families and proved expression of only 10 randomly selected miRNAs by quantitative RT-PCR. The same study indicates that 433 potential target genes of 120 miRNAs are related to metabolism, TFs, growth and development, and other important physiological traits (Din et al., 2014).

2.8 Studies on miRNAs that Play a Role in Regulating Abiotic Stress Response of Potato Plant

Up to now, studies have been carried out on detection of miRNAs playing their role in regulating drought tolerance only within abiotic stress. The results of these studies were first published in 2011, according to available literature few studies have been reported concerning miRNAs that regulate drought tolerance in potato.

Hwang et al. (2011) identified 3 miRNAs named stu-miR156a, stu-miR157a, and stu-miR396, which are expressed in the potato drought stress conditions. stu-miR396 expression was high from first to sixth hour after drought application. Expression of stu-miR159a and stu-miR157a increased to 6th hour when it decreased in first and third hours. Hwang et al. (2011b) investigated expression alteration of miRNAs including miR171 family in drought stress conditions in another study and observed that miR171a, miR171b and miR171c change in no water stress. They carried out drought application by adding 15% PEG 6000 to medium and by air dyring in a temperature controlled room. In air dry condition, the expression of miR171a decreased at first hour of stress application and then increased until sixth hour. In drought with PEG, miR171a decreased after 1 hour, then increased after 3 hours and remained same for 48 hours. Expression of miR171b declined in 1st hour of outdoor air application, increased to control level in 3rd hour and exceeded the control level in 6th hour. In PEG application, a slight decrease in expression of miR171b was observed up to 6th hour but from 12th hour, expression of miR171b was higher than control. The expression pattern of miR171c in outdoor drying was identical to miR171a. In PEG application, after 1 hour, expression decreased, then slightly increased and reached control level after 48 hours. Hwang et al. (2011c) measured the changes in expression levels of stu-miR172c, stu-miR172d, and stu-miR172e miRNAs in response to drought stress. Six miRNAs families consisting of a total of 11 miRNAs involved in regulating expression of genes responsible for proline elevation under water deficit conditions were estimated (Yang et al., 2013). Ten of the 11 miRNAs predicted by qRT-PCR analyzes made were successfully detected, and the expression of one of them increased while the expression of 9 decreased in arid conditions. Resultantly, the findings were obtained that miR172, miR396a, miR396c and miR4233 may be associated for the regulation of the expression of gene (P5CS), for proline synthesis. In addition, other genes responsible for proline

synthesis, *P5CR* and *ProDH* genes, may be regulated by miR2673 and miR6461, respectively (Yang et al., 2013). A study determined that potato miRNAs involved in regulating tolerance regulation, 674 novel and 458 known miRNAs in control samples, and 471 known 566 new miRNAs in the drought-treated samples were detected (Zhang et al., 2014). Same study preserved miRNAs whose expression was altered by more than 2 quarts in drought stress conditions was selected and a decrease in the expression of 100 of them and an increase in the expression of 99 were observed. Of the new miRNAs selected in the same way, the expression of 119 was higher, while the expression of 151 was lower. Zhang et al. (2014) predicted 214 novel miRNAs and 246 known miRNAs. Expression analyzes carried out on miRNAs and target genes have identified miRNAs as miR835, miR4398, miR811 and miR814 named miRNAs that play a role for drought tolerance. The targets of mentioned miRNAs were WRKY and MYB TF, hydroxylproline-rich glycoprotein, aquaporin, respectively. Expressions of mRNAs and miRNAs related with water deficit stress correlated negatively. A recent study identified change in large no of miRNA profile in response to single and combined drought and heat stress conditions in two potato cultivars (Unica and Russet Burbank) using sRNA sequencing (Gokçe et al., 2021). In this study stress was applied after 40 days of planting (tuber development stage). Drought stress was applied by cutting off water for 23 days and for heat stress a growth chamber was used to apply temperature ranging from 24/18 °C to 39/27 °C for 12 days. They concluded that eight miRNAs (Novel_105, Novel_9, Novel_8, miR162a-3p, miR156d-3p, miR172b-3p, miR398a-5p and miR160a-5p) were targeting the genes playing important role in respons to abiotic stress. Their results indicated that these miRNAs may play a vital role at different post transcriptioan levels against heat and drought stress in sensitive plant species. Şanlı, B.A. and Gökçe, Z.N.Ö. (2021) used a transgenic approach to overexpress miR160 in two potato cultivars (Unica and Russet Burbank) and applied drought and heat stress individually and their combination as well. Alongwith the confirmation of Auxin response factor 16 (ARF16) as target gene of miR160a-5p, study was also aimed to understand their function under stress. Transgenic plants showed improved tolerance to abiotic stress factors with improved physiological and biochemical traits. They reported that as miR160a-5p is involved in ABA pathways to increase tolerance of stress, it may also be involved in regulating heat shock proteins to confer heat stress for stabilization of proteins. Yalcin, M. and GÖKÇE, Z.N.Ö., (2021) overexpressed novel_105 miRNA in contrasting potato cultivars (Unica and Russet

Burbank) and applied single and combined drought and heat stress on wild type and transgenic plants. They observed an increase in novel_105 miRNA and a decline in expression of its predicted target *E3 ubiquitin-protein ligase XBAT35* in transgenic plants. Transgenic plants showed improved physiobiochemical functions of both cultivars under single and combined stress conditions. In transgenic plants minimal membrane damage and higher proline contents proved that the tolerance mechanism was activated with over expression of novel_105 miRNA. In a related study Asim et al. (2021) also reported that overexpression of miR172b-3p in two potato cultivars (Unica and Russet Burbank) is involved in front line tolerance mechanism against individual and combined drought and heat stress. Through indicators of biochemical, physiological and molecular analysis they proved *Ethylene responsive transcription factor RAP2-7-like* as target gene of miR172b-3p and also reported that they are probably involved in individual and combined stress tolerance in potato.These results can be interpreted as molecular evidence that miRNAs participate in response or tolerance mechanism of potato and can be used for development of abiotic stress resilient potato cultivars.

Transgenic studies elucidated functions of miRNAs associated with abiotic stress tolerance were performed on other plants, although not on potato. For example, under drought stress the expressional levels of miR169 is decresaed in Arabidopsis and expressional levels of core factor YA5 (*NFYA5*), which is target of miR169, is significantly stimulated (Li et al., 2008). Similarly, they also observed silenced nfya5 plants by knock-out technique and in transgenic plants expressing high levels of miR169a, leaf water loss was increased, and they were more susceptible to drought stress than non-transgenic control plants. In contrast, a decrease in plant water loss was reported in Arabidopsis transgenic plants with high *NFYA5* expression, and these transgenic plants were found to be more tolerant to drought stress than non-transgenic plants (Li et al., 2008). Similarly, soybean *GmNFYA3* gene as target of miR169, was transferred to Arabidopsis and transgenic Arabidopsis plants decreased water loss and increased tolerance to drought stress (Ni et al., 2013). However, in Arabidopsis overexpression of *GmNFYA3* has led to increased salinity stress and increased susceptibility to exogenous ABA (Ni et al., 2013). In tomato (*Solanum lycopersicum*), opposite observations of above findings were obtained. In drought stress conditions, expression of miR169 was increased in contrast to that in Arabidopsis and expression *NF-YA1/2/3 and MRP1* genes as its targets decreased. Constant overexpression of

tomato miR169c causes stoma openings, reduction in transpiration rate that also limits water loss via leaf, conferring drought tolerance (Zhang et al., 2011). Transgenic tomato plants with miR169 showed no visible developmental and morphological changes under field conditions. This suggested that miR169 or its mRNA target may have potential for stress tolerance (Zhang et al., 2011). Interestingly, high level of miR169 increases no water stress tolerance in tomato, while increasing susceptibility in Arabidopsis. From these evidences, it can be concluded that same miRNAs have different regulatory effects on different plant species under same conditions. For this reason, regulatory effects of specific miRNAs must be identified in each plant species and miRNA-based molecular regulation strategies should be developed based on species of plants.

The expression of miR319 in Arabidopsis was reported by Liu et al. (2008), sugar cane Thiebaut et al. (2011), and rice Lv et al. (2010) that was altered by cold stress. In cold stress exposure of rice, showed that expression of miR319 was decreased and expression of target genes as *OsPCF5,6,7,8 and OsTCP21* reduced (Lv et al., 2010). Detailed transgenic studies were performed and it was determined that tolerance of plants to cold (4 °C) after acclimatization to cold (12 °C) was increased due to high expression of Osa-miR319 gene in the transgenic rice (Yang et al., 2013). However, severe regression was observed in development of miR319 transgenic rice plants. To prevent the pleiotropic effect of miR319, two RNAi lines for *OsTCP21*and *OsPCF5*, two target genes of miR319, have been developed. These RNAi lines showed both better tolerance to cold than those of wild type controls, provided that they were acclimated to chilling, as well as phenotypically normal development.

Zhou et al. (2013) investigated the regulation of tolerance of *Agrostis stolonifera*, a preferred grass species in golf courses, to drought and salinity via miR319. Transgenic *A. stolonifera* plants transfected with rice miR319 gene (*Osa-miR319*) and carrying out high level of miR319 expression increased tolerance to drought and salinity and this tolerance increase was associated with high leaf intake and water retention and less uptake of sodium. As a result of gene expression analyzes carried out in the same study, a significant reduction in expression of four predicted target genes of miR319 (*TCP-AsPCF5, TCP-AsPCF6, TCP-AsPCF8* and *TCP-AsPCF14*) was observed and increased abiotic stress tolerance could be associated with decrease in the expression of these genes (Zhou et al., 2013). However, in transgenic *A. stolonifera* plants, increased

expression of Osa-miR319 resulted in undesirable properties of *A. stolonifera* plant such as leaf size increase, stem expansion, and decrease in shoot number. These results show that miRNA DNA sequences can be transferred to other plants to increase abiotic stress tolerance in plants. However, attention should also be paid to effects of miRNA expression changes on other characteristics besides stress tolerance.

Guan et al. (2013) reported a new thermo tolerance mechanism of plant, particularly related to reproductive organs. In this mechanism, expression of miR398 is increasing, thus reducing expression of the Cu/Zn SOD genes (*CSD1* and *CSD2*) which are target of miR398 and *CCS* gene (gene encoding Cu chaperone for *CSD1* and *CSD2*) (Guan et al., 2013). In the same study, it was found that *csd1, csd2* and *ccs* mutant plants showed an increase in accumulation of heat shock protein and temperature transcription factor and showed high temperature tolerance than wild (non-mutant) plants (Guan et al., 2013). It was reported that tolerance of summer plants to high temperature, especially those having flowering season with high temperatures, can be increased by molecular modifications of miR398 or its targets.

Yang et al. 2016 reported that miRNAs of plants have significant role in endogenic gene regulation which controls expression of genes at post-transcriptional level. Conducted studies established that NF-YA transcription factors which have role in development of plants and response to stress are regulated by family members of miR169. Specifically in this research, family members of four novel stu-miR169 in potato are forecasted according to the sequence data of potato genome. Sequences of Stu-miR169 have a target of potato 5 of StNF-YA genes according to the target prediction of miRNA, and assay of RNA ligase-mediated 5′RACE (5′ RLM-RACE) proved three of them. The study of stu-miR169 expression patterns and expected genes of their target resulted in down regulation of stu-miR169 in reaction against drought stress. Throughout the different stages of samples that were drought-treated, some StNF-YA target genes with mature stu-miR169 displayed unfavorable expression. The research results of the expression motifs from mature stu-miR169 and their expected target genes resulted that mature stu-miR169 response to the stress drought was down regulated. Some StNF-YA target genes that displayed dismissive expression motif with mature stu-miR169 throughout different stages of samples treated with drought. Moreover, the declined stu-

miR169 expression may have effect on over expression of family members of NF-YA which have relations based on resistance to drought stress.

Teotia et al. 2017 reported that miRNAs of plants control hundreds genes of target consisting of transcription factors that regulate stress response, growth regulation and development. Although, concrete functions of miRNA cannot be interpreted because each family of miRNA in the genome have different loci, that's why functionally unessential. For that reason, better way to investigate functions of miRNA family is simultaneous expression silencing of all members, which is hard job. Although, that may be partly done by TM - Target Mimic approach that allows to knockdown the whole miRNA family. Addition and developed way of the TM approach is STTM. The successful blocking of miRNA functions in dicots and monocots was done by usage of STTMs. It is established that miR159 differentially controlled by numerous abiotic stresses as well as ABA in different plants. In this work they describe in depth the protocol for constructing STTMs that are used to stop up functions of miR159 in Arabidopsis, with the possibility to use this method on different miRNAs which are stress regulated in plants.

Wyrzykowska et al. (2016) reported that usage of artificial microRNAs (miRNAs) is the recent technique of molecular biology that found different applications in sciences. This work describes CBP80/ABH1 gene silencing in Solanum tuberosum with the help of miRNAs. The Cap Binding Complex (CBC) consists of the CBP80/ABH1 protein as a part of its complex, which has a role in drought stress response. The plants transformed with the lower levels of CBP80/ABH1 protein show increased tolerance to conditions with reduced water. Designing of miRNAs with the help of Web MicroRNA designer platform was described in detail. Moreover, we describe how to conduct all stages of a procedure directed to receive transgenic potatoes with the help of prepared miRNA, using callus tissue culture and the strain LBA4404 of Agrobacterium tumefactions was used as a transgene carrier.

Shin et al. (2017) reported that MicroRNAs (miRNAs) considered as non-coding RNAs that act as negative controllers at post transcriptional level throughout the growth phases of the plant in response to both abiotic and biotic stresses. Main aim of this research was to apply next generation technique - miRNA-sequencing to investigate the drought

receptive miRNAs of potato (Solanum tuberosum L.). After experimentally generating small RNAs under aeration in the absence of watering for 1-3-6 hours contrasted to 0 hours of control, NGS was applied to find out drought receptive miRNAs. The selection of miRNAs was done according to the drought response, from that were selected 19 novel miRNAs and 21 known miRNAs. Among these samples 38 were examined to analyze expression level in four abiotic stress (drought, salt, cold, and abscisic acid) via northern blot, on the other hand two novel miRNAs were subjected to qRT-PCR to examine the level of expression. Analyze of the effect of abiotic stresses like salt, cold and ABA treatments were also conducted on other 38 miRNAs responsive to drought. Computational analysis was done to determine supposed gene target regulatory network of novel miRNAs.

Qiao et al. (2016) reported that light is a main natural factor that has impact on metabolic pathways and triggering building the potato secondary metabolites. Although, elastic changes in metabolic pathways of potato and physiological roles started due to light can be described by changes in expression of gene. Extensive studies at transcriptional level were conducted in controlling potato secondary metabolic pathways but on the other hand, the system of post-transcriptional regulation by miRNAs is not studied well. A homogenized omics (transcriptome and sRNAome) analysis was done to potato plants under the stimulus of light to investigate light-receptive miRNAs/mRNAs and develop supposed metabolism pathways controlled by the miRNA–mRNA pairs. They identified differentially expressed total 31 and 48 miRNAs in the tubers and leaves. It is established that among differentially expressed genes total 1841 genes from the tubers and 1353 genes in the leaves are upregulated, 897 genes in the tubers and 1595 genes in leaves were down regulated be the impact of the light. The analyses of Mapman enrichment identified up regulated metabolism of flavonoids, carotenoids, and phenyl propanoids and alkaloids-like that connected to MVA pathway genes, although CHO metabolism related genes appeared in the tubers and leaves. This research gave a major view to expression profiles of miRNA and mRNA in responsive to light potatoes, this result concludes that miRNAs may have important part in pathways of secondary metabolic, particularly in biosynthesis of glycoalkaloid. Results helped to understand genetic control of secondary metabolite pathways and open the way for further usage of genetically modified potato.

Chen et al. (2009) reported that overexpressing of miR1916 in tomato (OE-1) declined drought tolerance. While significant incline in drought resistance was observed in transgenic plants (ST-1 and Anti-7) having miR1916-silenced. The modified plants of tobacco have the same result in showing drought tolerance. Through physiological analysis revealed that osmoregulation and accumulation of reactive oxygen species (ROS) was affected by miR1916. Moreover, level of transcripts in the miR1916 genes of target, synthase of strictosidine (STR), histone deacetylases (HDAC) declined in miR1916-overexpressed modified tobacco plants. The results showed that miR1916 is submissive regulator in the plant drought tolerance and has a role in responses to abiotic stress in Solanaceae family.

Yang et al. (2013) reported that one of the main abiotic stresses of the thermophilic plants is low temperature that affects to the development and growth. The molecular mechanism of cold stress response in Eggplants is unclear. MicroRNAs are group of endogenic non-coding RNAs which play important role in plant growth and response to stress. On the other hand, the impact of various plant miRNAs in providing chilling toleration has been proved, but the mechanisms of chilling tolerance of eggplant is not fully studied.

Xie et al. (2017) reported that sweet potato (Impomoea batatas L.) is economically a major crop with a possibility of being used in pharmacy and turning into a bioenergy. Thus, investigating molecular basis of the tuber root development mechanisms and storage is so significant. In this research, they sequenced sweet potato degradome and all small RNAs with the help of high-throughput deep technology of sequencing to learn about reaction to chilling stress. There were identified 191 novel miRNAs and 190 known microRNAs, and 184 identified miRNAs also target 428 transcripts. Moreover, findings include differentially expressed 26 miRNAs under conditions of control and chilling stress. qRT-PCR confirmed expression of miRNAs and its targets. The analysis of RNAs and degradome sequencing showed that signaling of SA, response pathways of ROS and ABA-dependent are miRNA-mediated.

2.9 miR156d Discovery and Function

Reinhart et al. (2002) reported the discovery of miR156 in *Arabidopsis thaliana* and described its differential expression patterns in development. They reported that during development of Arabidopsis embryos already described roles of CARPEL FACTORY in floral meristems and leaves. Aung et al. (2015) described that in plant kingdom, miR156 and its target *SPL* genes are highly conserved and to control various aspects of plant growth an extensive gene regulatory network is being formed by them together. Cui et al. 2014 reported involvement of miR156 in integrating environmental signals to ensure intime flowering in *Arabidopsis thaliana*. They reported that blocking miR156 signaling pathway through target mimicry supported the sensitivity of the plant against stress treatment. They identified downstream genes of miR156, i.e. DIHYDROFLAVONOL-4-REDUCTASE (*DFR*) and SQUAMOSA PROMOTER BINDING PROTEIN LIKE 9 (*SPL9*) which influences the metabolism of anthocyanin.

Stief et al. (2014) reported that miR156 and its isoforms are highly induced to cope with heat stress for adoption at physiological and molecular levels. The miR156-SPL module regulates the response to elevated temperature in *Arabidopsis thaliana* and may serve to integrate development with stress responses.

Wang et al. (2011) reported that in maize and Arabidopsis miR156 is majorly present in seedlings and decreased at stage of juvenile to adult transition. Overexpression of miR156 in transgenic *populus x canadensis* caused reduction in *SPL* genes and miR172, and it prolonged juvenile phase. They concluded that miR156 is an evolutionarily conserved regulator of vegetative phase change in annual herbaceous plants.

Overexpression of miR156 in maize, rice and Arabidopsis caused prolonged vegetative phase together with the enhancement of biomass accumulation due to production of significantly higher number of total leaves (Schwab et al., 2015; Chuck et al., 2007; Xie et al., 2006). In rice, miR156 was reported as a respondent to drought stress and was down-regulated (Zhou et al., 2010). However, Ding et al. (2009) reported that in case of salt stress of maize, miR156 was not involved in salt response. Sun et al. 2012 indicated that the expression of miR156 was significantly induced under high drought condition in switch grass.

Liu et al. (2018) reported response of miR156 and their roles in tomato drought stress by by high-throughput sequencing. They compared miRNA profiles pre and post drought application in two tomato genotypes: IL2-5, a drought tolerant introgression line and M82, and a drought sensitive cultivated tomato (*Solanum lycopersicum*). They reported contrasting expression levels of miR156d between both genotypes. The miR156d expression downregulated in IL2-5 but was induced in M82.

2.10 miR172b Discovery and Function

Park et al. (2002) reported firstly the presence of miR172 in *Arabidopsis thaliana*. Another miRNA, such as miR172 regulated expressional levels of few AP2-like TFs and perform functions for developmental stages.

Zhu et al. (2011) reported that the expression level of AP2-like transcription factors is regulated by miR172. Among monocotyledons and dicotyledons, miR172 functions in specifying floral organ identity and regulating the transitions between developmental stages. Additionally, miR172 also plays its roles in specialized species-specific functions such as tuberization and cleistogamy.

Aukerman et al. (2003) reported that miR172 plays its role in support of early flowering and disrupting specification of floral organ identity. In Arabidopsis, overexpression of miR172 confirmed the subfamily of APETALA2 transcription factor genes as its target gene. Loss-of-function and gain-of-function analyses indicated down regulation of AP2 like target genes and confirmed miR172 roles in regulation of flowering time. Martin et al. 2009 reported the involvement of mir172 in support of tuberization in potato by down streaming photoreceptor phytochrome B (PHYB) and up streaming tuberization promoter homeodomain protein BEL5.

Zou et al. (2013) reported miR172 as a critical regulator during post-germinative growth and it is involved in ABA sensitivity during osmotic stress. While Bouaziz et al 2015 proved that in potato, alignment of AP2/ERF led to the identification of six StERFs and four StDREBs. In response to drought stress, the StDREB1 and StDREB2 genes showed increment in their expression and this DREB family is directly involved in regulation of reactive oxygen species production.

Zhu et al. (2009) reported that in rice delayed transition was resulted due to miR172b over-expression and it showed defects in development of floret. Elevated levels of miR172 resulted in repression of SNB and one of the target genes.

2.11 Target Gene of miRNA novel 8

Target genes of MiRNA novel 8 include PhotosystemII core complex proteins psbY, chloroplast and mitochondrial transcription termination factor family protein.

Surosa et al. (2007) reported that higher plant photosystem II (PSII) is oxygen-evolving complex (OEC) and is made of PsbP, PsbQ, PsbO, three nuclear-encoded proteins and an inorganic Mn 4 Ca cluster. They reported that Psbo is involved in binding calcium and GTP and being a manganese-stabilizing protein, it possesses a carbonic anhydrase activity. PsbP and PsbQ the two isoforms of the PsbO protein in Arabidopsis thaliana are not only involved in providing chloride and calcium ion concentration for water splitting but they also seem to have an additional role in grana stacking for the formation of PSII super complexes.

Shi et al. (2012) reported that Photosystem II is capable of absorbing splitting water and light. Either transiently or stably, around more than 40 proteins bind to the complex. Unusually high content of low molecular mass proteins is another special feature of this complex that represents more than half of the proteins. They found that as compared to the nuclear localized genes, chloroplast-localized genes which are involved in encoding PSII proteins show different response to environmental stress conditions.

Robels et al. (2012) reported that mda (mitochondrial transcription termination factor defective in *A*rabidopsis) mutations increased osmotic and salt stress tolerance and during seedling establishment altered sugar responses, which is possibly as a result of reduction in sensitivity of ABA. Up-regulation of the RpoTp/SCA3 was observed due to loss of MDA which is involved in nuclear gene encoding a plastid RNA polymerase and modified the levels of chloroplast gene transcripts. They reported a new mTERF factor in plants and showed that probably through ABA signaling, its role is in abiotic stress

responses, in maintaining connection of chloroplast gene expression, activity of ABA and adaptation of plant to stress.

Quesada et al. (2016) reported that abiotic stress effect plant development and growth, leading to devastating yield losses. During evolution process, plants adopt various strategies to adapt to such stresses, which may cause functional diversification or expansion of different genes. In response to abiotic stress, few novel genes may perform diverse functions. The mitochondrial transcription termination factor (MTERF) family is originally characterized and identified in metazoans; MTERFs regulate DNA replication in vertebrate mitochondria, transcription and translation. Nonetheless, just eight plant *MTERFs* have been classified, that encode chloroplast/ mitochondrial proteins.

Shevetsov et al. (2018) reported that MTERF family genes has emerged as a key player for mitochondrial gene expression. They reported characterization of mTERF22 in Arabidopsis thaliana that functions to regulate mtDNA transcription and highlighted that mTERF22 have role in numerous mitochondrial genes in plants.

Because potato plant is generally susceptible to abiotic stress, significant yield losses occur when conditions of one or several of adverse environmental conditions arise. Mittler (2006) reported that in the United States between 1980 and 2004, there was 20 billion dollars loss in agricultural production due to only drought, and in the same years, the loss caused by drought and hot air wave coming together has reached 120 billion dollars.

Regarding average temperature of 59 years in Niğde and Nevşehir regions where potato farming is common, it is observed that average maximum temperature in July and August is 28-29 °C (Anonymous, 2014). In Niğde and Nevşehir, during the last 59 years, the highest temperatures in July and August were between 38.0-39.5 °C (Anonymous, 2014). As can be understood from these meteorological data, hot weather fluctuations occur in Niğde and Nevşehir where potato production is the most in Turkey and during July-August months covered by potato production period. It is estimated that these hot weather waves affect potato yield negatively, because potato is known to be the abiotic strass sensitive plant. All studies conducted in Turkey in recent years show

that irregularities in rainfall regime increase due to global climate change and drought risk increases especially in the southern and central Anatolia due to decrease in rainfall (Aküzüm et al., 2010; Bahadır, 2011, Kapluhan, 2013; Öztürk, 2002; Turkeş, 2012). Turkeş (2012) stated that annual total rainfall in a major part of our country is decreasing, and the risk of drought and desertification is increased in the study conducted using data obtained from various climate models, and he brought a series of suggestions as a precaution, including breeding of tolerant varieties to drought.

There are many ways to overcome agricultural damage caused by abiotic stress conditions, but the most effective method is applying abiotic stress tolerant plant varieties. Recently, potato varieties used for agricultural production have been highly sensitive to drought and high temperature (Levy and Veilleux, 2007). Concerns about abiotic stress due to global climate change have begun to increase throughout the world in recent years, and development of drought and heat-tolerant varieties of potatoes has become one of the priority breeding goals (Monneveux et al. 2013).

CHAPTER III

MATERIALS AND METHODS

An abiotic stress-tolerant variety, Unica, was used as plant material to produce miRNA overexpressed transgenic potato plants in this study. Transgenic plants were produced by Agrobacterium-mediated gene transfer technique, using a plant expression vector harboring the relevant pre-miRNA's cDNA as insert. Various over-expressed potato transgenic lines for stu-miR156d-3p, stu-miR172b-3p, and miRNA novel 8 and a non-transgenic Unica cultivar were investigated under three abiotic stress conditions (drought, heat, combined drought+heat) for determination of the functions of the relevant miRNAs. For that, some physiological, biochemical, and molecular studies were carried out on the transgenic and wild type Unica plants grown under the control and the abiotic stress conditions. The details of vector construction, transgenic plant production, physiological, biochemical, and molecular studies were presented below.

3.1 Construction of Plant Expression Vectors Harboring cDNA of Relevant miRNAs

3.1.1 cDNA synthesis of pre-miRNAs and adding *NcoI* and *BstEII* digestion sites to the cDNAs

In this study all three selected miRNAs were found to be related with abiotic stress conditions in potato. Identification, confirmation and importance (post-transcriptional) of these miRNAs and their target genes against abiotic stress conditions is briefly explained in one of our previous study (Gökçe et al., 2021). A total RNA sample was extracted from Unica cultivar grown under greenhouse conditions with 16/8 hours of light dark and a temperature of 25±2 ℃ by using Trizol solution (Invitrogen, Catalog number: 155926). It was used as template for cDNA synthesis. The cDNA samples were synthesized from pre-miRNAs of stu-miR156d-3p, stu-miR172b-3p, and miRNA novel 8 (Table 3.1, Figure 3.1) using SuperScript™ III Reverse Transcriptase (Catalog No: 18080044) and miRNA-specific stem-loop primers (Table 3.2, Table 3.3).

Table 3.1. Sequence of pre-miRNA

	Pre-miRNA sequence
miRNA novel 8	UAGCCAAGGAUGACUUGCCUAAAACCCUUUUUA UGAAGGGUCAUUAAUUAAUUAAACUUUAGUUAC UAAAAUUAAAGUUUAUAUUAUAUGAUCCUAAAA UUGGGAUUUUAGGCGUUGUCUGAGGCUAAUC
stu-miR156d-3p	UUGACAGAAGAUAGAGAGCACUAAUGAUGAUAU GCUAAUUUCAUUCAGCAAAAGCAUCUCACUUCA UUUGUGCUCUCUAUGCUUCUGUCAUCA
stu-miR172b-3p	GCAGCACCAUCAAGAUUCACAUAGAAAAUAUGG ACUAUGAAAUGAAAUAUGCCCAAUUUUUGAAUA CAUGAGAAUCUUGAUGAUGCUGCAU

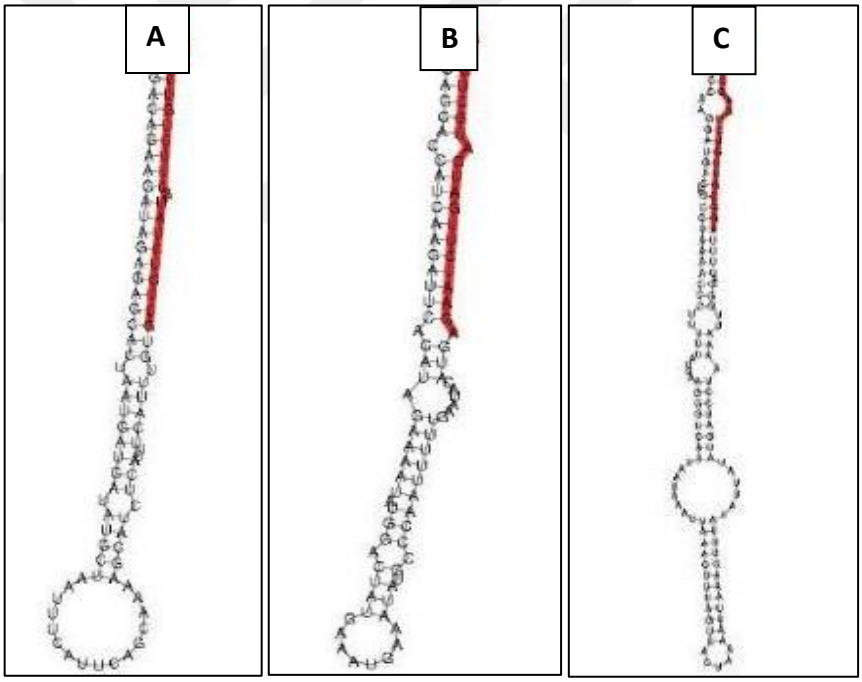


Figure 3.1. Structure of (A) miR156d-3p pre-miRNA, (B) miR172b-3p pre-miRNA, (C) MiRNA novel 8 pre-miRNA

Table 3.2. cDNA synthesis from total RNA using miRNA-specific stem-loop primers

Ingredients	**Amount (µl)**
miRNA-specific stem-loop primer	1.5
Total RNA (1µg/µl)	5.0
dNTPmix (10 mM)	1.0
dH_2O	12.5
Total	20.0
Incubation in water bath at 65 °C (5 min)	
Incubation on ice (5-10 min)	
First-Strand Buffer 5X	4.0
DTT (Invitrogen) 0.1 M	2.0
RNaseOUT Ribonuclease (5000 U) (Invitrogen)	1.0
Incubation at 42 °C for 2 min in heater block	
1 µl SuperScript™ III Reverse Transcriptase (200 U/µl)	
Incubation at 42 °C (1 h)	
Incubation at 70 °C (15 min)	

BstEII and NcoI digestion sites were added to the cDNAs of the pre-miRNAs by PCR, using pre-miRNA-specific reverse and forward primers (Table 3.3). While the NcoI digestion site was added to 5′ end of the cDNAs, using forward tailed-primer, the BstEII digestion site was added to 3′ end of cDNAs, using reverse tailed-primer. The PCR content is given in Table 3.4. The PCR conditions for stu-miR156d-3p, stu-miR172b-3p, and miRNA novel 8 were indicated in Table 3.5. Annealing temperature and amplicon length of the digestion site added cDNAs are shown in Table 3.6.

Table 3.3. miRNA-specific stem-loop primers to synthesize cDNA and pre-miRNA-specific primers to add digestion sites

Primer name	Sequences	Usage
stu-miR156d-3p-SL-RT	5′GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGACTGATGA- 3’	cDNA synthesis
stu-miR172b-3p-SL-RT	5′GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGACATGCAG- 3’	cDNA synthesis
novel_8-SL-RT	5′GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGACGATTAG- 3’	cDNA synthesis
stu-miR156d-3p	F- GCACCATGGAGATCTTTGACAGAAGATAGAGA GC	Ligation control
	R- GCAGCTAGCGGTNACCTGATGACAGAAGCATA GAGA	
stu-miR172b-3p	F- GCACCATGGAGATCT GCAGCACCATCAAGATTCA	Ligation control
	R- GCAGCTAGCGGTNACC ATGCAGCATCATCAAGATTC	
miRNA novel 8	F- GCACCATGGAGATCTTAGCCAAGGATGACTTG CCTA	Ligation control
	R- GCAGCTAGCGGTNACCGATTAGCCTCAGACAA CGCC	

*underline part of primer is restriction site added F and R at the beginning of the sequence indicate forward and reverse primer, respectively.

Table 3.4. PCR content for adding the digestion sites

Ingredients	**Amount (µL)**
PCR buffer with $MgSO_4$(10×) (Thermo Scientific)	2.5
dNTP (10mM) (Thermo Scientific)	0.5
Forward primer (5 µM)	2.5
Reverse primer (5 µM)	2.5
Pfu DNA Polymerase (2.5 U/µl) (Thermo Scientific)	0.2
dH_2O	13.8
cDNA (1:10 diluted)	3.0
Total	25

Table 3.5. PCR conditions for adding the digestion sites

Step	Function	Temperature (°C)	Duration (h:m:s)	Cycle
1	Initial Denaturation	94	00:02:00	-
2	Denature	94	00:00:15	40
3	Anneal	target-specific	00:00:15	40
4	Extend	72	00:00:20	40
5	Final extension	72	00:10:00	-
6	Keep	4	∞	-

Table 3.6. Annealing temperature and amplicon length of digestion site-added cDNAs

miRNA	**Annealing Temperature**	**PCR Amplicon+restriction enzyme sites (bp)**
stu-miR156d-3p	60 C°	93+30
stu-miR172b-3p	58 C°	91+30
miRNA novel 8	65 C°	130+30

3.1.2 The purification of cDNAs having the digestion sites from agarose gel

The cDNA samples having the digestion sites were run onto an agarose gel. For that, 0.5X TBE with 2.5% of agarose gel was prepared, after that samples and 4 µL DNA size marker (Thermo Scientific, 50 bp Gene ruler) was loaded to the gel. The cDNA samples were run for 1 h at 7 V/cm of electric potential, later, the cDNA bands in the gel were checked under UV light. Expected bands were excised from agarose gel. For purification of the cDNAs having the digestion sites, the cDNA samples in the excised bands were extracted with Thermo Scientific GeneJET Gel Extraction and DNA Cleanup Micro Kit (#K0832) by following procedure:

1) A 200 mg of agarose gel including amplified cDNA fragments was excised by surgical blade and was placed into a 1.5 mL tube.
2) A 200 µL of extraction buffer was added and mixed thoroughly by pipetting.
3) The gel was incubated at 58 °C for 30 mins in heater-shaker or until complete dissolution of gel slice.
4) A 200 µL of ethanol ≥ 99% was added and mixed by pipetting.
5) The mixture was transferred to the DNA Purification Micro Column preassembled with a collection tube (was incubated for 10-15 min). The column was centrifuged for 1 min at 14,000×g.
6) A 200µL of Prewash Buffer was added to the DNA Purification Micro Column and then centrifuged for 1 min at 14,000 × g. Later, the flow was discarded.
7) Wash Buffer (700µL) was added to the DNA Purification Micro Column and centrifuged at 14,000 × g for 1 min. Later, the flow was discarded.
8) Step 7 was repeated.
9) The empty DNA Purification Micro Column was centrifuged for an additional 1 minute at 14,000 × g to completely remove residual Wash Buffer.
10) The DNA Purification Micro Column was transferred into a clean 1.5 mL micro centrifuge tube, 20 µL of elution buffer was added into the DNA Purification Micro Column and centrifuged for 1 min at 14,000 × g. The elution buffer was heated at 55 °C before adding into the column.
11) Spectrophotometer was used to measure final concentration of eluted DNA

3.1.3 Restriction digestion of cDNAs

cDNA fragments were subjected to restriction digestion by using NcoI and BstEII restriction enzymes to produce overhangs so that cDNA fragments can ligate with plasmid vector. Content of digestion reaction of inserts (amplified pre-miRNAs) is given in Table 3.7. Digestion reaction conditions is indicated in Table 3.8.

Table 3.7. Content of digestion reaction of inserts

Chemicals	**Amount (µL)**
Insert (50 ng/µl) (stu-miR1 56d-3p, stu-miR172b-3p, miRNA novel 8)	15.0
10X Fast Digest buffer (ThermoScientific Lot00262799)	2.0
NcoI (10 U/µl) (ThermoScientific FastDigest #FD0573 Lot:00276217)	0.3
BstEII (10 U/µl) (BioLabs #R0162S Lot 0851604)	0.03
dH_2O	2.67
Total	20.0

Table 3.8. Digestion Reaction Conditions

Steps	Function	Temperature	Duration	Cycle No
1	Digestion	37 °C	1 h	1
2	Stop reaction	65 °C	5 min	1
3		4 °C	~	

3.1.4 Extraction of pCAMBIA1301 plasmid vector from *E. coli*

After restriction digestion of cDNA fragments, next step was to extract plasmid vector to be further used for its restriction digestion and ligation. For this purpose, colonies of *E. coli* (DH5α) bacteria including pCAMBIA vector were taken from Luria-Bertani

Agar (LB Agar) plate and were inoculated in 10 ml of liquid Luria-Bertani broth (LB broth) with 10 µl antibiotic (kanamycin 50µg/ml). These cultures were than incubated overnight at 37 °C and 200 rpm. pCAMBIA1301 vector was extracted from cultures. Thermo Scientific GeneJET Plasmid Miniprep Kit #K0503 was used to extract plasmid, following the procedure below:

1) Bacteria culture was centrifuged at >12000 × g. The pelleted cells were suspended in 250 µL of suspension solution and the cell suspension was transferred to a micro centrifuge tube.

2) The Lysis Solution (250 µL) was added and mixed completely by inverting the tube 4-6 times it becomes viscous and clear.
3) A 350 µL of the Neutralization Solution was added and mixed immediately and thoroughly by inverting the tube 4-6 times.
4) The mixture was centrifuged for 5 min to pellet cell debris and chromosomal DNA.
5) The supernatant was transferred to GeneJET spin column by pipetting (Incubation at room temperature for 5-10 min after transfering the supernatant to the column).
6) The column was centrifuged for 1 min. The flow was discarded, and the column was placed back into the same collection tube.
7) A 500 µL of the Wash Solution was added to the GeneJET spin column, centrifuged for 1 min and the flow was discarded. The column was placed back into the same collection tube.
8) The wash procedure was repeated using 500 µL of the Wash Solution.
9) The flow was discarded and additional 1 min centrifuge was carried out to remove residual Wash Solution.
10) GeneJET spin column was transferred into a fresh 1.5 mL micro centrifuge tube. A 50 µL of the Elution Buffer was added to the center of GeneJET spin column membrane (elution buffer was heated at 55°C before adding) to elute the plasmid DNA, incubated for 30 min at room temperature and centrifuged for 2 min.
Eluted pure pCAMBIA1301 vector was loaded on 1% agarose gel which was prepared with 0.5 X Tris/Borate/EDTA (TBE). A 4 µL of DNA size marker (ThermoScientific GeneRuler 1 kb Plus DNA Ladder #SM0103) were loaded with samples. The agarose gel was runned for 1 h at 7 V/cm, later, DNA vector in the gel was checked under UV light.

3.1.5 Digestion of pCAMBIA1301 vector by NcoI and BstEII restriction enzymes

Extraction of pCAMBIA1301 was followed by its digestion to remove GUS site and for ligation of amplified cDNA fragments. NcoI and BstEII restriction enzymes were used to digest pCAMBIA1301 vector (Figure 3.2) for obtaining required overhangs to be able to ligate inserts (stu-miR156d-3p, stu-miR172b-3p, miRNA novel 8) which were added NcoI and BstEII restriction digestion sites by PCR (Figure 3.3). The content of digestion reaction of pCAMBIA1301 is given in Table 3.9. Digestion reaction conditions indicated in Table 3.10.

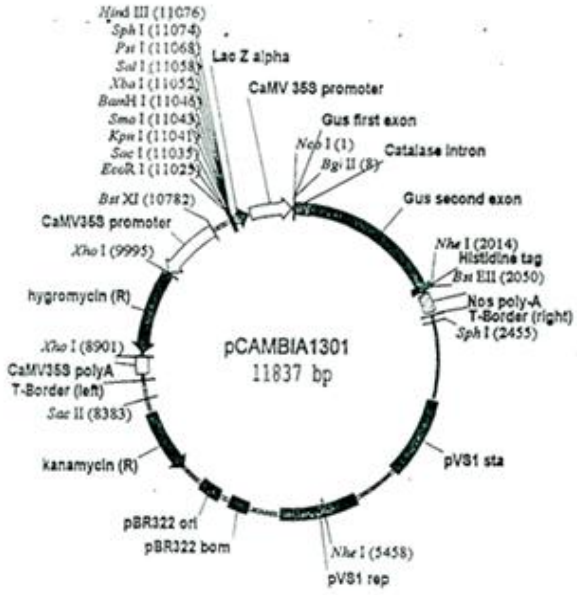


Figure 3.2. pCAMBIA1301 vector

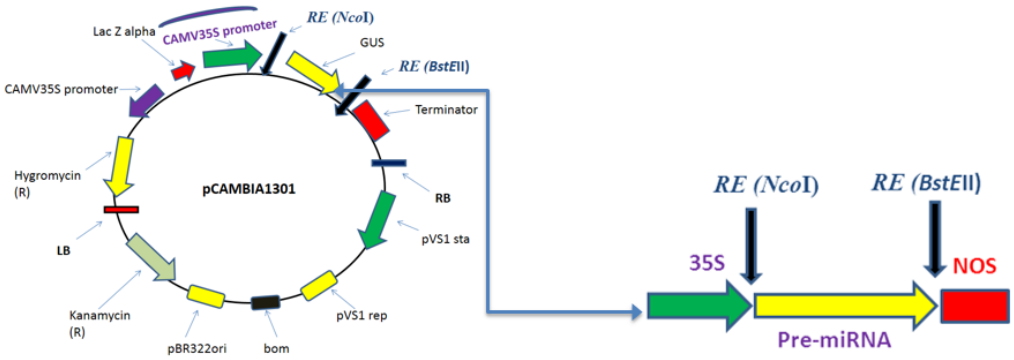


Figure 3.3. Schematic image for modification of pCAMBIA1301 vector.

After replacing GUS gene expected sizes of the vectors for stu-miR156d-3p, stu-miR172b-3p, and miRNA novel 8 were 9937bp, 9935 bp, and 9974 bp, respectively. Pre-stu-miRNAs were inserted to upstream NOS poly-A tail terminator. The vector contains *Hygromycin phosphotransferase (hptII)* to encode resistance against Hygromycin that was used a plant selectable marker whereas it contained Kanamycin for bacterial selection.

Table 3.9. Content of digestion reaction of pCAMBIA1301

Chemicals	**Amount (µL)**
pCAMBIA1301 (2µg/ µl)	35.0
10× Fast Digest buffer (ThermoScientific Lot00262799)	5.0
NcoI (10 U/µl) (ThermoScientific FastDigest #FD0573 Lot00276217)	2.0
BstEII (10 U/µl) (BioLabs #R0162S Lot 0851604)	0.25
dH_2O	7.75
Total	50

Table 3.10. Digestion Reaction Conditions of pCAMBIA1301

Steps	Function	Temperature	Duration
1	Digestion	37 °C	1 h
2	Stop reaction	65 °C	5 min
3		4 °C	~

Digested plasmid with NcoI and BstEII restriction enzymes were loaded in agarose gel. A 1% agarose gel was prepared with 0.5 × Tris/Borate/EDTA (TBE), then samples and 4µL DNA size marker (ThermoScientific Gene Ruler 1 kb Plus DNA Ladder #SM0103) were loaded to the agarose gel and run for 1 h at 7 V/cm, later, DNA vector in the gel was checked under UV light. Desired band of digested plasmid was extracted from 1 % agarose gel by ThermoScientific Gene JET Gel extraction and DNA cleanup micro kit #K0832. Gel extraction protocol was explained in detailed above.

Digested/gel eluted pCAMBIA1301 vector was loaded onto 1% agarose gel which was prepared with 0.5 × Tris/Borate/EDTA (TBE) to check concentration and elution. 4µl marker of Thermo Scientific Gene Ruler 1 kb Plus DNA Ladder #SM0103 on gel with samples.

3.1.6 Ligation of digested inserts (stu-miR156d-3p, stu-miR172b-3p, miRNA novel 8) with digested pCAMBIA1301 vector

Digested inserts (stu-miR156d-3p, stu-miR172b-3p, miRNA novel 8) and vector overhangs were ligated by using T4 DNA ligase (Promega). 100:12.5 vector:insert ratio was applied in ligation reaction. Content of ligation reaction and ligation conditions are given in Table 3.11 and 3.12, respectively.

Table 3.11. Content of Ligation Reaction of plasmid and insert

Chemicals	Amount (µL)
Digested pCAMBIA1301 (50 ng/µl)	2.0
Insert (stu-miR156d-3p, stu-miR172b-3p, miRNA novel 8) (5ng/µl)	0.25
T4 DNA Ligase 10× Reaction Buffer (Promega C126A)	1.0
T4 DNA Ligase (Promega, cat. no. M1808) (3U/µl)	1.0
dH_2O	5.75
Total	10

Table 3.12. Ligation conditions

Step	Function	Temperature	Duration
1	Ligation	22 °C	1h
2	Ligation	14 °C	4h
3	Ligation	4 °C	15 h

After ligation, the three different constructs were named as pCAMBIA1301+miR156, pCAMBIA1301+miR172, and pCAMBIA+miRNA novel 8.

3.1.7 Ligation Confirmation

After ligation the next step was to confirm either pre-miRNA fragments are ligated into the vector pCAMBIA1301 and PBB primers were used for this purpose. PBB primers were designed outward of cloning region of inserts on the vector pCAMBIA. PBB primers to check ligation are given in Table 3.13. Content of ligation confirmation PCR with PBB primers is indicated in Table 3.14. Conditions of ligation confirmation PCR are shown in Table 3.15.

Table 3.13. PBB primers to check ligation

	Sequence of primers
PBB-Forward	F-GAGAACACGGGGGACTCTTG
PBB-Reverse	R- TAATCATCGCAAGACCGGCA

Table 3.14. Content of Ligation Confirmation PCR

Chemicals	**Amount(µL)**
Ligated Plasmid pCAMBIA with insert (50 ng/µl)	1.0
Tag pol Green MM mix (2X)	10.0
PPB forward primer (5 µM)	2.5
PBB reverse primer (5 µM)	2.5
dH_2O	9.0
Total	25

Table 3.15. Conditions of Ligation Confirmation PCR

Step	**Function**	**Temperature (ºC)**	**Duration (H:m:s)**	**Cycle**
1	Initial Denaturation	94	00:03:00	-
2	Denature	94	00:00:15	40
3	Anneal	55	00:00:15	40
4	Extend	72	00:00:15	40
5	Final extension	72	00:10:00	-
6	Keep	4	∞	-

After ligation confirmation PCR, PCR products were run on 2.5% agrose gel to confirm the size of ligated plasmid with insert. For this purpose, 2.5% agarose was prepared with 0.5× Tris/Borate/EDTA (TBE), then PCR products and 4µL DNA size marker (ThermoScientific 50bp DNA ladder #SM0371) were loaded and run-on agarose gel for 1 h at 7 V/cm and checked under UV light.

3.1.8 Transformation of constructs to *E.coli*

After confirmation by PCR reaction, ligation mixture was transformed into *E.coli* (DH5α) cells by following standard protocol steps of transformation. After that competent cell were thawed on ice for 20-30 mins after taking out from -80 °C. Meanwhile LB medium plates having 25 µg/ml antibiotic (kanamycin) were placed from 4 ºC at room temperature (25-28 ºC). Then ligation product (4 µl of 10-100 ng) and 50 µL of competent cells were mixed by gently tapping the eppendorf tube. Then the DNA mixture or competent cells were allowed to incubate for 30 mins on ice. Afterwards, for heat shock the tubes were placed into 42 ºC water bath for 30 to 60 secs and then again putted on ice for approximately 2 min. LB media without antibiotic (500 µl) was putted in tubes having bacteria and kept at 37ºC in shaking incubator for 45 to 60 min. Afterwards, media was spread on plates containing LB agar with 25 mg/L kanamycin and were incubated overnight at 37 ºC. Following day, few grown colonies were observed and randomly selected for performing colony PCR to confirm presence of construct inside.

3.1.9 Colony confirmation

Selected colonies were checked with miRNA-specific primers by colony PCR whether harboring pCAMBIA1301+insert constructs. Primers and PCR content of colony PCR s given in Table 3.16 and 3.17 respectively. Conditions of colony PCR are mentioned in Table 3.18.

Table 3.16. miRNA-specific primers

Primer name	Sequence of primers
156d-3pF	F- GCACCATGGAGATCTTTGACAGAAGATAGAGAGC
156d-3pR	R- GCAGCTAGCGGTNACCTGATGACAGAAGCATAGAGA
172b-3pF	F- GCACCATGGAGATCTGCAGCACCATCAAGATTCA
172b-3pR	R- GCAGCTAGCGGTNACCATGCAGCATCATCAAGATTC
novel-8F	F- GCACCATGGAGATCTTAGCCAAGGATGACTTGCCTA
novel-8R	R- GCAGCTAGCGGTNACCGATTAGCCTCAGACAACGCC

Table 3.17. PCR content of Colony PCR

Chemicals	Amount(µL)
Selected Colony	
Taq pol Green MM mix (2X)	7.5
miRNA specific reverse primer (5 µM)	2.5
miRNA specific forward primer (5 µM)	2.5
dH_2O	7.5
Total	20

Table 3.18. Conditions of colony PCR

Step	Function	Temperature (^oC)	Duration (H:m:s)	Cycle
1	Initial Denaturation	94	00:02:00	-
2	Denature	94	00:00:15	40
3	Anneal	60,58,65 (miRNA156,172,novel-8)	00:00:15	40
4	Extend	72	00:00:20	40
5	Final extension	72	00:10:00	-
6	Keep	4	∞	-

PCR products were analyzed by agarose gel analysis. A 2.5% agarose gel was prepared with 0.5 X Tris/Borate/EDTA (TBE), then PCR products and 4 µL DNA size marker (ThermoScientific 50bp DNA ladder #SM0371) were loaded and runned at agarose gel for 1 h at 7 V/cm, later, the gel was checked under UV light. Next day, the constructs were extracted from cultures by Thermo Scientific GeneJET Plasmid Miniprep Kit #K0503. (Plasmid purification procedure is explained above. 3.1.4)

3.1.10 Transferring of pCAMBIA1301+insert constructs into *Agrobacterium* LBA4404 strain

pCAMBIA1301+miR156, pCAMBIA1301+miR172, and pCAMBIA+miRNA novel 8 constructs were extracted from *E. coli* cultures then transferred into *Agrobacterium tumefaciens* LBA4404 strain via electroporation. Competent cells of LBA4404 (OD: 0.5 to 0.7 at 600 nm) were added to plasmid (4 µL of 10-20 ng) and kept on ice. The electroporation device was set at 2.4 kV voltage, 25 µF capacitance with a resistance of 200 ohms. After electroporation, LB (500 µl) was added to the mixture and was incubated at 28ºC in shaking incubator for 3 hours at 600rpm. Cells were then spread on LB agar plates having 25 µg/ml kanamycin and were incubated for 24-48 hours at 28ºC. Following day, few grown colonies were observed and randomly selected for performing colony PCR using miRNA-specific primers as described in 3.1.9 to confirm presence of construct.

Isolated positive pCAMBIA1301+insert constructs concentration was measured by nanodrop (UV-vis Spectrophotometer, SHIMADZU). Spectrophotometer measurement results of positive pCAMBIA1301+insert constructs are indicated in Table 3.19. Sequence of positive clones was verified by sequencing.

Table 3.19. Concentrations of pCAMBIA1301+insert constructs

Sample name	Concentration (ng/µL)	OD260/280	OD260/230
stu-miR156d-3p	34.35	1.74	0.69
stu-miR172b-3p	54.97	1.97	1.58
miRNA-novel8	64.54	1.94	1.55

Confirmed colonies (*Agrobacterium* strain LBA4404 harboring pre-miRNA constructs) were taken from the selective agar plate and were inoculated in 10 ml of liquid Luria-Bertani broth (LB broth) with antibiotic (kanamycin 25 µg/ml) until reach to optimum optical density (0.6 at 600nm). Glycerol stocks (*Agrobacterium* LBA4404) having pre-miRNA constructs were prepared by addition of 500 ul of 50% glycerol to 500 ul of overnight cultured bacteria and stored at -80 ℃ for future use.

3.2 Explant Propagation

3.2.1 Tuber sterilization

The tubers of Unica cultivar were used to grow donor plants under sterile tissue culture conditions. Tubers were cleaned first with tap water having some dish washing detergent to clean their surface and to avoid contamination problem in future plants (Figure 3.4).

Figure 3.4. Water tapped clean tubers to be used for surface sterilization

After that, following steps were practiced inside laminar flow to surface sterilize potato tubers (Figure 3.5).

1) Tubers were placed in jars having distilled water and were cleaned with pure Tween-20 for 1-2 mins twice.
2) Tubers were cleaned with 70% Ethanol for 2 min.
3) After ethanol washing tubers were treated with antifungal (Mancozeb) solution (2gm/L) for 10-15 min.
4) Again, washing with 70% of ethanol was done for 1 min.
5) Tubers were rinsed with sterile water for 2-3 times.

6) Drying of the tubers were done on tissue paper (70% ethanol sprayed) followed by their placement in autoclaved jars.

7) Jars were sealed with para film and placed in growth chamber.

The plants obtained from the eyes of these tubers were used to multiply and increase our stock of explants (Figure 3.6).

Figure 3.5. Surface sterilization of tubers

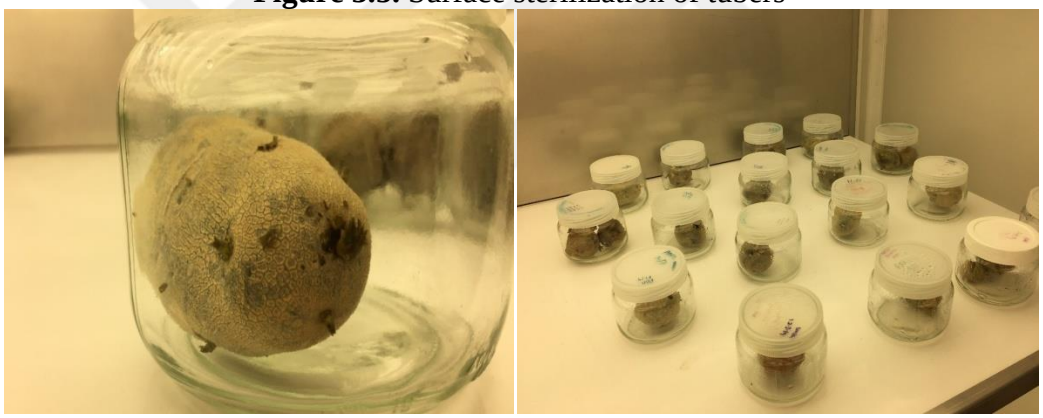


Figure 3.6. Sprouted eyes from surface sterile potatoes

Jars containing surface sterile tubers were placed in growth chamber 16/8-hour day/night, 25±2 °C temperature to get sprouted eyes. The sprouts were excised and placed in Murashige and Skoog (MS) medium containing mineral salts 4.4 g/l, 30g/l sucrose and 7-8 g/l agar for further plant obtaining and multiplication (Figure 3.7).

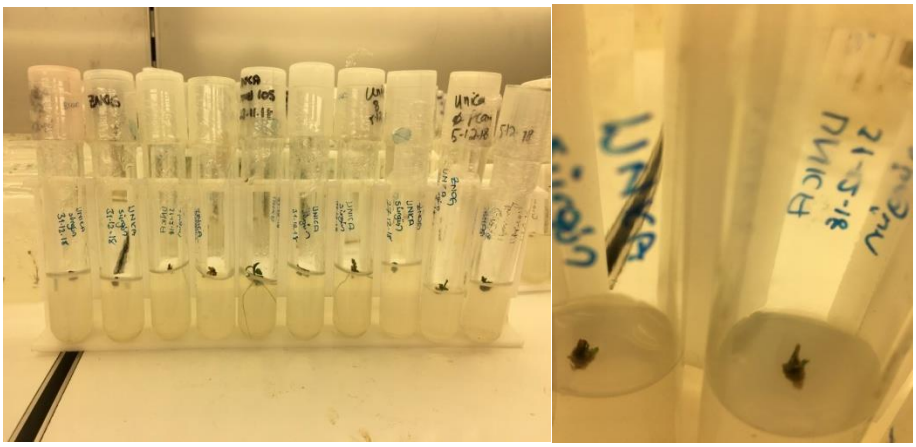


Figure 3.7. Plant growth initiation from tuber eyes

Cultures were incubated at photoperiod of 16 h at 22±1 °C. Plants normally took around 3 to 4 weeks to be able to be used for transformation (Figure 3.8). Single node cuts were practiced every 3 weeks by regenerating shoots and these nodes have been subcultured for micro propagation.

Figure 3.8. In-vitro grown plants for transformation experiments

3.3 Agrobacterium Culture Preparation

Two days before transformation, confirmed colonies (by colony PCR) from selective agar plate (*Agrobacterium* strain LBA4404 harboring pre-miRNA constructs) were inoculated in 10 ml of LB broth with 50mg/ml of kanamycin in 50 ml falcon tube.

Prepared cultures were overnight incubated in thermo-shaker at 28 °C and 200 rpm (Figure 3.9).

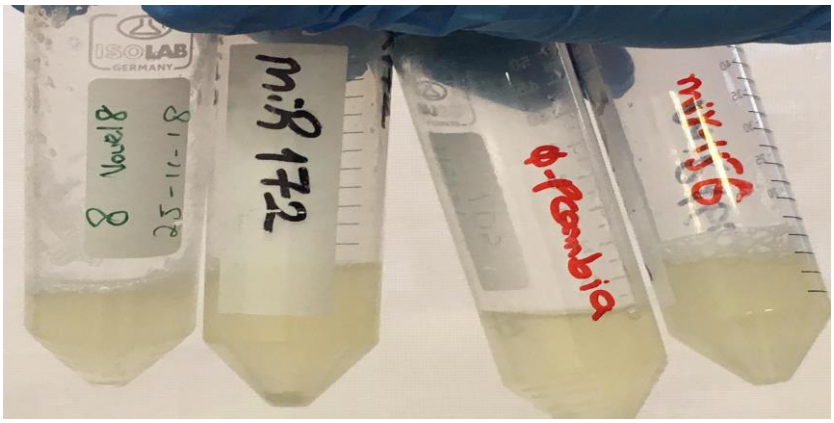


Figure 3.9. Agrobacterium (LBA4404) culture including pCAMBIA1301+insert

3.4 Transferring of pre-miRNAs to Potato Plants and Production of Transgenic Plants

3.4.1 Co-cultivation medium

Unica plants with 4-5 nodes were subjected to genetic transformation mediated by Agrobacterium, using leaf discs and internodal parts as explants. Agrobacterium strain containing constructs (pCAMBIA1301+insert constructs of stu-miR156d-3p, stu-miR172b-3p, miRNA novel 8) were inoculated with explants for around 45 minutes (Figure 3.10, Figure 3.11). Explants were placed onto solid cocultivation medium (Figure 3.12) and incubated for two days in chamber having standard conditions at 16 h day and 8 h night, 25±2 °C temperature, and 47 µmol/m2/s light intensity. 1L Co-cultivation medium consisted of 4.4 gm/L MS salts having 30gm/L sucrose, plant agar (8 gm/L) and 1ml acetosyringone (50 µM). The pH of medium was adjusted to 5.7, and after autoclave sterilazation, acetosyringone was added in to the medium.

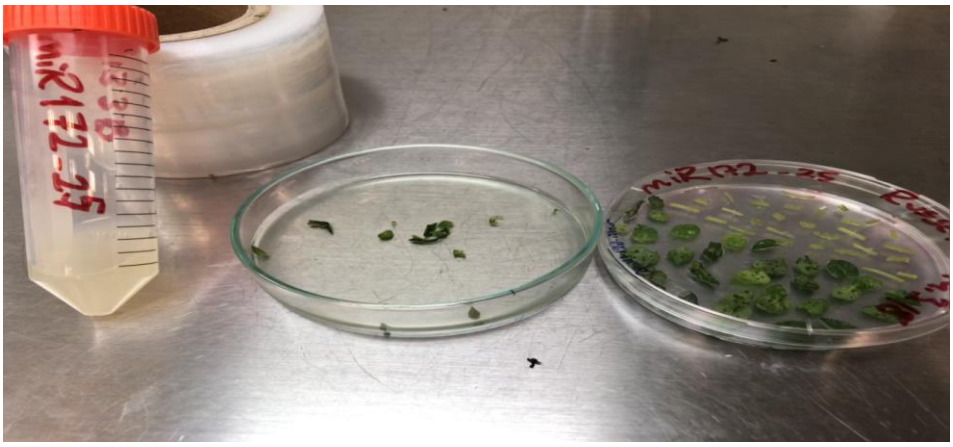


Figure 3.10. Explant inoculation with Agrobacterium (LBA4404) suspension harboring pCAMBIA1301+miRNA

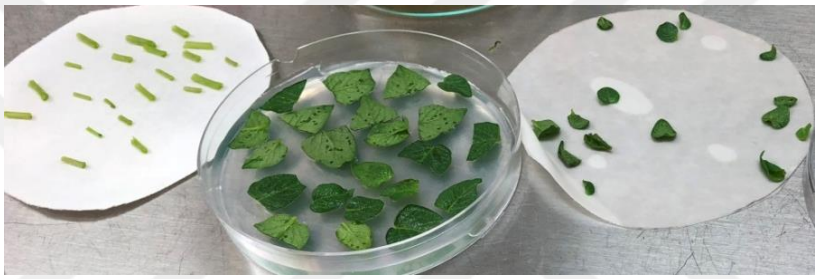


Figure 3.11. Drying of explants on sterile tissue paper

Figure 3.12. Explants on co-cultivation medium

3.4.2 Regeneration Selection Medium (RSM)

After co-cultivation, an antibiotic (Sulcid 500 mg/L) was used to wash the explants for nearly 15 min. Then the explants were dried on an autoclaved filter paper followed by their placement on callus induction medium (MS media supplemented with NAA 0.2

mg/L, BAP 2 mg/L, Trans-Zeatin 2 mg/L, Kinetin 1 mg/L, Sulcid 500 mg/L (to suppress agrobacterium) and hygromycin 4 mg/L (for selection of plant) (Figure 3.13).

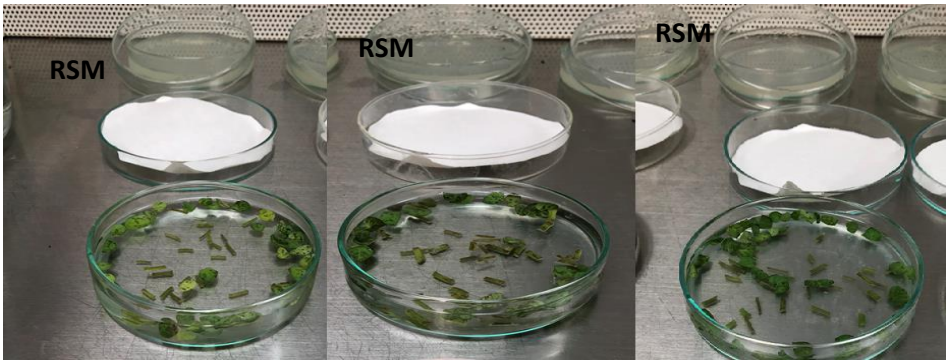


Figure 3.13. Explant transfer to RSM from co-cultivation medium after washing with antibiotic and drying on sterile filter paper

3.5 Confirmation of Transgenic Plants

For confirmation of transgenic plants, DNA was isolated from the top leaves of the plantlets grown under tissue culture conditions, and PCR was conducted using Hygromycin primers (Yang et al., 2011). Extracted DNA was also used to perform a PCR under same conditions as hygromycin with a difference of using 35S as forward and NOS poly-A tail as reverse primers having 53.8 ^{o}C annealing temperature. Contents and temperature conditions used for the PCR were following (Table 3.20. and Table 3.21)

Table 3.20. Contents used for the PCR

Ingredient	Amoun t(µL)
DNA (85 ng/µl)	2
MM PCR mix 2x	6
Forward hygro primer (5 µM)	2
Reverse hygro primer (5 µM)	2
dH_2O	8
Total	20

Table 3.21. Temperature conditions of the PCR for transgenic confirmation

Step	Temperature (°C)	Duration (H:m:s)	Cycle
1	94 °C	4 min	
2	94 °C	20 s	34 cycle
3	55 °C	20 s	
4	72°C	45 s	
	72°C	7 min	
5	4 °C	∞	

PCR products were analyzed by agarose gel analysis. A 2.0 % agarose gel was prepared with 0.5 X TBE, then PCR products and 4 µL DNA size marker (Bio Labs 50bp DNA ladder) were loaded and runned at agarose gel for 1 h at 7 V/cm, finally, the gel picture was captured under UV light.

3.6 Calculation of transformation efficiency

For determining transformation efficiency (TE), formula 3.1 was used:

$$\mathrm{TE}(\%) = (\mathrm{nTPR}/\mathrm{tnEU}) \times 1 \quad (3.1)$$

Where, TE is transformation efficiency, nTPR is number of transgenic plants regenerated, tnEU is explants total number used.

3.7 Plant Growth and Stress Treatments

After confirmation of transgenic plants through PCR, the next step was to multiply the plants according to the required number of plants following this work plan given below. Two different miR172b-3p over-expressed transgenic lines, two different miRNA novel 8 over-expressed transgenic lines, one miR156d-3p over-expressed transgenic line, and Unica cultivar (non-transgenic) were used in the further studies for investigating the functions of the relevant miRNAs (Table 3.22).

Table 3.22. work plan for grown plants

Genotype for Unica	**Treatment**	**Number of Pot**	**Number of Plant per pot**
Unica_Wt	Control	4	2
Unica_Wt	Drought	4	2
Unica_Wt	Control	4	2
Unica_Wt	Heat	4	2
Unica_Wt	Drough+Heat	4	2
miR156	Control	4	2
miR156	Drought	4	2
miR156	Control	4	2
miR156	Heat	4	2
miR156	Drough+Heat	4	2
miR172.1	Control	4	2
miR172.1	Drought	4	2
miR172.1	Control	4	2
miR172.1	Heat	4	2
miR172.1	Drough+Heat	4	2
miR172.2	Control	4	2
miR172.2	Drought	4	2
miR172.2	Control	4	2
miR172.2	Heat	4	2
miR172.2	Drough+Heat	4	2
miRNA-novel8.1	Control	4	2
miRNA-novel8.1	Drought	4	2
miRNA-novel8.1	Control	4	2
miRNA-novel8.1	Heat	4	2
miRNA-novel8.1	Drough+Heat	4	2
miRNA-novel8.2	Control	4	2
miRNA-novel8.2	Drought	4	2
miRNA-novel8.2	Control	4	2
miRNA-novel8.2	Heat	4	2

miRNA-novel8.2	Drough+Heat	4	2

Total Number of Pots	120
Total Number of Plants	240

In-vitro grown transgenic and wild type plantlets with 5-6 nodes were transferred to pots (5 L pots having 22.5 cm diameter top, 16.5 cm diameter base, and18 cm depth). All plants were regularly irrigated until stress application, and 100 g fertilization of equal concentration of N-P-K (18% each) was applied once to all pots. Three different stress experiments for drought (D), heat (H) and heat+dought (HD) with wild type cultivar were set up after 40 days of transplantation. Each experiment had its own control (nonstress) and stress treatment. Each experiment was conducted with four replications, and each pot containing two plants represented one replication. Drought (D) experiment was carried out in the net house (Figure 3.14) (temperature was 24–26 °C day/16–18°C night, the humidity was 55%–65% with no artificial light conditions), while the heat (H) and heat+drought (HD) combined stress experiments were executed in the environmentally controlled walk-in growth chambers. In the drought experiment, drought was given to one group of plants by withdrawing the watering for 20 days whereas the plants in the control group were irrigated regularly under net house conditions. For heat and combined heat-drought experiments, growth chambers were used side-by-side, one for the control and the other for the stress treatments. Control conditions of heat and combined drought-heat experiments were set up as 24 °C day and 18 °C night of temperature with a photoperiod of 14 h and relative humidity of 60–70%. In the other chamber, the temperature was gradually increased for heat treatment to plants. The gradual temperature increase was from 24 °C day and 18 °C night to 39 °C day and 27 °C night for total 9 days, then; a constant heat of 39/27°C was applied for 3 days (Table 3.23). Therefore, plants were exposed to heat for 12 days. The plants in the heat treatment group were irrigated regularly while the plants in the combined heat-drought treatment group were exposed to drought by withdrawing the irrigation for 12 days. Physiological traits (photosynthesis, stomatal conductance, transpiration rate, chlorophyll contents, and relative water contents) were measured on plants at some intervals during stress treatments. For further biochemical (proline, malondialdehyde, hydrogen peroxide and antioxidant enzyme activity) and molecular analyses (miRNA

and mRNA expression levels), the upper third and fourth leaves on the plants were harvested and immediately frozen in liquid nitrogen and stored at –80 °C.

Figure 3.14. Initial stage of transgenic plants grown in greenhouse

Above mentioned optimum conditions were applied to control plants until sample harvesting time.

Table 3.23. Plan for high temperature application

	0th day	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day	11th day	12th day Harvest
Day Temperature 14hours	24.0	25.0	26.0	27.0	29.0	31.0	33.0	35.0	37.0	38.0	39.0	39.0	39.0
Night Temperature 10hours	18.0	19.0	20.0	21.0	22.0	23.0	24.0	25.0	26.0	26.0	27.0	27.0	27.0
Average Temperature	21.5	22.5	23.5	24.5	26.1	27.7	29.3	30.8	32.4	33.0	34.0	34.0	34.0

3.8 Physiological Measurements

LI-6400 XT Licor a portable photosynthesis system was used to measure rate of photosynthesis, stomata conductance and transpiration rate in control and stress treatments of transgenic and non-transgenic plants of Unica genotype in mid-day hours. Constant light energy of portable system was 1000 µmol/m^2/sec, air flow was 500 µmol/s and CO_2 amount were arranged at 400 µmol. Upper third or fourth leaflets were used to take measurements from each replication. Measurements were taken at 0^{th} ,6^{th}, 8^{th}, 10^{th}, 12^{th}, 16^{th}, 18^{th}, and 20^{th} day of stress factor applications. While heat only and its combination with drought were applied upto12 days, the drought was applied up to 20 days.

3.8.1 Chlorophyll index

Chlorophyll contents were estimated by using non-destructive device (SPAD-502 chlorophyll-meter) from control and stress treated plants during stress application time in mid-day hours for each replication. Observations were taken at 0^{th} ,6^{th}, 8^{th}, 10^{th}, 12^{th}, 16^{th}, 18^{th}, and 20^{th} day of stress application. While heat only and its combination with drought were applied upto12 days, the drought was applied up to 20 days.

3.8.2 Leaf temperature (°C)

An infrared thermometer (IRT) instrument (MASTECH BM380) was used to measure leaf temperature in plants of control and stress treatments during stress application time in hours of mid-day from each replication. The apical leaflet of upper leaves (third or fourth) was used for recording plant temperature. Observations were recorded at 0^{th}, 6^{th}, 8^{th}, 10^{th},12^{th},16^{th},18^{th} and 20^{th} day of stress application. While heat only and its combination with drought was applied upto12 days, the drought was applied up to 20 days.

3.8.3 Relative water content (RWC)

Relative water content (RWC) was measured in replications of control and stress treatments by using apical third leaves of plants. Fresh weight of leaves was immediately noticed after harvesting at sensitive weighat scale. Leaves were placed in pure water for 16 hours to get turgid weight of leaves. After taking turgid weight, the leaves were dried by placing them in microwave for 10 mins followed by keeping them at 90°C for 3 hours in dry oven to ensure complete drying for taking correct dry weight of leaves.

RWC values of plant leaves were calculated by using following equation 3.2.

$$\text{RWC}\ (\%) = [(\text{Fresh weight} - \text{Dry weight})/(\text{Turgor weight} - \text{Dry weight})] \times 100 \quad (3.2)$$

Measurements were recorded at 0^{th} ,6^{th}, 8^{th}, 10^{th},12^{th}, 16^{th}, 18^{th} and 20^{th} day of stress factor applications. While heat only and its combination with drought were applied upto12 days, the drought was applied up to 20 days.

3.9 Leaf Sample Collection

At last day of stress application, upper 3^{rd} and 4^{th} compound leaves were collected from four replicates. Collected leaf samples were immediately putted in liquid nitrogen and kept at -80 °C. The leaf samples were used for further biochemical and molecular analyses.

3.10 Biochemical analyses

3.10.1 Proline content

Proline content at the last day of the stress treatments was measured in non-transgenic and transgenic Unica potato plants. The protocol developed by Bates et al. (1973) was used with minor modifications.

Following steps were practiced to measure proline content:

1. A 2 mL of sulfosalicylic acid (3%) was used to grind 100 mg leaf sample followed by their mixing.
2. A 20-minute centrifugation was practiced at 4 °C and 10,000 × g for precipitation of sample.
3. A 1.5 mL-volume centrifuge tubes were used to transfer 0.2 mL of the upper supernatant.
4. A 0.2 ml of freshly prepared ninhydrin solution (1.25 g ninhydrin, 20 mL 6M orthophosphoric acid, 30 mL glacial acetic acid) was added onto the supernatant.
5. The mixture tube was inverted for 15 seconds, then incubated at 90 °C for 1 hour. The reaction was inhibited by placement of the tube on ice for 10 min.
6. A 1ml toluene was added to each reaction mixture followed by vortexing for 15 sec.
7. The mixture was incubated at room temperature in dark for 20 min.
8. A 300 µl of the pink supernatant and 700 µl of tolune were added into a 1ml-quartz cuvette, and the absorbance of the samples was measured at 520 nm wavelength by using a spectrophotometer.
9. A 1 mL toluene was used as blank measurement.
10. Standard curve (Figure 3.15) and following equation 3.3 were used to calculate concentration of proline accumulation in leaf samples of plants

$$(A520 + 0.0108) / 0.0103) \times \text{Dilution Factor} \times (1/\text{tissue weight g}) / 115.13 \quad (3.3)$$

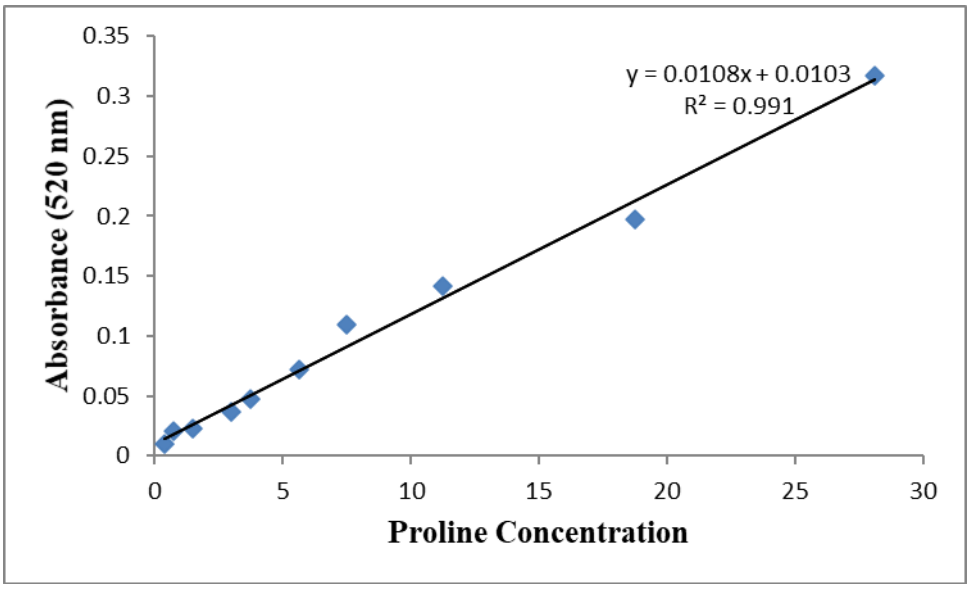


Figure 3.15. Standard curve used for measurement of proline

3.10.2 MDA content

The concentration of malondialdehyde (MDA) at the last day of the stress treatments was measured in transgenic and non-transgenic Unica potato plants. The protocol developed by Health and Packer (1968) was used with minor modifications.

Following steps were practiced to measure MDA content:

1. A 200-300 mg of leaf tissue was homogenized in 2 ml of 0.1 % (w/v) trichloroacetic acid (TCA).

3. The homogenate was centrifuged at 10000 × g and 4.0 °C for 20 min.

4. The supernatant (≈2ml) was transferred into a new 15ml tube.

5. A 2ml of the solution including 0.5% TBA and 20 % TCA was added onto the mixture in the 15ml tube.

6. The final mixture was incubated at 90-100 °C for 30 min.

7. The reaction was terminated by incubating on ice (≈10 min. until tubes get cold)

9. The mixture was centrifuged 10000 × g and 4.0 °C for 5 min.

10. The absorbance of the samples was measured at 532 and 600 nm wavelengths using a spectrophotometer.

The solution including 0.5% TBA and 20 % TCA was used as blank in the spectrophotometer.

The equation 3.4 was used to measure MDA accumulation.

$$\text{MDA (µmol/g FW)} = [(A532-A600)/155] \times 10^3 \times \text{dilution factor} \quad (3.4)$$

3.10.3 H_2O_2 content

The content of hydrogen peroxide (H_2O_2) at the last day of the stress treatments was measured in transgenic and non-transgenic Unica potato plants. Following steps were practiced to measure H_2O_2:

1. A 200-300 mg leaf samples were homogenized in 2 ml of 0.1% Trichloroacetic acid (TCA) (w/v) at 4°C.
2. The homogenate was centrifuged at 10,000 × g for 20 min at 4 °C.
3. A 0.75 ml of the supernatant, 0.75 ml of 10 mM K-phosphate buffer (pH 7.0), and 1.5 ml of 1M KI was mixed in a quartz cuvette.
4. The absorbance of the sample was measured at 390 nm of wavelength.
5. A mixture of 1.5 ml of 1M KI, 0.75 ml of 10 mM K-phosphate buffer (pH 7.0) and 0.75 ml of 0.1% TCA (w/v) was used as blank.

Standard curve absorbance values were used to calculate H_2O_2 concentration of accumulation in leaf samples of plants (Figure 3.16).

$$H_2O_2\ (\mu mol\ gFW^{-1}) = (A390 + 0.0139) / 0.0315) \times \text{Dilution Factor} \times (1/\text{tissue weight g}) \quad (3.5)$$

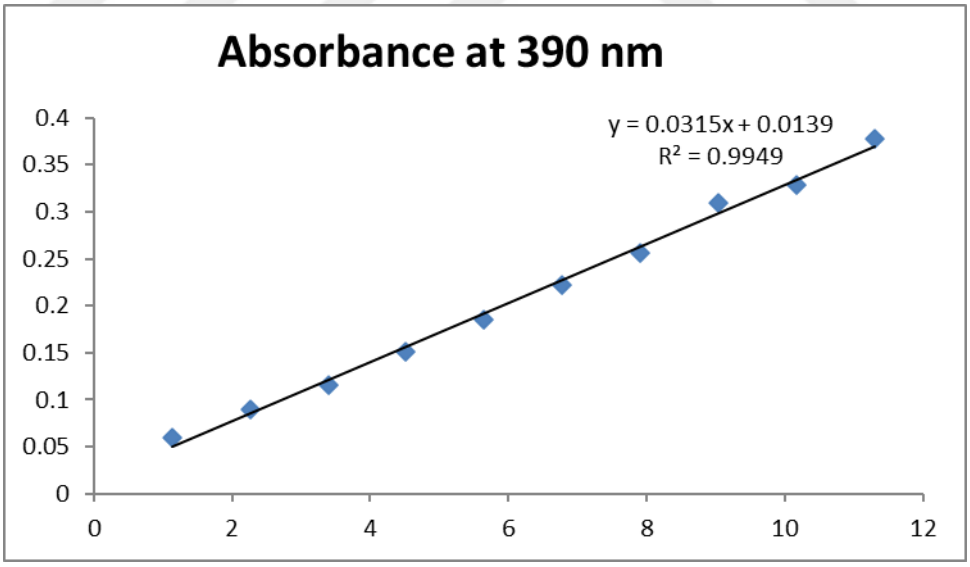


Figure 3.16. Standard curve used for measurement of H_2O_2

3.11 Antioxidant Enzyme Activity

At last day of stress treatments, leaf samples were harvested and were used to measure antioxidant enzymes such as catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD), and superoxide dismutase (SOD) from all plants (transgenic and wild-type).
Leaf samples (200 mg) were grinded by using mortar and pestle and putted in 4 ml tube with 50 mM K-phosphate buffer and polyvinyl–polyvinylpyrrolidone 1% (w/v) was added and mixed. Samples were centrifuged for 10 min at 10,000 × g (4°C), the supernatants were transfered to new tubes. The enzyme extracts were stored at −80°C until measurement of enzyme activity as described below. Three independent biological replicates were used to assay each enzyme sample.

3.11.1 Ascorbate peroxidase (APX)

A 70 µl of enzyme extract was used to measure the activity of APX. Enzyme extract was homogenized in 2900 µL of APX solution containing 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA and 0.5 mM ascorbic acid (Nakano and Asada, 1981). 10 mM hydrogen peroxide (30 µL) was added to initiate the reaction in a 3 ml cuvette. The change in absorbance was measured at 290 nm by observing the change in the rate of absorbance at 0 min and 2 min by spectrophotometer (UV-1800 Shimadzu).

3.11.2 Catalase (CAT)

Leaf CAT antioxidant enzyme was defined as µmol of H_2O_2 per min per milligram H_2O_2 breakdown and oserved as a change in absorbance up to 0.01, practicing the method given by Chance and Maehly et al. (1955). A 30 µL of enzyme extract was used to prepare reaction mixture with the addition of 2670 µL of 50 mM potassium phosphate buffer (pH 7.0) and 100 mM H_2O_2 (300 µL) was added at the end to initiate the reaction. The absorbance was measured at 240 nm via spectrophotometer (UV-1800 Shimadzu). Reaction mixture was used as blank with no enzyme extract.

3.11.3 Superoxide dismutase (SOD)

SOD measurement was practiced by following a method provided by Giannopolitis and Ries et al. (1977). The decline of nitroblue tetrazolium (NBT) was observed at 560 nm. A 50 µl of enzyme extract was used in reaction mixture. Alongwith enzyme extract, 13 mM methionine, 0.1 mM Na-EDTA, 50 mM of potassium phosphate buffer (pH 7.0) and 75 µM riboflavin were taken in a test tube. To initiate the reaction, test tubes were kept for 5 minutes under fluorescent light. The reaction was stopped by removing light and application of dark conditions. Reaction mixture was used as blank sample except enzyme extract. The increase in absorbance (ΔA) was recorded at 560 nm.

3.11.4 Peroxidase (POD)

A 90 µl of enzyme extract was used to measure the activity of POD. Enzyme extract was homogenized in 2840 µl of 10 mM K-phosphate buffer (ph 7.0) and 50 µl of 20 mM guaiacol. 20 µl of 40 mM H_2O_2 was also added to initiate the reaction in 3ml cuvette. The change in absorbance was measured at 470 nm at 0 min and 2min by spectrophotometer (UV-1800 Shimadzu). Same reaction mixture lacking enzyme extract was used as blank.

3.12 Molecular Studies

At last day of stress treatment, leaf samples were harvested and were placed at -80°C. These leaves were used to isolate total RNA to carry on molecular studies of transgenic and non transgenic plants.

3.12.1 Total RNA isolation

Total RNA was isolated from leaf samples using TRIzol® reagent. The details of the total RNA isolation protocol are given below.

1. A 200-300 mg of leaf tissue was homogenized by mortar and pestle using liquid nitrogen until sample become powder.
2. Add powdered sample into a 2ml-volume centrifuge tube, up to ≈500 µl level

3. Add 300 µl TRIzol® reagent and homogenize for about 40 s by vortex.
4. Add 700µl TRIzol® reagent, mix by inversion and incubate at room temperature (R/T) for 10 min.
5. Centrifuge at 13,000 rpm, 4 ℃ for 5 min.
6. Carefully remove 1000 µl of the supernatant and transfer it to a clean tube.
7. Add 220 µl chloroform and mix by vortex for 10 s.
8. Incubate at room temperature for 5 min.
9. Centrifuge at 13,000 rpm for 15 min.
10. While centrifugation is going on, prepare labelled tubes by adding 600 µl isopropanol to each tube.
11. Carefully take 600 µl of supernatant from the centrifuged samples, add it into the appropriate tube containing isopropanol.
12. Incubate the samples at −20 ℃ for 10-30 min.
13. Centrifuge at 13,000 rpm, 4 ℃ for 15 min.
14. Carefully pour off the supernatant. Gently tap inverted tube on tissue to remove any excess liquid. Place samples on ice
15. Add 100 µl sterile distilled water (sdH_2O) onto pellet and re-suspend by pipetting.
16. Add a further 350 µl sdH_2O.
17. Add 450 µl acidic phenol or chloroform pH:5.2 (not on ice).
18. Vortexing was done for 30 s.
19. Centrifuge at 13,000 rpm, 4 ℃ for 5 min.
20. Take 400 µl of supernatant and transfer it into a new centrifuge tube.
21. Add 1ml 70% ethanol (generally at -20^0 C but may use R/T). Invert tubes a couple of times.
22. Centrifuge at 13,000 rpm, 4 ℃ for 5 min.
23. Carefully pour off ethanol and tubes were inverted on paper towel for drying of pellet.
24. Dry the pellet in a laminar flowhood for 5 min.
25. Add 20 µl sdH_2O and re-suspend the pellet by pipetting or vortexing.
26. Add 30 µl DNaseI reaction mix (5µl 10x Buffer, 2µl DNaseI, 1.25µl RNase Out, 21.75µl ddH_2O) on a 20 µl RNA sample
27. Incubate at 30 ℃ for 15 min.
28. Add 400µl sdH_2O.

29. Vortex for 30s
30. Centrifuge at 13,000 rpm, 4 ℃ for 5 min.
31. Carefully take 400 µl of the supernatant from the centrifuged tube and transfer it into ta new tube
32. Mix by inversion for 10-15 times.
33. Incubate at −20 ℃ for a minimum of 30 min.
34. Centrifuge at 13,000 rpm, 4 ℃ for 10 min.
35. Carefully pour off liquid taking care not to disturb any pellet. Leave the tube inverted on paper towel while doing others. Tap gently and flick lid to remove remnants from lid.
36. Add 40 µl ddH_2O and re-suspend the pellet by pipetting or vortexing.
37. Prepare a 1.2% agarose gel to check RNA quality.
38. Store re-suspended sample at -80 ^{0}C.

3.12.2 cDNA synthesis

Extracted total RNA was used to synthesize cDNA of miRNA and their target mRNA (target genes). Omniscript Reverse Transcription Kit (Qiagen, Catalog No: 201511) was used for cDNA synthesis for miRNA and their target genes.

3.12.3 cDNA synthesis of miRNA

100 µg of each RNA sample taken from wild-type and transgenic plants under stress and control conditions were used to prepare 1st mix for synthesis of cDNA of different miRNA. Stem-loop primers of miRNA (0.05µM) were added in 1st mix followed by incubation of the samples of miRNA for 5 min at 65 °C. A 2nd mix for each miRNA sample was prepared containing 1X of RT reaction buffer (5X), 1.6U of RNAse Out (40 U/µl), 0.5 mM of dNTP (10 mM), 8 U of revert aid reverse transcriptase (200 U/µl) and 100 ng/µl of RNase free dH_2O. After centrifugation, 2nd mix was added to 1st mix until a final volume of 10 µl per miRNA sample. Samples were incubated at 37 °C for 1 hour and then reaction was inactivated for 10 min at 70 °C. Finally, cDNA was diluted 8X per sample and was stored at -20°C. Primers used for cDNA synthesis of miRNA are given in Table 3.24

Table 3.24. Primers used for cDNA synthesis of miRNA

Primer Name	Sequence (5'-3')
miRNA novel 8-SL-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGATTAG
stu-miR156d-3p-SL-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGATGA
stu-miR172b-3p-SL-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGCAG
U6_cDNA	CCAGTGCAGGGTCCGAGGTATTTGGACCATTTCTCGAT

3.12.4 cDNA synthesis of mRNA

For each RNA sample, 500 ng of RNA sample in 10 µl dilution was used to prepare 1st mix for synthesis of cDNA. Oligo-dT primer (0.05 µM) were added in 1st mix followed by incubation of the samples for 5 min at 70 °C. A 2nd mix for each RNA sample was prepared containing 5 µl of RT reaction buffer (5X), 1.25 µl of dNTP (10 mM), 1.6 U of RNAse Out (40 U/µl), 1 µl of reverse transcriptase (200 U/µl) and 100 ng/µl of RNase free dH_2O. After brief centrifugation of both mixes, 2nd mix was added to 1st mix. Samples were incubated for 1 hour at 37 °C and then reaction was inactivated for 10 min at 70 °C. Finally, cDNA was diluted 5X per sample and was stored at -20°C.

3.12.5 RT-qPCR analysis

Real time PCR was performed to investigate gene expressional change of miRNAs and their target genes under stress conditions. For cDNA synthesis from the miRNAs, miRNA-specific stem-loop primers were used. While for RT-qPCR, miRNA-specific

primers were used as forward and a universal reverse primer was used as reverse primer for expression analysis of miRNA (Table 3.25). RT-qPCR contents included 5 µl of total Syber green master mix ThermoFisher scientific (2X), 0.4 µl of forward primer (2 µM) and reverse primer (2 µM), RNase-free water 1.7 µl and 2.5 µl of diluted cDNA as template (1:10). RT-qPCR temperature cycle was set at 95 °C (5 min), 30 cycles at 95 °C 40 sec, specific annealing temperature of primers for 60 sec, 72 °C for 45 and 4 °C for unlimited time. Annealing temperature was 54 °C, 56 °C and 60 °C for miR156, miR172 and miRNA- novel8, respectively. In case of target genes, oligo-dT primers were used for cDNA synthesis of target genes. While for RT-qPCR of target genes, all qPCR contents were same as in case of miRNA except using gene specific forward and reverse primers for expression analysis of target genes (Table 3.26). Temperature cycle was also same as described before in case of miRNA where annealing temperature was 58 °C for all target genes. The elongation factor 1-α (ef1α) was used as reference gene for quantifying the expression of miRNA and target genes (Nicot et al. 2005). Three biological and three technical repeats were used for each miRNA and target genes. The Ct values of samples were analyzed by Rotor-Gene Q (QIAGEN) RT-PCR. The $2^{-\Delta\Delta Ct*}$ proportional calculation method was used for the calculation of gene expression values (Livak and Schmittgen, 2001).

Table 3.25. Primers used for qRT-PCR of miRNA

Primer Name	**Forward Primers (5' -3')**	**Reverse Primers (5'-3')**
stu-miR156d-3p-F	CGGGCTCTCTATGCTTCTGTCATCA	
stu-miR172b-3p-F	GGCGGAGAATCTTGATGATGCTGC	
miRNA novel 8-F	GGAGGCGTTGTCTGAGGCTAATC	
miRNA-R-Universal		CCAGTGCAGGGTCCGAGGTA

Table 3.26. Primers used for qRT-PCR for target genes

Primer Name	Forward Primers (5'-3')	Reverse Primers (5'-3')
Phospholipid-transporting ATPase	CTTCACATCGCTTCCTGTGA	AATTGGTGGTGCCAAAGAAG
AP2 transcription factor SlAP2d	TAGGTACCTCCCACGGACAC	TGTTGGGACCAGACATTTGA
Photosystem II core complex proteins psbY, chloroplast	TGCTTTGCTTTGGGTAGCTT	CATATGGCCAAACGCCTACT
Mitochondrial transcription termination factor family protein	GACAAGCTAAGGCCTGCAAC	TTGTGGCAATTTCCGTCATA

3.13 Statistical Analysis

ANOVA was performed on the physiological dataset based on the randomized complete block design considering stress treatments (Control, D, Control of H or HD, H, and HD) and genotypes (Unica and transgenic Unica) as the main factors. Since genotypes consist of transgenics and non-transgenics with their control groups, ANOVA was performed on each genotype with a complete set of stress treatments. Gene expression values were also analysed by ANOVA, in this case, a comparison of genotypes (transgenic, wild-type), samples of control, heat, and drought treatments, and the interaction between the two was practiced. Statistical differences among treatments were estimated by the Least significant difference test (LSD) at a probability threshold level of $P<0.05$. All statistical analyses were done using Statistix 8.1 software. Paired t-test was conducted to calculate the significant difference ($P<0.05$) between transgenics and non-transgenics at each stress treatment using SPSS software. XLSTAT was used for principal components analysis (PCA), to summarize comprehensive variation among the samples; a sample correlation matrix was used to assure equal weight distribution to all biochemical variables.

CHAPTER IV

RESULTS

4.1 cDNA Synthesis of pre-miRNAs and Addition of Digestion Sites

The cDNAs of pre-miRNAs of stu-miR156d-3p, stu-miR172b-3p, miRNA novel-8 were synthesized by using miRNA-specific stem-loop primers. Then the NcoI and BstEII digestion sites were added to cDNAs by PCR. The restriction sites added and amplified cDNAs were confirmed by agarose gele analysis (Figure 4.1). Expected sizes of the cDNAs for stu-miR156d-3p, stu-miR172b-3p, and miRNA novel-8 were 123bp (93 bp for original cDNA + 30 bp for added tail), 121 bp (91 bp for original cDNA + 30 bp for added tail), and 160 bp (130 bp for original cDNA + 30 bp for added tail), respectively. (Figure 4.1, 4.2).

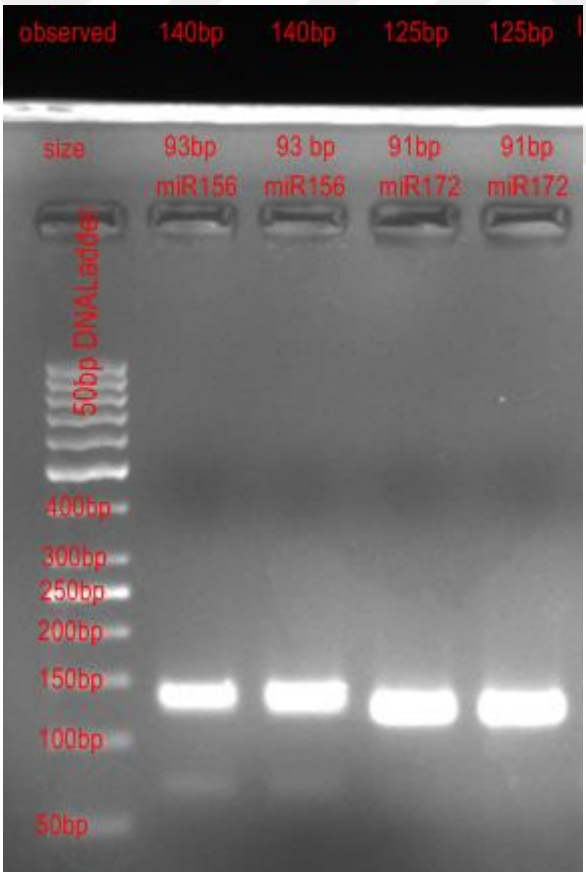


Figure 4.1. Agarose gel image of tail-added and amplified cDNA of pre-miRNAs (miR156d-3p, miR172b-3p)

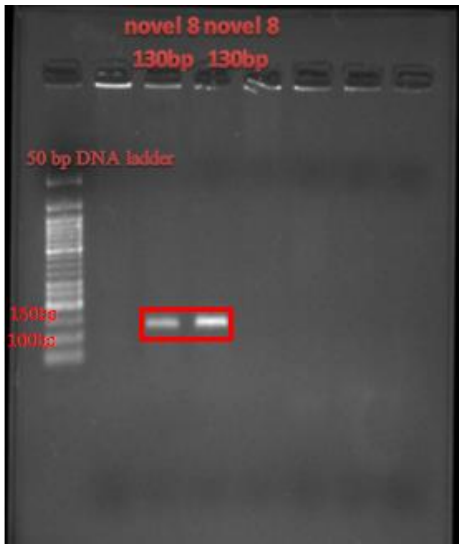


Figure 4.2. Agarose gel image of tail-added and amplified cDNA of pre-miR novel-8

4.2 Purification of the cDNAs and Insertion to the Plasmid Vector

The restriction site added and amplified cDNA samples (pre-miRNAs of stu-miR156d-3p, stu-miR172b-3p, miRNA novel-8) were run on agarose gel to confirm the tail addition and amplification. The bands on same agarose gel were exciced and the cDNA samples were prufied to insert them to the pCAMBIA1301 plasmid vector.

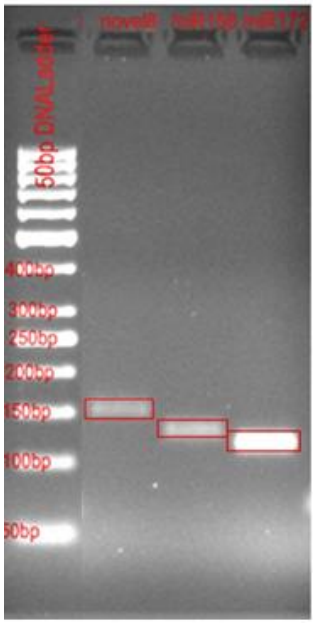


Figure 4.3. Gel extraction results of amplified cDNA fragments of pre-miRNAs

4.3 Isolated Plasmid pCAMBIA from *E. coli* (DH5α) Cell Suspension

Agarose gel image of isolated pure pCAMBIA without any insert is indicated in Figure 4.4.

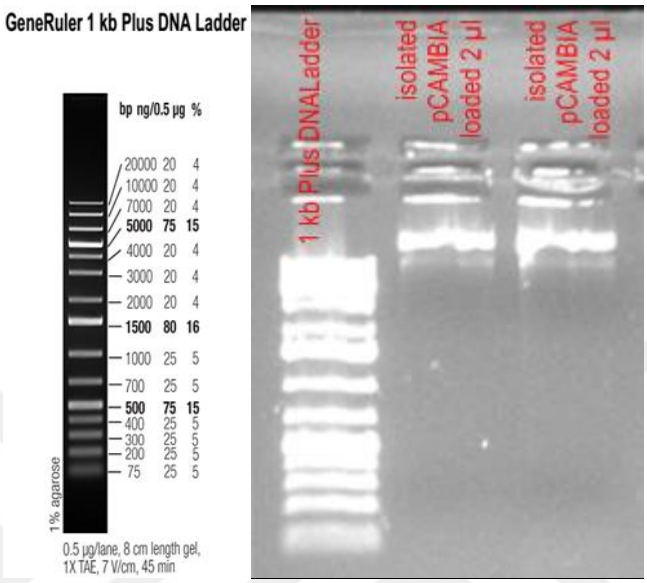


Figure 4.4. Extracted plasmid pCAMBIA1301

4.4 Agarose Gel Image of digested pCAMBIA1301 vector by *BstEII* and *NcoI* restriction enzymes

Agarose gel image of digested pCAMBIA1301 vector by *BstEII* and *NcoI* restriction enzymes is shown in Figure 4.5. The bottom band is GUS gene that was discarded from vector. The bands in the frame were eluted from gel. Digested and gel eluted bands that were loaded on agarose, are indicated in Figure 4.6.

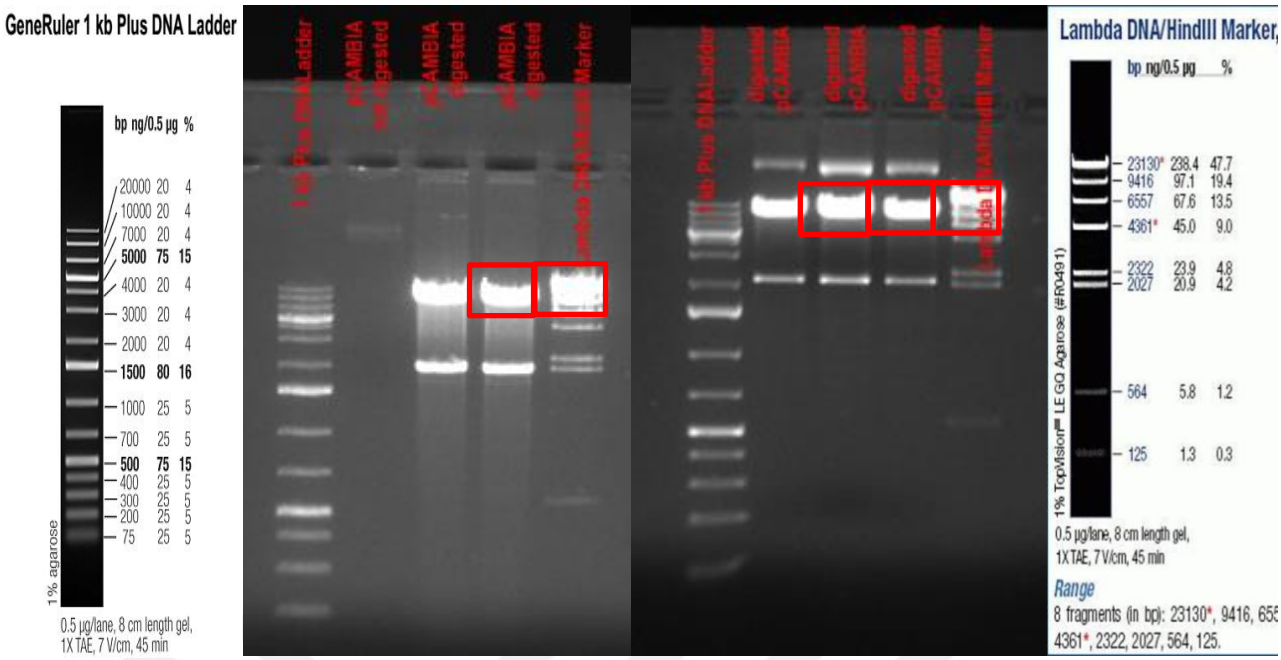


Figure 4.5. Image (agarose gel) of digested pCAMBIA1301 vector by *NcoI* and *BstEII* restriction enzymes

ThermoScientific Lambda DNA/HindIII Marker #SM1331 and ThermoScientific GeneRuler 1 kb Plus DNALadder #SM0103(1 % agarose gel, 0.5 X TBE).

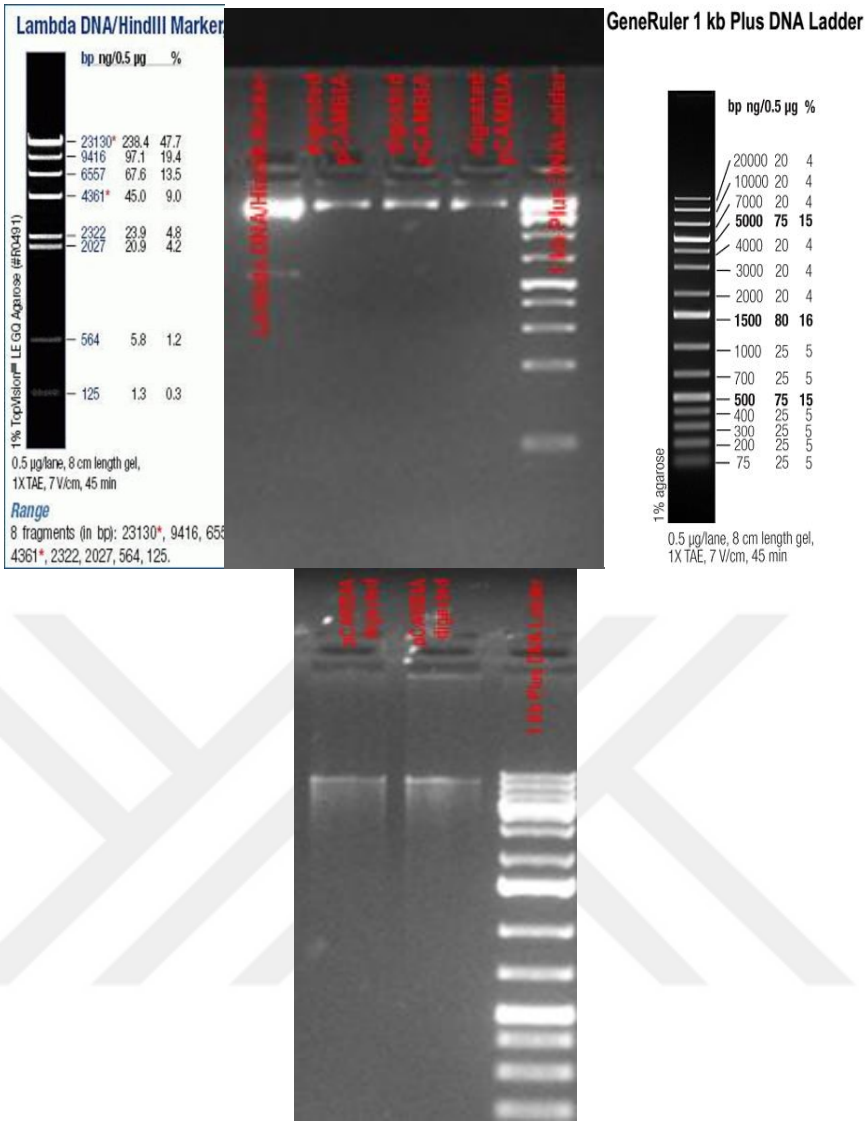


Figure 4.6. Agarose gel image of digested/gel eluted pCAMBIA vector

ThermoScientific GeneRuler 1 kb Plus DNALadder #SM0103, (ThermoScientific GeneRuler 1 kb Plus, 1 % agarose gel, 0.5 X TBE)

4.5 Agarose Gel image of Ligation confirmation PCR

Agarose gel image of ligation confirmation PCR with PBB primers is indicated on Figure 4.7. The amplicon sizes were larger than the amplified stu-miR156d-3p, stu-miR172b-3p, miRNA novel 8 by PCR, because PBB primers were designed outward of cloning region of inserts on the vector pCAMBIA.

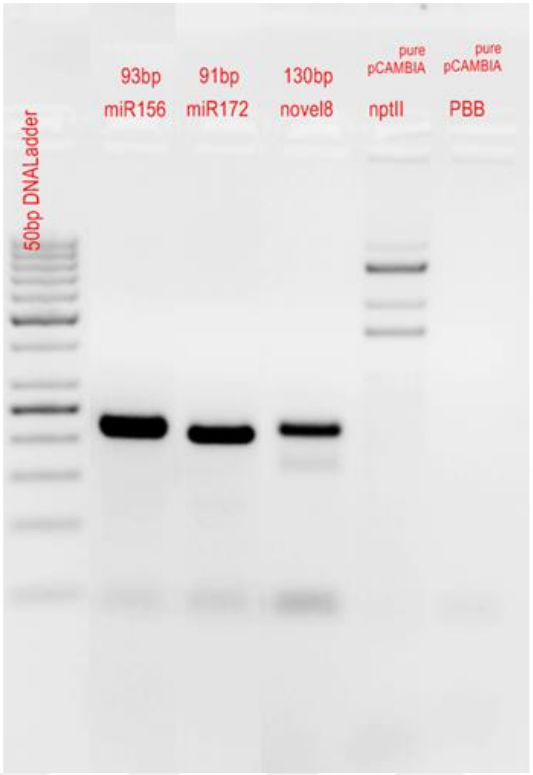


Figure 4.7. Agarose gel image of ligation confirmation PCR

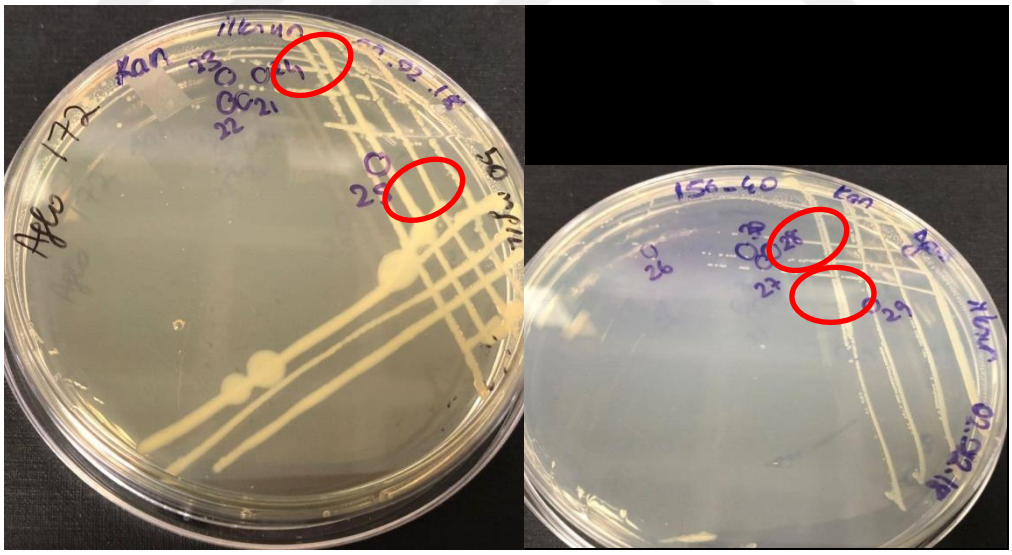


Figure 4.8. Positive colonies of Agrobacrium (LBA4404) harbouring pCAMBIA1301+insert constructs of stu-miR172b-3p and stu-miR156d-3p

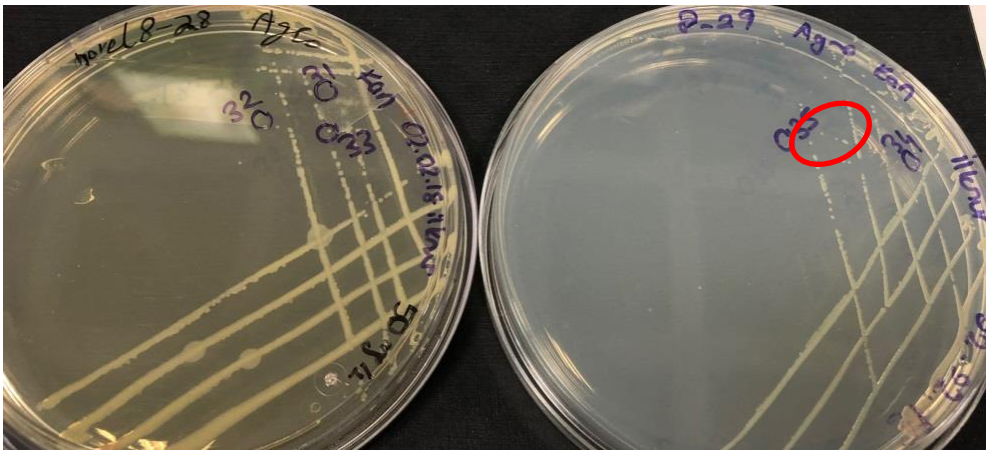


Figure 4.9. Positive colonies of Agrobacrium (LBA4404) harbouring pCAMBIA1301+insert constructs of miRNA novel 8

4.6 Colony conformation PCR with miRNA specific primers

Agarose gel image of colony PCR confirmation of pre-miRNAs of stu-miR156d-3p, stu-miR172b-3p, miRNA novel 8 with specific primers is given in Figure 4.10. Expected size of stu-miR156d-3p is 93 bp. As seen in Figure 4.10, all bands of stu-miR156d-3p is around 93 bp. Expected size of miR172b-3p was 91 bp. Expected size of novel8 is 130 bp however one band at same size of novel8 is observed as nearly 150 bp on agarose gel. The reason behind can be addition of cutting sites of restriction enzyme during primer designing. An addition of 15bp was done to each primer for cutting site of restriction enzyme.

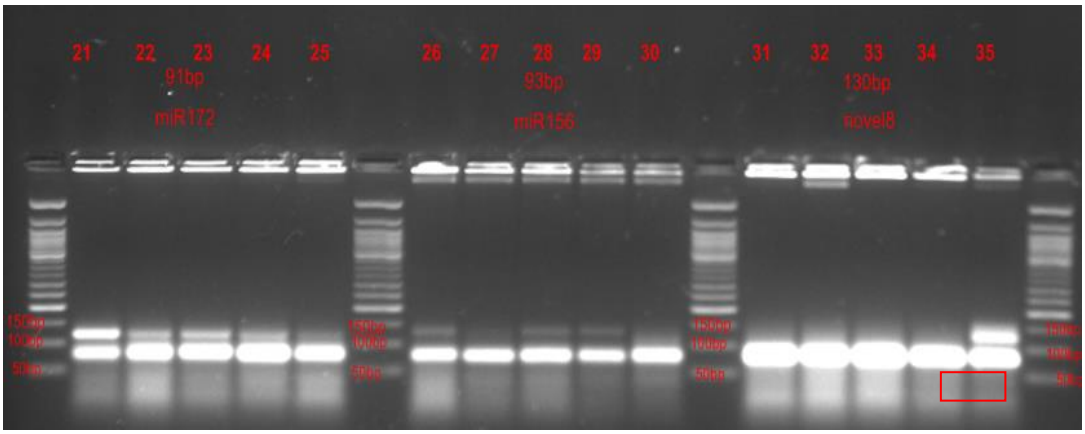


Figure 4.10. Colony PCR Confirmation of stu-miR156d-3p, stu-miR172b-3p, miRNA novel 8

4.7 Trans callus

Explants of Unica were cut, wounded, shifted to co-cultivation medium followed by their placement at regeneration selection medium for initiation of calli formation. After callus formation, calli were shifted to medium for shoot formation (Figure 4.11).

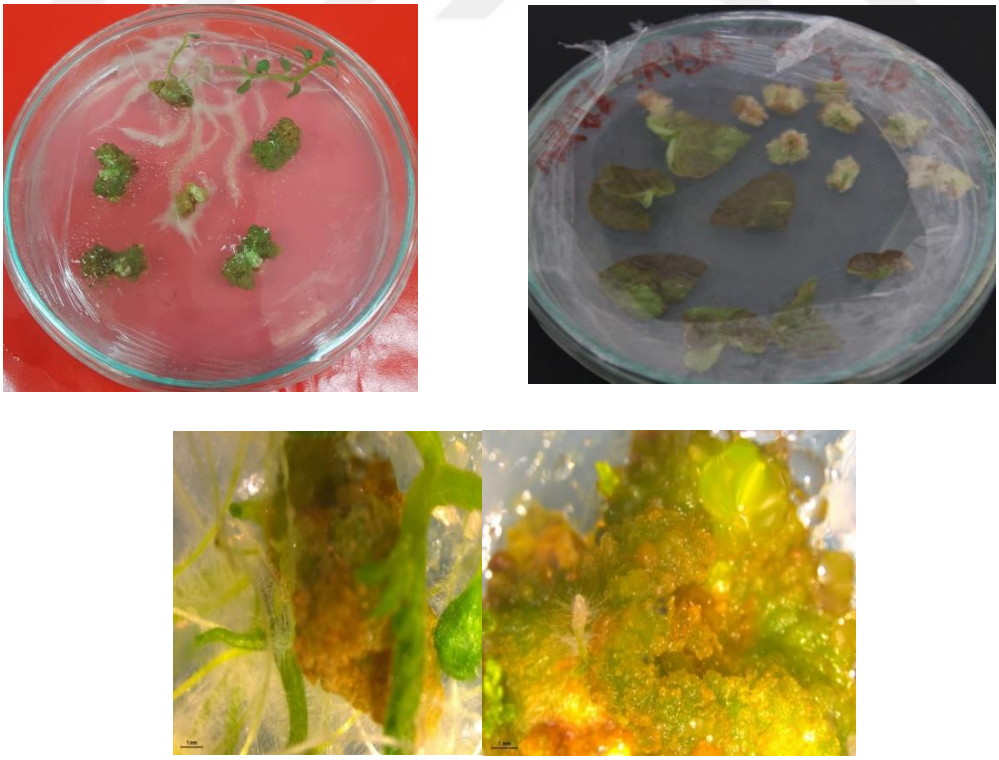


Figure 4.11. Shoot initiation from callus

Shoots were shifted to MS-0 medium in chamber at 25±2 °C of temperature for 16 hours of photoperiod.

Figure 4.12. Putative transgenic plants at MS-0 medium

As putative transgenic plants grew about 15 cm long, leaves were harvested to isolate DNA using Gene Jet Plant DNA Purification Kit (Thermo Fisher Scientific).

4.8 Confirmation of putative transgenic plants (PCR techniques)

For confirmation of transgenic plants having pre-stu-miRna inside, PCR was conducted using hygromycin primers.
Result of this PCR is following.

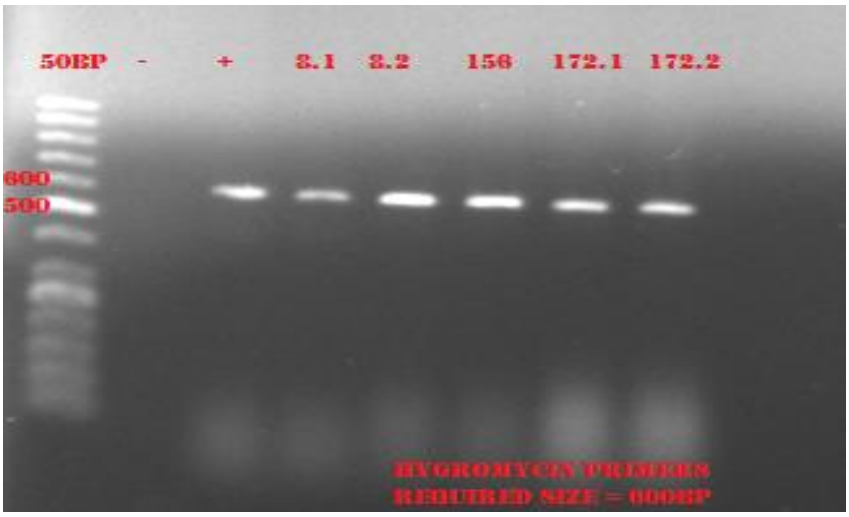


Figure 4.13. PCR with hygromycin primers from expected transgenic Unica plants harbouring miRNA novel 8, stu-miR156d-3p and stu-miR172b-3p

For confirmation of transgenic plants having Agrobacterium (LBA4404) suspension harbouring pCAMBIA1301+ pre-stu-miRNA, PCR was conducted using 35S as forward and Nos Poly-A tail as reverse primer.

Expected size of band was 961 bp (35S promotor region = 549 bp, Nos Poly-A tail region = 252 bp, miRNA novel 8 = 160 bp) in both transgenic lines harbouring miRNA novel 8. Result of this PCR is shown in following figures (4.14, 4.15).

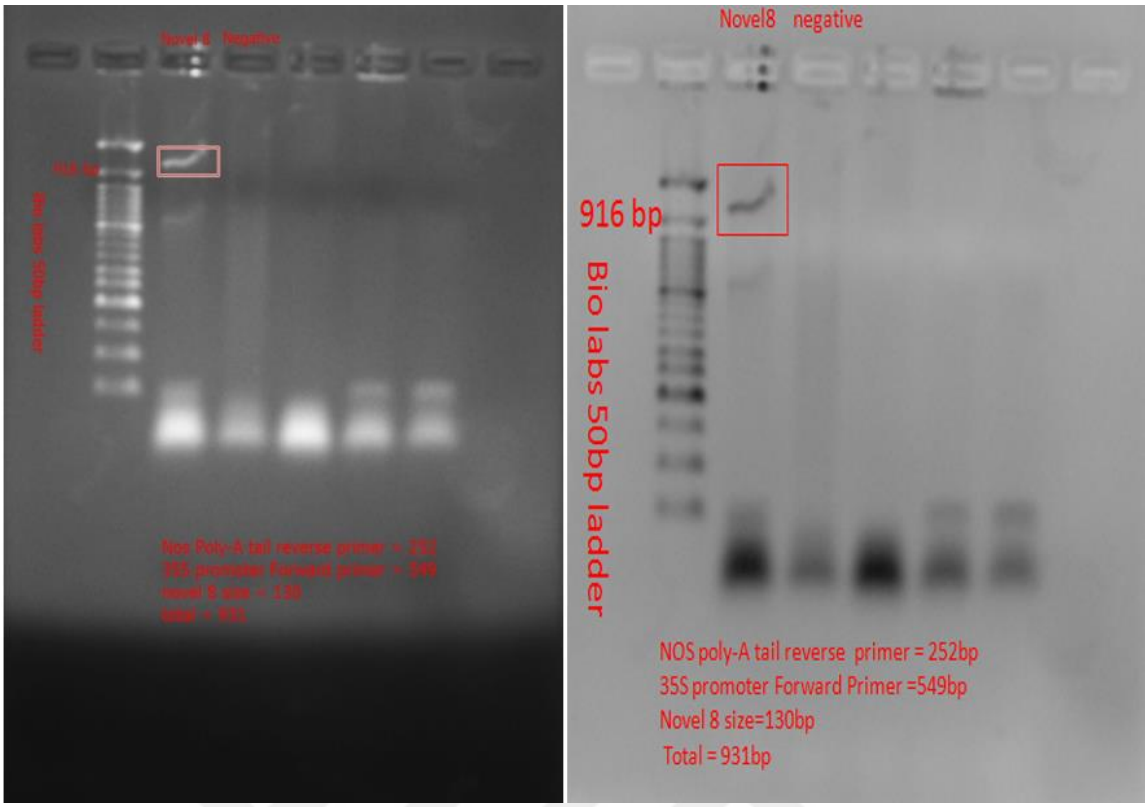


Figure 4.14. PCR from 1st transgenic line of unica plants harboring miRNA novel 8

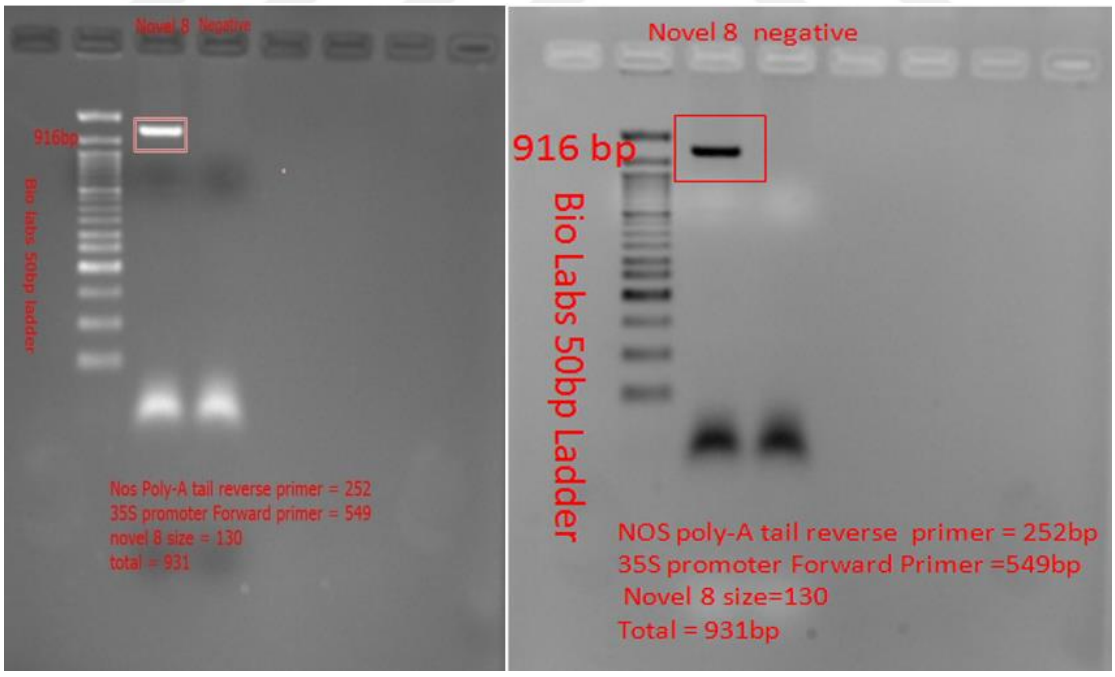


Figure 4.15. PCR from 2nd transgenic line of unica plants harboring miRNA novel 8

Expected size of band was 924 bp (35S promotor region = 549 bp, Nos Poly-A tail region = 252 bp, miR156-3p = 123 bp) in transgenic line harbouring miR156-3p. Result of this PCR is shown in following figure (4.16).

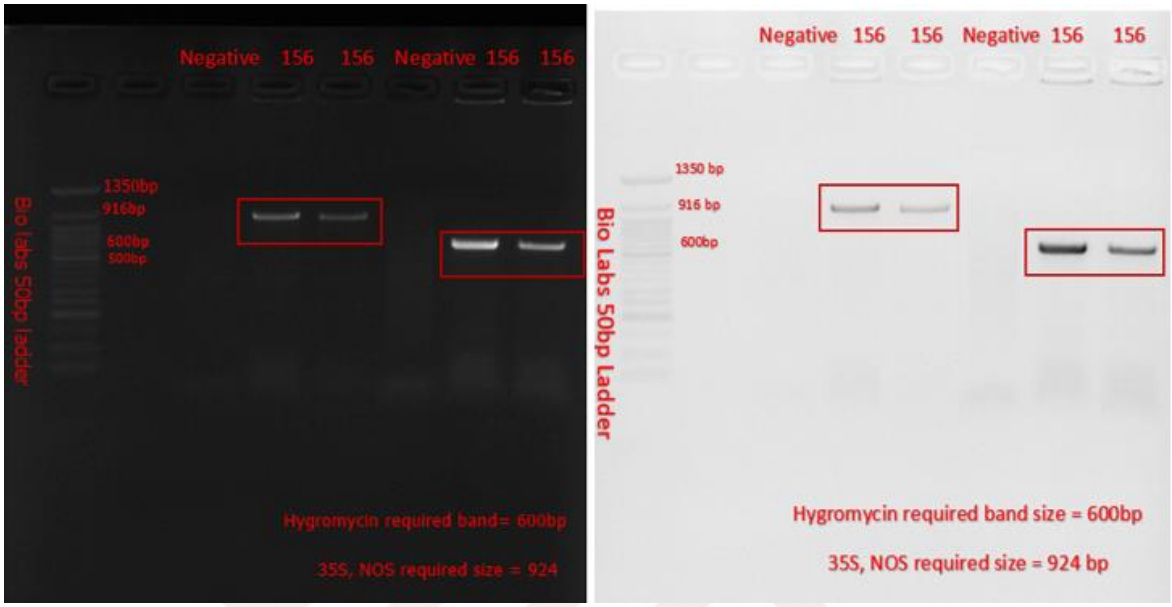


Figure 4.16. PCR from transgenic line of unica plants harboring stu-miR156-3p

Expected size of band was 922 bp (35S promotor region = 549 bp, Nos Poly-A tail region = 252 bp, miR172-3p = 121 bp) in both transgenic lines harbouring miR172-3p. Result of this PCR is shown in following figures (4.17, 4.18).

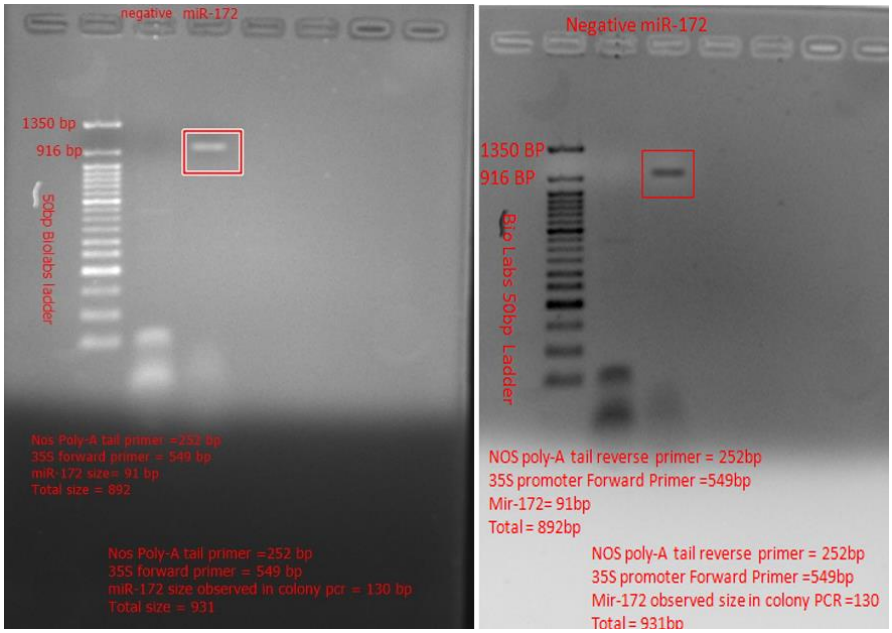


Figure 4.17. PCR from 1st transgenic line of unica plants harboring stu-miR172-3p

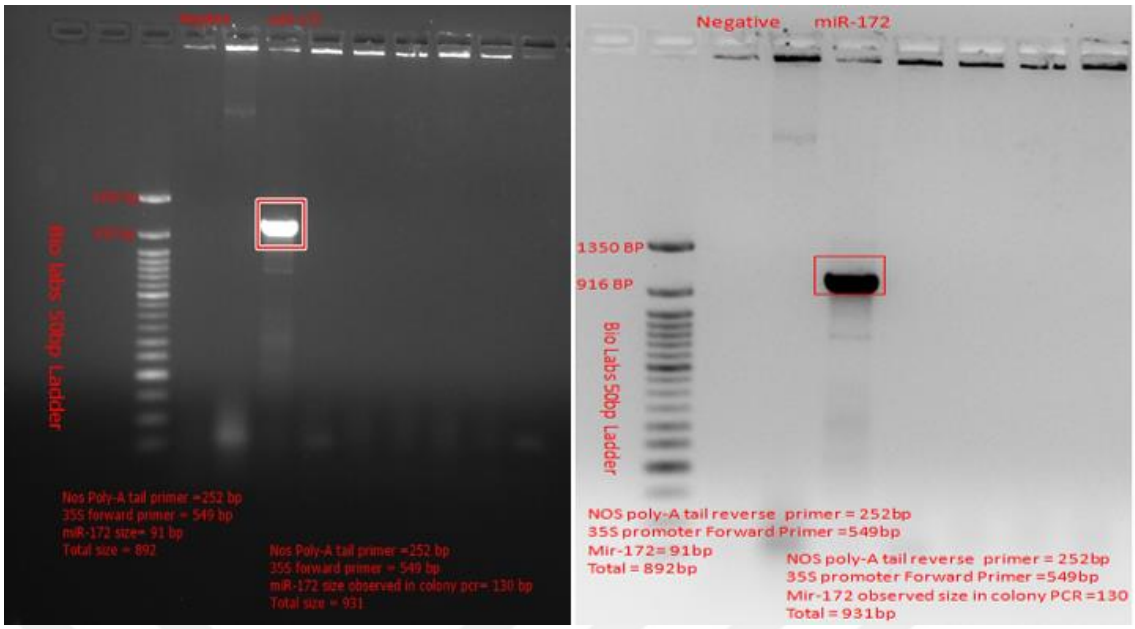


Figure 4.18. PCR from 2nd transgenic line of unica plants harboring stu-miR172-3p

4.9 Transformation Efficiency

Efficiency of transformation of transgenic plants was calculated as 0.94 %, 0.79 % and 0.90 % for, miRNA novel 8, miR156d-3p and miR172b-3p respectively as mentioned in Table 4.1.

Table 4.1. Transformation efficiency

miRNA novel 8		**miR156d-3p**		**miR172b-3p**	
Total Internodes	**Transgenic plants**	**Total Internodes**	**Transgenic plants**	**Total Internodes**	**Transgenic plants**
211	2	126	1	221	2
0.94 % transformation efficiency		0.79 % transformation efficiency		0.90% transformation efficiency	

4.10 Growth of Plants in Greenhouse and Stress Treatment

After confirmation of transgenic plants through PCR, next step was to multiply the plants according to the required number of plants. When plants grew up to 6 leaves stage, they were shifted to greenhouse. The main purpose to measure physiological

responses (photosynthesis rate) was to observe influence of stress on plants so that variability in stress-induced responses could be distinguished.

4.11 Physiological responses of transgenic miR156d-3p plants to Stress Conditions

Physiological responses were primarily measured to spot the exact period at which plants encountered stress conditions. Transgenic plants harboring miR156d-3p were exposed to continuous drought, heat alone and combination of heat+drought stress for 20 and 12 days respectively. Figure 4.19, and Figure 4.20 represents plants after exposure to stress treatments. Combined heat and drought stress showed major loss of leaves as compared to other stress conditions and control plants.

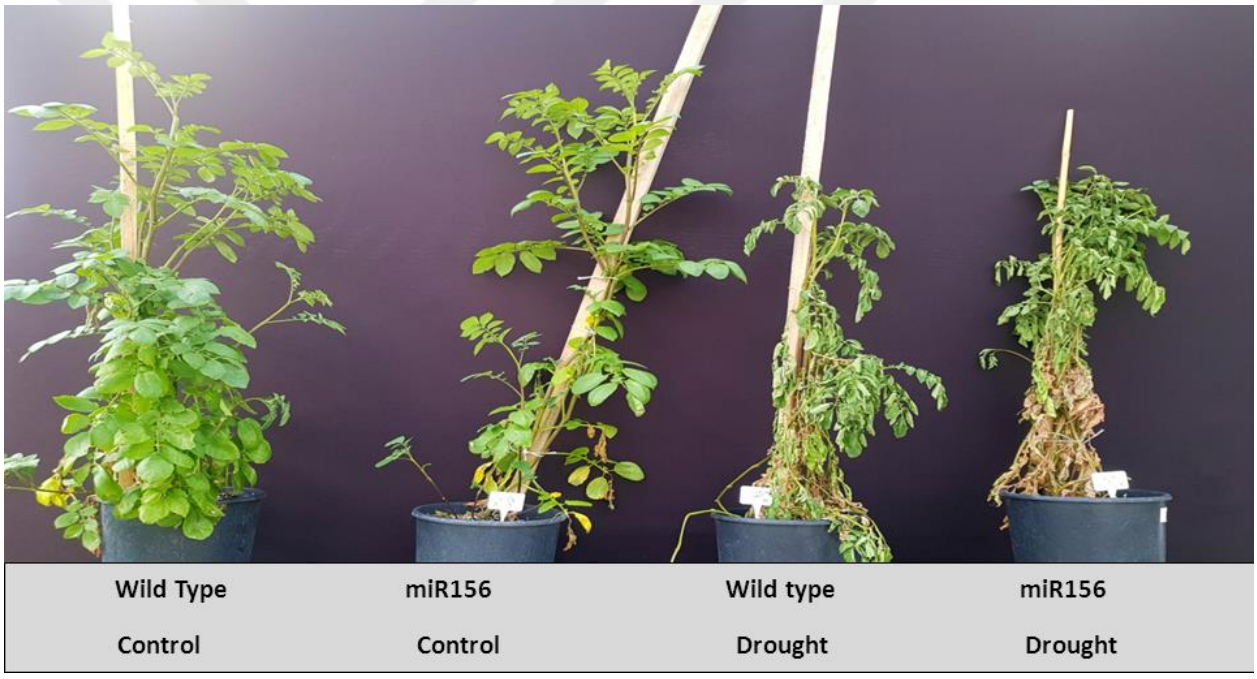


Figure 4.19. Above ground phenotype of wild-type and transgenic plants after 20 days of drought treatment

Treatments were control of wild-type plants, control of transgenic plants, the drought of wild-type plants, and drought of transgenic plants.

Figure 4.20. Above ground phenotype of wild-type and transgenic potato plants at 12 days after heat treatment and combined heat+drought treatment

Treatments were control of wild-type plants, heat of wild-type plants, combined heat-drought of wild-type plants, control of transgenic plants, the heat of transgenic plants, and combined heat drought of transgenic plants.

Generally, stress treatments caused a gradual reduction in photosynthetic rates (Pn) in wild-type and transgenic plants from the 10th day to the end of treatments, however no reduction in photosynthesis rate was observed in either wild-type or transgenic under heat treatment (Figure 4.21). Under drought condition a significant reduction of 71.2% (from 32.6 to 9.4 µmol m^{-2} s^{-1}) and 66.8% (from 36.78 to 12.18 µmol m^{-2} s^{-1}) was observed in Pn of wild-type and transgenic plants, respectively. However, the first significant decrease in Pn under the combined heat and drought treatment was observed on the 10^{th} day of the treatment for wild-type plants. While both wild-type and transgenic plants kept on fixing carbon on the 12^{th} day of combined stress treatment (Figure 4.21). The photosynthetic rate of wild-type and transgenic plants was reduced by 76.36 % (from 15.1 to 3.6 µmol m^{-2} s^{-1}) and 62% (from 21.39 to 7.94 µmol m^{-2} s^{-1}) compared to their control plants on the 12th day of the combined stress treatment. Since

the lowest and the significant decrease in Pn was observed on the 20th day for drought treatment and the 12th day for heat only and combined heat-drought, we decided to consider these days as the last day of the stress treatments and further investigation was performed on the plants at the last days of the treatments.

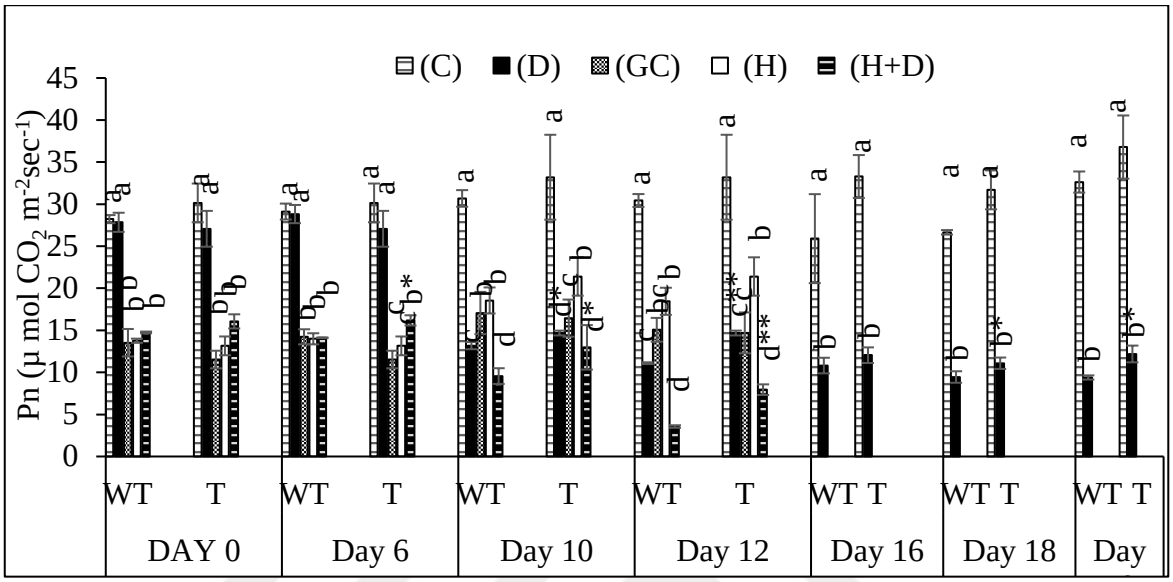


Figure 4.21. Influence of abiotic stress on the photosynthetic rate of potato genotypes

Transgenic (T) and wild-type (WT) plants were classified as control ( ), drought ( ), control for heat and combined heat-drought stresses ( ), heat ( ), and combined heat-drought ( ). Data shows as mean ± SD. Asterisks (*,**) showed the significant difference of treatments in transgenics from wild-type plants at $P<0.05$ and $P<0.01$ respectively.

The stomatal conductance rate of both wild-type and transgenic plants exhibited a significant reduction under drought and combined heat and drought conditions (Figure 4.22a). Despite that, stomatal conductance was significantly higher in WT and transgenic plants under heat treatment than in control plants. The stomatal conductance rate increased significantly by 35 % (0.14 to 0.19 mol H_2O m^2 s^{-1}) and 44 % (0.09 to 0.13 mol H_2O m^2 s^{-1}) in response to heat stress in transgenic and WT plants (Figure 4.22a). In accordance with stomatal conductance, the transpiration rate decreased significantly in WT and transgenic lines with the exposure to drought only and combined stress. Contrarily it exhibited higher transpiration rate by 10 % (3.16 to 3.48

H_2O m^2 s^{-1}) in WT, while 117% (3.88 to 8.43 H_2O m^2 s^{-1}) under HS conditions in transgenic plants (Figure 4.22b).

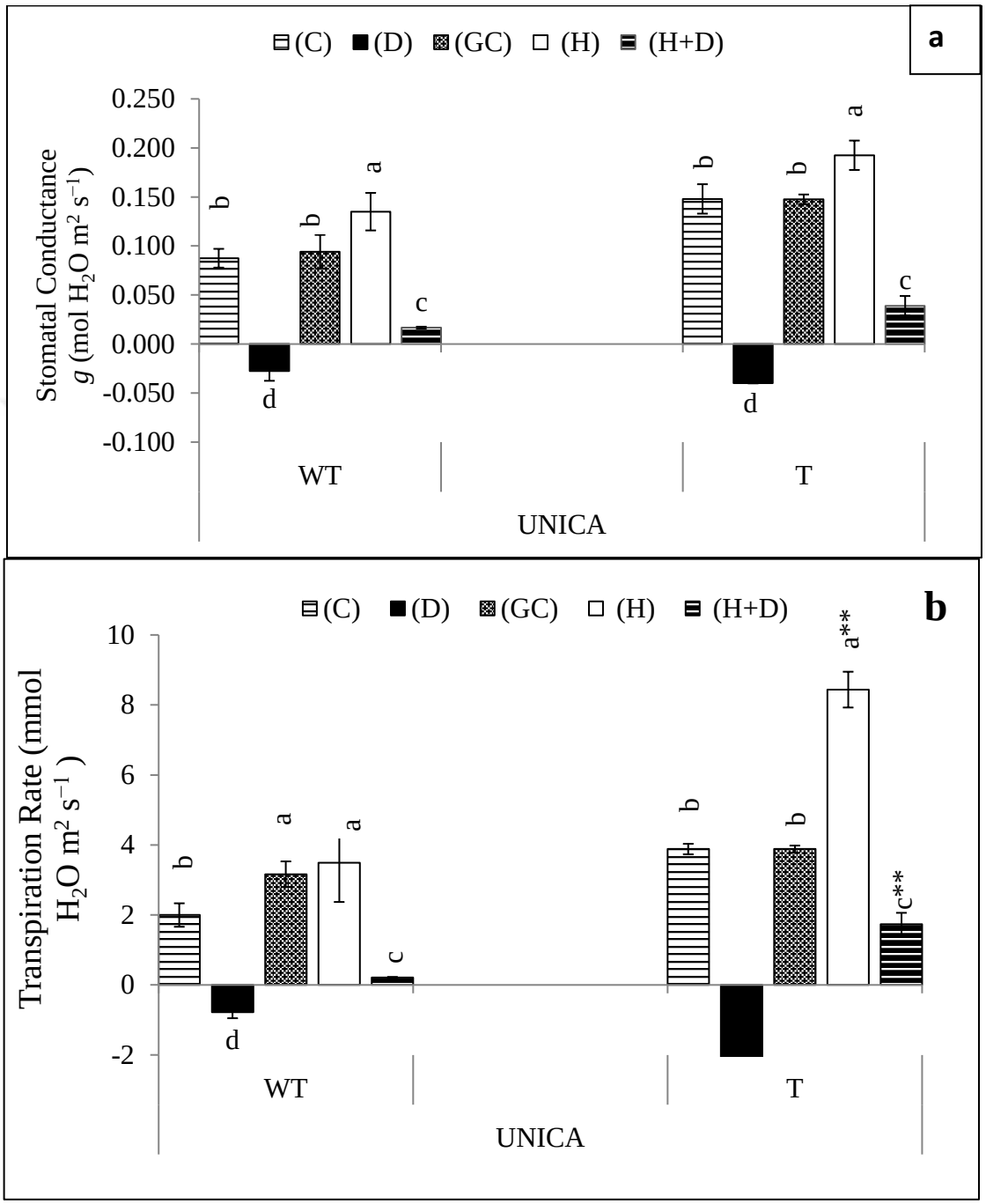


Figure 4.22. Influence of abiotic stress on physiological traits of potato genotypes

Transgenic (miR156d-3p) (T) and wild-type (WT) plants were exposed to optimum temperatures and water level as control (C, ▤) or drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □) and combined heat-drought (HD, ▬) stresses. Stomatal conductance (**a**), Transpiration rate **(b),** were measured as

outlined in the text. Data shows as mean ± SD Asterisks (*,**) showed the significant difference of treatments in transgenics from wild-type plants at $P<0.05$ and $P<0.01$ respectively.

Figure 4.23 shows the chlorophyll content and temperature of plant leaves under control and stress conditions. Plants under drought stress showed no change in their chlorophyll content. While plants under heat and combined stress conditions showed significant decrease in chlorophyll content. Leaf temperature of all plants showed and increase under heat and combined stress conditions while under DS leaf temperature showed significant increase in transgenic plants.

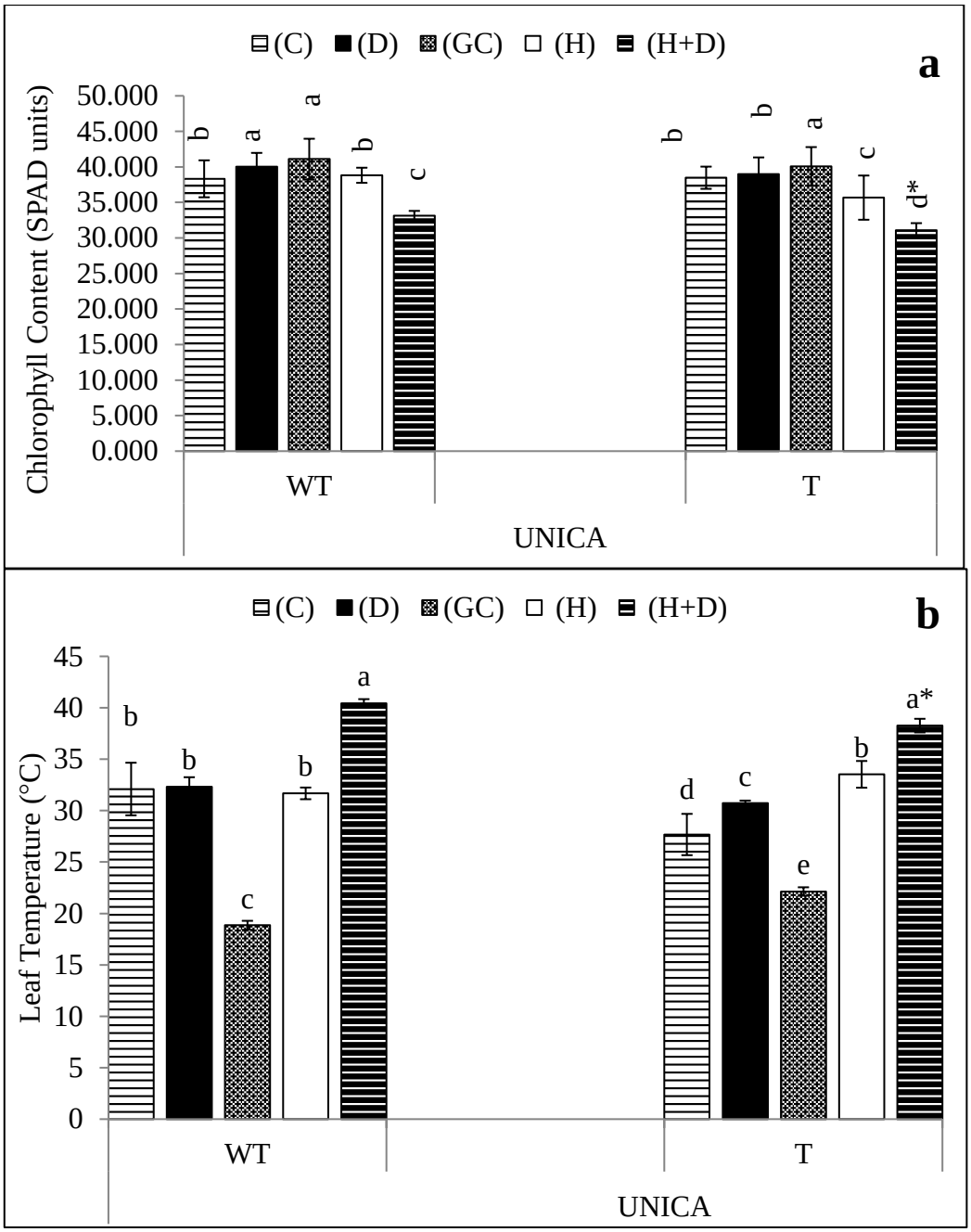


Figure 4.23. Effect of abiotic stress on physiological traits of potato genotypes

Transgenic (T) and wild-type (WT) plants were exposed to optimum temperatures and water level as control (C, ▤) or drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □) and combined heat-drought (HD, ▬) stresses. Relative chlorophyll content (**a**), Leaf Temperature **(b).** Data shows as mean ± SD. Asterisks (*,**) shows the significant difference of treatments in transgenics from wild-type plants at P<0.05 and P<0.01 respectively.

Figure 4.24 shows the relative leaf water content of plants under control and stress conditions. Under all stress conditions imposed, a significant reduction was recorded in leaf water content of wild-type and transgenic plants as compared to their controls.

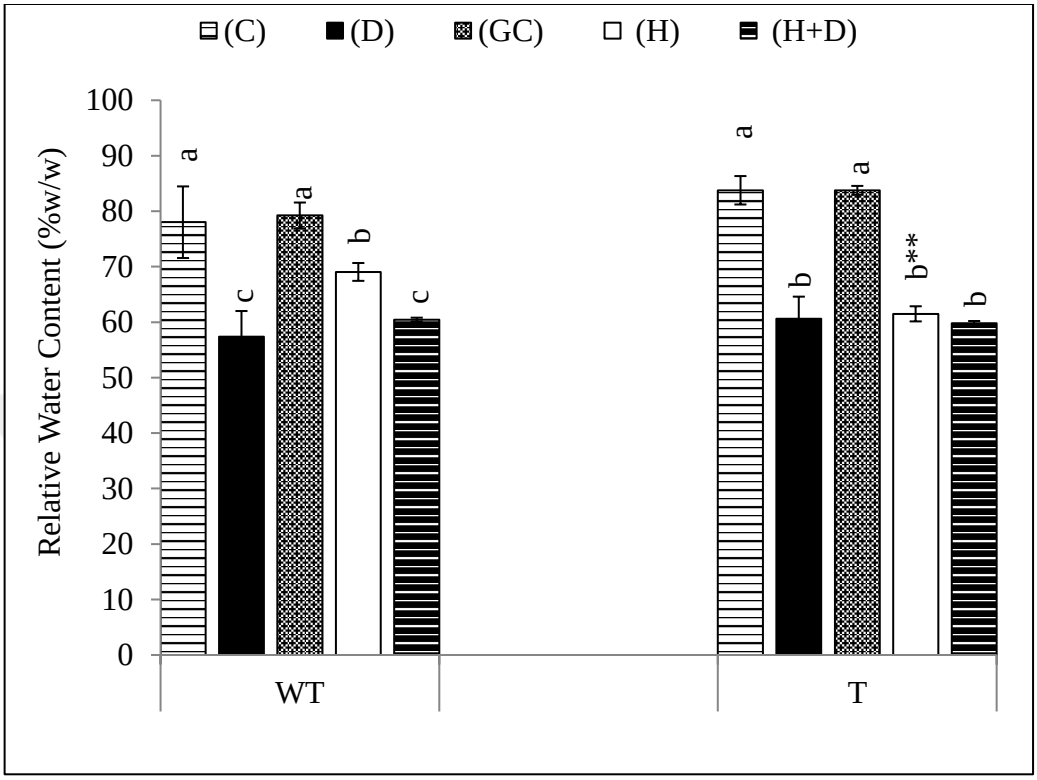


Figure 4.24. Effect of abiotic stress on physiological traits of potato genotypes

Transgenic (T) and wild-type (WT) plants were exposed to optimum temperatures and water level as control (C, ▤) or drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □) and combined heat-drought (HD, ▬) stresses. Data shows as mean ± SD. Asterisks (*,**) shows the significant difference of treatments in transgenics from wild-type plants at $P<0.05$ and $P<0.01$ respectively.

Overall, the data proved that wild-type and transgenic plants altered their response to the drought, heat, and their combination. The change in photosynthetic rate and transpiration rate agreed with the stomatal conductance during the stress period showing that photosynthesis and transpiration activities were dependent on the stomatal conductance.

4.12 Biochemical Responses to Stress Conditions

Wild type and transgenic plants having overexpression of miR156d-3p were exposed to continuous drought, heat alone and combination of heat with drought stress for 20^{th} and 12^{th} days, respectively, and at the last day of stress, leaf samples were collected from control and under stress plants for measurement of proline, MDA, H_2O_2 and activity of antioxidant enzymes (APX, CAT, SOD, POD). Proline has potential to serve as signaling molecules for the modulation of mitochondrial functions, influencing cell proliferation, triggering gene expression, that play vital role in plant recovery. It directly protects the essential proteins and integrity under stress. Heat and combined stress caused a significant increase in proline accumulation in WT and T plants. WT and transgenic plans under DS showed significantly huge increment of approximately 7 times (1.36 to 10 µmol/g FW) and 11 times (1.60 to 17.96 µmol/g FW) in proline accumulation (Figure 4.25). No significant difference in MDA content was found in WT and T plants under drought and combined stress except a significant reduction in transgenic plants under heat only condition (Figure 4.26a). WT and T plants showed an increase of 23.34% (12.08 to 14.90 µmol/g FW) and 10.30% (12.03 to 10.79 µmol/g FW) in accumulation of MDA under HD respectively. Higher H_2O_2 concentration is harmful to cells, resulting in localized oxidative damage, lipid peroxidation, disruption of metabolic function, and loss of cell integrity. In both wild-type and transgenic plants, we observed an elevating trend in H_2O_2 accumulation under drought conditions, whereas decreasing trend was observed under heat (59.05 %, 45.77 %) and combined heat and drought (9.87 %, 24.07 %) conditions (Figure 4.26b). The transgenic plants showed a similar amount of H_2O_2 accumulation to the wild-type plants under heat conditions.

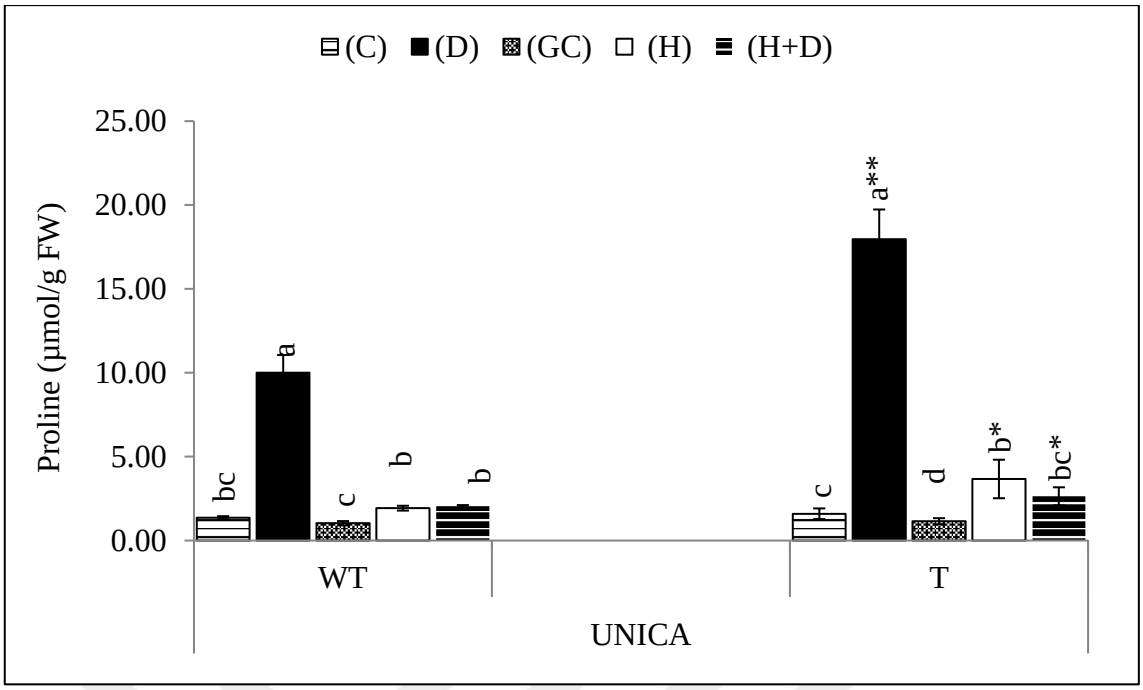


Figure 4.25. Effect of abiotic stress on biochemical traits of potato genotypes

Transgenic(T) and wild-type (WT) plants were classified as control (C, ▤), drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □), and combined heat-drought (HD, ▬) stresses. Data shows as mean ± SD. Asterisks (*,**) shows the significant difference of treatments in transgenics from wild-type plants at $P<0.05$ and $P<0.01$ respectively.

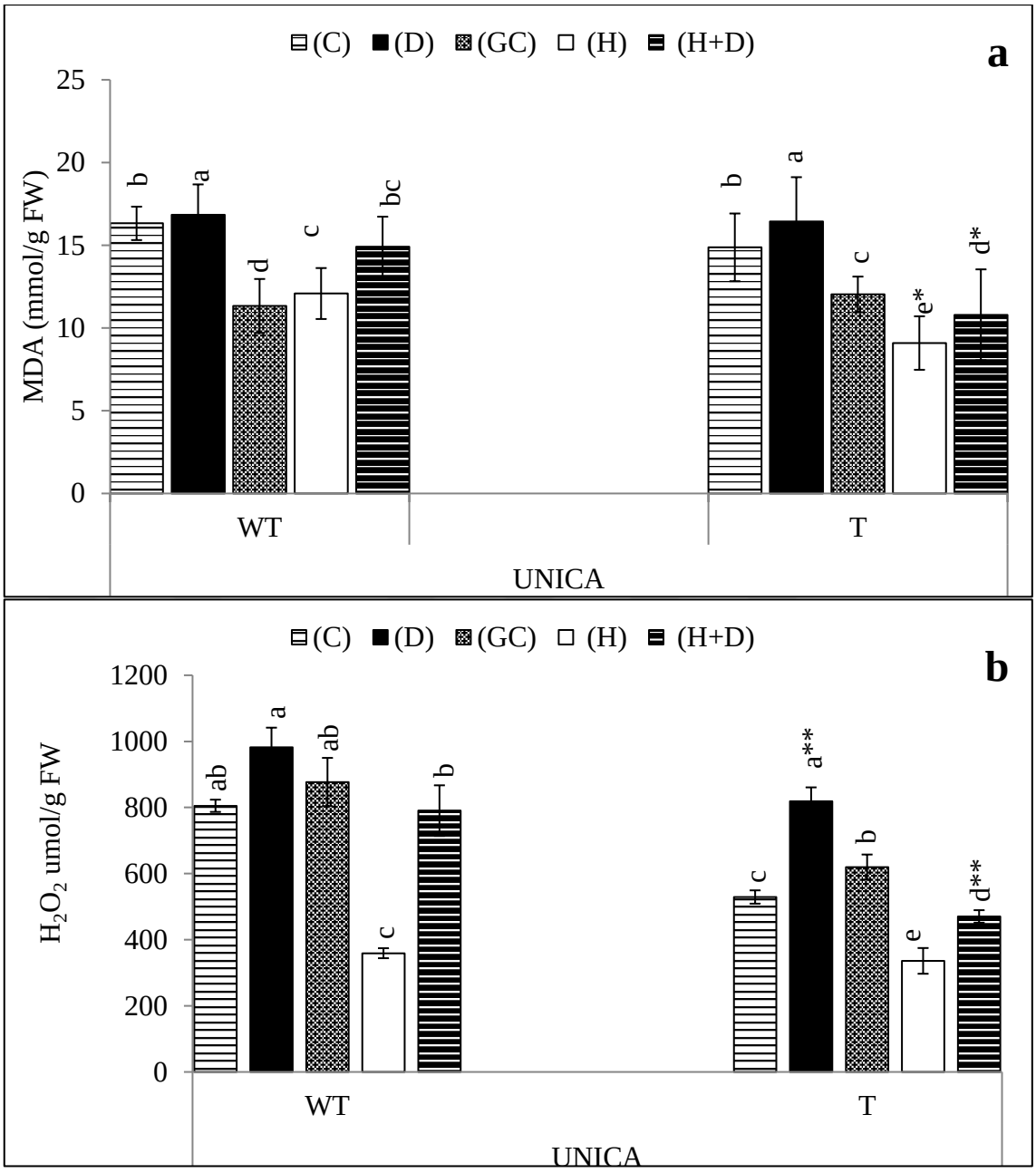
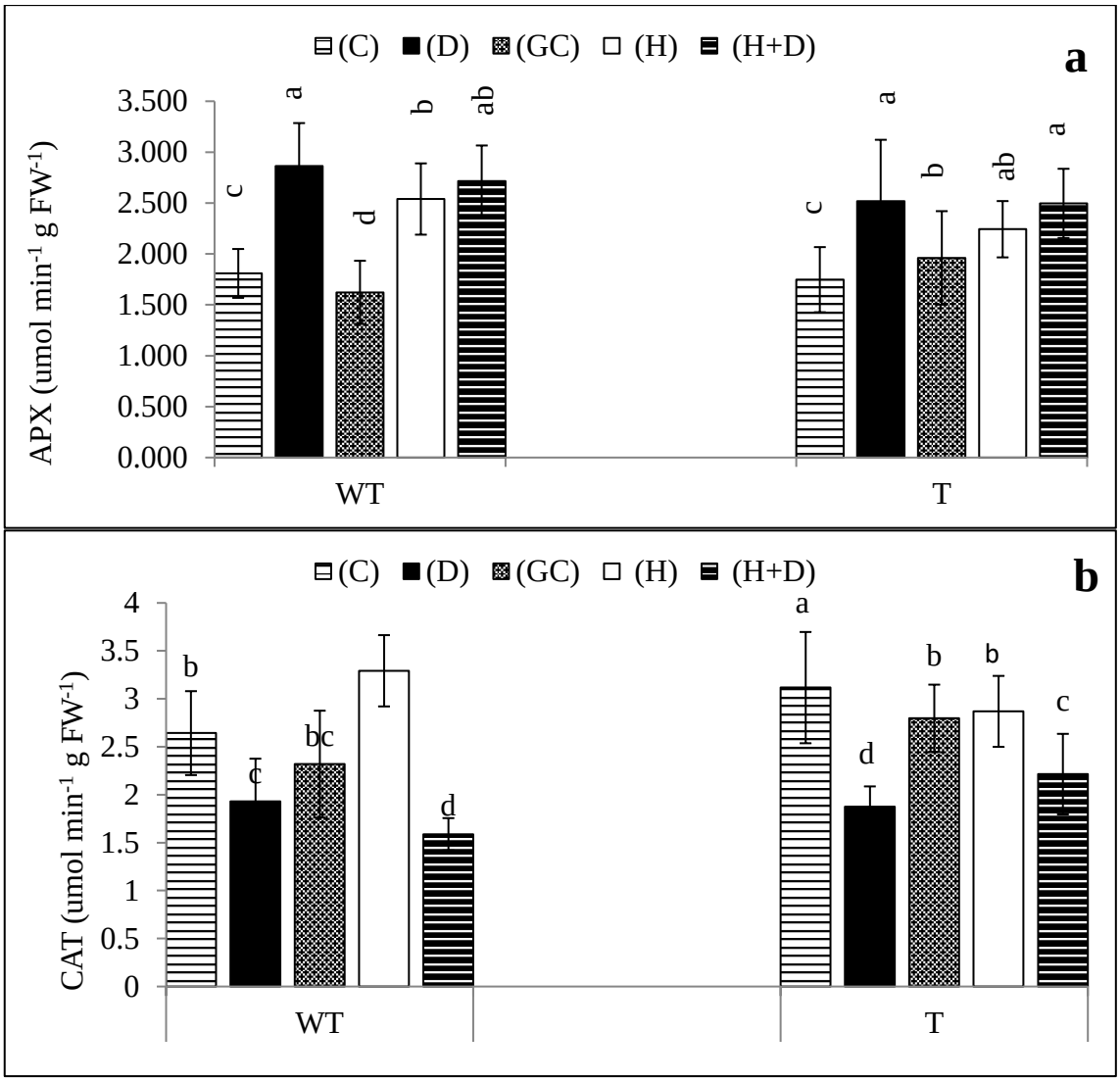


Figure 4.26. Effect of abiotic stress on biochemical traits of potato genotypes

Transgenic(T) and wild-type (WT) plants were classified as control (C, ▤), drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □), and combined heat-drought (HD, ▬) stresses. Malondialdehyde (MDA) contents **(a),** and H_2O_2 **(b).** Data shows as mean ± SD. Asterisks (*,**) shows the significant difference of treatments in of transgenics from wild-type plants at $P<0.05$ and $P<0.01$ respectively.

Activity of APX, CAT, POD and SOD antioxidant enzymes was measured under control and stress in WT and transgenic plants having over expression of miR156d-3p

(Figure 4.27). Under all stress conditions a significant increase in wild type and transgenic plants was observed in accumulation of APX. Under drought and combined stress conditions CAT showed a significant decline in WT under drought (27 %) and combined stress (31 %) and transgenic plants drought (39.87 %) and combined stress (20.78 %) conditions. Incase of CAT, wild type plants showed a signifcant increase of 42 % under HS, whereas transgenic plants showed no significant change. Under drought and combined stress a trend of significant increase in POD accumulation was observed in WT under drought (61 %), and combined stress (28 %) and transgenic plants under drought (27.84 %), combined stress (15.05 %) conditions. However, heat showed no signifcant change in POD content in WT and T plants. WT and transgenic plants did not show any significant change in SOD contents under heat and combined stress. Under drought stress, WT plants did not show any significant change but T plants showed a decline of 13.24 % compared to their control.


(C) (D) (GC) (H) (H+D)
a
APX (umol min-1 g FW-1)
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
c a d b ab
c a b ab a
WT
T
(C) (D) (GC) (H) (H+D)
b
CAT (umol min-1 g FW-1)
4
3.5
3
2.5
2
1.5
1
0.5
0
b c bc d
a d b b c
WT
T

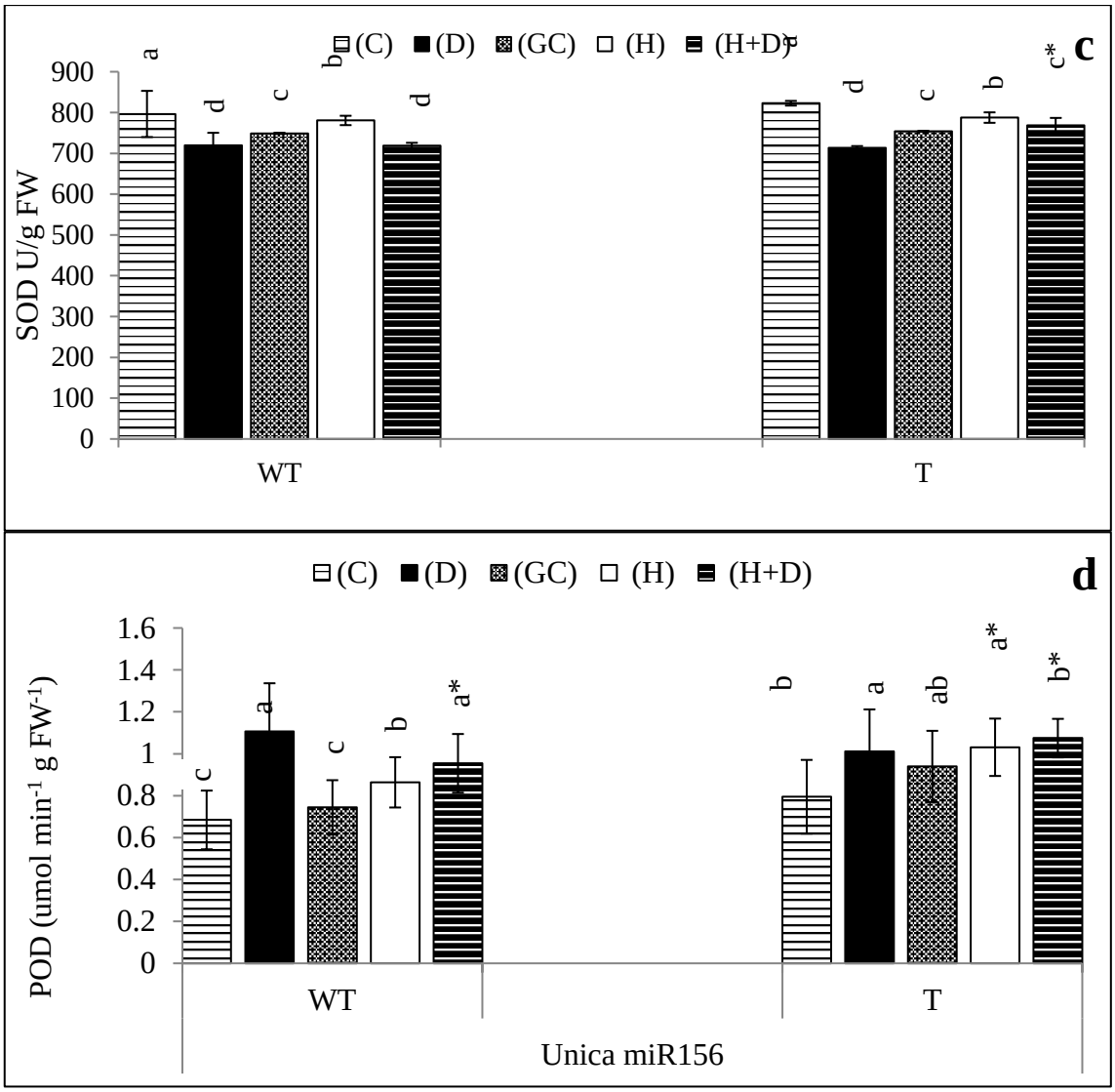


Figure 4.27. Effect of abiotic stress on biochemical traits of potato genotypes

Transgenic (T) and wild-type (WT) plants were classified as control (C, ▤), drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □), and combined heat-drought (HD, ▬) stresses. APX **(a),** CAT (b), SOD (c) and POD **(d).** Data shows as mean ± SD. Asterisks (*,**) shows the significant difference of treatments in transgenics from wild-type plants at $P<0.05$ and $P<0.01$ respectively.

4.13 Morphological and Physiological Responses of Transgenic miR172b-3p Plants to Stress Conditions

Plants of both independent transgenic lines harboring miR172b-3p were exposed to continuous drought, heat alone and combination of heat with drought stress for 20th and 12th days respectively. Figure (4.28, 4.29) represents plants of both independent lines after exposure to stress treatments. Combined heat and drought stress showed major loss of leaves as compared to other stress conditions and control plants.

Figure 4.28. Above ground phenotype of wild-type and transgenic plants after 20 days of drought treatment

Treatments were control of wild-type plants, control of transgenic plants, the drought of wild-type plants, and drought of transgenic plants.

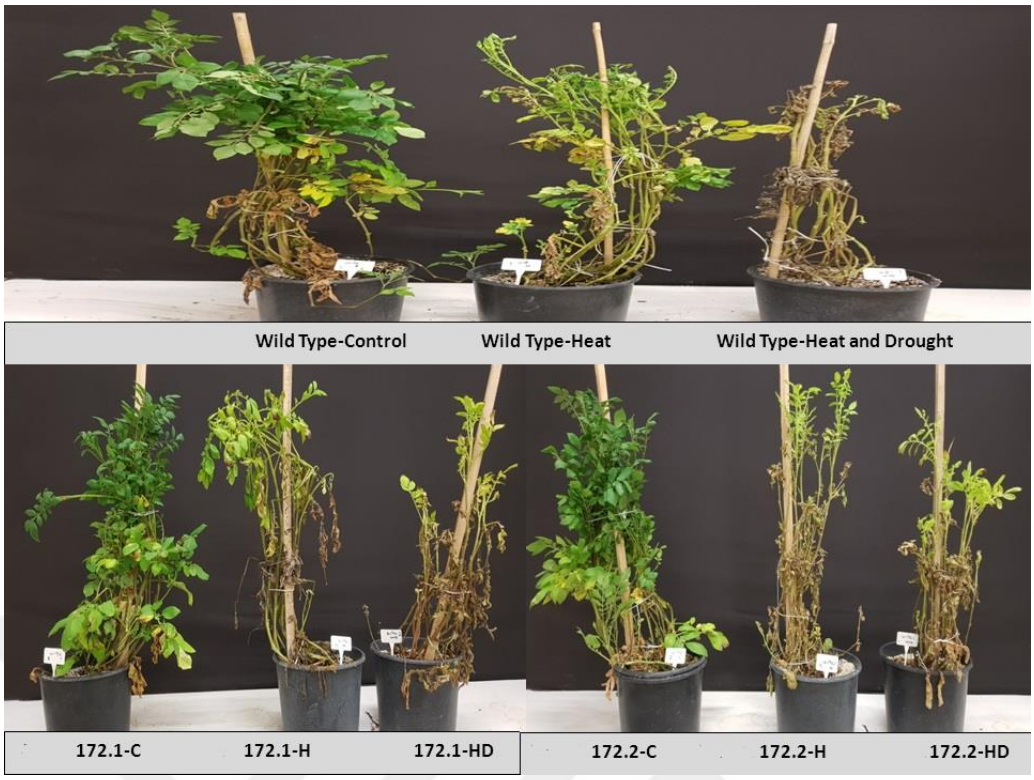


Figure 4.29. Above ground phenotype of wild-type and transgenic potato plants at 12 days after heat treatment and combined heat-drought treatment

Treatments were control of wild-type plants, heat of wild-type plants, combined heat-drought of wild-type plants, control of transgenic plants, the heat of transgenic plants, and combined heat-drought of transgenic plants. Generally, stress treatments caused a gradual reduction in photosynthetic rates in wild-type and transgenic plants having miR172b-3p transgenic plants of both lines from the 10th day to the end of treatments, however no reduction in photosynthesis rate was observed in either wild-type or transgenic under heat treatment (Figure 4.21). Under drought condition a significant reduction of 71.2% (from 32.6 to 9.4 µmol m^{-2} s^{-1}), 77.03% (from 38.71 to 8.89 µmol m^{-2} s^{-1}) and 66.0% (from 42.53 to 14.18 µmol m^{-2} s^{-1}) was observed in photosynthesis rate of wild-type and transgenic plants of line 1 and 2, respectively. However, the first significant decrease in photosynthesis rate under the combined heat and drought treatment was observed on the 10^{th} day of the treatment for wild-type plants. While both wild-type and transgenic plants showed significant decline in photosynthetic rate on the 12^{th} day of combined stress treatment (Figure 4.30). The photosynthetic rate of transgenic lines and wild-type plants reduced by 77.1 % (from 19.76 to 5.62 µmol m^{-2} s^{-1}), 69.54 % (from 18.19 to 5.54 µmol m^{-2} s^{-1}) and by 76.4 % (from 15.1 to 3.6 µmol

m^{-2} s^{-1}) respectively, compared to their control plants on the 12th day of the combined stress treatment (Figure 4.30a). Since the lowest and the significant decrease in photosynthesis rate was observed on the 20th day for drought treatment and the 12th day for heat only and combined heat-drought. These days were decided as last days of the stress treatments and further investigation was performed on the plants at the last days of the treatments.

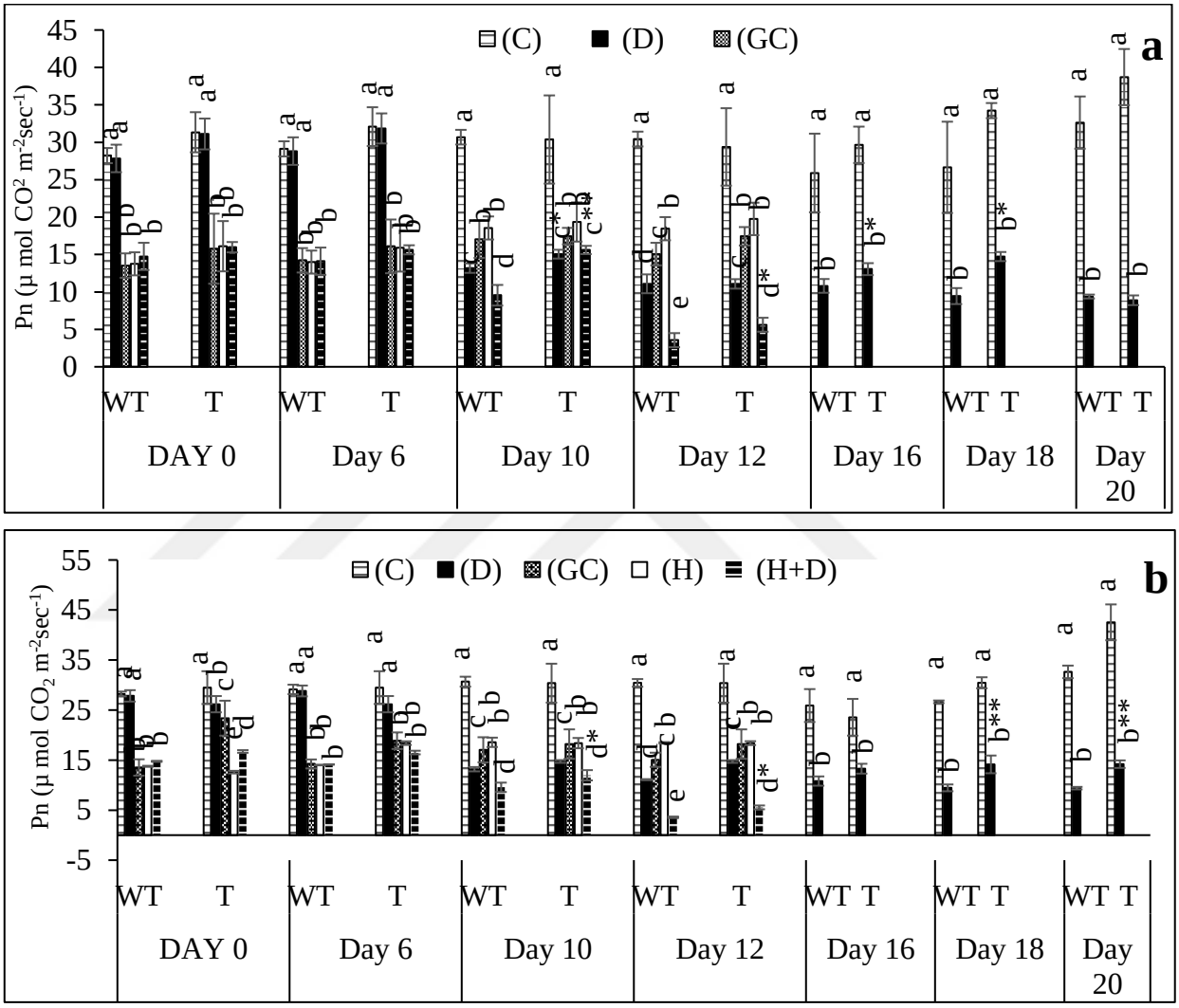


Figure 4.30. Effect of abiotic stress on the photosynthetic rate of potato genotypes

Transgenic (T) and wild-type (WT) plants were classified as control ( ), drought ( ), control for heat and combined heat-drought stresses ( ), heat ( ), and combined heat-drought ( ). The photosynthetic rate was measured on plants. Data shows as mean ± SD. Asterisks (*,**) shows the significant difference of treatments in transgenics from wild-type plants at $P<0.05$ and P<0.01 respectively.

The stomatal conductance rate of both wild-type and transgenic plants of both lines exhibited a significant reduction under drought and combined heat and drought conditions (Figure 4.31a). Despite that, stomatal conductance was significantly higher in wild-type and both lines of transgenic plants under heat treatment than in control plants. The stomatal conductance rate increased significantly by 42.10 % (0.19 to 0.27 mol H_2O m^2 s^{-1}) and 58.82 % (0.17 to 0.27 mol H_2O m^2 s^{-1}) in response to HS, while of wild-type showed an increase of 44 % (0.09 to 0.13 mol H_2O m^2 s^{-1}). In accordance with stomatal conductance, the transpiration rate decreased significantly in WT and transgenic lines with the exposure to drought only and combined stress. Contrarily it exhibited higher transpiration rate by 10% (3.16 to 3.48 H_2O m^2 s^{-1}) in WT, while 52.81 % (3.73 to 5.71 H_2O m^2 s^{-1}) and 62.96 % (3.51 to 5.72 H_2O m^2 s^{-1}) under HS conditions in both transgenic lines (Figure 4.31b).

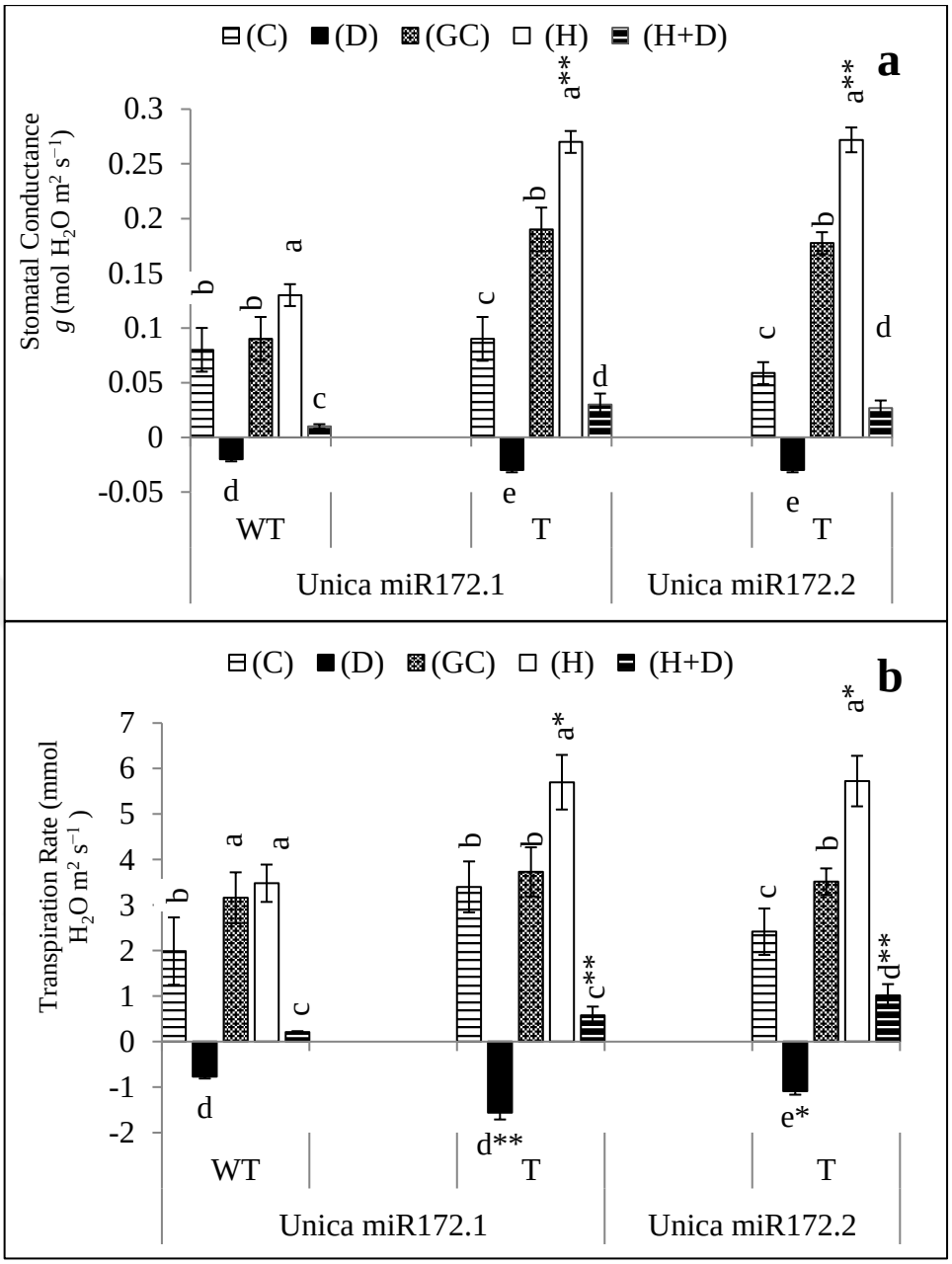


Figure 4.31. Effect of abiotic stress on physiological traits of potato genotypes

Transgenic (T) and wild-type (WT) plants were exposed to optimum temperatures and water level as control (C, ⊟) or drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □) and combined heat-drought (HD, 〓) stresses. Stomatal conductance (**a**), Transpiration rate **(b)**. Data shows as mean ± SD. Asterisks (*,**) shows the significant difference of treatments in transgenics from wild-type plants at $P<0.05$ and $P<0.01$ respectively.

Figure 4.32 shows the chlorophyll content and temperature of plant leaves from both independent transgenic lines of miR172b-3p under control and stress conditions. Plants under DS showed no significant change in chlorophyll content of WT and first transgenic line while significant increase in second transgenic line was found. While all transgenic and wild-type plants didn't show any significant change under heat but showed significant decline in response to combined stress conditions. Leaf temperature of all plants showed an increase under heat and combined stress conditions while under DS leaf temperature showed significant increase in first while no change in second transegnic line and wild-type plants.

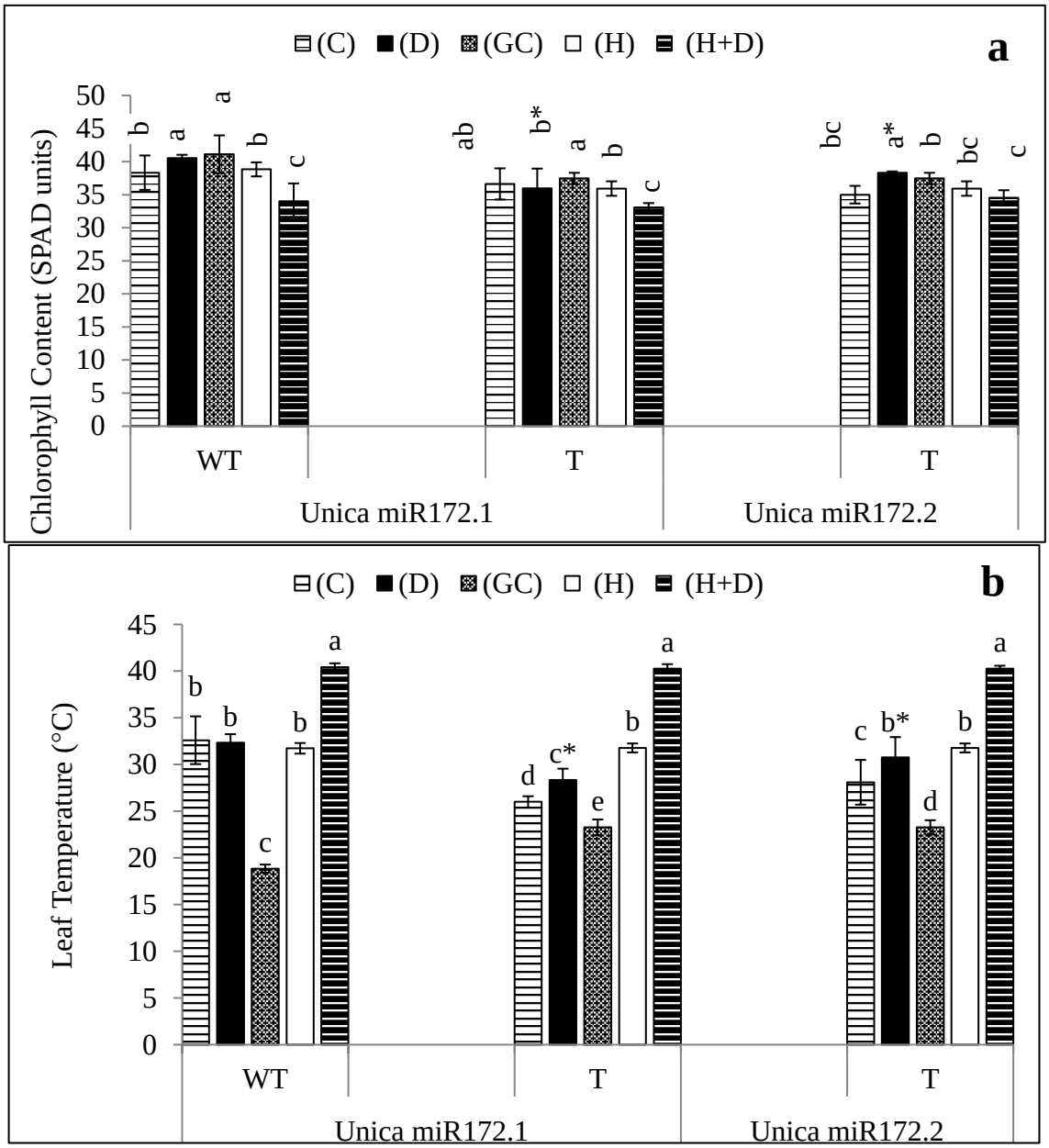


Figure 4.32. Effect of abiotic stress on physiological traits of potato genotypes

Transgenic (T) and wild-type (WT) plants were exposed to optimum temperatures and water level as control (C, ▤) or drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □) and combined heat-drought (HD, 〓) stresses. Relative chlorophyll content (**a**), Leaf Temperature **(b)**. Data shows as mean ± SD. Asterisks (*,**) shows the significant difference of treatments in transgenics from wild-type plants at P<0.05 and P<0.01 respectively.

Figure 4.33 shows the relative leaf water content of plants under control and stress conditions. Under all stress conditions imposed, a significant reduction was recorded in leaf water content of wild-type and transgenic plants as compared to their respective controls.

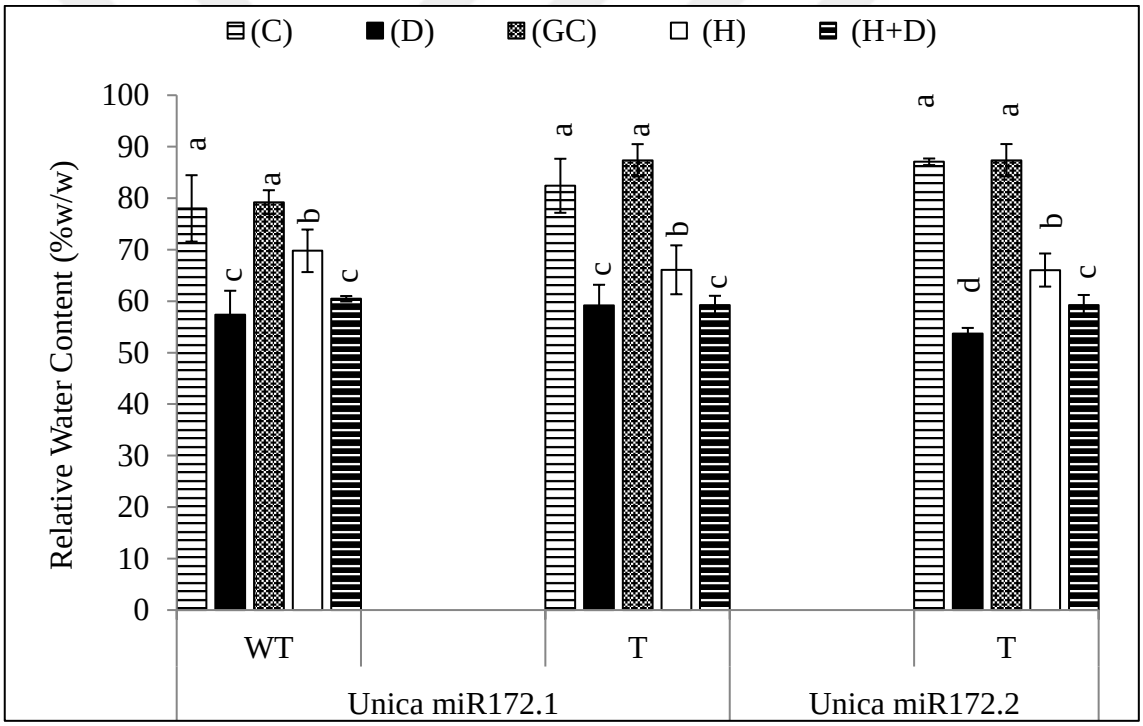


Figure 4.33. Effect of abiotic stress on physiological traits of potato genotypes

Transgenic (T) and wild-type (WT) plants were exposed to optimum temperatures and water level as control (C, ▤) or drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □) and combined heat-drought (HD, 〓) stresses. Data shows as mean ± SD. Asterisks (*,**) shows the significant difference of treatments in transgenics from wild-type plants at *P<0.05* and P<0.01 respectively.

Overall, the data proved that wild-type and transgenic plants altered their response to the drought, heat, and their combination. The change in photosynthetic rate and transpiration rate were in agreement with the stomatal conductance during the stress period showing that photosynthesis and transpiration activities were dependent on the stomatal conductance.

4.14 Biochemical Responses of Non-transgenic Plants to Stress Conditions

Transgenic lines having overexpression of miR172b-3p were exposed to continuous drought, heat alone and combination of heat with drought stress for 20 and 12 days respectively and at last day of stress, leaf samples were collected from control and under stress plants for measurement of proline, MDA, H_2O_2 and activity of antioxidant enzymes (APX, CAT, SOD, POD). It directly protects the essential proteins and integrity under stress. Heat and combined stress caused a significant increase in proline accumulation in WT and T plants. WT and both transgenic lines under DS showed significantly huge increment of 635 % (1.36 to 10 µmol/g FW), 700.77 % (2.57 to 20.58 µmol/g FW) and 807.02 % (1.85 to 16.78 µmol/g FW) in proline accumulation (Figure 4.34). No significant difference was found in WT and T plants under drought stress, while heat and combined stress caused a significant increase in MDA content (Figure 4.35a). WT plants showed an increase of 7 % (11.36 to 12.09 µmol/g FW) and 30 % (11.34 to 14.75 µmol/g FW) in accumulation of MDA under H and HD respectively. While transgenic plants of both lines showed a decline of 16.31% (11.34 to 9.49 µmol/g FW) and 14.37 % (11.06 to 9.47 µmol/g FW) under heat respectively. Combined stress showed no significant change in MDA contents in transgenic plans of both lines. In both wild-type and transgenic plants, we observed an elevating trend in H_2O_2 accumulation under drought conditions, whereas decreasing trend was observed under heat and combined heat and drought conditions (Figure 4.35b). The transgenic plants of both transgenic lines showed a similar amount of H_2O_2 accumulation to the wild-type plants under heat stress conditions.

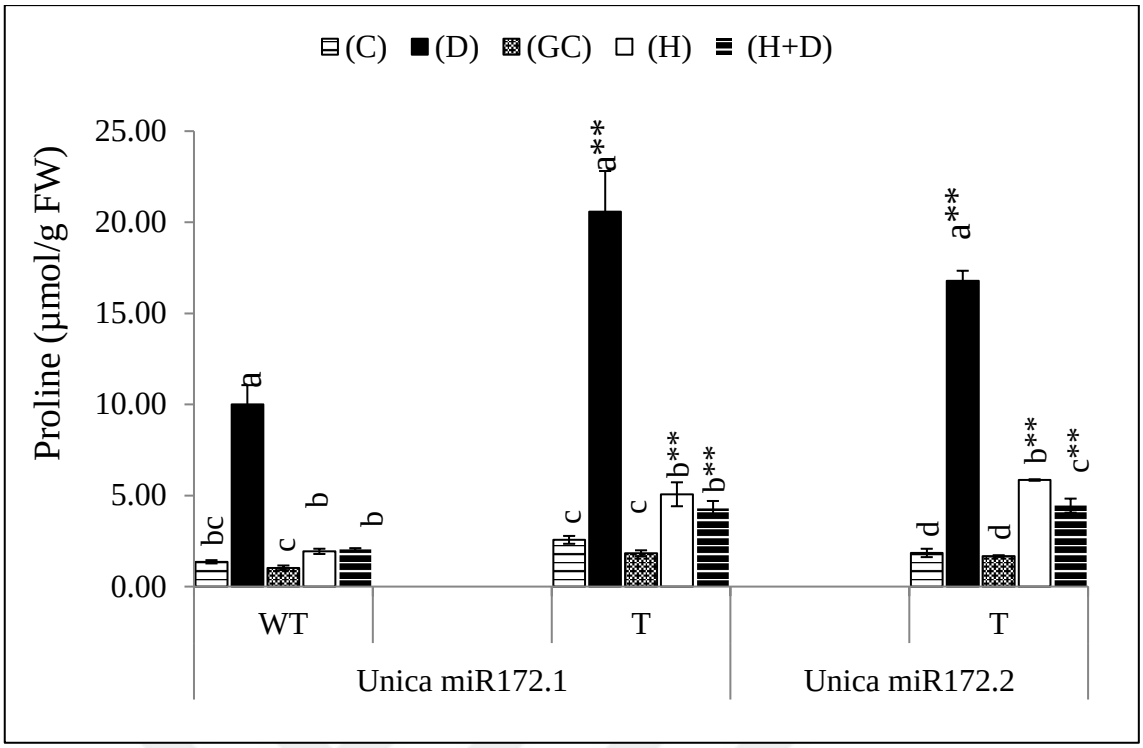


Figure 4.34. Influence of abiotic stress on biochemical traits of potato genotypes

Transgenic (T) and wild-type (WT) plants were classified as control (C, ▤), drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □), and combined heat-drought (HD, 〓) stresses. Data shows as mean ± SD. Asterisks (*,**) shows the significant difference of treatments in transgenics from wild-type plants at $P<0.05$ and $P<0.01$ respectively.

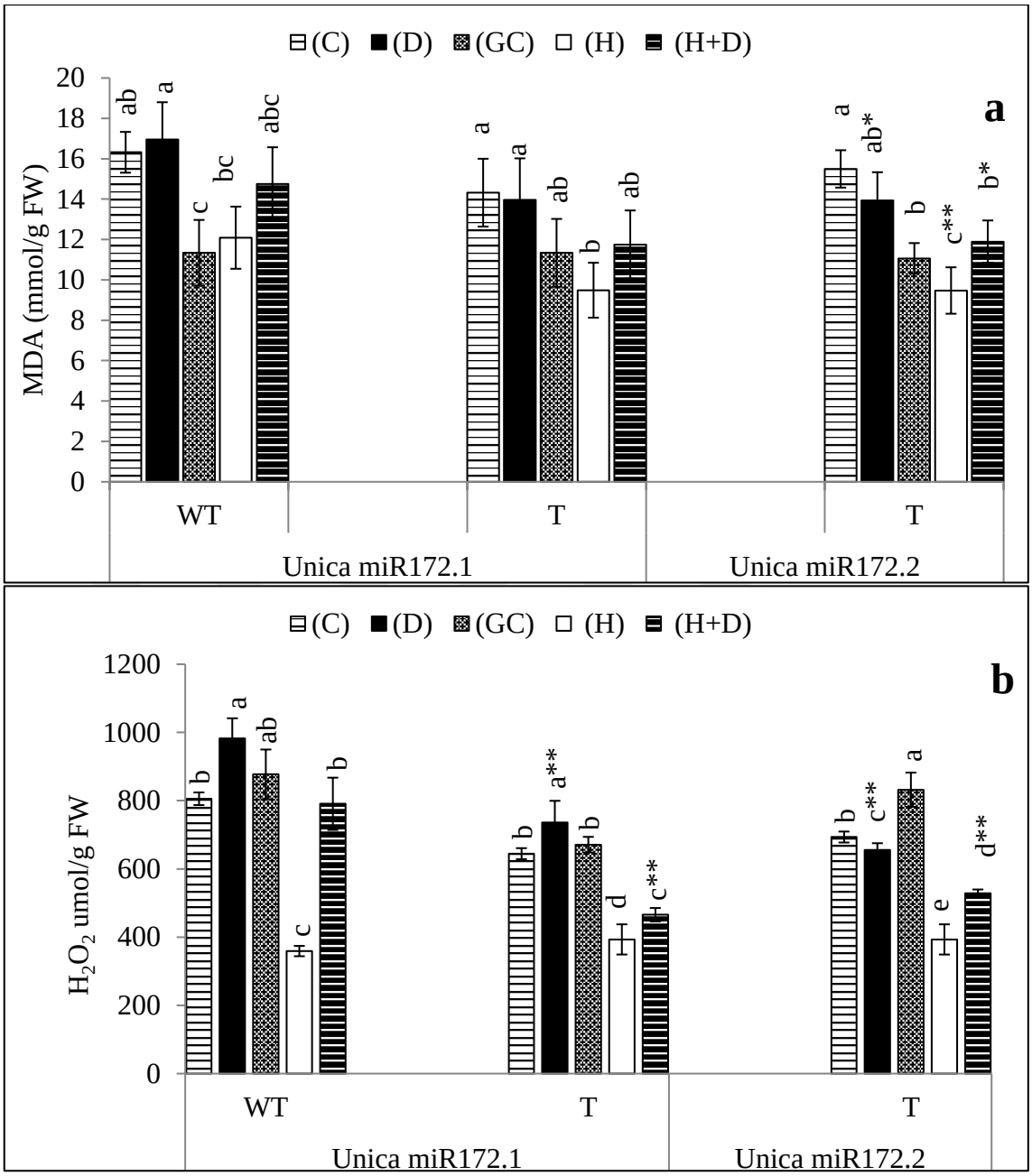
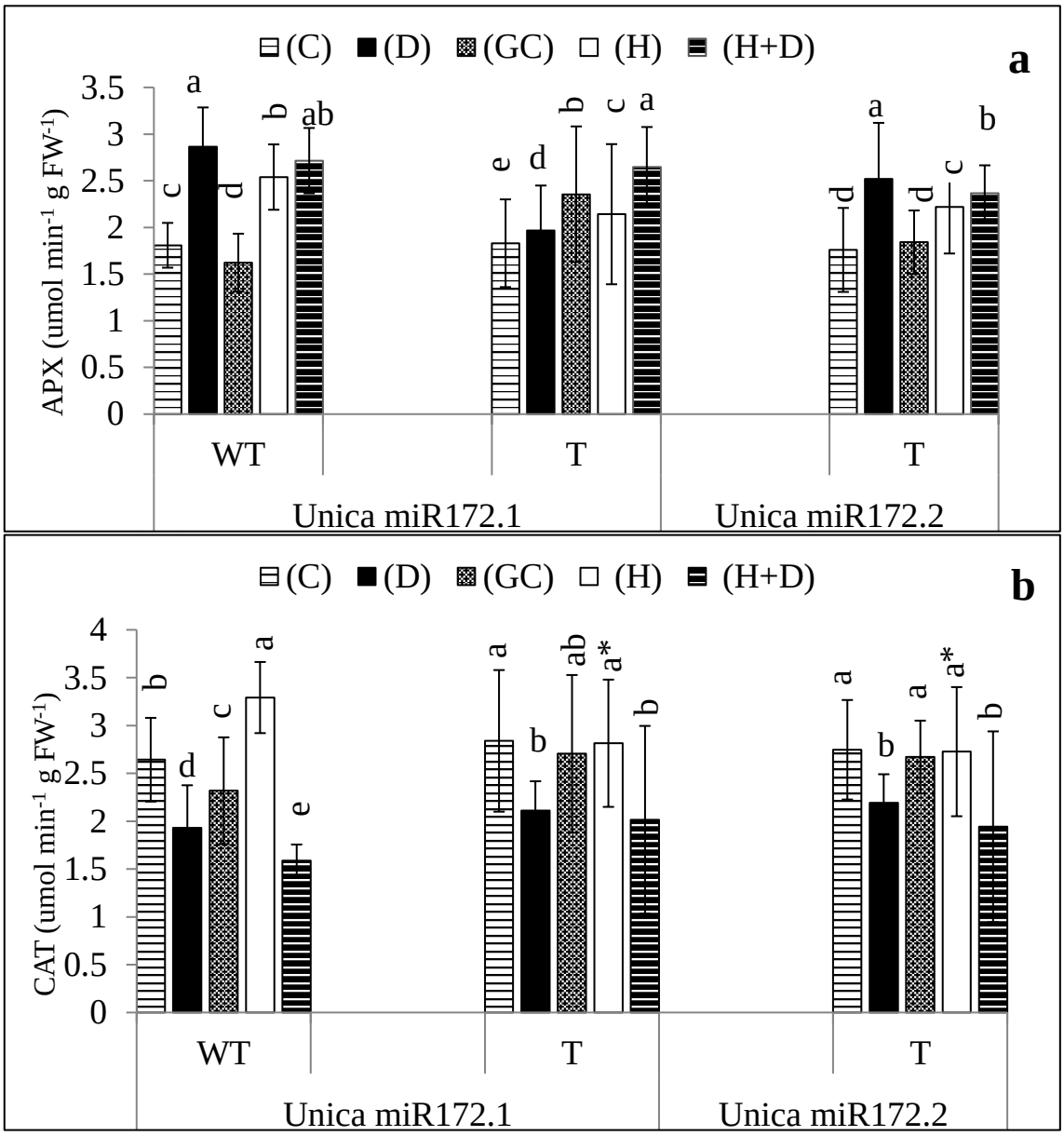


Figure 4.35. Effect of abiotic stress on biochemical traits of potato genotypes

Transgenic (T) and wild-type (WT) plants were classified as control (C, ▤), drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □), and combined heat-drought (HD, ▬) stresses. Malondialdehyde (MDA) contents **(a),** and H_2O_2 **(b)**. Data shows as mean ± SD. Asterisks (*,**) shows the significant difference of treatments in transgenics from wild-type plants at $P<0.05$ and P<0.01 respectively.

Activity of APX, CAT, POD and SOD antioxidant enzymes was measured under control and stress in WT and transgenic plants of both independent lines having over expression of miR172b (Figure 4.36). Under all stress conditions a significant increase in wild type and transgenic plants was observed in accumulation of APX except no change in transgenic plants of both lines under heat stress. Under drought and combined stress conditions CAT showed a significant decline in WT (27 %, 31 %) and transgenic plants (L1 25.44 %, 25.55 %, L2 5.10 %, 27.34 %) . Incase of CAT, wild type plants showed a signifcant icrease of 42 % under HS, Whereas plants of both transgenic lines showed no significant change. Under drought and combined stress a trend of significant increase in POD accumulation was observed in WT (61 %, 28 %) and transgenic plants (L1 47.61 %, 49.36 %, L2 66.66 %, 53.33 %) . However, heat showed no signifcant change in POD content in WT and T plants. WT and transgenic plants did not show any significant change in SOD contents under drought and combined stress. While under HS, WT and plants of transgenic line did not show any significant change as compared to their respective control.


(C) (D) (GC) (H) (H+D)
a
APX (umol min-1 g FW-1)
3.5
3
2.5
2
1.5
1
0.5
0
c a d b ab
e d b c a
d a d c b
WT
T
T
Unica miR172.1
Unica miR172.2
(C) (D) (GC) (H) (H+D)
b
CAT (umol min-1 g FW-1)
4
3.5
3
2.5
2
1.5
1
0.5
0
b d c a e
a b ab a* b
a b a a* b
WT
T
T
Unica miR172.1
Unica miR172.2

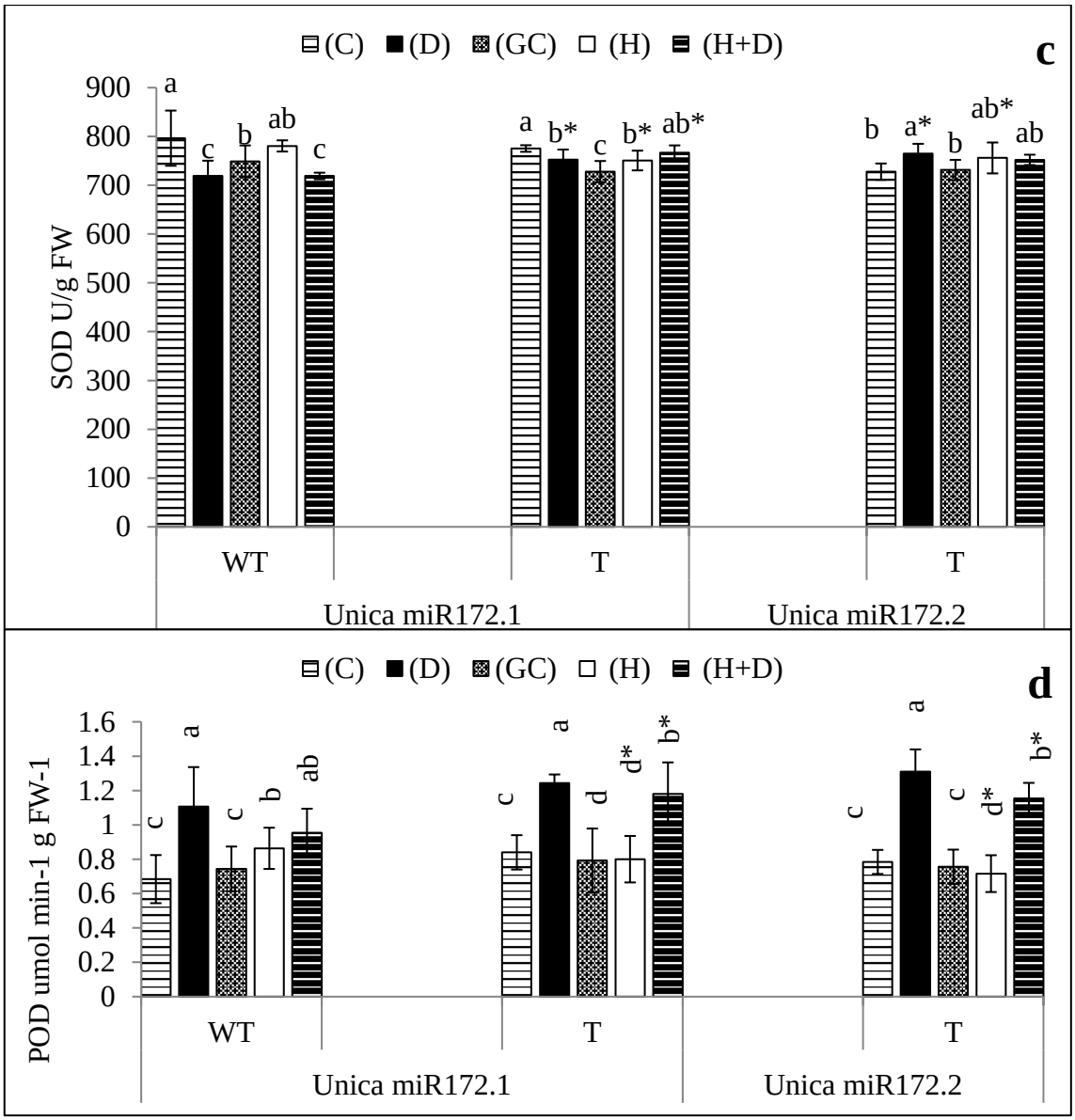


Figure 4.36. Effect of abiotic stress on biochemical traits of potato genotypes

Transgenic (T) and wild-type (WT) plants were classified as control (C, ▤), drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □), and combined heat-drought (HD, ▬) stresses. APX (a), CAT (b), POD (c) and SOD (d). Data shows as mean ± SD. Asterisks (*,**) shows the significant difference of treatments in transgenics from wild-type plants at $P<0.05$ and P<0.01 respectively.

4.15 Physiological Responses of Transgenic miRNA novel 8 Plants to Stress Conditions

Physiological responses were primarily measured to spot the exact period at which plants encounter stress conditions. Plants of both independent transgenic lines harboring

miRNA novel 8 were exposed to continuous drought, heat alone and combination of heat with drought stress for 20 and 12 days respectively. Figure (4.37, 4.38) represents plants of both independent lines after exposure to stress treatments. Combined heat and drought stress showed major loss of leaves as compared to other stress conditions and control plants.

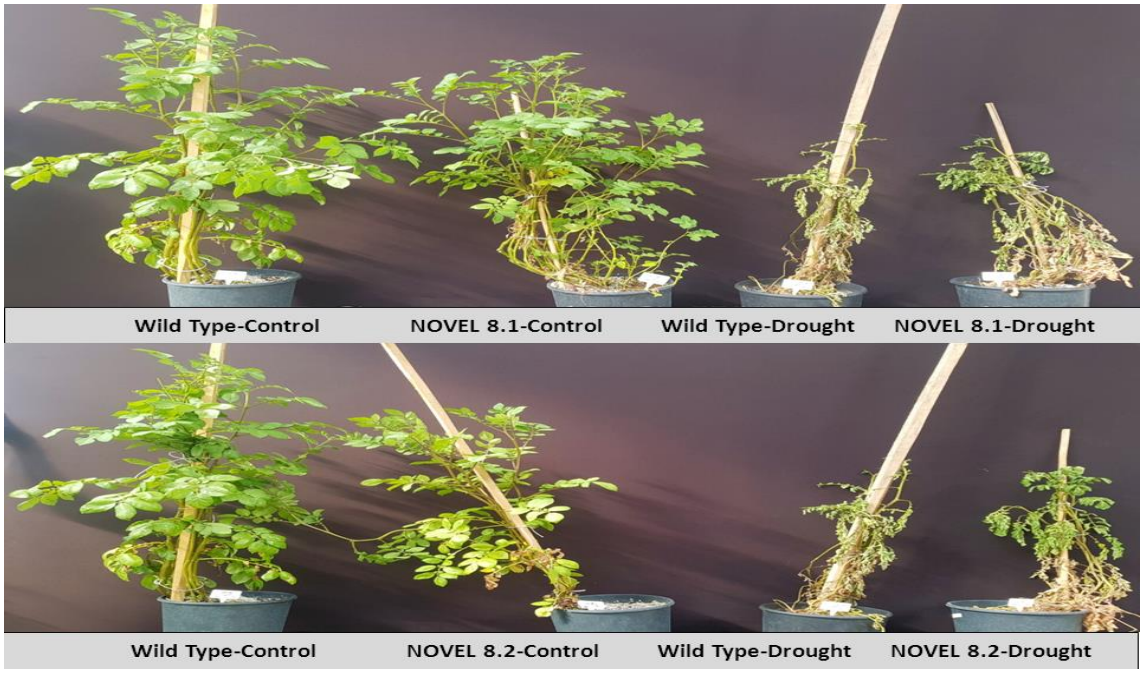


Figure 4.37. Above ground phenotype of transgenic and wild-type potato plants after 12 days of drought treatment

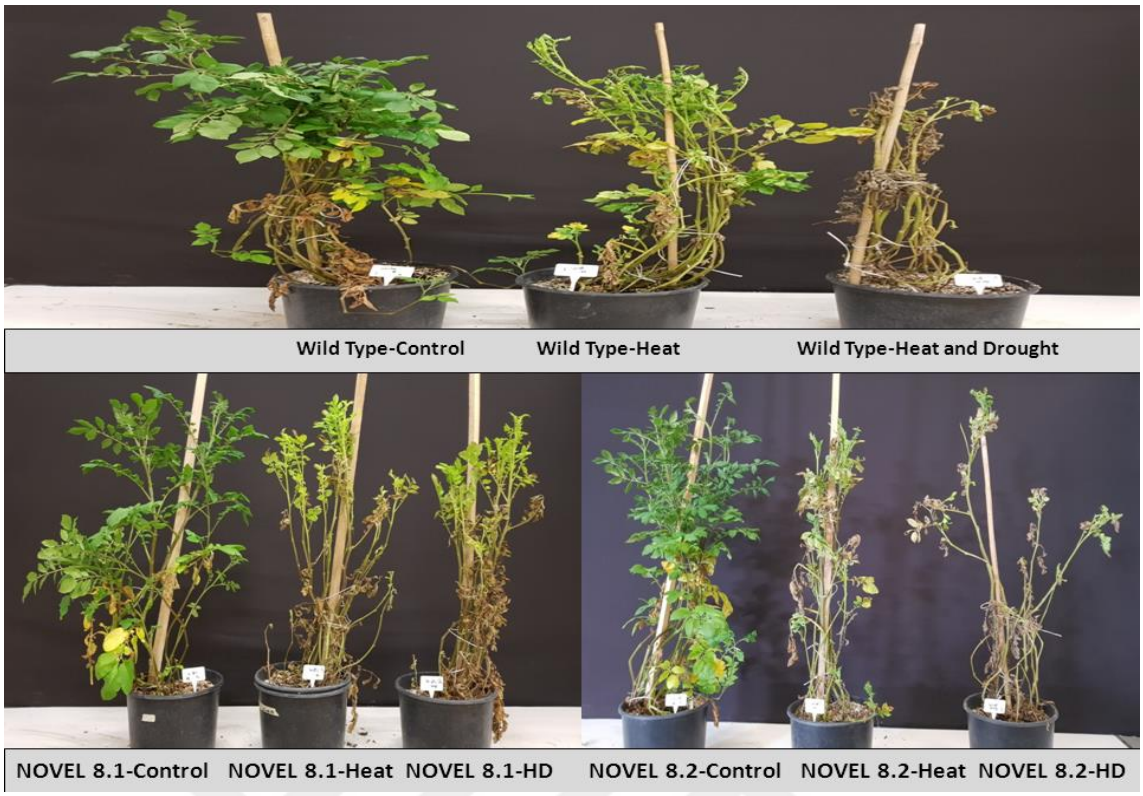


Figure 4.38. Above ground phenotype of wild-type and transgenic lines of potato plants at 12 days after heat treatment and combined heat-drought treatment

Generally, stress treatments caused a gradual reduction in photosynthetic rates in wild-type and transgenic miRNA novel 8 transgenic plants of both lines from the 10th day to the end of treatments, however no reduction in Pn was observed in either wild-type or transgenic under heat treatment (Figure 4.21). Under drought condition a significant reduction of 71.2% (from 32.6 to 9.4 µmol m^{-2} s^{-1}), 67.0% (from 40.52 to 13.25 µmol m^{-2} s^{-1}) and 68.0% (from 43.42 to 14.00 µmol m^{-2} s^{-1}) was observed in Pn of wild-type and transgenic plants of line 1 and 2, respectively. However, the first significant decrease in Pn under the combined heat and drought treatment was observed on the 10th day of the treatment for wild-type plants. While both wild-type and transgenic plants kept on fixing carbon on the 12th day of combined stress treatment (Figure 4.39). The photosynthetic rate of transgenic lines and wild-type Unica plants reduced by 54% (from 15.77 to 7.22 µmol m^{-2} s^{-1}), 68% (from 17.77 to 5.75 µmol m^{-2} s^{-1}) and by 76.4% (from 15.1 to 3.6 µmol m^{-2} s^{-1}) respectively, compared to their control plants on the 12th day of the combined stress treatment. Since the lowest and the significant decrease in Pn was observed on the 20th day for drought treatment and the 12th day for heat only and combined heat-drought, we decided to consider these days as the last day of the

stress treatments and further investigation was performed on the plants at the last days of the treatments.

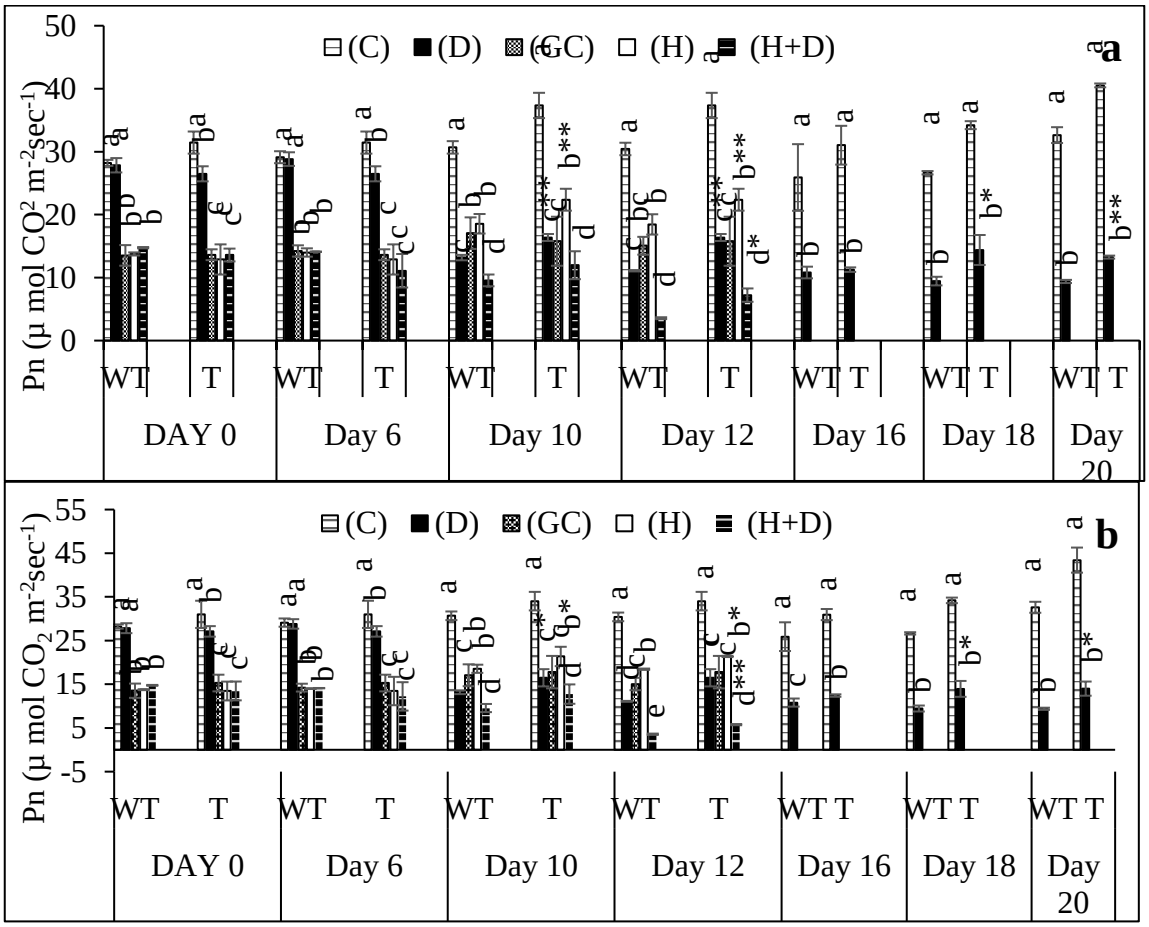


Figure 4.39. Influence of abiotic stress on the photosynthetic rate of potato genotypes

Transgenic (T) and wild-type (WT) plants were classified as control ( ), drought ( ), control for heat and combined heat-drought stresses ( ), heat ( ), and combined heat-drought ( ). Unica 1st transgenic **(a)** and Unica 2nd transgenic **(b).** Data are shown as mean ± SD. Asterisks shows significant difference of treatments in transgenics from wild-type plants at $P<0.05$ and P<0.01 respectively.

The stomatal conductance rate of both wild-type and transgenic plants of both lines exhibited a significant reduction under drought and combined heat and drought conditions (Figure 4.40a). Despite that, stomatal conductance was significantly higher in wild-type and both lines of transgenic plants under heat treatment than in control

plants. The stomatal conductance rate increased significantly by 48% (0.25 to 0.37 mol H_2O m^2 s^{-1}) and 75% (0.20 to 0.35 mol H_2O m^2 s^{-1}) in response to HS, while of wild-type showed an increase of 44% (0.09 to 0.13 mol H_2O m^2 s^{-1}) (Figure 4.40a). In accordance with stomatal conductance, the transpiration rate decreased significantly in WT and transgenic lines with the exposure to drought only and combined stress. Contrarily it exhibited higher transpiration rate by 10% (3.16 to 3.48 H_2O m^2 s^{-1}) in WT, while 323% (2.29 to 9.68 H_2O m^2 s^{-1}) and 54% (3.78 to 5.44 H_2O m^2 s^{-1}) under HS conditions in both transgenic lines (Figure 4.40b).

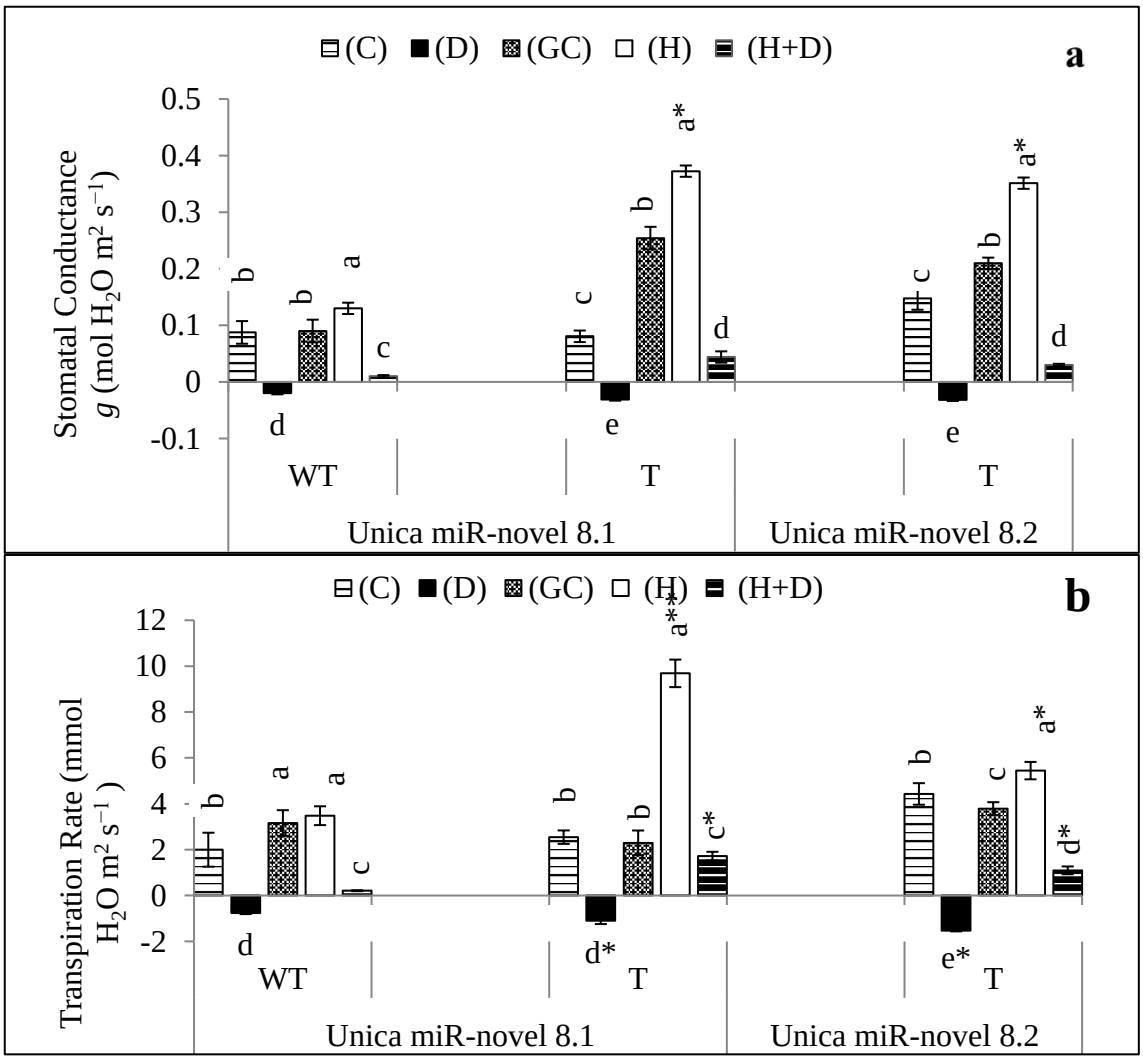


Figure 4.40. Influence of abiotic stress on physiological traits of potato genotypes

Transgenic (T) and wild-type (WT) plants were exposed to optimum temperatures and water level as control (C, ▤) or drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, ☐) and combined heat-drought (HD, 〓) stresses. Stomatal conductance (**a**), Transpiration rate **(b)**. Data are shown as mean ± SD. Asterisks (*,**) shows significant difference of treatments in transgenics from wild-type plants at $P<0.05$ and P<0.01 respectively.

Figure 4.41 shows the chlorophyll content and temperature of plant leaves from both independent transgenic lines of miRNA novel 8 under control and stress conditions. Plants under DS showed significant increase in first and significant decrease in second transgenic line in their chlorophyll content compared with wild-type plants. While plants under heat and combined stress conditions showed significant decrease in chlorophyll content of both transgenic lines. Leaf temperature of all plants showed and increase under heat and combined stress conditions while under DS leaf temperature showed significant increase in first while no change in second transegnic line.

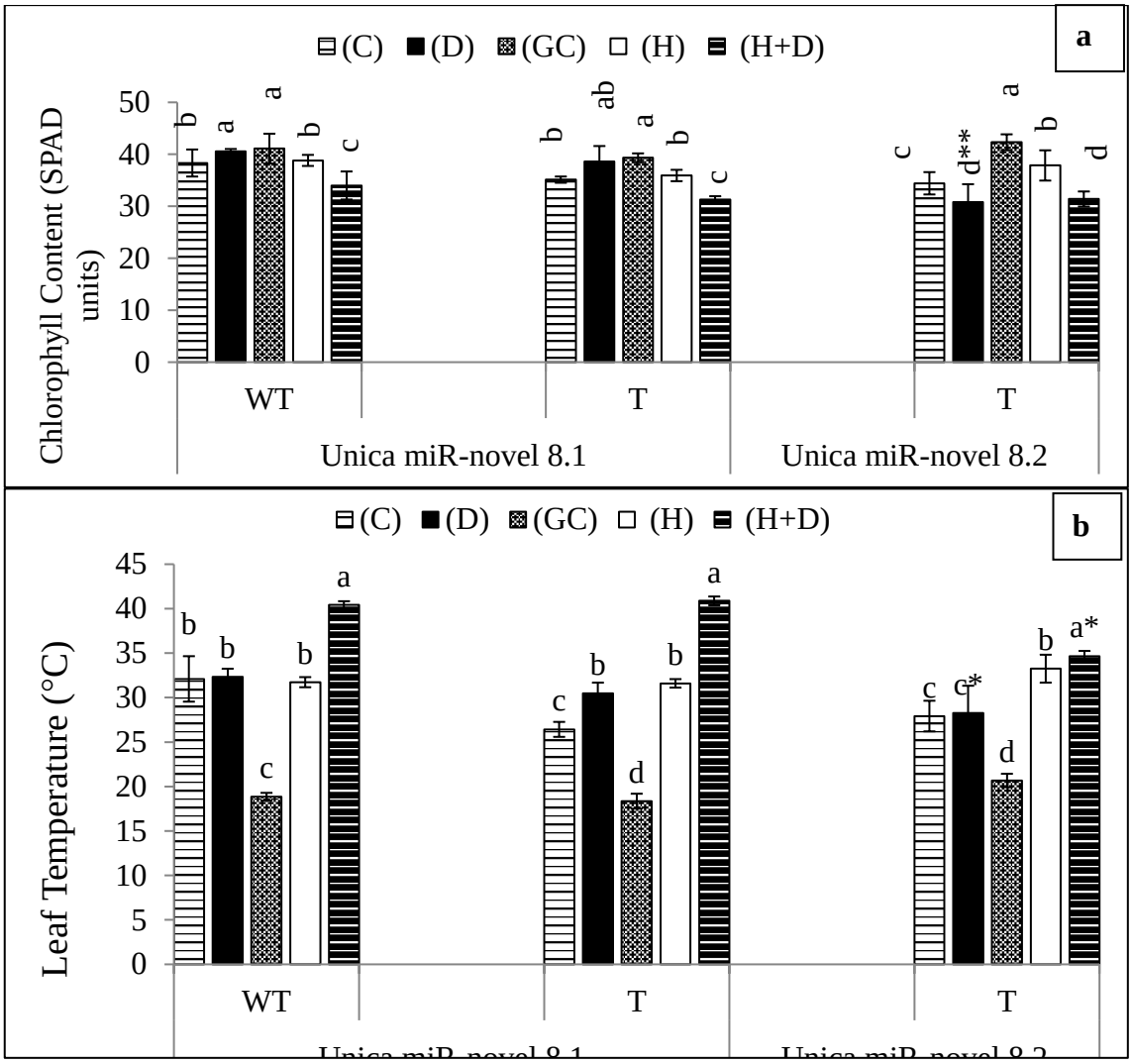


Figure 4.41. Influence of abiotic stress on physiological traits of potato genotypes

Transgenic (T) and wild-type (WT) plants were exposed to optimum temperatures and water level as control (C, ▤) or drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □) and combined heat-drought (HD, ▬) stresses. Relative chlorophyll content (**a**), Leaf temperature **(b)** were measured as outlined in the text. Data are shown as mean ± SD. Asterisks (*,**) showed the significant difference of treatments in transgenics from wild-type plants at $P<0.05$ and P<0.01 respectively.

Figure 4.42 shows the relative leaf water content of plants under control and stress conditions. Under all stress conditions imposed, a significant reduction was recorded in leaf water content of wild-type and transgenic plants as compared to their respective controls.

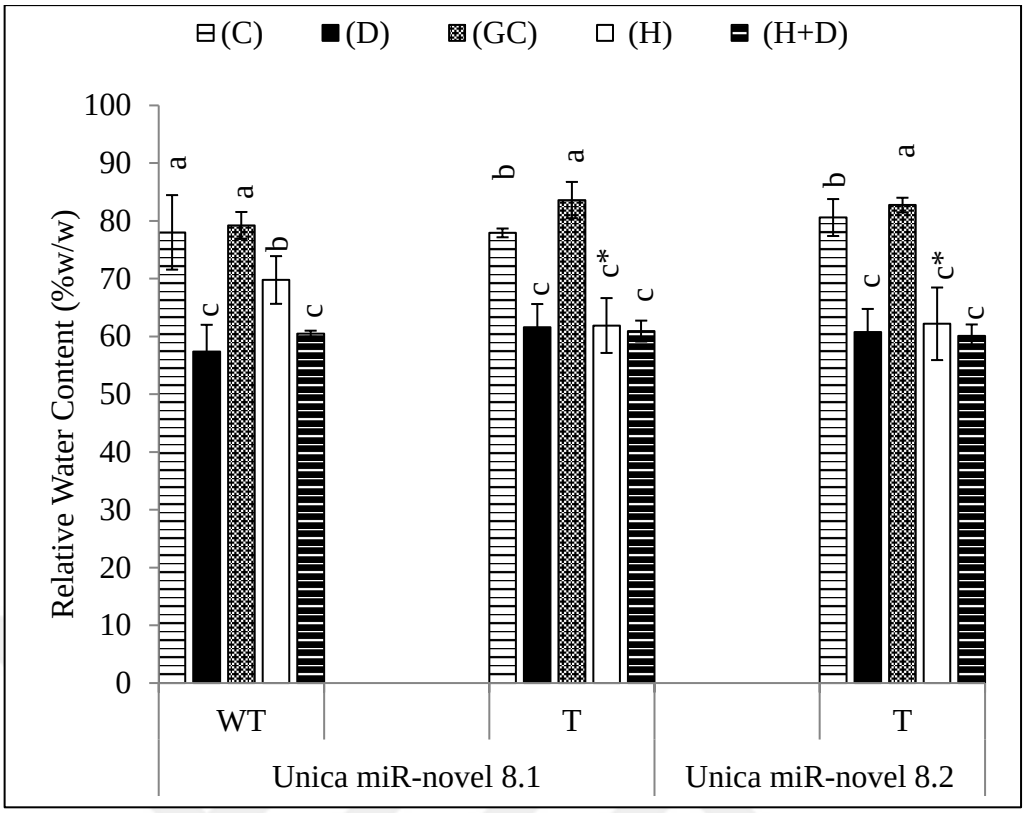


Figure 4.42. Influence of abiotic stress on physiological traits of potato genotypes

Transgenic (T) and wild-type (WT) plants were exposed to optimum temperatures and water level as control (C, ⊟) or drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □) and combined heat-drought (HD, 〓) stresses. Data are shown as mean ± SD. Asterisks (*,**) shows significant difference of treatments in transgenics from wild-type plants at $P<0.05$ and P<0.01 respectively.

Overall, the data proved that wild-type and transgenic plants altered their response to the drought, heat, and their combination. The change in photosynthetic rate and transpiration rate were in agreement with the stomatal conductance during the stress period showing that photosynthesis and transpiration activities were dependent on the stomatal conductance.

4.16 Biochemical Responses of Non-transgenic Plants to Stress Conditions

Transgenic lines having overexpression of miRNA novel 8 were exposed to continuous drought, heat alone and combination of heat with drought stress for 20 and 12 days respectively and at last day of stress, leaf samples were collected from control and under stress plants for measurement of proline, MDA, H_2O_2 and activity of antioxidant

enzymes (APX, CAT, SOD, POD). Proline has potential to serve as signaling molecules for the modulation of mitochondrial functions, influencing cell proliferation, triggering gene expression, that play vital role in plant recovery. It directly protects the essential proteins and integrity under stress. Heat and combined stress caused a significant increase in proline accumulation in WT and T plants. WT and both transgenic lines under DS showed significantly huge increment of 635% (1.36 to 10 µmol/g FW), 997% (1.44 to 15.79 µmol/g FW) and 836% (1.38 to 12.91 µmol/g FW) in proline accumulation (Figure 4.43). No significant difference was found in WT and T plants under drought stress, while heat and combined stress caused a significant increase in MDA content (Figure 4.44a). WT plants showed an increase of 7% (1.36 to 10 µmol/g FW) and 30% (11.34 to 14.75 µmol/g FW) in accumulation of MDA under H and HD respectively. While transgenic plants of both lines showed an increase of 52% (11.13 to 15.89 µmol/g FW) and 77% (11.13 to 19.73 µmol/g FW) under heat and 39% (13.51 to 18.72 µmol/g FW) and 51% (13.51 to 20.35 µmol/g FW) under HD respectively. Higher H_2O_2 concentration is harmful to cells, resulting in localized oxidative damage, lipid peroxidation, disruption of metabolic function, and loss of cell integrity. In both wild-type and transgenic plants, we observed an elevating trend in H_2O_2 accumulation under drought conditions, whereas decreasing trend was observed under heat and combined heat and drought conditions (Figure 4.44b). The transgenic plants of both transgenic lines showed a similar amount of H_2O_2 accumulation to the wild-type plants under heat conditions.

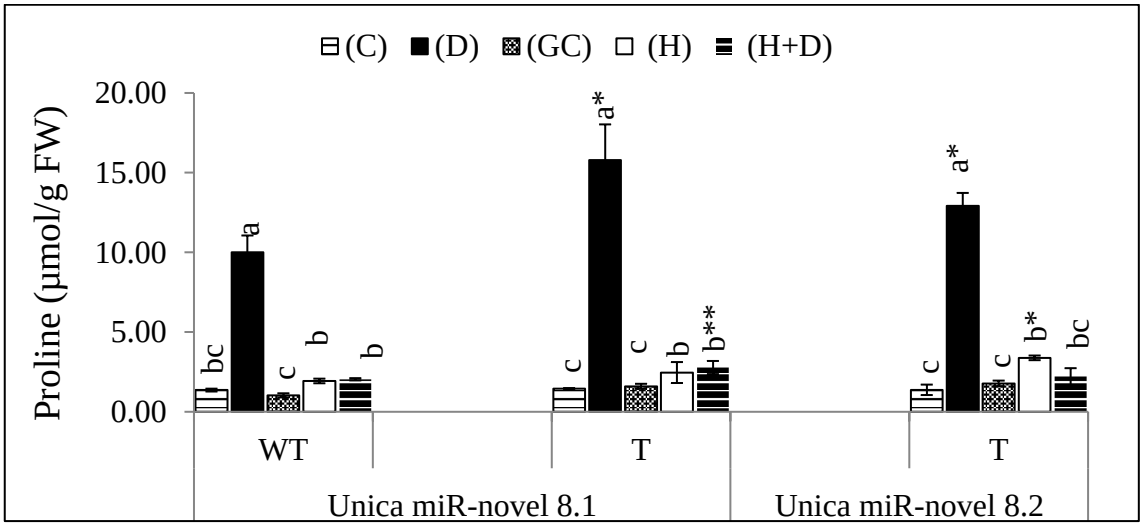


Figure 4.43. Influence of abiotic stress on biochemical traits of potato genotypes

Transgenic (T) and wild-type (WT) plants were classified as control (C, ▤), drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □), and combined heat-drought (HD, ▬) stresses. Data shows as mean ± SD. Asterisks (*,**) shows the significant difference of treatments in transgenics from wild-type plants at *P<0.05* and P<0.01 respectively.

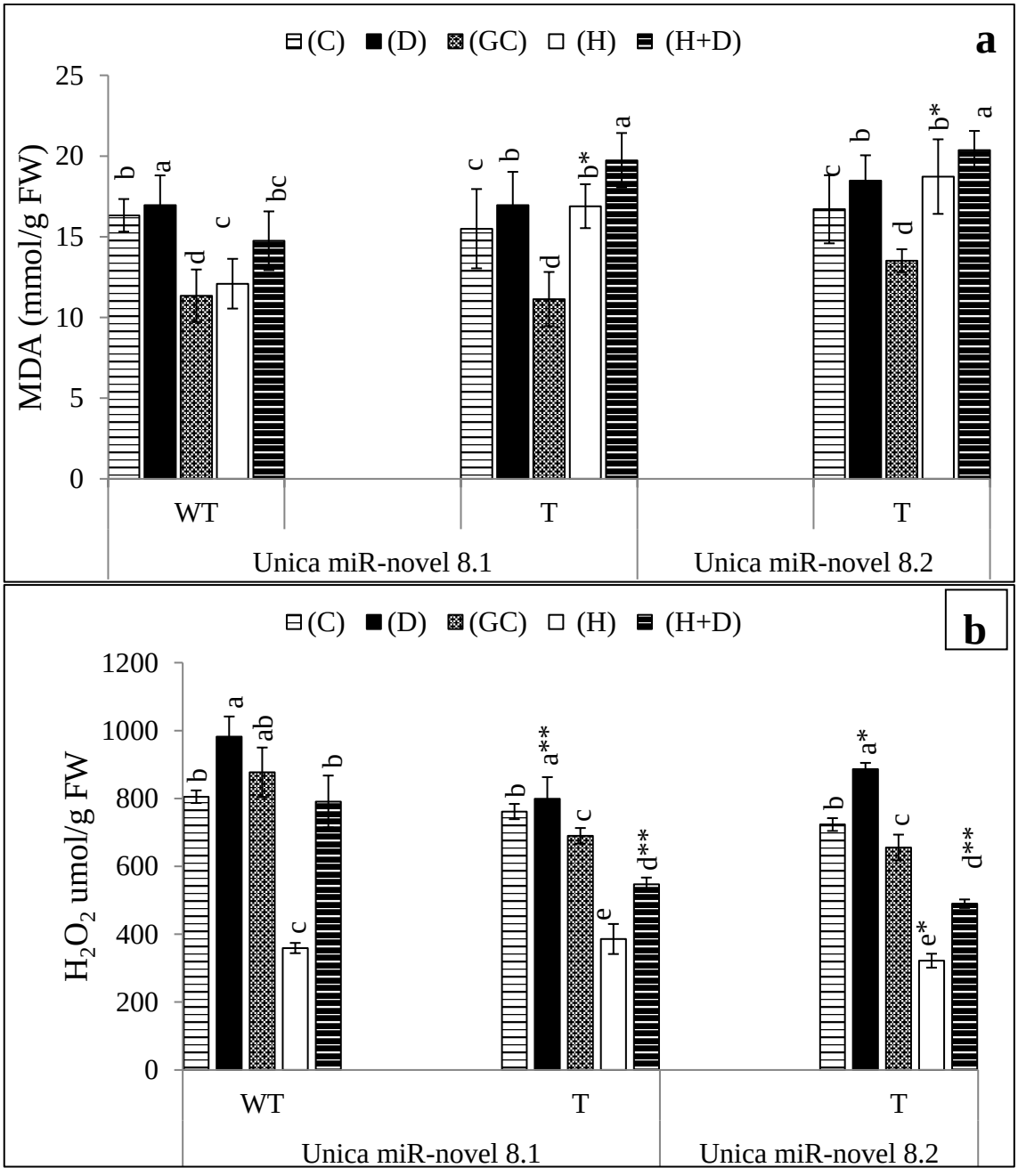
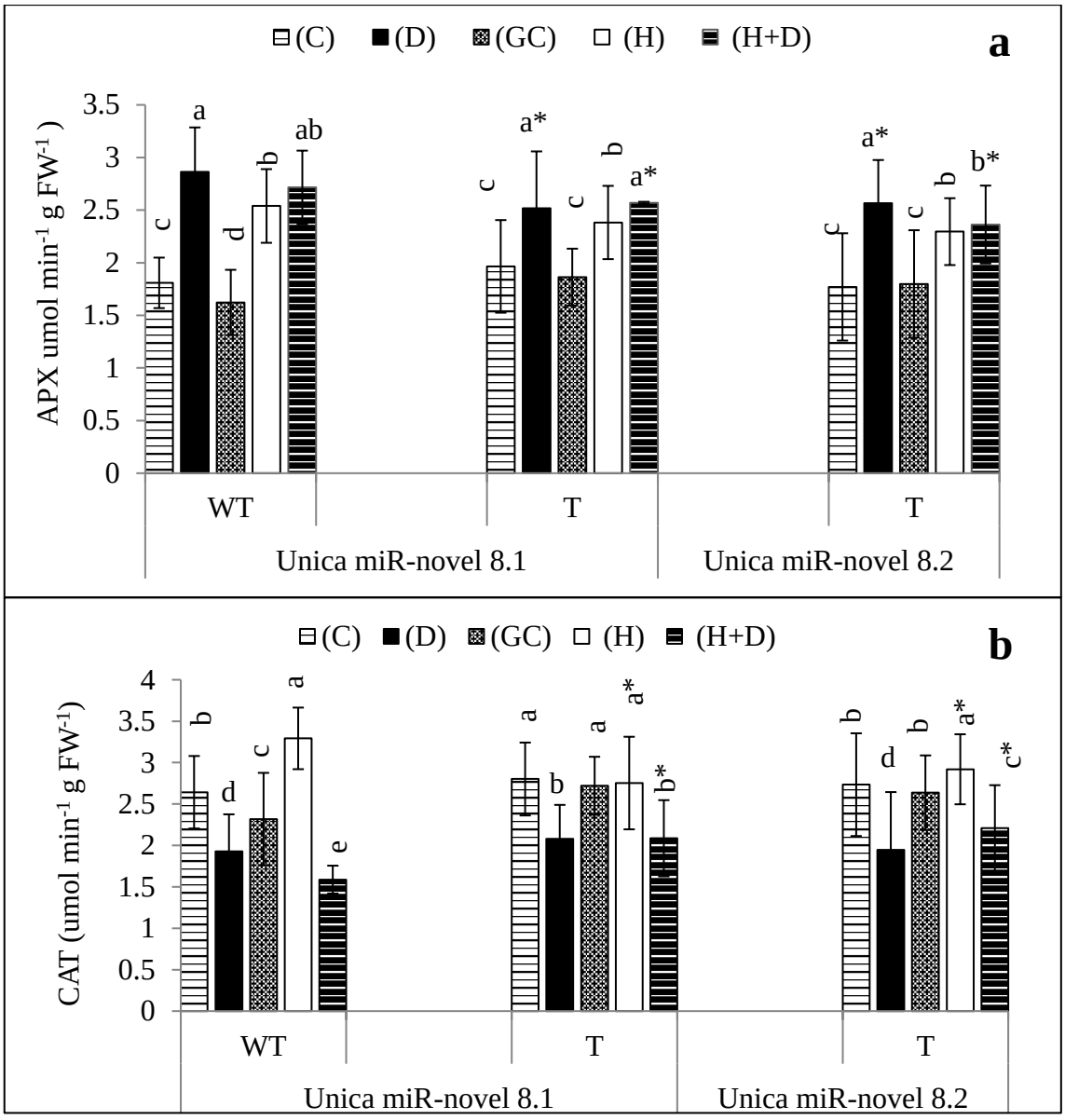


Figure 4.44. Effect of abiotic stress on biochemical traits of potato genotypes

Transgenic(T) and wild-type (WT) plants were classified as control (C, ▤), drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □), and combined heat-drought (HD, 〓) stresses. Malondialdehyde (MDA) contents **(a),** and H_2O_2 **(b)**. Data shows as mean ± SD. Asterisks (*,**) shows the significant difference of treatments in transgenics from wild-type plants at $P<0.05$ and $P<0.01$ respectively.

Activity of APX, CAT, POD and SOD antioxidant enzymes was measured under control and stress in WT and transgenic plants of both independent lines having over expression of miRNA novel 8 (Figure 4.45). Under all stress conditions a significant increase in wild type and transgenic plants was observed in accumulation of APX. Under drought and combined stress conditions CAT showed a significant delcine in WT(27%, 31%) and transgenic plants (L1 26%, 23%, L2 28%, 16%) . Incase of CAT, wild type plants showed a signifcant icrease of 42% under HS, Whereas plants of both transgenic lines showed no significant change. Under drought and combined stress a trend of significant increase in POD accumulation was observed in WT (61%, 28%) and transgenic plants (L1 60%, 41%, L2 51%, 44%) . However, heat showed no signifcant change in POD content in WT and T plants. WT and transgenic plants did not show any significant change in SOD contents under drought and combined stress. While under HS, WT and plants of second transgenic line did not show any significant change but plants of first transgenic line showed a significant decline (10.73%) as compared to control.


(C)
(D)
(GC)
(H)
(H+D)
a
APX umol min-1 g FW-1)
3.5
3
2.5
2
1.5
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0.5
0
a
ab
b
d
c
a*
b
a*
c
c
c
a*
b*
b
c
WT
T
T
Unica miR-novel 8.1
Unica miR-novel 8.2
(C)
(D)
(GC)
(H)
(H+D)
b
CAT (umol min-1 g FW-1)
4
3.5
3
2.5
2
1.5
1
0.5
0
b
d
c
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e
a
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a*
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WT
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Unica miR-novel 8.1
Unica miR-novel 8.2

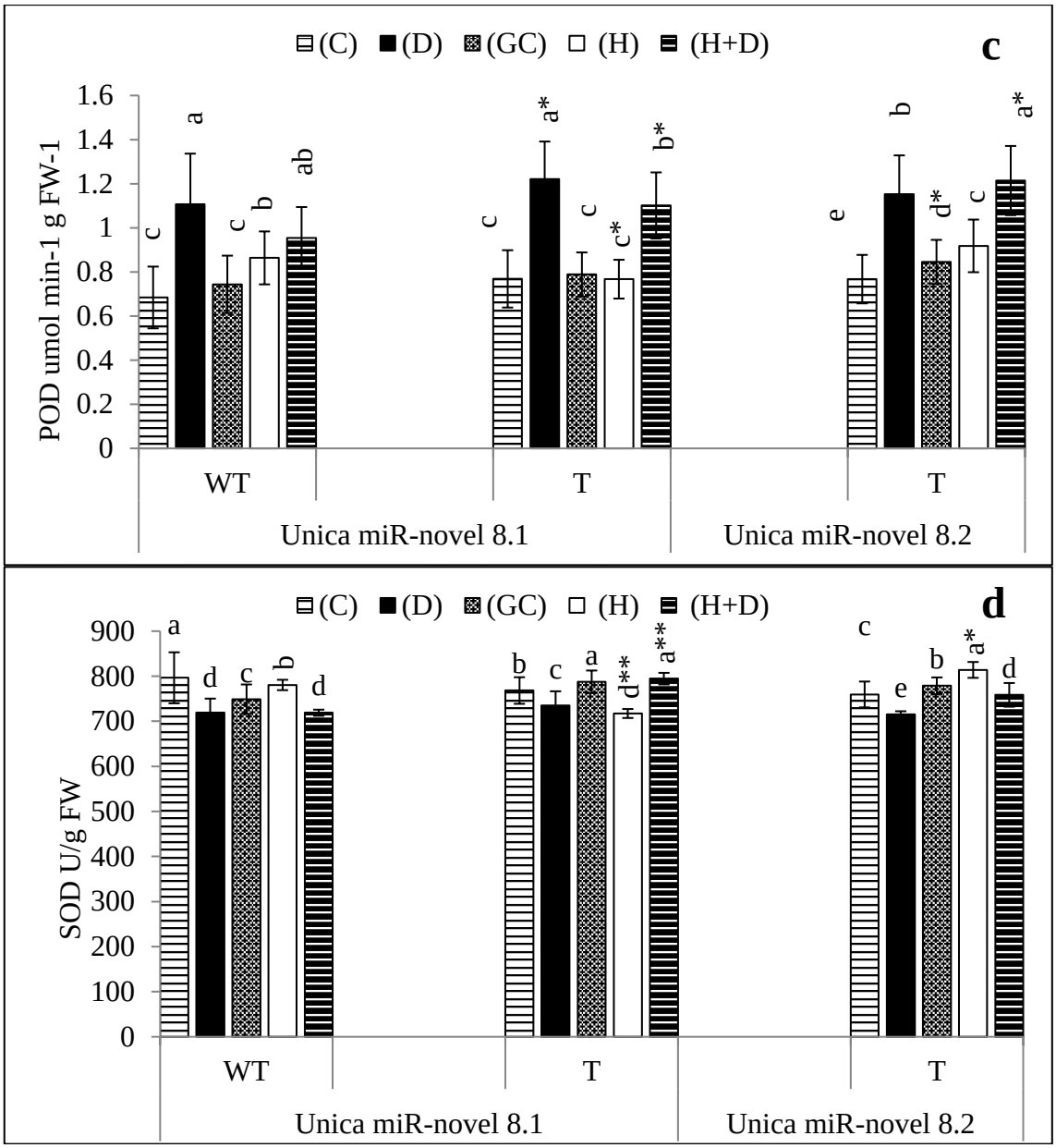


Figure 4.45. Influence of abiotic stress on biochemical traits of potato genotypes

Transgenic(T) and wild-type (WT) plants were classified as control (C, ▤), drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □), and combined heat-drought (HD, ▬) stresses. APX **(a),** CAT (b), POD (c) and SOD **(d).** Data shows as mean ± SD. Asterisks (*,**) shows the significant difference of treatments in transgenics from wild-type plants at P<0.05 and P<0.01 respectively.

4.17 Principal Component Analysis (PCA)

It reduced the dimensionality of the data by considering few components to explain the variation in the data. It enhances the ease of data interpretation and at the same time minimizes the loss of information (Figure 4.46).

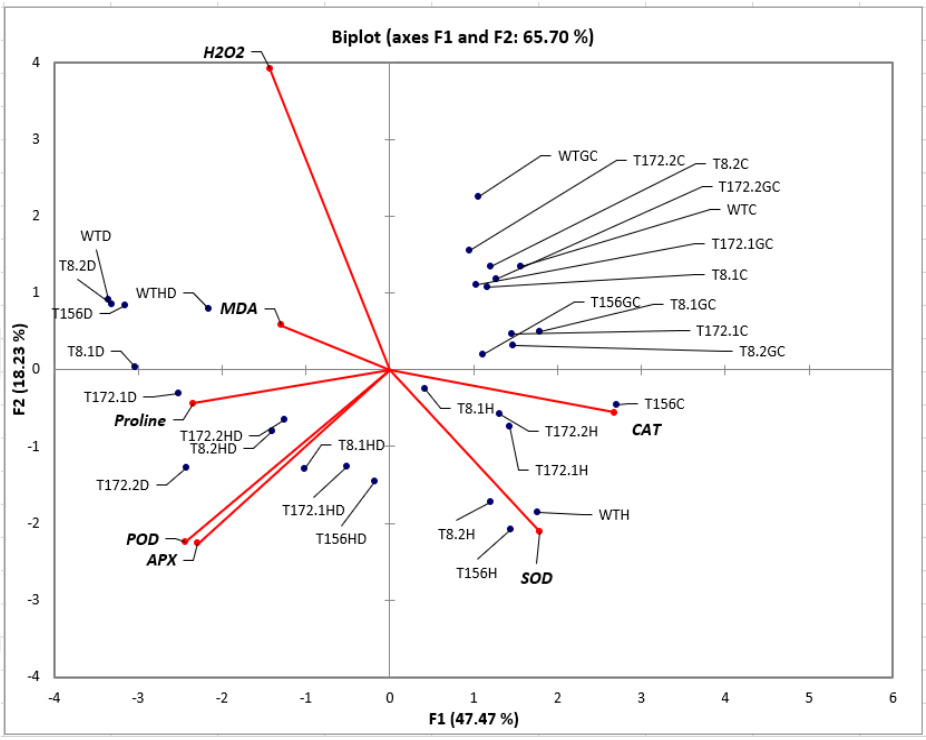


Figure 4.46. Principal component analysis

PCA biplot of our current study showed that the first two components explained 65.70% of total variation (PC1; 47.47% and PC2; 18.2%) in the data. All control treatments are grouped together in the positive quadrat irrelevant to any variables elucidating their non-responsiveness. The transgenic lines having over expression of miR172 for drought only and combined stress whereas transgenic line of miRmiRNA novel 8 under combined stress responded same and average to proline and POD. However, transgenic lines having over expression of miR172b under drought stress showed high proline accumulation. The transgenic lines having overexpression of miR156d, and miRNA novel 8 showed high performance of SOD under heat only stress. POD and APX activity were higher in all transgenic plants of all 3 miRNAs under combined stress conditions. The biplot further predicts that transgenic lines having overexpression of miR156d, and miRNA novel 8 showed more accumulation of MDA compared to other

treatments. CAT activity was high in both independent transgenic lines having overexpression of miR172b as compared to any other treatment.

4.18 Total RNA Isolation for Non-transgenic and Transgenic Lines of Unica After Stress.

Total RNA was isolated before expression analysis of target genes and miRNA and with qRT-PCR in non-transgenic and transgenic, (Table 4.2). 1µg RNA/sample was diluted (500ng/µL) and checked on TBE agarose gel (Figure 4.47). For cDNA synthesis and qRT-PCR, these samples were used.

Table 4.2. Spectrophotometer results after RNA isolation of non-transgenic and all transgenic lines

Control	**Concentration ng/µL**	**260/280**	**260/230**	**Gel figure 4.52**
Non-transgenic	2121.12	1.99	1.174	1
Novel 8.1	1101.04	1.939	0.697	2
Novel 8.2	981.76	1.98	1.091	3
miR156	1585.28	1.998	0.931	4
miR172.1	618.32	2.016	0.894	5
miR172.2	1714.28	2.029	1.109	6
Drought				
Non-transgenic	796.2	2.064	1.727	7
Novel 8.1	771.92	2.045	1.061	8
Novel 8.2	1677.24	2.056	1.332	9
miR156	2005	2.051	1.004	10
miR172.1	867.44	2.078	1.158	11
miR172.2	1752.32	2.095	1.242	12
Heat				
Non-transgenic	1530.92	2.088	1.177	13
Novel 8.1	1344.36	2.074	1.339	14
Novel 8.2	1811.96	2.075	1.258	15
miR156	2111.2	2.058	1.094	16

miR172.1	2127.72	2.042	1.261	17
miR172.2	714.72	2.063	0.993	18
Drought+Heat				
Non-transgenic	1695.5	2.08	1.636	19
Novel 8.1	1521.16	2.055	1.115	20
Novel 8.2	1041.12	2.078	1.171	21
miR156	764.14	2.098	1.121	22
miR172.1	941.72	2.098	1.282	23
miR172.2	1498.6	2.093	1.139	24
Drought+Heat Control				
Non-transgenic	964.28	2.046	0.908	25
Novel 8.1	2082.92	2.002	0.896	26
Novel 8.2	1247.64	2.056	1.153	27
miR156	1176.2	2.034	0.687	28
miR172.1	1085.8	2.076	0.932	29
miR172.2	870.8	2.072	1.154	30

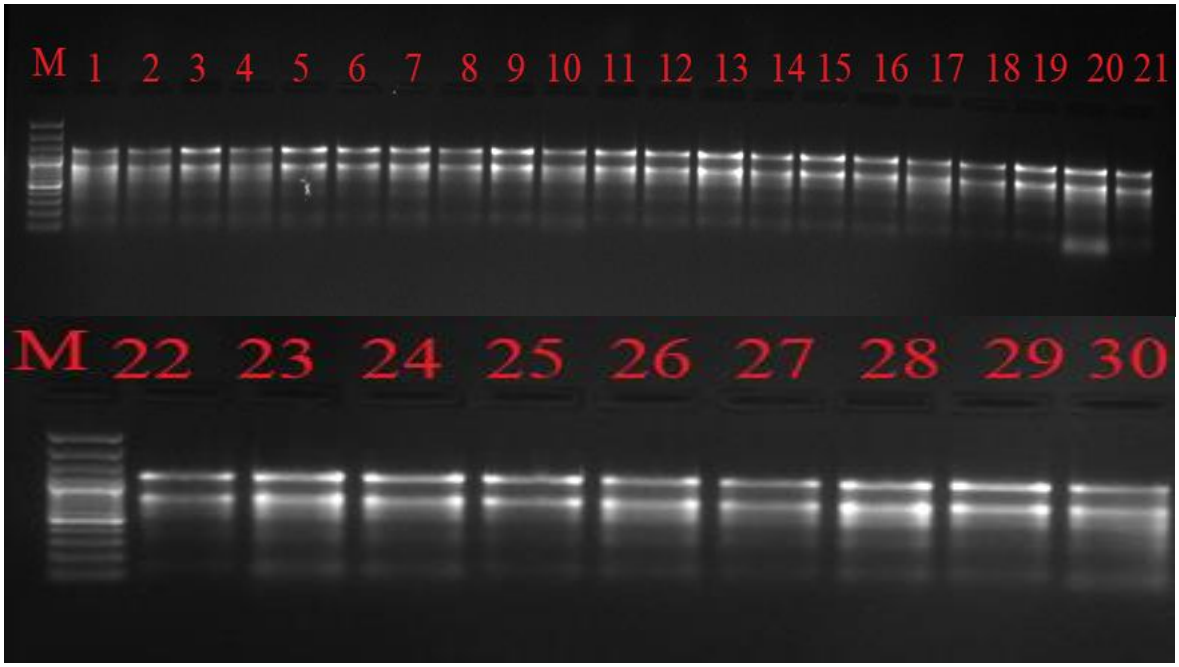


Figure 4.47. RNA samples (500ng/µL) on agarose gel, M 100 bp+ marker

4.19 Changes in Transcript Levels of miR156d-3p and Target Gene

The expression value of wild-type plants showed nonsignificant change under drought and heat while a significant increase in miR156d was observed under combined stress conditions (Figure 4.48a).

Transgenic plants showed a significant increase in the expression value of miR156d-3p under control and all stress conditions in comparison to wild type plants. Transgenic plants showed an increase of 2.83 and 3.60 folds in miR156d expression under control and drought conditions, respectively, compared to the wild-type plants under the same conditions. Whereas 2.9, 3.66, and 3.99 folds of increase was observed under control, heat and combined stress, respectively. Wild-type plants depicted a significant increase in the expressional values of Phospholipid-transporting ATPase nuclear ribonucleoprotein 1-like (*PPTATP*) under all applied stress conditions (Figure 4.48b). Despite this, the expression of *PPTATP* in transgenic plants dramatically downregulated under all stress conditions compared to their transgenic and wild-type controls. Transgenic plants showed a decline of 2.21 and 4.95 folds in the expression of *PPTATP* under control and drought, respectively, compared to wild-type plants under the same conditions, whereas a 2.26, 4.40, and 5.05-folds downregulation was observed under the control, heat, and combined stress conditions in comparison to their wild-type plants under the same conditions. Overall, a negative correlation was observed between the expression of miR156d in potato under control and different abiotic stress conditions.

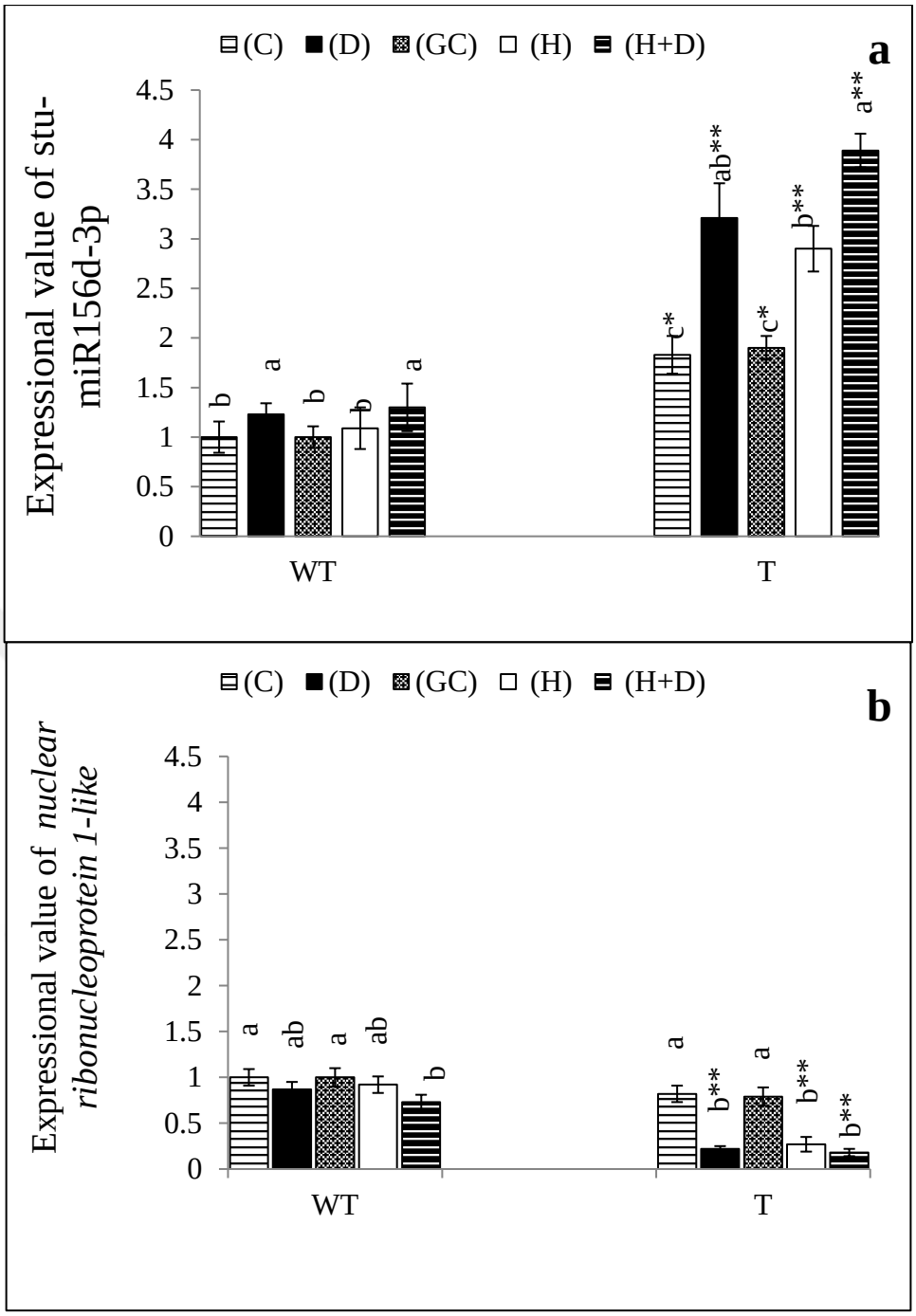


Figure 4.48. Relative transcript level (compared to wild-type control) of miRNA (stu-miR156d-3p) and its target gene (nuclear ribonucleo protein 1-like)

Transgenic (T) and wild-type (WT) plants were classified as control (C, ▤), drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □), and combined heat-drought (HD, ▬). Relative expression levels of miR156d-3p **(a)** and nuclear ribonucloe protein 1-like **(b)** were quantified by the $2^{-\Delta\Delta Ct}$ method. Data shows

as mean ± SD. Asterisks (*,**) shows the significant difference of treatments in transgenics from wild-type plants at P<0.05 and P<0.01 respectively.

4.20 Changes in Transcript Level of miR172b and Target Gene

The expression value of wild-type plants showed nonsignificant change under drought while a significant decline in miR172b was observed under heat and combined stress conditions (Figure 4.49a).

Transgenic plants of both lines showed a significant increase in the expression value of miR172b under control and all stress conditions in comparison to wild type plants. Transgenic plants of first line showed an increase of 3.12 and 4.30 folds in miR172b expression under control and drought conditions, respectively, compared to the wild-type plants under the same conditions. Whereas 3, 5.74, and 5.49 folds of increase was observed under control, heat and combined stress, respectively. Similarly, second transgenic line showed an increase of 2.04 and 4.16 folds in miR172b expression under control and drought, respectively, whereas it exhibited an increase of 2.97, 5.70, and 5.80 folds under control, heat, and combined stress conditions, respectively. Wild-type plants depicted a significant increase in the expressional values of ethylene-responsive transcription factor RAP2-7-like (*ERTF*) under all applied stress conditions (Figure 4.49b). Despite this, the expression of *ERTF* in first transgenic line dramatically downregulated under all stress conditions compared to their transgenic and wild-type controls. First transgenic line showed a decline of 2.32 and 5.64 folds in the expression of *ERTF* under control and drought, respectively, compared to wild-type plants under the same conditions, whereas a 2.21, 9.25- and 8.45-folds downregulation was observed under the control, heat, and combined stress conditions in comparison to their wild-type plants under the same conditions. Following the same pattern, the expression of *ERTF* was downregulated in second transgenic line by 2.29 and 6.80 folds under control and drought, respectively, whereas a downregulation of 2.31, 11.32, and 11.04 folds were observed under control, heat, and combined stress conditions.

Overall, a negative correlation was observed between the expression of miR172b and ERTF in potato under control and different abiotic stress conditions.

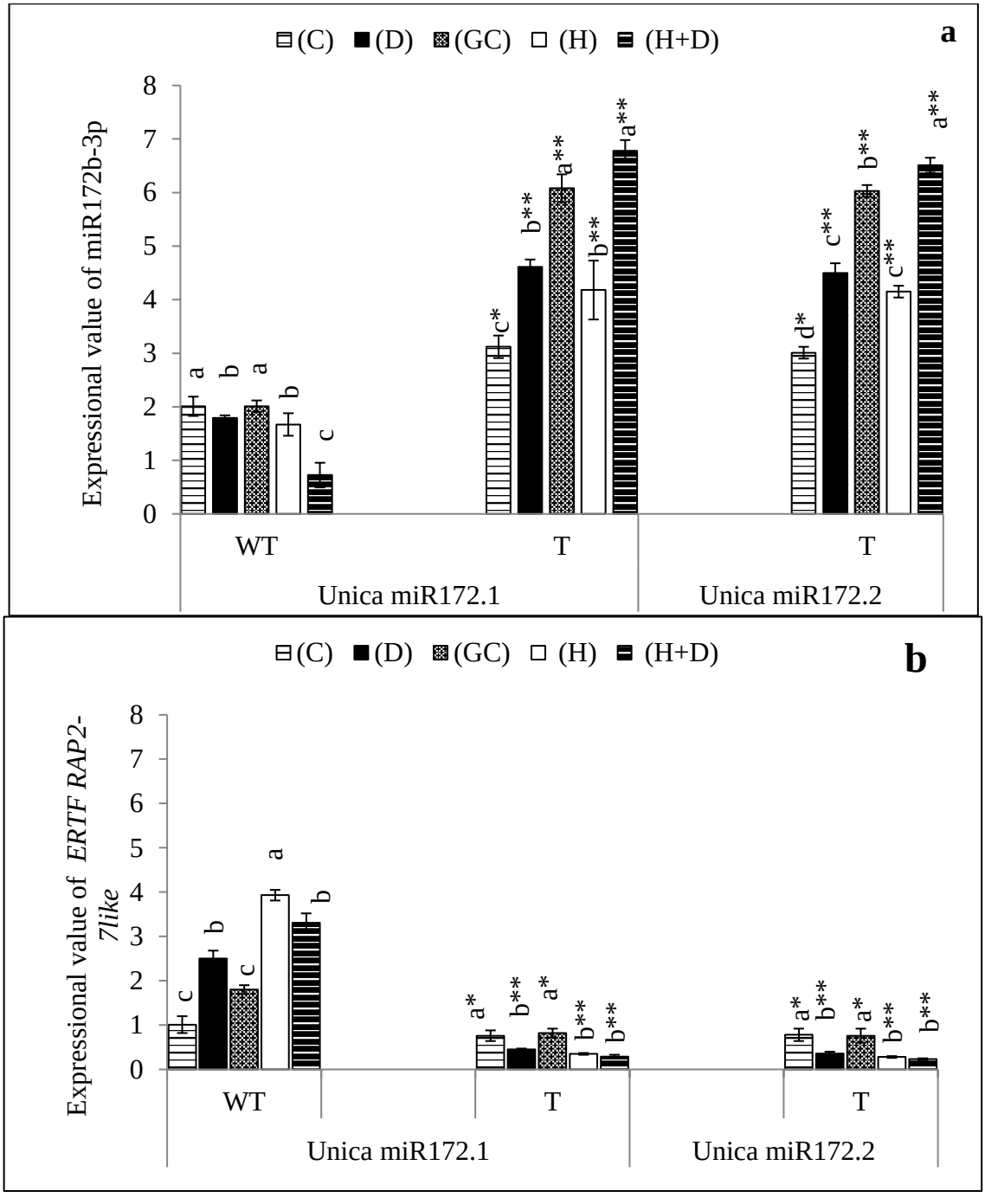


Figure 4.49. Relative transcript level (compared to wild-type control) of miRNA (stu-miR172b-3p) and its target gene (ERTF RAP2-7-like)

Transgenic (T) and wild-type (WT) plants were classified as control (C, ▤), drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □), and combined heat-drought (HD, ▬). Relative expression levels of miR172b-3p **(a)** and ERTF RAP2-7 like **(b)** were quantified by the $2^{-\Delta\Delta Ct}$ method. Data shows as mean ±

SD. Asterisks (*,**) shows the significant difference of treatments in transgenics from wild-type plants at P<0.05 and P<0.01 respectively.

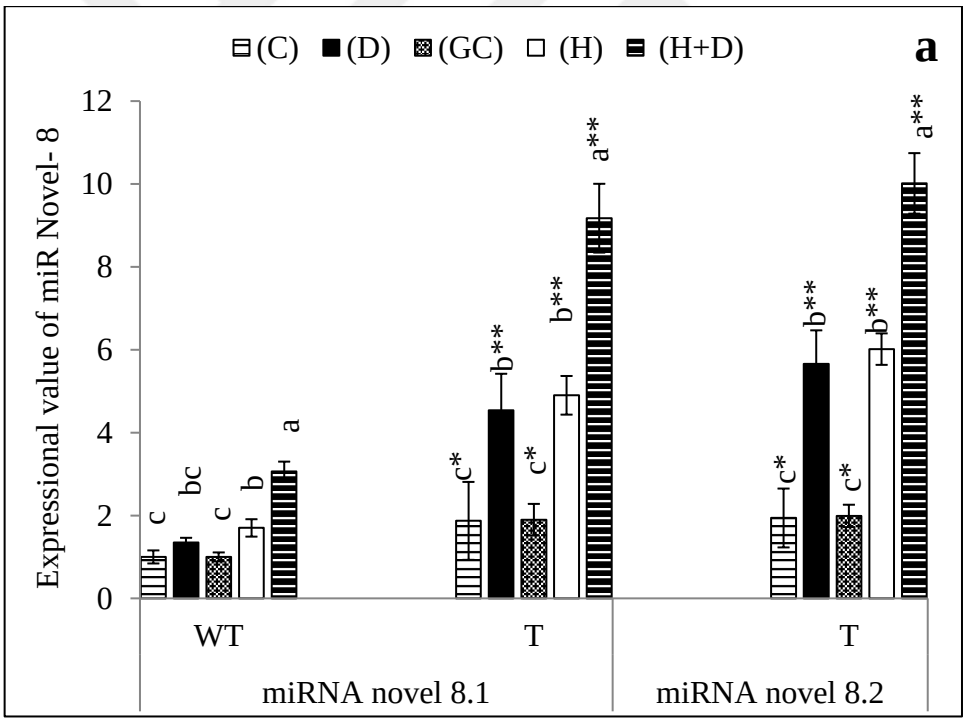
4.21 Changes in Transcript Levels of miRNA novel 8 and Target Genes

The expression value of wild-type plants showed nonsignificant change under drought while a significant increase in miRNA novel 8 was observed under heat and combined stress conditions (Figure 4.50a).

Transgenic plants of both lines showed a significant increase in the expression value of miRNA novel 8 under control and all stress conditions in comparison to wild type plants. Transgenic plants of first line showed an increase of 2.95 and 3.94 folds in miRNA novel 8 expression under control and drought conditions, respectively, compared to the wild-type plants under the same conditions, whereas 2, 3.40, and 3.15 folds of increase was observed under control, heat and combined stress, respectively. Similarly, second transgenic line showed an increase of 3.04 and 4.17 folds in miRNA novel 8 expression under control and drought, respectively, whereas it exhibited an increase of 2.99, 3.52, and 3.44 folds under control, heat, and combined stress conditions, respectively. Wild-type plants depicted a significant increase in the expressional values of photosystem II core complex protein (PCCP) under all applied stress conditions (Figure 4.50b). Despite this, the expression of PCCP in first transgenic line dramatically downregulated under all stress conditions compared to their transgenic and wild-type controls. First transgenic line showed a decline of 2.32 and 3.54 folds in the expression of PCCP under control and drought, respectively, compared to wild-type plants under the same conditions, whereas a 2.21, 9.25-, and 8.45-folds downregulation was observed under the control, heat, and combined stress conditions in comparison to their wild-type plants under the same conditions. Following the same pattern, the expression of PCCP was downregulated in second transgenic line by 2.29 and 6.80 folds under control and drought, respectively, whereas a downregulation of 2.31, 11.32, and 11.04 folds were observed under control, heat, and combined stress conditions.

Wild-type plants depicted a significant increase in the expressional values of mitochondrial transcription termination factor family (MTTF) under all applied stress conditions (Figure 4.50c). Despite this, the expression of MTTF in first transgenic line

dramatically downregulated under all stress conditions compared to their transgenic and wild-type controls. First transgenic line showed a decline of 2.25 and 5.92 folds in the expression of MTTF under control and drought, respectively, compared to wild-type plants under the same conditions, whereas a 2.28, 8.07, and 11.42-folds downregulation was observed under the control, heat, and combined stress conditions in comparison to their wild-type plants under the same conditions. Following the same pattern, the expression of MTTF was downregulated in second transgenic line by 2.28 and 6.17 folds under control and drought, respectively, whereas a downregulation of 4.22, 9.90, and 11.95 folds were observed under control, heat, and combined stress conditions. Overall, a negative correlation was observed between the expression of miRNA novel 8 and its target genes (PCCP, and MTTF) in potato under control and different abiotic stress conditions. Overall, a negative correlation was observed between the expression of miRNA novel 8 in potato under control and different abiotic stress conditions.



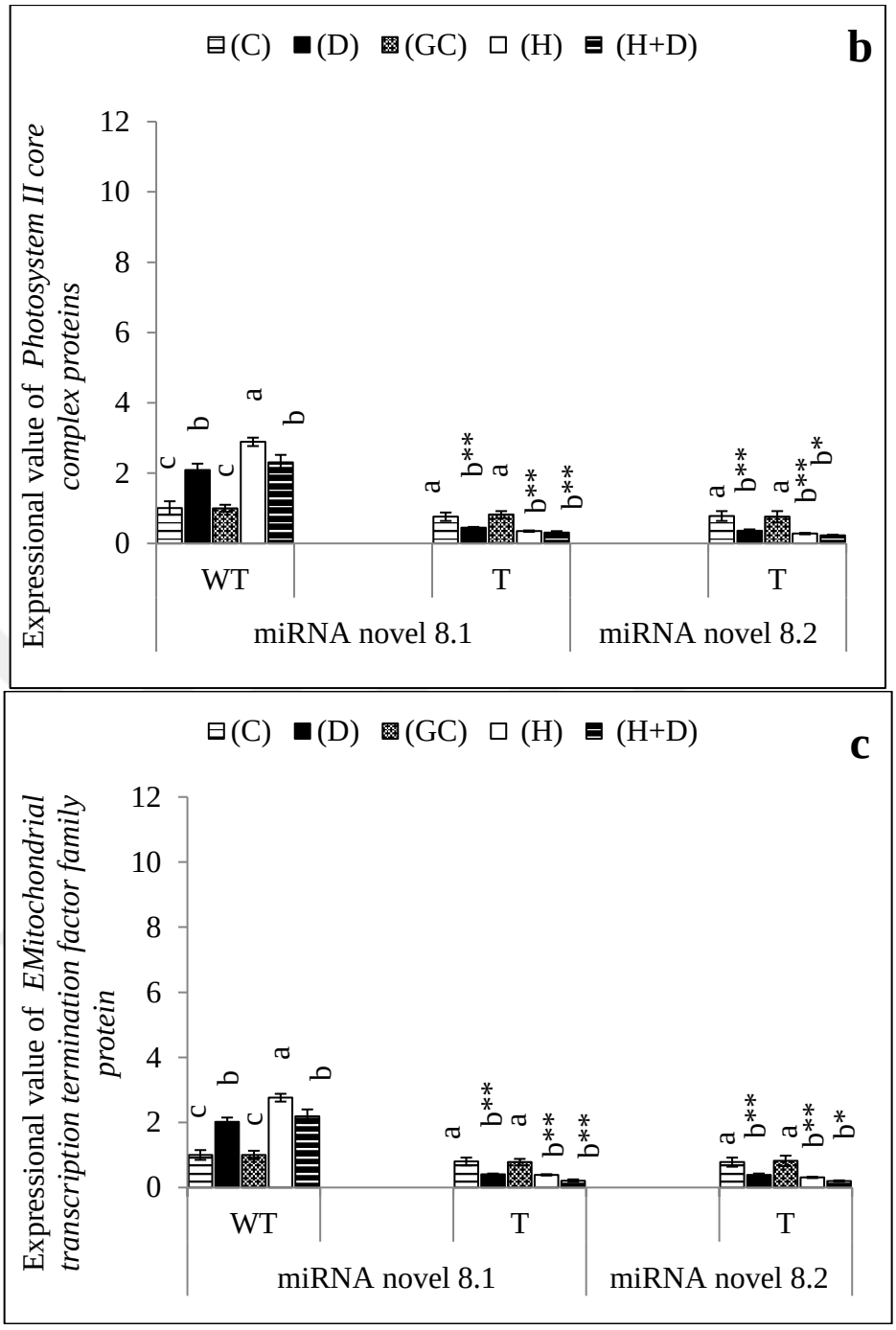


Figure 4.50. Relative transcript level (compared to wild-type control) of miRNA (miR-novel8) and its target genes (Photosystem II core complex proteins psbY and mitochondrial transcription termination factor family protein)

Transgenic (T) and wild-type (WT) plants were classified as control (C, ▤), drought (D, ■), control for heat and combined heat-drought stresses (GC, ▩), heat (H, □), and combined heat-drought (HD, 〓). Relative expression levels of miRNA novel 8 **(a),** Photosystem II core complex proteins psbY **(b)** and mitochondrial transcription termination factor family protein (**c**) were quantified by the $2^{-\Delta\Delta Ct}$ method. Data shows as mean ± SD. Asterisks (*,**) shows the

significant difference of treatments in transgenics from wild-type plants at $P<0.05$ and $P<0.01$ respectively.

Fig 4.51 represents an example for miRNAs and their target genes to be seen in two different peaks after amplification to clarify successful amplification for miRNAs and their target genes.

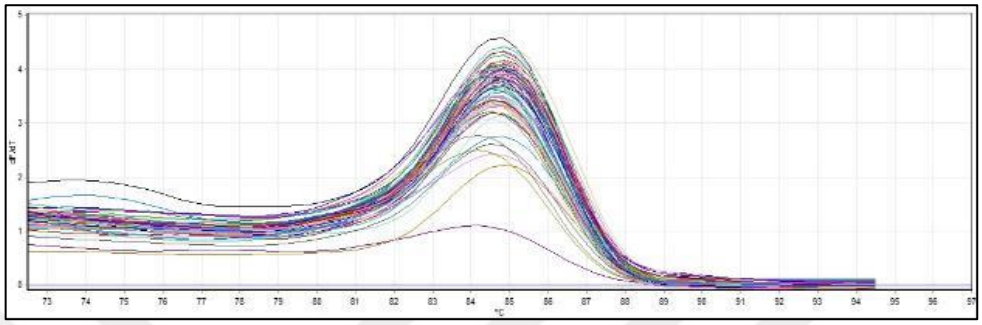


Figure 4. 51. Example of qRT-PCR melting curves of miRNAs and their target genes

CHAPTER V

DISCUSSION

MicroRNAs are master regulators in most biological processes of growth and various abiotic and biotic stresses by having a multidimensional interaction with their targets at both post-transcriptional and transcriptional level in plants and animals (Song et al., 2019). Plant miRNAs commonly showed a negative correlation with their target genes (Bagga et al., 2005). Some miRNAs and their target genes function in the tolerance mechanisms under stress conditions. An imperative way to understand the biological function of miRNAs is to identify the target genes they regulate (Marmisolle et al., 2020). In plants, bioinformatics is successful and helpful to identify high complementary targets of most of the known miRNAs (Jones-Rhoades and Bartel, 2004). Individual drought, heat, and their combination are among the abiotic stress factors that are difficult to control because of their complex and poorly understood mechanisms and interactions in potato. Major abiotic factors adversely affecting the yield of marketable potato include drought and heat (Monneveux et al., 2013; Aksoy et al., 2015) and usually occurrence of at once in prone zones lead to major crop loss (Mittler, 2006; Zandalinas et al.,2018). In an earlier study, many miRNAs were identified in response to individual and combined drought and heat stress factors by high-throughput sequencing in two contrasting (abiotic stress Russet Burbank (susceptible) vs. Unica (tolerant) cultivars of potato (Gökçe et al., 2021; Şanlı and Gökçe, 2021; Yalçın and Gökçe, 2021; Asim et al., 2021). Among 24 potential miRNAs, miR172b-3p become prominent as its expression was suppressed after stress application. Transgenic plants overexpressing pre-miR172b-3p from both were generated to further characterize the functions of miR172b-3p in potato. Through analysis of the expression level of mature miR172b-3p and target gene together with the various physiological and biochemical indicators suggested that miR172b-3p was involved in potato defense response to abiotic stress factors. The aim of the research was to determine the role and function of selected miRNAs (miRNA novel 8, stu-miR156d-3p and Stu-miR172b-3p) in potato under drought, heat and combined heat and drought stress conditions. For this aim, a transgenic approach was used to overexpress pre-miRNA in potato stress tolerant cultivar Unica (Gökçe et al., 2021). After stress treatments the level of selected miRNAs and their target genes were determined by

qRT-PCR technique. Through analysis of the expression level of mature miRNAs and target genes together with various physiological and biochemical indicators suggested that miRNAs might be involved in potato defense response to abiotic stress factors.

5.1 Plants having Overexpression of miR156d-3p

Transgenic plants having overexpression of miR156d-3p showed physiological difference compared with wild-type Unica plants. Drought stress caused less loss of leaves as compared to other stress conditions whereas under heat and combined stress conditions the loss of leaves was less in transgenic plants as compared to wild-type plants. Photosynthetic activity showed a decline with the increase in stress intensity and period. Photosynthesis rate in transgenic plants under drought only and combined stress was significantly high as compared to wild-type plants. Although heat stress showed not any significant effect in photosynthetic activity in transgenics and wild-types, the reason might be disruption of functional stomatal activity due to continuous water availability to support leaf transpiration and gaseous exchanges. Same phenomenon was observed and reported by Rizhsky et al. (2002, 2004). They elaborated that in Arabidopsis and tobacco under combined drought and heat stress conditions plants were not able to open stomata, while contrasting behaviour of plant was obseed under heat stress to enhance cooling via transpiration. Proline content showed high accumulation in transgenic lines under drought conditions as compared to non-transgenic plants under same stress conditions. Drought stress only showed significantly higher accumulation of proline content in transgenic plants as compared to wild-type plants. Same results were reported by Kang et al., (2019) in transgenic lines of tobacco having over expression of miR156. This higher accumulation of proline may also be attributed to improve tolerance against abiotic stress as proline is not only osmoprotectant but also acts as non-enzymatic antioxidant and downturns lipid peroxidation and reactive oxygen species (Chen et al. 2020; Margutti et al. 2017). Transgenic plants also showed less lipid peroxidation under heat and combined stress conditions as compared to wild-type plants. Overexpression of miR156d causes downturn of SPL genes and SPL is involved in disrupting formation of transcription factors MYB-bHLH -WD40 complex. MYB-bHLH -WD40 activates genes such as DFR and FLAVONOID3’-HYDROXYLASE (F3’H) (Gou et al., 2011). Overexpression of miR156 and its association with reduced SPL9 resulting in increased anthocyanin content and increased abiotic stress tolerance in Arabidopsis has already

been reported (Cui et al., 2014). Matthews, C., (2018) reported that plants having overexpression of miR156 showed high tolerance against heat stress, our expressional results of miR156d under drought, heat and combined stress are also in accordance with this phenomenon. miR156/SPL gene network is already known to be contributing for the regulation of antioxidants such as anthocyanins and carotenoids (Yin et al., 2019; Arshad et al., 2017; Wei et al., 2012; Wei et al., 2010) so this can be a possible reason behind this for high and less activity of antioxidant enzymes under only heat, combined stress and drought stress respectively. Results of first defensive line enzymes APX and CAT are also in accordance with this logic and same results were also reported by Arshad et al., (2017). Comparison of lipid peroxidation activity and H_2O_2 content in transgenic plants gives us a direction that miR156d plays supportive role for genes which are involved in playing protective role under heat stress. The contrast in accumulation of APX and CAT under different stress conditions gives us a direction that their role may be a due to transgenic plants that showed more tolerant behavior under stress conditions as both have been already reported as H_2O_2 scavenging enzymes (Liu et al., 2018; Wang et al., 2019).

Taken together, these results suggest that miR156 can be temporarily engineered to improve plant resistance to abiotic stress in potato, while same stress tolerant behavior was observed in Arabidopsis, rice and alfalfa (Cui et al., 2014; Gou et al., 2011; Matthews, C., 2018; Yin et al., 2019).

5.2 Plants having Overexpression of miR172b-3p

Transgenic plants having overexpression of miR172b-3p showed physiological difference compared with wild-type Unica plants. Same phenotypic deviation was observed by (Zou et al., 2013) in Arabidopsis. Under all stress conditions loss of leaves was less in transgenic plants as compared to non-transgenic plants. Interesting observation was late wilting of leaves in transgenics under drought only stress.

miR172b-3p has been abundantly studied in the development and control of flowering time (Lauter et al., 2005; Nair et al., 2010), but recent studies have suggested its roles in biotic (Li et al., 2014; Gai et al., 2014) and abiotic stress tolerance (Kuang et al., 2019). The results of this study revealed that expression levels of miR172b-3p and its target

gene *ERTF RAP2-7-like* were changed and negatively correlated with each other after the application of abiotic stress conditions. The results of other studies also showed that the expression levels of miR172b-3p were affected by stress applications (Gai et al., 2014; Luan et al., 2018; Kuang et al., 2019) while the expression levels of target genes were downregulated after stress application (Luan et al., 2018). *RAP2-7-like* protein, being a part of the AP2/ERF transcription family, plays a major role in the regulation of transcription directly by binding to the TBSF motif in the promoter of immune receptor gene FLAGELLIN-SENSING2 (FLS2) and inhibits its activity (Sreenivasulu et al., 2007). This model elaborates that overexpressing miR172b-3p causes suppression of TOE1 (ERTF RAP2-7), ultimately enhancing the activity of FLS2-mediated immunity during plant development in Arabidopsis (Zou et al., 2018). The miR172b-TOE1 module has been reported as a major integrator to coordinate plant development, immunity, and timing of flowering against environmental factors (Zou et al., 2018). Therefore, its regulation under abiotic stresses is essential for the survival of the plants and eliminating the yield losses in crops. Physiologically, in the case of individual drought treatment, an improvement in carbon assimilation was observed in transgenic plants of sensitive Russet Burbank but not in resistant Unica as compared to their wild-types, indicating an enhancement in the photosynthetic capacity due to greater tolerance (Asim et al., 2021). Transgenic Unica showed a significantly high photosynthetic rate (Pn) and transpiration rate (E) as compared to their wild-type plants under combined stress. The reason for this observation may be the maintenance of functional stomatal activity due to continuous water availability to support transpiration rate (E) and photosynthetic rate (Pn). Photosynthesis activity showed a decline with the increase in stress intensity and period. At last stages of stress photosynthesis rate in transgenics under drought and combined stress was significantly higher in contrast to wild type plants. Although heat stress caused not any significant effect on photosynthetic activity in transgenics and non-transgenics, the reason behind may be functional stomatal activity due to continuous water availability to support leaf transpiration and gaseous exchanges (Rizhsky et al., 2002, 2004). Although miR172b has been abundantly studied in flowering time control and development (Lauter et al. 2005; Nair et al. 2010), but recent studies have suggested its roles in biotic (Li et al. 2014; Gai et al. 2014) and abiotic stress tolerance (Kuang et al. 2019). A lower leaf temperature was detected in the plants under single heat stress as compared to the plants kept under combined stress conditions. This feature might be attributed to the continuity of E under heat-only

conditions. Similar results were also concluded in Arabidopsis and tobacco studies (Rizhsky et al., 2002, 2004).

In the cells, membrane damage can be determined by measuring the MDA levels, which is a marker of the oxidized product of lipid peroxidation. In our study (Asim et al., 2021), it was observed that transgenic Unica under heat-only stress resulted in significantly less membrane damage in the former genotype whereas the latter showed stable lipid peroxidation as compared to their respective wild-type plants. Interestingly in contrast to wild-types, Pn activity conformed with the MDA levels showing stability and significant improvement in transgenic Unica and RBB, respectively. In the case of H_2O_2 accumulation, no significant difference was observed among wild-type and transgenic plants of both genotypes under heat stress. This phenomenon suggested that under heat conditions, potato plants improve water use efficiency to stabilize cellular membrane and balance photosynthesis and transpiration activity for their survival but under combined stress conditions unavailability of water with high heat may cause more adverse effects at physiological, and biochemical level (Zhao et al., 2014). The transgenic Unica did not show a significant difference from wild-type plants; however, it showed a change of equal MDA accumulation according to their respective controls. On the other hand, a significant decrease in H_2O_2 accumulation in transgenic Unica than wild-type plants under drought and combined stress conditions. In transgenic Unica, a decrease in H_2O_2 accumulation agrees with improved Pn under combined stress. This data suggests that combined stress causes activation of drought protective mechanisms in plants of both genotypes. Moreover, an overexpression of miR172b-3p may cause transgenic plants to acclimatize the stress by enhancing the capacity of the light-harvesting complex thereby reducing the cellular damage caused by the overproduction of reactive oxygen species (ROS) in the photosynthetic electron transport chain (Ruban, 2016). Similar results of less MDA and H_2O_2 accumulation after stress application were reported in transgenic S. lycopersicum having overexpression of miR172a and miR172b (Luan et al., 2018) and in transgenic Arabidopsis having overexpression of soybean miR172c (Li et al., 2016). In contrast to heat and drought individual treatments, under combined stress-specific expression of few transcripts and high expression with synergistic interaction of many transcripts (mainly related to drought) related to acute or acclamatory stress responses is also a reason for more adverse effects of combined heat and drought stress observed in potato, tobacco, and Arabidopsis (Rizhsky et al., 2002,

2004; Demirel et al., 2020). One possible reason can be the affection of ABA-responsive genes, as it has already been reported that overexpression of soybean miR172c increased ABA sensitivity in Arabidopsis (Li et al., 2016). However, yet it needs to be unveiled that under combined stress conditions, how come the interaction of transcripts undergo modification depending upon the potential of genotype and alters their role in complex defensive networking. Taken together, these observations indicate that drought and heat individual and combined stress responsive genes regulated by miR172b-3p may involve in defense mechanisms to enhance stress tolerance. The involvement of miR172-3p in the regulation of drought tolerance in tobacco and rice has also been reported (Frazier et al., 2011; Zhou et al., 2010; Ferdous et al., 2015). Suppression of TOE1 and WRKY44 due to overexpression of miR172b-3p is one of the reasons behind this tolerance behavior against drought stress. Han et al. (2013) reported that WRKY44 is involved in sugar metabolism and signaling, they performed yeast two-hybrid screening to detect the interaction of WRKY44 and TOE1 and resulted that miR172 can suppress WRKY44 and TOE1, which encodes interactive proteins. This suppression leads to drought escape and tolerance by affecting sugar signaling in Arabidopsis having overexpression of miR172, which agrees with the behavior of potato plants observed in the present study. Heat stress-causing downregulation of miR172 (Xin et al., 2010; Khaksefidi et al., 2015) and upregulation of TOE1 (Zhao et al., 2016) have already been reported and the same results were observed in potato in our previous study (Kaplan, 2017). High temperature causing TOE1 upregulation due to downregulation of miR172 is reported to have an association with miR156-regulated Squamosa Promoter-Binding Protein-Like (SPL) genes to modulate developmental transitions by regulating their expression (Wu et al., 2009; Nonogaki, 2010; Jung et al., 2011; Stief et al., 2014; Zhao et al., 2016; Matthews et al., 2019). Downregulation of SPL genes due to miR156 causes phenotypic and biochemical advantages and improvements in tolerance of heat (Matthews et al., 2019) and abiotic stress (Cui et al., 2014). High temperature causes alteration in expressions of all components in miR156-SPL-miR172- AP2 (TOE1) (Zhao et al., 2016). According to this module and our findings, overexpression of miR172b-3p and downregulation of TOE1 may ultimately cause downstream of SPL genes, which agrees with the working pattern of miR156 and results in an improvement in tolerance against high temperature. As opposed to H_2O_2, the accumulation of proline was high in wild-type plants under drought stress, but it was strongly enhanced in transgenic plants of both cultivars as compared to their wild-types.

Similar results were reported in earlier findings in potato (Knipp et al., 2006; Demirel et al., 2020) and Arabidopsis (Rizhsky et al., 2004). It has already been proved that apart from being an osmoprotectant, proline also acts as a potent nonenzymatic antioxidant (Smirnoff and Cumbes, 1989; Mohanty and Matysik, 2001; Szabados and Savouré, 2010; Giberti et al., 2014; Rejeb et al., 2014; Chen et al., 2020) and downturns MDA, H_2O_2 (Ozden et al., 2009; Sobahan et al., 2016; Margutti et al., 2017) and free radicals in potato (Knipp et al., 2006). A comparison of proline content with H_2O_2 under all stresses in transgenic plants also indicates that an increase in proline content is also the reason for the lower accumulation of H_2O_2. Compared with wild type, the same results of maximum increase in proline in transgenic Arabidopsis plants having overexpression of miR172c resulted in a downturn of H_2O_2 and this increase causes an improvement in tolerance against drought stress (Li et al., 2016). Overall, these results imply that in Solanum tuberosum the miR172b-3p-ERTF RAP2-7-like acts as a module, which may regulate antioxidants to reduce the ROS accumulation and prevent cell membrane damage during drought and heat stresses. In summary, unique changes were identified at physiological, and biochemical levels under individual heat and drought stresses and in their combination. A negative feedback loop of ERTF RAP2-7- like in the result of overexpression of stu-miR-172b-3p in transgenic plants. In this study it was noticed that potato plant having overexpression of miR172b-3p countered to stress by i) enhancing the light-harvesting capacity and limiting nonphotochemical quenching, and ii) compensating the photosynthesis reduction by deflection in metabolism to maintain development and growth phases, and iii) by reducing ROS, MDA and increasing proline accumulation under different stress conditions. It was observed and confirmed that the downregulation of ERTF RAP2-7-like may cause behavioral variation in potato because of combined and single stresses. Under adverse environmental conditions, developmental remodeling can be fine-tuned by miR172b-3p and ERTF RAP2-7-like. Mizoi et al., (2012) and Bouaziz et al., (2015) have also reported that in potato major parts of AP2/ERF TF domain are DREB (StDREB1- StDREB4) and ERF (StERF1- StERF6) and they play crucial role in response to drought stress factors mainly. DREB2A is a member of DREB family and is already reported as a key regulator in increasing tolerance against drought response (Shinozaki and Yamaguchi-Shinozaki 2000; Sakuma et al., 2006; Reis et al., 2014; Agarwal et al., 2017;) and has been directly linked to H_2O_2 response (Rizhsky et al. 2004; Vanderauwera et al., 2005; Haag et al.,2013). One reason behind less accumulation of ROS can be overexpression of stu-

miR172b-3p may cause severe down regulation of DREB2A while the other reason can be increase in activity of antioxidant enzymes. POD activity was also high in drought only and its combination with heat while this was not the case in heat only stress. APX which is known to be first line defensive antioxidant enzyme to maintain thylakoid membrane (Wang et al., 2019) also showed less accumulation in drought only stress. APX activity can also be a reason for transgenic plants to show more tolerance against drought only stress as compared to its combination with heat. Taken together the results evolved that miR172b is involved in high tolerance response under drought only stress due to the activity of StDREB1 and StDREB2 as AP2/ERF transcription factors. These factors have capability to play dual role under drought stress in potato (Bouaziz et al., 2015).

Taken together, results suggest that miR172 and its target genes may involve in the abiotic stress resopnse of potato through the regulation of DREB and ERF genes, while same drought stress response was reported in potato (Hwang et al., 201, Bouaziz et al., 2015) and in Arabidopsis (Li et al.,2016).

5.3 Plants having Overexpression of miRNA novel 8

Transgenic independent lines having overexpression of miRNA novel 8 showed physiological difference compared with non-transgenic unica plants. Plant height and number of leaves per plant were less on transgenic plants compared with non-transgenic plants. Same results were observed by Fischer et al., (2008) in PsbO potato mutant. Pawłowicz et al., 2012 confirmed the protective function of PsbO by giving stability to PSII against drought stress in forage grasses. Drought stress caused less loss of leaves as compared to other stress conditions where combined stress conditions caused severe negative effects and high loss of leaves compared with other stress conditions. Morphologically, transgenic plants under all stress conditions showed less adverse effects as compared to wild-type plants. Photosynthesis activity showed improvement with the significant increase under drought only and combined stress. Although heat stress showed not any significant effect on photosynthetic activity, the reason behind may be functional stomatal activity due to continuous water availability to support leaf transpiration and gaseous exchanges. Transgenic plants under drought stress showed significantly less and nonsignificant change in chlorophyll content in first and second

line of plants. Whereas Gururani et al. 2013 have reported high chlorophyll contents during drought stress in potato transgenic plants having less expression of PSII. Proline content showed high accumulation in transgenic lines under stress conditions, as compared to wild-type plants under all stress conditions. This high accumulation of proline may also be the reason behind improvement in tolerance against abiotic stress as proline is not only osmoprotectant but also acts as non-enzymatic antioxidant and downturns lipid peroxidation and reactive oxygen specie (Chen et al. 2020; Margutti et al. 2017). H_2O_2 content was less in transgenics as compared to wild-type plants under all stress conditions. A significant decline in MDA accumulation in transgenic plants compared with wild-type plants was observed under heat only conditions. Compared to wild-types, in transgenic plants activity of ROS scavenging enzyme APX was significantly less under drought and combined stress, but no change was observed in case of heat only stress. So, this phenomenon gives us a direction that overexpression of miRNA novel 8 have linkage with the genes related to abiotic stress tolerance. Involvement of PSII in different responses to drought stress in Arabidopsis was also reported by Chen et al., 2016. Activity of all antioxidant enzymes was also in agreement with the amount of H_2O_2 content under different stress conditions especially APX and CAT activity was high under heat only stress condition to cop up with H_2O_2. Both are first line defensive enzymes and have been already reported as H_2O_2 scavenging enzymes to maintain thylakoid membrane and normal plant growth (Liu et al., 2018; Wang et al., 2019).

Taken together, physiologically, and biochemically the transgenic plants showed involvement of miRNA novel 8 in abiotic stress tolerance. While these lines showed resistance to individual and combined stress that may be because of the suppression of mitochondrial transcription termination factor family which have been widely reported as its involvement in tolerance of heat stress (Quesada et al., 2016).

CHAPTER VI

CONCLUSION

The purpose of thesis was investigation of overexpressed miRNA novel 8, miR156d-3p and miR172b-3p roles in potato cultivar (Unica) by transgenic approach under heat, drought, and combined drought+heat abiotic stresses. Generation of pre-miRNA harbouring transgenic plants in Unica were achieved which is already declared as abiotic stress tolerant cultivar. Transformation efficiency in Unica was 0.94 %, 0.79% and 0.90% for transgenic plants having overexpression of miRNA novel 8, miR156d-3p and miR172b-3p respectively. Three stress conditions as drought and heat alone and combined were applied to plants including wild-type and transgenic lines. Comparative studies at morphological, physiological, biochemical, and molecular levels were performed between wild-type and transgenic plants. These comparative analyses depicted those transgenic plants showed variance in their response to different abiotic stress conditions. qRT-PCR outcomes indicated that level of all three miRNAs (miRNA novel 8, miR156d-3p and miR172b-3p) were highly overexpressed in their respected transgenic lines whereas their target genes (Photosystem II core complex proteins psbY, Mitochondrial transcription termination factor family protein, Phospholipid-transporting ATPase and ERTF RAP2-7like protein) had low level in transgenic plants. Their expression levels of all miRNAs were not uniform under different abiotic stress conditions indicating that these target genes are not controlled by single miRNA. According to all analyses, miRNA novel 8 and miR172b-3p and miR156d-3p can be engineered to improve plant resistance against only heat and combined (heat and drought) stress in potato.

To the best of our knowledge, this research presents the effects of miRNA novel 8 and miR172b-3p and miR156d-3p overexpression under individual and combined stress in potato. Hence along with this provided information, many questions are yet to be answered. For example, how the postgerminative development and adaptation are fine-tuned by various target genes of miRNAs? What are the regulatory networks modulated by ABA signals after their transduction to miR172b-3p in potato under single or combined stress conditions? In future research, answering these questions will provide a novel vision into the miRNA-mediated signal transduction during different

developmental stages in response to adverse abiotic conditions and will ultimately enhance multistress tolerance in potato.

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APPENDICES

APPENDIX-A

Results of ANOVA (Analysis of variance) for all observed variables

Observed Variables	SOV	Df	Mean Square	F value	P value	CV
Stomata Conductance	C	5	0.0170	4.8057	0.000***	47.099
	S	4	0.2828	79.769	0.000***	
	C:S	20	0.0138	3.9116	0.000***	
Transpiration Rate	C	5	19.918	25.815	0.000***	23.485
	S	4	294.057	381.133	0.000***	
	C:S	20	10.481	13.584	0.000***	
RWC	C	5	184.10	22.4673	0.000***	4.0040
	S	4	2948.78	359.8575	0.000***	
	C:S	20	136.45	16.6520	0.000***	
Leaf Temperature	C	5	11.20	3.8749	0.0033 **	5.7684
	S	4	735.79	254.6736	0.000***	
	C:S	20	20.58	7.1240	0.000***	
Chlorophyll content	C	5	223.99	9.9743	0.000***	13.821
	S	4	1055.69	47.0099	0.000***	
	C:S	20	114.46	5.0970	0.000***	
Proline	C	5	32.88	3.475	0.000***	15.443
	S	4	853.71	52.220	0.000***	
	C:S	20	9.32	1355.875	0.000***	
MDA	C	5	95.309	9.2096	0.000***	22.663
	S	4	81.590	7.8840	0.000***	
	C:S	20	15.344	1.4827	0.000***	
H_2O_2	C	5	103279	27.5761	0.000***	9.83
	S	4	662900	176.9981	0.000***	
	C:S	20	20733	5.5357	0.000***	
APX	C	5	0.1570	0.260	0.933 ^{NS}	35.49
	S	4	2.7971	4.639	0.0019 **	
	C:S	20	00772	0.128	0.999 ^{NS}	
CAT	C	5	4.449	0.181	0.968 ^{NS}	30.412
	S	4	0.140	7.845	0.000***	
	C:S	20	0.567	0.247	0.999 ^{NS}	
POD	C	5	0.0332	1.301	0.271 ^{NS}	16.931
	S	4	0.8252	32.286	0.000***	
	C:S	20	0.0324	1.271	0.220 ^{NS}	
SOD	C	5	1816.1	2.812	0.021*	3.358
	S	4	6121.2	9.479	0.000***	
	C:S	20	3621.3	5.609	0.000***	
miRNA expression	C	4	21.2987	7.55	0.0001**	46.84
	S	4	43.6521	15.47	0.0000***	
	C:S	16	6.1213	2.17	0.0199*	
Target gene	C	4	0.05669	6.37	0.0003**	20.29

expression	S	4	1.15355	129.63	0.0000***	
	C:S	16	0.05401	6.07	0.0000***	
Photosynthesis 0 day	C	5	25.14	6.47	0.0000***	9.65
	S	4	1484.85	382.27	0.0000***	
	C:S	20	19.09	4.92	0.0000***	
Photosynthesis 6 day	C	5	31.04	9.85	0.0000***	8.69
	S	4	1538.20	488.25	0.0000***	
	C:S	20	12.80	4.06	0.0000***	
Photosynthesis 10 day	C	5	24.24	3.56	0.0056**	13.37
	S	4	1491.03	219.11	0.0000***	
	C:S	20	10.56	1.55	0.0847 ^{NS}	
Photosynthesis 12 day	C	5	47.14	8.13	0.0000***	13.49
	S	4	2264.25	390.37	0.0000***	
	C:S	20	10.47	1.81	0.0323*	
Photosynthesis 16 day	C	5	10.29	3.57	0.0055**	20.60
	S	4	3916.07	1358.96	0.0000***	
	C:S	20	11.55	4.01	0.0000***	
Photosynthesis 18 day	C	5	20.12	22.51	0.0000***	10.56
	S	4	4683.96	5242.33	0.0000***	
	C:S	20	9.22	10.32	0.0000***	
Photosynthesis 20 day	C	5	26.84	18.97	0.0000***	11.52
	S	4	4882.70	4882.70	0.0000***	
	C:S	20	12.75	9.01	0.0000***	

Acronyms: DF: Degree of freedom; SS: Sum of square; MS: Mean square

*** Significant at P<0.001
** Significant at P<0.01
* Significant at P<0.05
NS = Non-significant

APPENDIX-B

Effect of different stress treatments on photosynthesis rate at different stress days, stomata conductance, transpiration rate, leaf temperature, chlorophyll content and relative water content measured in six different cultivars (one non-transgenic and five transgenic lines)

Photosynthesis 0 Day	C1	C2	C3	C4	C5	C6
Control	28.22 **a**	31.34 **a**	29.4875 **a**	30.1475 **a**	31.4425 **a**	31.01 **a**
Drought	27.84 **a**	31.11 **a**	26.1725 **b**	27.07 **a**	26.4825 **b**	27.105 **b**
Control (H,HD)	13.52 **b**	15.78 **b**	23.38 **c**	11.5275 **b**	13.635 **c**	15.2825 **c**
Heat	13.76 **b**	16.11 **b**	12.59 **e**	13.1525 **b**	12.8825 **c**	13.4525 **c**
Heat+Drought	14.75 **b**	15.98 **b**	16.7275 **d**	16.0625 **b**	13.59 **c**	13.475 **c**

Photosynthesis 6th Day	C1	C2	C3	C4	C5	C6
Control	29.12 **a**	32.09 **a**	29.4875 **a**	30.1475 **a**	31.4425 **a**	31.01 **a**
Drought	28.82 **a**	31.85 **a**	26.1725 **a**	27.07 **a**	26.4825 **b**	27.105 **b**
Control (H,HD)	14.23 **b**	16.09 **b**	18.88 **b**	11.5275 **c**	13.635 **c**	15.2825 **c**
Heat	13.99 **b**	15.89 **b**	18.39324 **b**	13.1525 **c**	12.8825 **c**	13.4525 **c**
Heat+Drought	14.11 **b**	15.63 **b**	16.5125 **b**	16.18 **b**	11.09 **c**	12.225 **c**

Photosynthesis 10th Day	C1	C2	C3	C4	C5	C6
Control	30.68 **a**	30.37 **a**	30.37783 **a**	33.2006 **a**	37.33701 **a**	34.0352 **a**
Drought	13.18 **c**	15.05 **c**	14.72826 **c**	14.6776 **d**	16.37157 **c**	16.4853 **c**
Control (H,HD)	17.06 **b**	17.46 **b**	18.19496 **b**	16.4454 **c**	15.77647 **c**	17.7764 **c**
Heat	18.55 **b**	19.35 **b**	18.39324 **b**	21.3973 **b**	22.36884 **b**	21.3688 **b**
Heat+Drought	9.56 **d**	15.61 **c**	11.96696 **d**	12.9733 **d**	11.97025 **d**	12.7522 **d**

Photosynthesis 12th Day	C1	C2	C3	C4	C5	C6
Control	30.43 **a**	29.38 **a**	30.37783 **a**	33.2006 **a**	37.3370 **a**	34.0352 **a**
Drought	11.09 **d**	11.08 **c**	14.72826 **c**	14.6776 **c**	16.3715 **c**	16.4853 **c**
Control (H,HD)	15.06 **c**	17.44 **b**	18.19496 **b**	14.6949 **c**	15.7764 **c**	17.7764 **c**
Heat	18.45 **b**	19.76 **b**	18.39324 **b**	21.3973 **b**	22.3688 **b**	21.3688 **b**
Heat+Drought	3.56 **e**	5.62 **d**	5.5475 **d**	7.9425 **d**	7.22 **d**	5.757 **d**

Photosynthesis 16th Day	C1	C2	C3	C4	C5	C6
Control	25.9 **a**	29.66 **a**	23.56 **a**	33.287 **a**	31.025 **a**	30.955 **a**
Drought	10.81 **b**	13.05 **b**	13.28 **b**	12.0425 **b**	11.265 **b**	12.415 **b**

Photosynthesis 18th Day	C1	C2	C3	C4	C5	C6
Control	26.65 **a**	34.23 **a**	30.4575 **a**	31.6975 **a**	34.2075 **a**	34.105 **a**
Drought	9.45 **b**	14.73 **b**	14.14 **b**	11.0875 **b**	14.38931 **b**	13.9425 **b**

Photosynthesis 20th Day	C1	C2	C3	C4	C5	C6
Control	32.63 **a**	38.71 **a**	42.53811 **a**	36.787 **a**	40.522 **a**	43.423 **a**
Drought	9.39 **b**	8.89 **b**	14.1875 **b**	12.2855 **b**	13.2525 **b**	14.0025 **b**

Effect of different stress treatments on Stomatal conductance

Stomatal conductance	C1	C2	C3	C4	C5	C6
Control	0.088 **b**	0.0806 **c**	0.1475**c**	0.148 **b**	0.09 **c**	0.059 **c**
Drought	-0.0275 **d**	-0.0311 **e**	-0.0325 **e**	-0.040 **d**	-0.03 **e**	-0.03 **e**
Control (H,HD)	0.0942 **b**	0.2541 **b**	0.209 **b**	0.1475 **b**	0.19 **b**	0.1775 **b**
Heat	0.135 **a**	0.372**a**	0.351**a**	0.1925 **a**	0.27 **a**	0.271 **a**
Heat+Drought	0.0166 **c**	0.0440**d**	0.03 **d**	0.0389 **c**	0.03**d**	0.026 **d**

Effect of different stress treatments on Transpiration rate

Transpiration rate	C1	C2	C3	C4	C5	C6
Control	1.9975 **b**	2.545496 **b**	4.428063 **b**	3.883 **b**	3.4 **b**	2.41554 **c**
Drought	-0.77 **d**	-1.09387 **e**	-1.53329 **d**	-2.48 **d**	-1.56 **d**	-1.09 **e**
Control (H,HD)	3.16 **a**	2.295 **c**	3.789 **e**	3.8825 **b**	3.73 **b**	3.514 **b**
Heat	3.48 **a**	9.684949 **a**	5.445 **a**	8.43736 **a**	5.7 **a**	5.725 **a**
Heat+Drought	0.21 **c**	1.71636 **d**	1.096667 **c**	1.736667 **c**	0.58 **c**	1.014 **d**

Effect of different stress treatments on Leaf Temperature

Leaf temperature	C1	C2	C3	C4	C5	C6
Control	32.1 **b**	26.425 **c**	27.925 **c**	27.675 **d**	26 **d**	28.1 **c**
Drought	32.32 **b**	30.475 **b**	28.25 **c**	30.725 **c**	28.35 **c**	30.75 **b**
Control (H,HD)	18.85 **c**	18.35 **d**	20.675 **d**	22.133 **e**	23.275 **e**	23.275 **d**
Heat	31.733 **b**	31.6 **b**	33.25 **b**	33.525 **b**	31.775 **b**	31.775 **b**
Heat+Drought	40.433 **a**	40.875 **a**	34.6666 **a**	38.267 **a**	40.25 **a**	40.266 **a**

Effect of different stress treatments on Chlorophyll content

Chlorophyll content	C1	C2	C3	C4	C5	C6
Control	38.325 **b**	35.125 **b**	34.425 **c**	38.475 **b**	36.625 **ab**	35 **bc**
Drought	40.55 **a**	38.6 **ab**	30.825 **d**	38.975 **b**	35.95 **b**	38.3 **a**
Control (H,HD)	41.1 **a**	39.325 **a**	42.325 **a**	40.075 **a**	37.45 **a**	37.45 **b**
Heat	38.825 **b**	35.925 **b**	37.8575 **b**	35.675 **c**	35.925 **b**	35.925 **bc**
Heat+Drought	34 **c**	31.26667 **c**	31.4 **d**	31.100 **d**	33.05 **c**	34.533 **c**

Effect of different stress treatments on Relative Water Content

Relative water content	C1	C2	C3	C4	C5	C6
Control	78.02 **a**	77.93 **b**	80.589 **b**	83.77717 **a**	82.41 **a**	87.072 **a**
Drought	57.4 **c**	61.615 **c**	60.745 **c**	60.6 **b**	59.18 **c**	53.715 **d**
Control (H,HD)	79.22 **a**	83.6125 **a**	82.745 **a**	83.76596 **a**	87.36 **a**	87.369 **a**
Heat	69.78 **b**	61.895 **c**	62.1925 **c**	61.5025 **b**	66.09 **b**	66.045 **b**
Heat+Drought	60.47 **c**	60.9325 **c**	60.11 **c**	59.815 **b**	59.23 **c**	59.232 **c**

Any two means not sharing a letter in common differ significantly at $P < 0.001$.
C1 is non-transgenic Unica, C2 and C3 are independent transgenic lines of miR172b, C4 is transgenic line of miR156d and C5 and C6 are independent transgenic lines of miRNA novel-8.

APPENDIX-C

Effect of different stress treatments on MDA, proline, H_2O_2, APX, CAT, POD, SOD, expression of miRNA and expression of target genes measured in six different cultivars (one non-transgenic and five transgenic lines).

Effect of different stress treatments on MDA

MDA	C1	C2	C3	C4	C5	C6
C1	16.324 **b**	15.49702 **c**	16.70008 **c**	14.8757 **b**	14.32 **a**	15.49694 **a**
D2	16.96 **a**	16.95952 **b**	18.47512 **b**	16.44535 **a**	13.96 **a**	13.93492 **ab**
GC3	11.34 **d**	11.13696 **d**	13.51452 **d**	12.03078 **c**	11.34 **ab**	11.06444 **b**
H4	12.09 **c**	16.89315 **b**	18.72547 **b**	9.09207 **e**	9.49 **b**	9.474462 **c**
HD5	14.75 **bc**	19.73548 **a**	20.3552 **a**	10.797 **d**	11.75 **ab**	11.89427 **b**

Effect of different stress treatments on Proline

Proline	C1	C2	C3	C4	C5	C6
C1	1.36 **bc**	1.44332 **c**	1.37748 **c**	1.5979 **c**	2.5651 **c**	1.8480 **d**
D2	10.01 **a**	15.79473 **a**	12.91098 **a**	17.962 **a**	20.5783 **a**	16.78 **a**
GC3	1.03 **c**	1.59564 **c**	1.78108 **c**	1.1512 **d**	1.8317 **c**	1.6769 **d**
H4	1.93 **b**	2.462758 **b**	3.38405 **b**	3.6705 **b**	5.0676 **b**	5.85 **b**
HD5	2.04 **b**	2.77612 **b**	2.20623 **bc**	2.6333 **bc**	4.2877 **b**	4.4433 **c**

Effect of different stress treatments on H_2O_2

H_2O_2	C1	C2	C3	C4	C5	C6
C1	805.1713 **b**	761.4443 **b**	723.1361 **b**	529.2135 **c**	644.71 **b**	693.4218 **b**
D2	982.3821 **a**	799.2168 **a**	886.405 **a**	819.011 **a**	736.12 **a**	655.6734 **c**
GC3	877.1663 **ab**	689.8901 **c**	655.439 **c**	619.6526 **b**	670.97 **b**	832.1683 **a**
H4	359.1908 **c**	385.9077 **e**	322.0347 **e**	335.9871 **e**	393.30 **d**	393.3063 **e**
HD5	790.9382 **b**	547.2279 **d**	489.9238 **d**	470.4522 **d**	465.69 **c**	528.895 **d**

Effect of different stress treatments on APX

APX	C1	C2	C3	C4	C5	C6
C1	1.808 **c**	1.965313 **c**	1.7701 **c**	1.7471 **c**	1.830569 **e**	1.759 **d**
D2	2.863 **a**	2.518115 **a**	2.5661 **a**	2.5175 **a**	1.968755 **d**	2.520 **a**
GC3	1.622 **c**	1.863569 **c**	1.7989 **c**	1.9613 **b**	2.355161 **b**	1.8417 **d**
H4	2.539 **b**	2.382535 **b**	2.2955 **b**	2.2429 **ab**	2.141841 **c**	2.2186 **c**
HD5	2.715 **ab**	2.569043 **a**	2.3630 **b**	2.496647 **a**	2.645866 **a**	2.3655 **b**

Effect of different stress treatments on CAT

CAT	C1	C2	C3	C4	C5	C6
C1	2.642343 **b**	2.801949 **a**	2.733712 **b**	3.116771 **a**	2.839525 **a**	2.746456 **a**
D2	1.929968 **d**	2.079457 **b**	1.946934 **d**	1.876228 **d**	2.110913 **b**	2.190801 **b**
GC3	2.318725 **c**	2.720602 **a**	2.6367 **b**	2.79762 **b**	2.70443 **ab**	2.670836 **a**
H4	3.292342 **a**	2.753251 **a**	2.919351 **a**	2.868996 **b**	2.814398 **a**	2.726608 **a**
HD5	1.587525 **e**	2.086779 **b**	2.20769 **c**	2.214694 **c**	2.014246 **b**	1.941381 **b**

Effect of different stress treatments on POD

POD	C1	C2	C3	C4	C5	C6
C1	0.684187 **c**	0.76849 **c**	0.767379 **e**	0.794846 **b**	0.84009 **c**	0.78429 **c**
D2	1.107165 **a**	1.221341 **a**	1.152618 **b**	1.011527 **a**	1.24383 **a**	1.309188 **a**
GC3	0.743879 **c**	0.788385 **c**	0.845366 **d**	0.93940 **ab**	0.79255 **d**	0.756019 **c**
H4	0.863605 **b**	0.767196 **c**	0.91806 **c**	1.030887 **a**	0.80022 **d**	0.716233 **d**
HD5	0.954187 **ab**	1.101333 **b**	1.214361 **a**	1.0746 **b**	1.18095 **b**	1.154584 **b**

Effect of different stress treatments on SOD

SOD	C1	C2	C3	C4	C5	C6
C1	796.509 **a**	768.2841 **b**	759.4937 **c**	822.7848 **a**	775.3165 **a**	727.8481 **b**
D2	719.493 **d**	734.8805 **c**	715.5415 **e**	713.7834 **d**	752.4613 **b**	764.7679 **a**
GC3	748.945 **c**	787.6231 **a**	778.8326 **b**	754.2194 **c**	727.8481 **c**	731.3643 **b**
H4	780.590 **b**	717.2996 **d**	813.9944 **a**	787.6231 **b**	750.703 **b**	755.9775 **ab**
HD5	719.057 **d**	794.6554 **a**	758.66 **d**	768.2841 **c**	766.526 **ab**	752.08 **ab**

Any two means not sharing a letter in common differ significantly at $P < 0.001$.
C1 is non-transgenic unica, C2 and C3 are independent transgenic lines of miR172b, C4 is transgenic line of miR156d and C5 and C6 are independent transgenic lines of miRNA novel-8.

APPENDIX-D

Effect of different stress treatments on miR172b and its target gene expression

miR172,TG	WT-miR172	T-miR172 L1	T-miR172 L2	WT-Tg	T-Tg L1	T-Tg L2
C1	2.01 **a**	3.12 **c**	3.01 **d**	1.01 **c**	0.76 **a**	0.78 **a**
D2	1.79 **b**	4.61 **b**	4.50 **c**	2.5 **b**	0.45 **b**	0.36 **b**
GC3	2.01 **a**	6.08 **a**	6.03 **b**	1.8 **c**	0.82 **a**	0.76 **a**
H4	1.67 **b**	4.18 **b**	4.15 **c**	3.93 **a**	0.35 **b**	0.28 **b**
HD5	0.73 **c**	6.78 **a**	6.51 **a**	3.31 **b**	0.29 **b**	0.23 **b**

Any two means not sharing a letter in common differ significantly at $P < 0.001$.

Where WT is wild type plant, T is transgenic plant, miR172 L1 is miR172b expression in first transgenic line, miR172 L2 is miR172b expression in second transgenic line, Tg is target gene (Ethylene-responsive transcription factor RAP2-7-like).

Effect of different stress treatments on miR156d and its target gene expression

miR156,TG	WT-miR156	T-miR156	WT-Tg	T-Tg
C1	1 **b**	1.83 **c**	1 **a**	0.82 **a**
D2	1.23 **a**	3.21 **ab**	0.87 **ab**	0.22 **b**
GC3	1 **b**	1.90 **c**	1 **a**	0.79 **a**
H4	1.09 **b**	2.90 **b**	0.92 **ab**	0.27 **b**
HD5	1.3 **a**	3.89 **a**	0.73 **b**	0.18 **b**

Any two means not sharing a letter in common differ significantly at $P < 0.001$.
Where WT is wild type plant, T is transgenic plant, Tg is target gene (Phospholipid transporting ATPase).

Effect of different stress treatments on miRNA novel-8 and its target genes expression

miR novel-8, TG	WT-miR novel-8	T-miR novel-8 (L1)	T-miR novel-8 (L2)	WT- P II	T-Photosystem II (L1)	T-Photosystem II (L2)	WT-MTTF	T-MTTF (L1)	WT-MTTF (L2)
C1	1 **c**	1.87 **c**	1.94**c**	1.01 **c**	0.76 **a**	0.78**a**	1 **c**	0.8 **a**	0.78 **a**
D2	1.35**bc**	4.54**b**	5.66**b**	2.09 **b**	0.45 **b**	0.36**b**	2.02 **b**	0.41 **b**	0.39 **b**
GC3	1 **c**	1.90 **c**	1.99 **c**	1 **c**	0.82 **a**	0.76**a**	1 **c**	0.78 **a**	0.82 **a**
H4	1.70 **b**	4.90**b**	6.01**b**	2.89 **a**	0.35 **b**	0.28**b**	2.76 **a**	0.39 **b**	0.31 **b**
HD5	3.06 **a**	9.17**a**	10.01**a**	2.31 **b**	0.31 **b**	0.23**b**	2.19 **b**	0.21 **b**	0.20 **b**

Any two means not sharing a letter in common differ significantly at $P < 0.001$.
Where WT is wild type plant, T is transgenic plant, T-miR novel-8 L1 is miRna novel-8 expression in first transgenic line, miR novel-8 L2 is miRna novel-8 expression in second transgenic line, Photosystem II is target gene (Photosystem II core complex proteins psbY) and MTTF is target gene (Mitochondrial transcription termination factor family protein).

CURRICULUM VITAE

Arslan ASIM was born on --------in ------, ------. He completed his secondary education from Noukhez Public High School, Multan in 2003. Afterwards, he joined Government Emerson College, Multan, --- and completed his higher secondary education in 2005. Then he was enrolled in Bahauddin Zakariya University, Multan for his undergraduate and postgraduate studies from Department of Horticulture, Bahauddin Zakariya University, Multan Pakistan in 2009 and 2012, respectively. He got admission in Graduate School of Natural and Applied Sciences, Department of Agricultural Genetic Engineering at Niğde Ömer Halisdemir University, Niğde, Turkey in September 2015 to pursue his PhD education under the supervision of Assoc. Prof. Dr. Ufuk DEMIREL. During his PhD thesis research, he worked on the response of some mirnas in potato to combined abiotic stress. He knows English, Urdu, Punjabi, Seraiki, and Turkish languages.

PUBLICATIONS PRODUCED DURING THESIS WORK

ASIM, A., GÖKÇE, Z. N. Ö., BAKHSH, A., ÇAYLI, İ. T., AKSOY, E., ÇALIŞKAN, S., Çalışkan, M.E., and DEMİREL, U. (2021). "Individual and combined effect of drought and heat stresses in contrasting potato cultivars overexpressing miR172b-3p", ***Turkish Journal of Agriculture and Forestry*** 45.5: 651-668, 2021.

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ARSLAN ASIM, Ufuk Demirel, Allah Bakhsh, and Zahide Neslihan Ozturk Gökçe, "Evaluation of miRNA mediated networking and feedback against drought, heat and combined stress tolerance in Potato (*Solanum tuberosum* L.)" in: International Congress on Invitro Biology (SIVB), **San Diego, California, USA** 6-10 June, 2020.