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BORON TOXICITY RESPONSIVE REGULATIONS OF ANTIOXIDATIVE METABOLISM AT PHYSIO-BIOCHEMICAL, TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL LEVELS IN PLANTS

DOĞA SELİN KAYIHAN
A THESIS SUBMITTED FOR THE DEGREE OF
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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

BİTKİLERDE BOR TOKSİSİTESİNE CEVABEN ANTİOKSİDATİF METABOLİZMADA GELİŞEN FİZYO-BİYOKİMYASAL, TRANSKRİPSİYONEL VE POST-TRANSKRİPSİYONEL SEVİYEDEKİ DÜZENLENMELER

DOĞA SELİN KAYIHAN
DOKTORA TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
PROF. DR. YELDA ÖZDEN ÇİFTÇİ

GEBZE
2018

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TEKNİK ÜNİVERSİTESİ

DOKTORA JÜRİ ONAY FORMU

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SUMMARY

Excess boron (B) is one of the major limiting factors for cereal production in the world. It is important to understand the underlying mechanisms of B toxicity responsive regulations in order to facilitate improving B tolerance in plants. Therefore, in this thesis, the antioxidant mechanisms at different biological organizations including physio-biochemical, transcriptional and post-transcriptional levels were elucidated in plants under B toxicity. For this purpose, oxidative damage level, accumulation of non-enzymatic antioxidants, expression levels of antioxidant enzymes and their respective activities as well as expression levels of some related microRNAs were determined in *Arabidopsis thaliana* grown on MS media containing 1 mM B (1B) and 3 mM B (3B) for 14 d. Increased contents of flavonoids, anthocyanins and proline and remarkable induction of total superoxide dismutase (SOD) activity provoked oxidative stress tolerance under 3B. The correlation was found between the expressions and activities of SOD and ascorbate peroxidase (APX). Rather than phi class glutathione *S*-transferase (GST) genes, *ATGSTU19* and *ATGSTZ1* can have role in dramatic increase of total GST activity under 1B. Expression levels of microRNAs related to jasmonate and ethylene metabolisms were induced remarkably by 1B. B toxicity might not affect cell wall modification at post-transcriptional level due to stable levels of *miR397* and *miR408* expressions. Striking results from *Arabidopsis thaliana* were screened in B-sensitive and -tolerant wheat cultivars. Accordingly, *miR172* and its target (*TOE1*) can have role in B tolerance mechanism in plants. These findings might provide new targets for breeding studies aimed for improving B tolerant plants.

Key Words: Boron toxicity, *Arabidopsis thaliana*, antioxidants, transcription, microRNA, wheat (*Triticum aestivum* L.)

ÖZET

Yüksek seviyede bor (B), dünyada tahıl üretimindeki başlıca sınırlayıcı faktörlerden biridir. Bitkilerde B toleransı geliştirmeye olanak sağlamak için B toksisitesine cevaben gelişen düzenlenmelerin altında yatan mekanizmaları anlamak önemlidir. Bu yüzden, bu tezde, B toksisitesi altındaki bitkilerdeki antioksidan mekanizmaları, fizyo-biyokimyasal, transkripsiyonel ve post-transkripsiyonel seviyeleri içeren farklı biyolojik organizasyonlarda aydınlatıldı. Bu amaçla, 14 gün boyunca 1 mM B (1B) ve 3 mM B (3B) içeren MS ortamında büyüyen *Arabidopsis thaliana*'daki oksidatif zarar seviyesi, enzimatik olmayan antioksidanların birikimi, antioksidan enzimlerinin ekspresyon seviyeleri ve aktiviteleri ve bazı ilgili mikroRNA'ların ekspresyon seviyeleri belirlenmiştir. Artan flavonoid, antosiyanin ve prolin miktarları ve total süperoksit dismutaz (SOD) aktivitesindeki dikkate değer artış, 3B koşulunda oksidatif stres toleransını provoke etmiştir. SOD ve askorbat peroksidaz (APX) enzimlerinin ekspresyonları ile aktiviteleri uyumlu bulunmuştur. 1B altında dramatik bir şekilde artan total glutatyon *S*-transferaz (GST) aktivitesinde, phi sınıfı GST genlerinden ziyade, *ATGSTU19* ve *ATGSTZ1* ekspresyon seviyelerinin rolü olabilir. Jasmonat ve etilen metabolizmaları ile ilişkili mikroRNA'ların ekspresyon seviyeleri 1B altında dikkat çekecek derecede uyarılmıştır. *miR397* ve *miR408* ekspresyonlarının sabit kalmasından dolayı, B toksisitesi hücre duvarı modifikasyonunu post-transkripsiyonel seviyede etkilemiyor olabilir. *Arabidopsis thaliana*'da elde edilen dikkat çekici sonuçlar, B-hassas ve B-dirençli buğday çeşitlerinde taranmıştır. Buna göre, *miR172* ve hedeflediği genin (*TOE1*), bitkilerdeki B tolerans mekanizmasında rolleri olabilir. Bu bulgular, B'a toleranslı tahıl bitkileri geliştirmeyi amaçlayan ıslah çalışmalarına yeni hedefler sunabilir.

Anahtar Kelimeler: Bor toksisitesi, *Arabidopsis thaliana*, antioksidanlar, transkripsiyon, mikroRNA, buğday (*Triticum aestivum* L.)

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LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Acronyms		**Explanations**
%	:	Percent
g	:	Gram
mg	:	Miligram
^{o}C	:	Centigrade degree
kg	:	Kilogram
µg	:	Microgram
µl	:	Microliter
bp	:	Base Pairs
ng	:	Nanogram
M	:	Molar
µM	:	Micromolar
mM	:	Milimolar
Mb	:	Megabase pairs
nm	:	Nanometer
µmol	:	Micromol
nmol	:	Nanomol
m	:	Meter
s	:	Second
h	:	Hour
min	:	Minute
cm	:	Centimeter
ml	:	Mililiter
nmol	:	Nanomol
O_2	:	Oxygen
$O_2^{\cdot -}$	:	Superoxide anion
$HO_2^{\cdot}$	:	Hydroperoxide

$OH^{\cdot -}$	:	Hydroxyl radical
$^1O_2$	:	Singlet oxygen
H_2O_2	:	Hydrogen peroxide
H_3BO_3	:	Boric acid
$H_4BO_4^-$	:	Borate anion
1B	:	1 mM H_3BO_3
3B	:	3 mM H_3BO_3
KI	:	Potassium iodide
HCl	:	Hydrogen chloride
$K\text{-}PO_4$	:	Potassium phosphate
Na_2EDTA	:	Disodium EDTA
$MgCl_2$	:	Magnesium chloride
Cu	:	Copper
ε	:	Extinction coefficient
ACT	:	Actin
ANOVA	:	One-way Analysis of Variance
AP2	:	Apetala2
APX	:	Ascorbate peroxidase
AsA	:	Ascorbate
AsA-GSH	:	Ascorbate-glutathione
ATP	:	Adenosine triphosphate
B	:	Boron
BAC	:	Bacterial Artificial Chromosome
B.C.	:	Before Christ
BLAST	:	Basic Local Alignment Search Tool
BOR	:	Boron transporter
BSA	:	Bovine Serum Albumin
CAT	:	Catalase
cDNA	:	Complementary DNA
CDNB	:	1-Chloro-2,4-dinitrobenzene

Chl a : Chlorophyll a

Chl b : Chlorophyll b

CSD : Copper-zinc superoxide dismutase

Cx+c : Carotenoid

DEPC : Diethyl pyro carbonate

DHAR : Dehydroascorbate reductase

DNA : Deoxyribonucleic acid

DTNB : Dithiobis-(2-nitrobenzoic acid)

EAT : Early Activation Tagged

EDTA : Ethylenediaminetetraacetic acid

ERF : Ethylene Responsive Factor

FW : Fresh weight

GAPDH : Glyceraldehyde-3-Phosphate Dehydrogenase

GR : Glutathione reductase

GSH : Glutathione

GSH1 : Glutamate cysteine ligase

GSH2 : Glutathione synthetase

GSSG : Oxidized form of GSH

GST : Glutathione *S*-transferase

JA : Jasmonic acid

LAC : Laccase

LSD : Least Significant Difference

MDA : Malondialdehyde

MDAR 2 : Monodehydroascorbate reductase 2

MDHAR : Monodehydroascorbate reductase

MIP : Major intrinsic protein

miRNA : MicroRNA

mRNA : Messenger RNA

MS : Murashige and Skoog medium

MSD : Manganese superoxide dismutase

MYB : Myeloblast

NAD : Nicotinamide adenine dinucleotide

NADPH : Nicotinamide adenine dinucleotide phosphate

NBT : Nitroblue tetrazolium

NF-Y : Nuclear factor Y

NIP : Nodulin 26-like intrinsic proteins

NPT : Non-protein thiol

PBT : Protein-bound thiol

PCR : Polymerase chain reaction

PCs : Phytochelatins

PCS : Phytochelatin synthase

POX : Peroxidase

PVP : Polyvinylpyrrolidone

qRT-PCR : Quantitative Real Time Polymerase Chain Reaction

RG II : Rhamnogalacturonan II

RNA : Ribonucleic acid

ROS : Reactive oxygen species

RT : Reverse Transcriptase

RT-PCR : Reverse Transcription-Polymerase Chain Reaction

SL : Stem-loop

SOD : Superoxide dismutase

Tae : Triticum aestivum

TAIR The Arabidopsis Information Resource

TBA : Thiobarbituric acid

TCA : Trichloroacetic acid

TCP : Teosinte branched 1, Cycloidea and Proliferating cell nuclear antigen factors

TCHQD : Tetrachlorohydroquinone dehalogenase

TIS : Total Insoluble Sugar

TOE : Target of EAT

TSP : Total soluble protein

TSS : Total soluble sugar

TT : Total thiol

YAC : Yeast Artificial Chromosome

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1. INTRODUCTION

Boron (B) is an essential micronutrient for plants [Warington, 1923]. However, high level of B is one of the important abiotic stress factors in the world and it negatively affects the plant development and crop yield mostly in arid areas [Landi et al., 2012]. Toxic B causes deposition of lignin and suberin in roots [Ghanati et al., 2005], reduced growth of shoots and roots [Lovatt and Bates, 1984; Nable et al., 1990] and inhibition of photosynthesis and stomatal conductance [Lovatt and Bates, 1984; Landi et al., 2013] as well as proton extrusion from roots [Roldan et al., 1992]. Along with these physiological defects, B toxicity can lead to oxidative stress [Cervilla et al., 2007; Ardıc et al., 2009].

Oxidative stress occurs due to overaccumulation of reactive oxygen species (ROS) which induce cell death via oxidizing lipids, pigments, proteins and nucleic acids as well as inactivating enzymes [Blokhina et al., 2003]. In order to overcome harmful effects of ROS, plants use ROS scavenging mechanisms including antioxidant enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (POX; EC 1.11.1.7), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), glutathione reductase (GR; EC 1.6.4.2) and non-enzymatic antioxidants such as glutathione (GSH) and ascorbate (AsA) [Mathews et al., 1984].

Regulation of the enzymatic and non-enzymatic antioxidants under B toxicity was examined in many plant species. For example, increased level of flavonoids and anthocyanins in tomato [Cervilla et al., 2012], higher phenolic content in sweet basil [Pardossi et al., 2015] and significant increases in activities of enzymatic antioxidants in chickpea [Ardıc et al., 2009] were determined under toxic B conditions. Similarly, higher B stress tolerance was provided by higher ROS scavenging capacity in soybean [Hamurcu et al., 2013]. Also, *Brassica* seedlings exhibited increased activity of antioxidative enzymes and enhanced proline concentration against oxidative damage occurred by toxic B supply [Archana, 2013]. However, Karabal and colleagues [2003] suggested that antioxidant enzymes do not play a role in B tolerance mechanism against toxic B level in barley since toxic B concentrations led to membrane damage in a ROS independent manner. Likewise, lipid peroxidation level and hydrogen peroxide content did not decrease depending on the changes in antioxidant enzyme activities in maize [Esim et al., 2012]. As a conclusion, in studies conducted to date, the correlation between B stress and antioxidative regulations has not been clearly revealed because of the variation in antioxidative responses in differents plants [Liu et al., 2005]. For this reason, it is very important to assess toxic B responsive regulations of antioxidative mechanisms at different levels of biological organizations.

1.1. Aim, Contribution and Scope of the Thesis

B toxicity is a lasting agricultural problem by causing important decreases in yield of cereals throughout the world. In Turkey, existence of soils high in B causes to yield losses of barley and wheat [Kalaycı et al., 1998; Torun et al., 2003]. Since soil amelioration is not a practical solution, developing tolerant cultivars is the only way to cope this condition [Roessner et al., 2006]. Even though there are B-tolerant cultivars, breeding programs have not sufficiently succeeded to solve this problem yet [Miwa et al., 2007].

B toxicity and accompanying oxidative stress has been investigated in numerous plants. Though, knowledge about how plants respond to B toxicity is still limited and contradictory [Liu et al., 2005]. One of the reasons of insufficient comprehension of B stress is that most of the examinations were conducted at only one biological organization level. However, a stress is expressed at different sub-organismal levels in the same time in a plant body including physiological, biochemical, cellular and molecular levels [Zhang, 2015]. In this sense, investigating the genes and their expression levels under stress condition confer new insights about stress response along with biochemical pathways, cellular processes and regulatory mechanisms [Clarke and Zhu, 2006]. Utility of a model organism provides to investigate molecular basis of responses against biotic or abiotic stresses [Sharma and Davis, 1997]. Therefore, *Arabidopsis thaliana*, as a model plant, is an excellent system to study B stress effects at different levels in the same time. Thus, the main objective of this thesis was to evaluate B stress responsive regulations of antioxidative mechanisms with an integrative approach including physiological, biochemical, transcriptional and post-transcriptional investigations in *Arabidopsis thaliana*. Secondly, the verification of striking results from *Arabidopsis thaliana* was aimed by using two wheat (*Triticum aestivum* L.) cultivars differing in B tolerance.

Firstly, in order to investigate the oxidative damage in B-stressed *Arabidopsis thaliana*, phenotypic differences, level of lipid peroxidation and content of hydrogen peroxide were determined in this thesis. Then, the concentration of total soluble proteins, proline, anthocyanins, flavonoids, photosynthetic pigments, total soluble and insoluble sugars, GSH, protein-bound, non-protein and total thiols as well as the activities of CAT, APX, GR, SOD, POX and glutathione *S*-transferase (GST, EC 2.5.1.18) were measured in order to identify the antioxidative responses of *Arabidopsis thaliana* under B stress. Afterwards, their regulations were determined at transcriptional and post-transcriptional levels under B toxicity. Finally, regulations of the selected genes were investigated in contrasting wheat cultivars for confirmation of their contribution to B stress-response in cereals. These data can be used for phytoremediation strategies in soils contaminated with excess B.

2. LITERATURE REVIEW

2.1. *Arabidopsis thaliana* as a Model Plant

Arabidopsis thaliana is a small, annual and dicot weedy plant belonging to Brassicaceae family of Brassicales order in Angiosperms. It was discovered in Harz Mountains by Johannes Thal, in 1577 [Kück, 2005]. The great importance of this small plant in molecular biology is arising from its utility as a model plant due to its small genome with 125 Mb pairs and 5 chromosomes, short life cycle, easy cultivation in restricted place, prolific seed production, availability of genetic and physical maps of chromosomes, its efficient transformation and availability of mutants [Sharma and Davis, 1997; Web 1, 2018].

Although suitability of *Arabidopsis thaliana* for genetic studies was proposed in 1940s [Laibach, 1943] and meanwhile, first X-rays induced mutants (in fact, the first *Arabidopsis* mutant was documented in 1873 by Alexander Braun) were isolated, plants were not seen as an experimental choice for molecular studies till 1970s. However, by the key improvements such as mutant screens [Meinke and Sussex, 1979a,b; Somerville and Ogren, 1980; Estelle and Somerville, 1986], discovery of the *Arabidopsis*' small size genome [Leutwiler et al., 1984], cloning of the first *Arabidopsis* gene [Chang and Meyerowitz, 1986], first *Agrobacterium*-based transformation of *Arabidopsis* [Lloyd et al., 1986] and publishing of the first comprehensive genetic map [Koornneef et al., 1983], *Arabidopsis thaliana* has been started to refer as a model system in 1980s [Meinke and Sussex, 1979a]. Since then, *Arabidopsis thaliana* has an increasing popularity along with an increasing number of the studies that combine genetics and molecular biology methods [Krämer, 2015].

The genome of *Arabidopsis thaliana* has been sequenced in 2000. Today, there are extensive cDNA collections, hence ESTs, as well as cDNA and genomic libraries including insert yeast and bacterial artificial chromosomes (YACs and BACs) belonging to *Arabidopsis thaliana*. Moreover, genetic and molecular biology data related to *Arabidopsis thaliana* have been collected in a number of public databases [Sharma and Davis, 1997]. One of them is The Arabidopsis Information Resource (TAIR), linking with "http://www.arabidopsis.org/", that allows to access to all genomic information. TAIR is organically bound to The Arabidopsis Biological

Resource Center where seed and DNA resources of *Arabidopsis thaliana* are collected, reproduced, preserved and distributed.

To conclude, all these resources and experimental advantages make *Arabidopsis thaliana* an excellent system for progressing in plant biology area [Sharma and Davis, 1997] which includes plant development, physiology, metabolism, cell biology, genetics, epigenetics and system biology as well [Krämer, 2015].

2.2. Wheat (*Triticum aestivum* L.) as a Crop

Wheat (*Triticum aestivum* L.) is a monocot plant belonging to *Triticum* genus in Poaceae family of order Poales. It is an annual grass and is widely cultivated for its grain which is mainly processed into human food. Besides the largest cultivation area, wheat is the second most-produced cereal with a 749 million tons production in the world, according to Food and Agriculture Organization in 2016. In fact, it is the most cultivated cereal in Turkey [Soylu et al, 2005].

Triticum is one of the first domesticated crops dating back to 9600 B.C. in the Middle East [Lev-Yadun et al., 2000]. Genetic analyses exhibited that wild einkorn wheat (*T. monococcum ssp. monococcum* L.) was first grown in the Karacadağ Mountains in Turkey.

Among the crops, wheat has the most complicated genetic structure. It can be grouped into three ploidy classes including diploid (2n = 2x = 14), tetraploid (2n =4x = 28), and hexaploid (2n = 6x = 42) (Provan et al., 2004). Most cultivated wheat in the world is the common or bread wheat (*Triticum aestivum* L.) which has an allohexaploid genome (AABBDD) with a size of ~16000 Mb and 90% repeated sequences [Li et al., 2004] whereas durum or pasta wheat is tetraploid (AABB). Most of tetraploid wheat species were originated from wild emmer (*T. dicoccoides*) whose hybridization occurred through natural selection long before domestication. However, hexaploid wheats were formed following farming activities [Hancock, 2004].

Wheat is cultivated in various regions throughout the world due to its high adaptation ability [Caverzan et al., 2016]. However, as in the case of other cereals, its production faces many environmental stresses such as drought, salt, cold, heat, water excess, chemicals and pathogens [Rahaie et al., 2013] as well as boron (B) toxicity.

2.3. Boron (B)

B element was discovered in 1808. Belonging to Group IIIA of the periodic table, B acts as a semi-metal and makes covalent bonds [Kot, 2009]. It is always associated with clay and other impurities as a mineral. Despite its low natural abundance, B has a wide distribution in both the hydrosphere and lithosphere [Morgan, 1980]. B concentration averages 10-20 mg B kg^{-1} in rocks and 1-10 mg B kg^{-1} in sea water [Power and Woods, 1997]. Soils are categorized as low B content when lower than 10 mg B kg^{-1} or as high B content in range 10-100 mg B kg^{-1}. Turkey, as the largest B producer, shares 72.8% (953.3 million tons) of total B reserve (1.31 billion tons of B_2O_3) in the world. Russia and United States of America (USA) follow us with 7.6% and 6.1%, respectively [Web 2, 2018]. Reserves of colemanite, major existing form of B in our country, appear around Kütahya, Balıkesir and Bursa, whereas borax reserves are found in Eskişehir.

B is widely used for industrial purposes including glass, ceramics, frit, detergents, soaps, textile, paper, medicine and cosmetic as well as agricultural purposes such as wood preservatives, fertilizers or pesticides [Web 3, 2018].

B chemistry has evolved based on B-oxygen (O) compounds. Borate ($H_4BO_4^-$) is the main natural compound of B, whereas, less often form is boric acid (H_3BO_3) (Figure 2.1). On the other hand, at physiological pH, more than 98% of B exists as H_3BO_3 and less than 2% as borate anion [Woods, 1996]. Both H_3BO_3 and $H_4BO_4^-$ form borate esters (Figure 2.1) with cis-hydroxyl groups of a wide variety of biomolecules [Loomis and Durst, 1992], such as sorbitol, apiose, ribose and serine [Tate and Meister, 1978]. These binding properties are critical to understand the functional roles of B in plants [Bolanos et al., 2004].

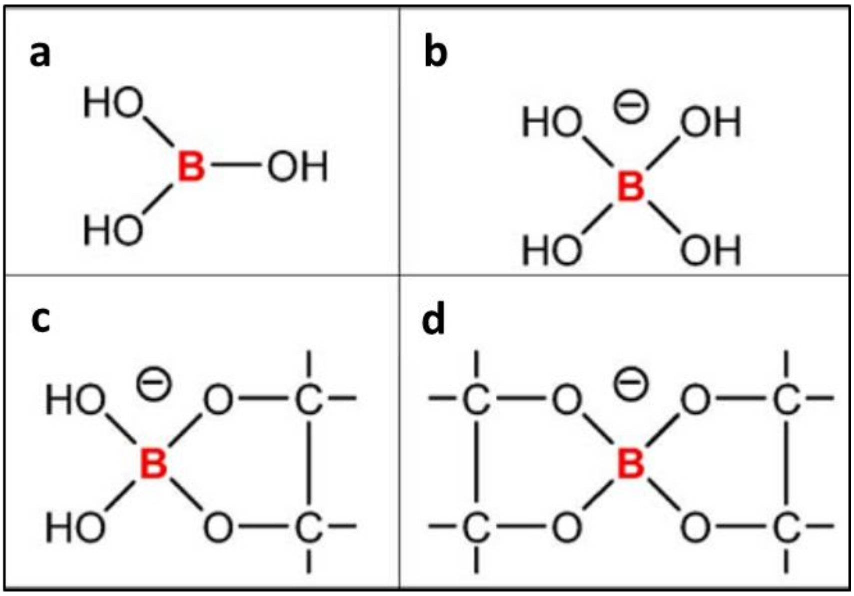

Figure 2.1: Shematic representation of chemical structures of boric acid (a), borate anion (b) and their diol esters (c, d).

2.3.1. B as a Nutrient for Plants

B is an essential micronutrient for vascular plants [Warington, 1923] Nevertheless, its physiological roles in plants are still less well understood than the other nutrients. However, a strong relationship between B and plant cell wall organization has been proposed since a B-polysaccharide complex was isolated and characterized from radish root cell walls [Matoh et al., 1993]. This complex, rhamnogalacturonan II (RG II), is a dimeric fraction in pectin and cross-linked by borate esters (Kobayashi et al., 1996). These B-cross-links occur between two apiose residues of each monomer in the polysaccharide chain [Loomis and Durst, 1992] (Figure 2.2). Furthermore, lack of B led to expansions of pore sizes in cell wall of *Chenopodium album* L suspension cells [Fleischer et al., 1998], whereas pores became smaller following B resupply [Fleischer, 1999]. In other words, beside cell wall structure, B also takes part in modification of the plant cell wall. However, this primary function of B remains insufficient to clarify all impairments seen in B deficiency. Supportively, B is also essential for animals which do not possess cellulosic cell walls. Thus, B is likely to have wider roles in living metabolisms [Brown et al., 2002].

Another crucial role for B is suggested as contribution to cell membrane transport system since B deficiency impaired phosphate and potassium uptake [Parr and Loughman, 1983; Schon et al., 1991, respectively] and H^+ pumping ATPases

[Pollard et al., 1977]. In fact, B may be involved in electron transport [Blaser- Grill et al., 1989] by affecting electrostatics on the plasma membrane. Indeed, B can bind to many membrane compounds including glycolipids, glycoproteins, galactose, mannose, tyrosine and serine due to their hydroxyl groups. Hence, as well as permeability, B is functional in both formation and stability of plant membranes [Cakmak and Römheld, 1997; Brown et al., 2002].

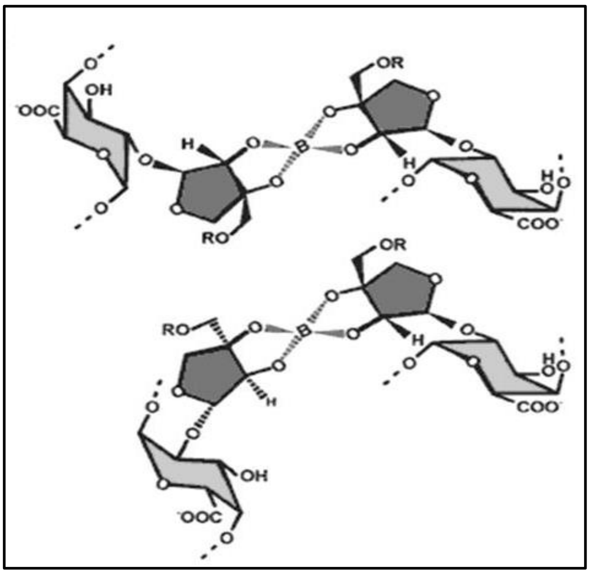

Figure 2.2: Configuration of RG II cross-linked by borate diol ester.

When looking at inside of the cell, behaviors of the cytoskeleton members are affected by B level [Yu et al., 2001]. On the other hand, B is involved in translocation of sugar or sugar-alcohols such as sorbitol and mannitol across the phloem [Hu et al., 1997]. Additionally, plants require sufficient B for reproductive growth containing germination and elongation of pollen tube, flowering, fruiting and seed yield [Blevins and Lukaszewski, 1998], however, contribution of B to especially pollen tube formation was suggested to be structural since high amounts of borate complexes existed in cell walls and membranes of pollens [Jackson, 1989]. Furthermore, B is functional in phenol and ascorbate metabolism as well as nitrogen fixation [Parr and Loughman, 1983] and auxin regulation [Blevins and Lukaszewski, 1998].

2.3.1.1. B Uptake and Transport in Plants

In the soil, 10% of total B is present as undissociated H_3BO_3, which is the main form of B plants uptake from the soil solution [Power and Woods, 1997]. Since high permeability of the cell membrane to B was suggested earlier [Raven, 1980], transfer of B from soil to root cells was thought to occur by only passive diffusion. However, Takano and colleagues [2006] exhibited facilitated B transport by a boric acid channel, NIP5;1, in *Arabidopsis thaliana* especially under limited B condition. NIP5;1 is a member of nodulin 26-like intrinsic proteins (NIP) subfamily belonging to major intrinsic proteins (MIP) family. The same group identified BOR1 protein, which was defined as active (energy-dependent) transport system, and these type proteins were generally named as BOR transporters [Takano et al., 2002]. Unlike NIP5;1, BOR1 is responsible for export of B out of root cells into xylem cells of *Arabidopsis thaliana* under low B treatment. Thus, under B deficiency, BOR1 possibly generates B concentration gradient between xylem and root cells and NIP5;1 subsequently provides increased B import into root cells from environment [Miwa and Fujiwara, 2010]. Thereafter, many AtBOR1-like genes were identified in *Arabidopsis thaliana* [Miwa et al., 2007] as well as other plants including rice [Nakagawa et al., 2007], barley and wheat [Reid, 2007], and even in *Saccharomyces cerevisiae* [Takano et al., 2002]. Moreover, contributions of these BOR1 homologs in toxic B tolerance were suggested due to their ability to reduce B concentration in cells by means of efflux mechanism (Miwa and Fujiwara, 2011).

In plant body, after loaded to xylem, B is likely distributed through transpiration stream [Brown and Shelp, 1997]. Accordingly, B accumulates in the old leaves where high rate of transpiration occurs. In contrast, higher B content was determined in young leaves than mature leaves in some plants. These plants possess great amount of sugar alcohols, by which B form stable complexes and is translocated through phloem [Brown and Shelp, 1997]. Supportively, higher B remobilization capacity was provided by elevated level of sorbitol in transgenic tobacco [Brown et al., 1999].

2.4. B Toxicity in Plants

Despite the great importance for plants, the optimum range of B levels in plant tissues is narrow [Blamey et al., 1997]. This means that B can be readily toxic for many plants even when its level is very slightly higher than that is necessary for normal growing [Mengel and Kirkby, 2001]. Many countries, especially having arid or semi-arid soils, are suffering from yield loss due to excess B [Nable et al., 1997]. For example, 30% of southern Australia soils contain high level of B. Hence, barley yield decreases by 17% in this area [Miwa et al., 2007]. On the other hand, in Turkey's Central Anatolia Plateau where B toxicity is considered as one of the most challenging factor for crop production [Sillanpaa, 1982], 10% of the sampled soils were determined to contain higher B than threshold value of toxicity [Gezgin et al, 2002]. Moreover, 15% of the barley samples taken from farming areas of this region showed light toxicity symptoms, whereas 6% showed medium-severe symptoms [Avcı and Akar, 2005].

B toxicity is mainly seen in low rainfall receiving regions [Power and Woods, 1997]. Some soils naturally contain high level of B [Severson and Gouch, 1983], whereas some areas have become toxic due to irrigation water [Branson, 1976] or fertilizers [Gupta et al., 1976] including high B content.

Typically, toxic B leads to inhibition of shoot and root growth, and to leaf burn in the tips and margins of old leaves which is distinguished by chlorotic or necrotic zones, as visible symptoms [Bennett, 1993; Fitzpatrick and Reid, 2009]. In case of plants transporting B by phloem, symptoms rather appear as bark necrosis as well as internal necrosis in fruits and stem dieback [Nable et al., 1997]. B toxicity symptoms do not occur in roots where lower B accumulates than the other parts of the plants [Nable, 1988].

B concentration within plants varies in a wide range depending on species and cultivars or genotypes in a species [Furlani et al., 2003; Torun et al., 2006; Cervilla et al., 2012] and even in different tissues or different developmental stages of a plant [Mengel and Kirkby, 2001; Roessner et al., 2006]. Grapes, apple, beans, figs and peach are the most sensitive crops to toxic B, whereas most tolerant ones are carrot, cotton, sugarbeet, alfalfa and turnips. Maize, barley, tomato, potato, peas and tobacco can be regarded as semi-tolerant [Mengel and Kirkby, 2001].

Disruption of the plant cell wall is suggested as one of the consequences of B toxicity [Reid et al., 2004]. In concordance with that, excess B led to deposition of suberin in cortical cell walls of soybean roots [Ghanati et al., 2005] and induced lignification rate in tomato roots [Cervilla et al., 2009]. Moreover, B can interfere in some metabolic events because of its binding ability to ribose containing molecules such as adenozin triphosphate (ATP), nicotinamide adenine dinucleotide (NAD), ribonucleic acid (RNA), and even to free ribose as well. Complexation with B inhibits metabolic functions of ATP, NADH and NADPH [Hunt, 2002]. Supportively, reaction capacity of NAD+, NADH and NADP-dependent enzymes were reduced under excess B condition [Reid et al., 2004]. Decreased capacity of ATP could directly arise from complexation with B or indirectly from reduced ability of NAD+ or NADH necessary for ATP synthesis. Furthermore, binding of B to ribose, which is either free or within RNA, could disrupt cell development and division [Reid et al., 2004]. On the other hand, a transient binding of B to the 3’ end of RNA was suggested as a reason for disruption of splicing mechanism [Reid, 2007; Shomron and Ast, 2003].

In addition to these negative effects of B toxicity, inhibition of photosynthesis due to decrease in stomatal conductance was suggested [Lovatt and Bates, 1984]. Limited photosynthesis rate might be arising from structural impairing of thylakoids [Pereira et al., 2000] or combined effect of oxidative damage, impaired electron transport capacity and decreased activity of some photosynthetic enzymes [Han et al., 2009]. However, underlying reasons of B toxicity causing photosynthetic damage are still not clear.

2.5. B Stress-Tolerance Mechanisms

Tolerance capacity to excess B differs depending on plants [Ferreyra et al. 1997] and on even cultivars of the same species [Nable, 1988]. Till now, three main approaches were suggested for plant tolerance to B toxicity including binding of cellular compounds to extra B [Reid et al., 2004], vacuolar compartmentation of extra B and active transport of B from the cell by a transporter [Hayes and Reid, 2004]. Overall, plant B tolerance seems likely correlated with ability of maintaining cellular B content at favorable concentration.

2.5.1. B Transporters

Previously, B tolerance in plants had been associated with restricted uptake of B by roots [Nable, 1988]. However, Hayes and Reid [2004] exhibited that after excess B supply, internal B concentration of a tolerant cultivar was lower than the sensitive one while their B permeability was same in each other. In other words, rather than reduced B uptake, active pumping of B provided tolerance in the tolerant cultivar. In 2002, investigation of the first B transporter, BOR1, in *Arabidopsis thaliana* demonstrated that B could be actively exported from the root cells into shoot cells [Takano et al., 2002]. However, overexpression of this efflux type transporter did not provide tolerance to B toxicity condition, but it gave tolerance for B deficiency in *Arabidopsis thaliana* [Miwa et al., 2006]. Because, under low B supply, BOR1 confers loading of B from root cells into xylem, and so, a concentration gradient between these cells, and subsequently uptake of B from the soil while it is degraded under toxic B [Takano et al. 2005].

After BOR1, another efflux type B transporter was identified and named as BOR4, which was not degraded under B toxicity, unlike BOR1 and its overexpression conferred tolerance to this condition [Miwa et al., 2007]. Furthermore, BOR1 homologs Bot1 and HvBor2 were determined in barley [Sutton et al., 2007; Reid, 2007] and TaBor2 was also found in wheat [Reid, 2007]. Higher expression levels of these genes provided higher B reduction capacity in root cells and resultant B tolerance of these plants [Miwa and Fujiwara, 2011].

Nevertheless, in a study that screened 70 durum wheat genotypes for B tolerance variation, a correlation between B accumulation in shoots and degree of the symptoms was not observed after excess B supply [Torun et al., 2006]. Similarly, elevated level of B concentration in B tolerant wheat cultivars exposed to high B was shown and symptom data were found to be more reliable than measuring B contents in plants [Kalaycı et al., 1998]. Supportively, different tolerance rates despite of similar B contents in tissues of two sweet basil cultivars were attributed to different phenol contents of these plants [Pardossi et al., 2015]. Therefore, rather than lower accumulation of B in tissues, internal mechanisms could also confer tolerance to B toxicity in plants.

2.5.2. Antioxidative Regulations in Response to B Toxicity

2.5.2.1. Oxidative Stress and Antioxidant System

In aerobic organisms, derivatives of atmospheric oxygen (O_2) are constantly formed during the aerobic metabolism [Halliwell and Gutteridge, 1989]. These partially reduced or excited forms of O_2 are defined as reactive oxygen species (ROS) which are continuously removed by scavenging mechanisms in the cells. Under stress conditions, these mechanisms remain inadequate and resultant imbalance between produced and removed ROS leads to oxidative stress [Finkel and Holbrook, 2000]. ROS involve radicals including superoxide ($O_2^{\cdot -}$), hydroperoxide ($HO_2^{\cdot}$) and hydroxyl radical ($OH^{\cdot -}$) as well as non-radicals such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) [Mutlu et al., 2011]. In spite of their deleterious effect, ROS also serve as signal molecules not only in stress condition but also in developmental processes of plants under normal conditions [Davletova et al., 2005].

Plants have an antioxidant system found in all cellular compartments where ROS are immediately scavenged by enzymatic or non-enzymatic members of this system. Thus, cooperation of ROS-producing enzymes and antioxidants maintains homeostasis of ROS through adjusting ROS level depending on the cellular requirement. In *Arabidopsis thaliana*, this network is expressed by at least 289 genes [Gechev et al., 2006].

Major sources of ROS in plants are chloroplasts where electron transport processes produce singlet oxygen and superoxide anion [Noctor and Foyer, 1998]. Another source of ROS is mitochondria in which superoxide anions produced by electron leakage from the electron transport chain. These superoxide anions are dismutated to H_2O_2 and O_2 by superoxide dismutase (SOD) activity. H_2O_2 also appears in apoplast through dismutation of superoxide anions generated by plasma membrane-bound NADPH oxidases [Torres et al., 2002]. Peroxisomes are other organelles where H_2O_2 is largely produced. H_2O_2 is mainly scavenged by ascorbate peroxidase (APX) and catalase (CAT) enzymes. APX detoxifies H_2O_2 by using ascorbate (AsA) as reductor or electron donor which is oxidized to monodehydroascorbate and subsequently to dehydroascorbate (DHA). Afterwards, AsA is reproduced from monodehydroascorbate and DHA by actions of monodehydroascorbate reductase

(MDHAR) and dehydroascorbate reductase (DHAR), respectively. Meanwhile, DHAR uses glutathione (GSH) as substrate and resultant GSSG (oxidized form of GSH) is then re-reduced to GSH by activity of glutathione reductase (GR). This recycling reaction serial is named as Halliwell-Asada pathway or AsA-GSH cycle. The enzymes in this cycle are represented as enzymatic antioxidants whereas AsA and GSH are represented as non-enzymatic antioxidants (Figure 2.3, [Gechev et al, 2006]).

Low levels of H_2O_2 are detoxified by APX or other peroxidases such as glutathione peroxidase and guaiacol peroxidase while its higher levels promote CAT which has a much lower affinity to H_2O_2 [Mittler and Zilinskas, 1991]. CAT is also distinguished from the other peroxidases due to no requirement for a reductant since its function is dismutation [Mhamdi et al 2010].

Elevated levels of ROS that antioxidants could not cope with, are harmful for cells in the way of oxidizing proteins, lipids and even nucleic acids, as well as causing membrane damage [Del Rio et al. 2003]. However, transiently increased moderate levels of ROS protect the biological systems from more severe oxidative stress and thereby provide stress tolerance [Gechev et al, 2006]. For example, H_2O_2 contributes to certain biological processes such as plant-pathogen interactions as a signal molecule [Mhamdi et al., 2010]. In fact, H_2O_2 was found to induce the expression of antioxidant defense genes [Desikan et al., 2001]. Moreover, GSH provides fine-tuned redox balance in either normal or stressed cells and thereby GSH stress signaling [Landi et al., 2012].

Another important but less well studied enzymatic antioxidant is glutathione *S*-transferase (GST) which appears as a ubiquitous detoxification enzyme in aerobic organisms [Dixon et al, 1998]. Although this enzyme has been studied in many species for approximately more than five decades, plant GSTs have taken attention after their protective activity against an herbicide was discovered in maize by Frear and Swanson in 1970, since when, GSTs have been clarified in many other plant species [listed by Öztetik, 2008]. The known primary role of multifunctional GST is to catalyze the conjugation of GSH molecule to electrophilic and usually hydrophobic toxic compounds from which non-toxic peptide derivatives are formed [Dixon et al, 1998]. This GSH transferase reaction is occurred by addition or substitution of GSH. Also, plant GSTs can perform GSH-dependent reactions including peroxidation, isomerization or oxidoreduction [Edwards and Dixon, 2005]. Furthermore, they can function as non-catalytic auxin- or cytokinin-binding proteins [Sappl et al, 2009].

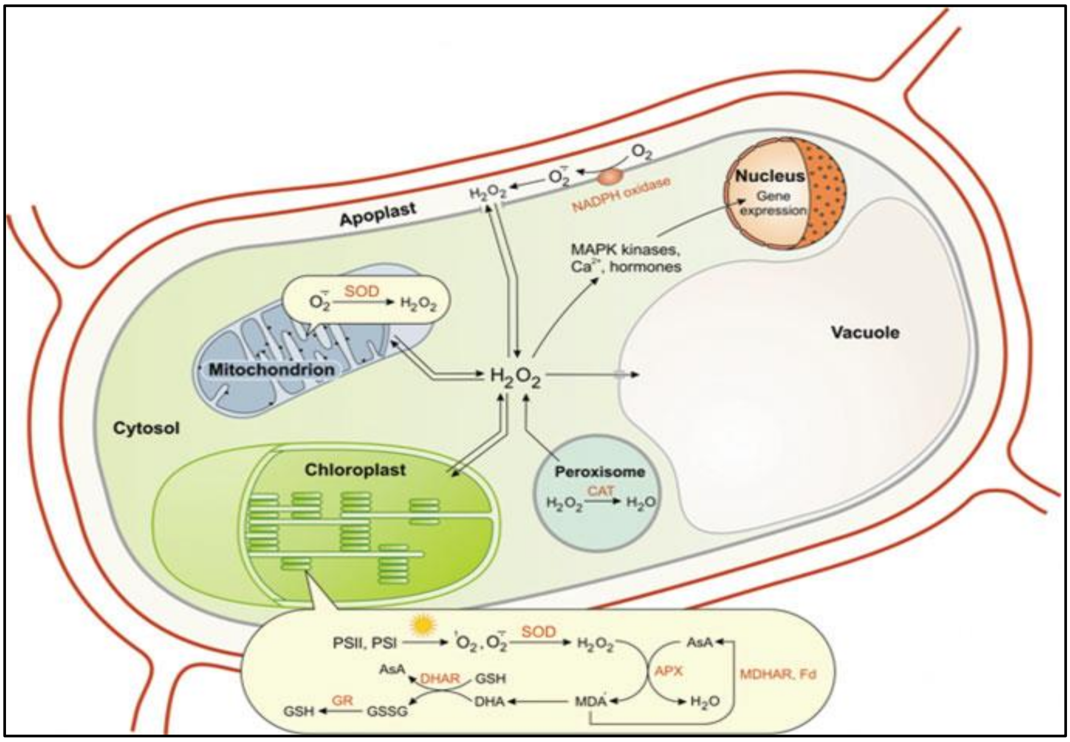

Figure 2.3: Schematic representation of major sources of ROS generation and scavenging enzymes in a plant cell.

There is also evidence for GST mediated non-enzymatic GSH-flavonoid conjugation that prevents oxidation and cross-linking of anthocyanins in cytoplasm and thus, allows vacuolar transport of them [Kitamura et al, 2004]. Stress signaling [Loyall et al., 2000] and regulation of apoptosis [Kampranis et al., 2000] are the other suggested roles for plant GSTs but they are less well known.

Uptill now, plant GST superfamily is classified into eight groups including phi (GSTF), tau (GSTU), zeta (GSTZ), theta (GSTT), lambda (GSTL), dehydroascorbate reductase (DHAR), tetrachlorohydroquinone dehalogenase (TCHQD) and an unclassified microsomal GST [Mohsenzadeh et al., 2011] among them, classes of phi, tau, lambda and DHAR are plant specific whereas theta and zeta classes are also found in animals and fungi [Dixon et al., 1998]. Phi, tau, theta and zeta classes of GSTs are dimeric proteins that stabilize GSH by the conserved serine residue within their active sites and they serve conjugation, peroxidation and isomerization reactions [Labrou et al., 2001]. On the other hand, DHAR and lambda classes of GSTs are monomers and possess cysteine residue in place of serine at the active site that shows lesser conjugation activity to GSH and act as oxidoreductase instead [Öztekin, 2008].

In brief, GST enzymes show a wide diversity in their functions based on their catalytic properties. Likewise, members of plant GST family could display diverse and sometimes individual regulations in response to different kind of stresses. GSTs are affected by amino acid starvation [Zhao et al., 1998], B deficiency [Kobayashi et al, 2004; Wang et al, 2010], heat [Lee et al., 2007], chilling, hypoxia, dehydration, wounding, pathogen attack, ethylene, hydrogen peroxide, salicylic acid and xenobiotics such as herbicides [reviewed by Sappl et al., 2009], nitroaromatics [Gunning et al., 2014], ozone [Sharma and Davis, 1994], salt [Moons, 2003], methyl jasmonate [Wagner et al., 2002] as well as heavy metals such as aluminium [Richards et al., 1998], copper [Smith et al, 2004], arsenic [Ahsan et al., 2008] and cadmium [Lee et al., 2010]. GSTs are believed to counteract the oxidative damage caused by these stress conditions via detoxifying endogenously generated components such as lipid and DNA peroxides by GSH dependent peroxidase activity or, direct conjugation of GSH to the toxic compound [Marrs, 1996], as mentioned above. However, the knowledge about the regulation of plant GSTs and their natural substrates and hence, their role in metabolism still remains insufficient [Bartling et al., 1993; Mohsenzadeh et al., 2011].

2.5.2.2. B Responsive Antioxidative Regulations

Toxic effect of B was reported in many plants. However, oxidative stress and occurring antioxidative responses against B toxicity have been studied only since beginning of 2000. High B level led to oxidative cell damage in various plant species including barley [Karabal et al., 2003; Inal et al., 2009], grapevine [Güneş et al., 2006], chickpea [Ardıc et al., 2009], apple rootstock EM 9 [Molassiotis et al., 2006], tomato [Cervilla et al., 2007; Oluk et al., 2012], *Brassica* [Archana, 2013], zucchini, cucumber [Landi et al., 2013], wheat [Eser and Aydemir, 2016], pepper [Sarafi et al., 2017] and citrus [Sang et al., 2017]. In general, antioxidative responses through B-provoked oxidative stress occur in plants. For instance, stringent control of SOD was suggested in tobacco [Garcia et al., 2001] as a response against excess B. Similarly, increased activity of CAT was determined in barley [Karabal et al., 2003], sunflower [Dube et al., 2000], tobacco [Garcia et al., 2001], hot pepper [Lee, 2006], tomato [Cervilla et al., 2007] and pear [Wang et al., 2011] under high B condition. Moreover, decreased activities of APX in grapevine [Güneş et al., 2006] and POX in sunflower [Dube et al., 2000] were accompanied by increased CAT activity following B toxicity. In contrast, APX was induced whereas CAT activity decreased in citrus [Han et al., 2009], tomato [Oluk et al., 2012] and soybean [Hamurcu et al., 2013] in response to high B. In addition, toxic level of B induced activities of SOD, POX and CAT in *Artemisia* [Aftab et al., 2010] and *Brassica* [Archana, 2013] as well as SOD, APX and CAT in chickpea [Ardıc et al., 2009], wheat [Eser and Aydemir, 2016] and *Arabidopsis thaliana* [Sürgün et al., 2016].

In case of non-enzymatic antioxidants, AsA level increased in potato [Mondy and Munshi, 1993], orange [Keles et al., 2004] and tomato [Cervilla et al., 2007]. On the contrary, high B caused to decrease in the level of AsA in citrus [Han et al., 2009]. Likewise, GSH synthesis was decreased by B toxicity in sunflower [Ruiz et al., 2003]. In contrast, increase in GSH content was determined in tomato [Cervilla et al., 2007] and pear [Wang et al., 2011] following excess B treatment.

Phenolics such as flavonoids and anthocyanins have antioxidative properties including stabilization and delocalization of unpaired electrons, chelation as well as acting as hydrogen or electron donor or as radical quencher [Landi et al., 2012]. High B supply induced phenol levels in tobacco [Ruiz et al., 1998]. Furthermore, in a recent work, complexation of anthocyanins with B and subsequently sequestration of this complex were proposed as B tolerance mechanism [Landi et al., 2014].

Much more examples related to B-responsive regulations of antioxidants can be given in plants. However, some researches demonstrated that plants had a low or non-response to B related oxidative stress [Martínez-Cuenca et al., 2015; Gimeno et al., 2012]. In fact, B toxicity-induced oxidative damage was suggested to occur in ROS-independent manner in barley [Karabal et al., 2003] and watermelon [Hamurcu et al., 2015]. Moreover, antioxidative enzymes were not found to be responsible for B stress tolerance [Karabal et al., 2003; Esim et al., 2012]. Supportively, B toxicity did not cause oxidative damage and remarkable induction of enzymatic antioxidants in wheat [Kayıhan et al., 2017]. Yet, many genes belonging to POX and GST families were differentially regulated in this study. Likewise, upregulation of GSTs was demonstrated by transcriptomic analyses in barley [Öz et al., 2009] and poplar [Yıldırım and Uylaş, 2016; Yıldırım, 2017] as well as by proteomic analysis in *Arabidopsis thaliana* [Chen et al., 2014]. In brief, there is an obvious inconsistency between the results of the studies performed for the changes in antioxidative regulations under high B stress [Liu et al., 2005]. These contradictory results are likely derived from differing antioxidant responses based on plant species [Liu et al., 2005] or even cultivar of a species [Cervilla et al., 2007; Ardıc et al., 2009; Cervilla et al., 2012; Oluk et al., 2012; Landi et al., 2014] as well as on developmental stage [Liu et al., 2005; Cervilla et al., 2012] or even tissue [Molassiotis et al., 2006; Roessner et al., 2006; Oluk et al., 2012] of a plant and even on concentration of B toxicity [Cervilla et al., 2012] and the method (e.g. biochemical or molecular approaches such as transcriptomics, proteomics) used for detection of differential regulations. Hence, the relationship between B toxicity and antioxidative regulations has not been clearly revealed.

2.5.3. miRNA: A New Approach for Understanding Regulation of B Stress Response

To date, numerous studies investigating components of B stress-responsive mechanisms in plants were conducted as mentioned before. These studies were focused on responses either at physio-biochemical level by means of biochemical assays or recently at molecular level via omics technologies. Molecular based experiments revealed many genes that participate to B toxicity response. However, answering the question how genes are regulated during the stress is as important as finding novel genes [Zhang, 2015]. Hence, microRNAs (miRNAs), as key regulators of gene expression in post-transcriptional manner, are good candidates for searching the stress network. These non-coding small RNAs, with a 21 to 24 nucleotide length, have a wide distribution in plants.

The basis of miRNA function is the negative regulation of the target gene expression through blocking its translation by binding to complementary region within the mRNA of this gene [Winter and Diederichs, 2011]. If miRNA align with target mRNA perfectly, fate of mRNA is cleavage whereas imperfect complementarity represses translation of the mRNA into protein due to inhibition of ribosome movement. In plants, inhibition of translation usually occurs as cleavage since most of the miRNAs bind their targets perfectly [Zhang et al., 2006].

After plant miRNAs were discovered in 2002 [Llave et al., 2002] and suggested to have important roles in plant growth and development [Bartel, 2004], further investigation revealing their contribution to environmental stress response [Jones-

Rhoades and Bartel, 2004; Zhang et al., 2005] made miRNAs one of the hot topics. Overexpression of miRNAs such as miR156 and miR169 provided tolerance against heat and drought stress, respectively [Zhang et al., 2011; Stief et al., 2014]. Drought tolerance was improved also by overexpressed miR319 [Zhou et al., 2013], miR394 [Ni et al., 2012] and miR395 [Kim et al., 2010]. In addition to drought, miR319 and miR395 enhanced the tolerance to salinity [Kim et al., 2010; Zhou et al., 2013] and moreover, chilling tolerance was provided by former [Yang et al., 2013] whereas tolerance to oxidative stress and heavy metal stress was provided by latter [Zhang et al., 2013]. Furthermore, some miRNAs were found as responsive to nutrient stress. For instance, upregulation of miR399 and miR395 under deficiencies of phosphorus and sulfate helped to maintain homeostasis of these elements [Kraft et al., 2005; Liang et al., 2010]. A recent work was conducted in *Citrus* exposed to B deficiency and thus, differential regulations of many miRNAs were determined [Lu et al., 2015].

Uptill now, only two studies related to B toxicity responsive miRNAs were performed in plants. These are high-throughput sequencing studies and the first one suggested a critical role for miR408 in B tolerance via regulation of the signal transduction mechanism within barley [Özhüner et al., 2013] whereas the second and very recent one proposed that miR397, targeting secondary cell wall related genes, had a pivotal role in B tolerance of *Citrus* [Huang et al., 2016]. However, there are still large number of miRNAs targeting different genes which need to be determined and their roles for B tolerance remain to be elucidated.

3. MATERIALS AND METHODS

3.1. Plant Material and Growth Conditions

3.1.1. *Arabidopsis thaliana*

Seeds of *Arabidopsis thaliana* ecotype Columbia were provided by Halbay Turumtay from Karadeniz Technical University in Turkey. First, the seeds were surface sterilized with 70% EtOH solution for 2 minutes and then with 15% NaOCl for 10 minutes. The seeds were rinsed three times with sterile distilled water and finally transferred to MS/2 media [Murashige and Skoog, 1962] including normal (100 µM) and toxic levels of B (1 mM and 3 mM H_3BO_3). Following the vernelization period for 3 days at 4 °C, germination and cultivation of plantlets were carried out at 22 ± 2 °C in the plant growth chamber providing supplementary irradiance (300 µmol m^{-2} s^{-1}) with 16-h light photoperiod and 50 ± 5% relative humidity. At the end of the growth period for two weeks, seedlings were harvested and used for further analyses.

3.1.2. Wheat (*Triticum aestivum* L.)

Seeds of the sensitive wheat cultivar Atay and the tolerant wheat cultivar Bolal [Kalaycı et al., 1998] were provided by Field Crops Central Research Institute, Ankara. After one-day imbibition, surface sterilization of seeds was performed with 40% NaOCl for 20 minutes and then, with sterile distilled water for 3 times. Then, seeds were dried and grown in Vitro Vent containers including sterile perlites. Their growth conditions were similar to *A. thaliana* as mentioned above. Seeds were irrigated by liquid MS/2 containing sufficient (100 µM) and toxic levels of B (1 and 3 mM H_3BO_3) for 10 d.

3.2. Physiological and Biochemical Analyses in *Arabidopsis thaliana* seedlings

At the end of the two weeks, phenotypic differences between control and B stressed plants were first determined and then, further physio-biochemical analyses were carried out.

3.2.1. Determination of MDA and H_2O_2 Contents

In order to determine the level of oxidative stress, levels of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) were determined according to Heath and Packer [1968], and Sergiev et al. [1997], respectively. Following recording the fresh weights of the seedlings, they were homogenized with 3 ml 0.1% trichloroacetic acid (TCA) and were centrifuged at 11,300 *g* for 15 min at 4 °C and supernatants were used for both MDA and H_2O_2 determination. For the determination of MDA content 0.25 ml of 0.1 M Tris/HCl (pH 7.6) and 0.5 ml of TCA-TBA-HCl reagent (15% TCA, 0.375% thiobarbituric acid (TBA), 0.25 M HCl) were added to 0.25 ml of the supernatant and incubated at 95 ^{o}C for 45 min and then these mixtures were put into into ice and subsequently centrifuged at 11,300 *g* for 5 min. Absorbance was measured at 532 and 600 nm. MDA level was calculated using its ε of 155 $mM^{-1}cm^{-1}$ and expressed as nmol/ml/g fresh weight (FW). For the determination of H_2O_2, 0.25 ml of 0.1 M Tris/HCl (pH 7.6) and 0.5 ml of 1 M KI were added to 0.25 ml of the supernatant and incubated for 90 min in dark. Absorbance was measured at 390 nm and concentration of H_2O_2 was calculated using its ε of 0.28 $\mu M^{-1}cm^{-1}$ and expressed as μmol/ml/g FW.

3.2.2. Proline Content

The proline content was determined according to the method of Bates et al. [1973]. The seedlings were homogenized in a mortar with 1 ml 3% sulphosalicylic acid. The extracts were centrifuged at 18,300 *g* for 5 min at 4 ^{o}C. 0.2 ml acid ninhydrin (freshly prepared 12.5 ml acid ninhydrin solution included 0.31 g ninhydrin, 7.5 ml acetic acid and 5 ml 6 M phosphoric acid), 0.2 ml 96% acetic acid, 0.1 ml 3% sulphosalicilic acid and 0.1 ml supernatant of the centrifuged extracts were mixed in a new tube. The tubes were incubated at 96 ^oC for 60 min and then 1 ml toluene was added into the tubes. Subsequently, tubes were centrifuged at 18,300 *g* for 5 min. Absorbance of the upper red phase of the centrifuged samples was read at 520 nm against toluene. The amount of proline was calculated using pure proline as standard curve (0.01 mM-1.5 mM) and the result was expressed as mmol/g FW.

3.2.3. Determination of Photosynthetic Pigments

Contents of photosynthetic pigments were determined according to Lichtenthaler [1987]. Two seedlings from each plate were transferred to tubes. Then, 2 ml of pure acetone was added onto each seedling in the tubes. They were incubated at 4 ºC for one night and releasing green color was read at 661.6, 644.8 and 470 nm. The contents of pigments were calculated according to the following equations and the result was expressed as µg/ml/g FW.

$$\text{Chlorophyll a (Chl a)} = (11.24 \times A661.6) - (2.04 \times A644.8) \tag{3.1}$$

$$\text{Chlorophyll b (Chl b)} = (20.13 \times A644.8) - (4.19 \times A661.6) \tag{3.2}$$

$$\text{Total chlorophyll (Chl a+b)} = (7.05 \times A661.6) + (18.09 \times A644.8) \tag{3.3}$$

$$\text{Carotenoid (Cx+c)} = [(1000 \times A470) - (1.9 \times \text{Chl a}) - (63.14 \times \text{Chl b})]/214 \tag{3.4}$$

3.2.4. Determination of Anthocyanin Content

The level of anthocyanin was measured according to Mancinelli [1975]. Frozen samples were homogenized with 1 ml extraction buffer (12 ml 100% methanol, 2.6 ml dH_2O, 400 µl 37% H_2O_2). The extracts were taken into tubes and incubated at room temperature for 10 min. After vigorous shaking, they were centrifuged at max speed for 5 min and absorbances of supernatants were read at 530 and 657 nm against buffer. The content of anthocyanins was found by following equation.

$$\text{Anthocyanin content} = [A_{530}-(A_{657}/3)]/FW \tag{3.5}$$

3.2.5. Flavonoid content

Flavonoid content was determined according to Krizek and Britz [1998]. Frozen samples were extracted in a mortar with 2 ml solvent of ethyl alcohol: acetic acid (99:1, v/v). The homogenates were centrifuged at 15,000 *g* for 10 min. The supernatants were transferred to new tubes and gently boiled for 10 min in a water bath at 80 °C. After cooling of samples, the absorbances were measured at 270, 300 and 330 nm against the solvent. Flavonoids contents were calculated cumulatively considering the ε of 33.000 M^{-1} cm^{-1} and results were expressed as μM/FW g.

3.2.6. Determination of Total Soluble and Insoluble Sugar Contents

Total soluble and insoluble sugar contents were determined by using anthrone reagent [Malik and Srivastava, 1979]. Weighed samples were ground in mortars with 2 ml 80% ethanol. The homogenates were collected in tubes and incubated for 30 min in a heatblock at 80 °C. After incubation, they were centrifuged at 3,000 *g* for 10 min. Supernatants were used for further analyses of soluble sugars (glucose, fructose, sucrose) while pellets were used for determination of insoluble sugar (starch). In order to determine soluble sugar content, 1.8 ml of 0.2% anthrone solution (0.3 g anthrone dissolved in 150 ml precooled sulphuric acid 98%) were added on to 0.2 ml supernatant in a tube. Then, tubes were incubated in boiling water for 7.5 min and cooled on ice. Absorbance was read at 625 nm and soluble sugar content was calculated using standard of D-glucose. The contents were expressed as mg/ml/g FW. In order to determine insoluble sugar content, 1.5 ml of precooled 52% perchloric acid were added onto pellets. After incubation at 100 °C in water bath for 10 min, samples were centrifuged at max speed. Then, 0.2 ml of supernatants were transferred into new tubes and mixed with 1.8 ml of 0.2% anthrone. Then, tubes were incubated in boiling water for 7.5 min and cooled on ice. Absorbance was read at 625 nm and insoluble sugar content was calculated using standard of D-glucose. The result was expressed as mg/ml/g FW. D-glucose standards (0.011, 0.022, 0.044, 0.066, 0.088, 0.11 mg/ml) were prepared from D-glucose stock solution (11mg/100ml). Then, 0.2 ml of standards were transferred into new tubes and mixed with 1.8 ml of 0.2% anthrone. The tubes were incubated in boiling water for 7.5 min. When cooled, standard absorbances were measured at 625 nm and used for generating standard curve.

3.2.7. Determination of Glutathione, Non-Protein, Protein-Bound and Total Thiol Contents

Total thiol analysis was carried out according to Cai and colleagues [2004]. Frozen seedlings were ground and homogenized with 0.02 M EDTA, and centrifuged at 16260 *g* at 4 ^{o}C for 10 min. The supernatants (0.4 ml) were mixed with 0.2 M Tris buffer (pH 8.2, 0.3 ml), 0.01 M 5-5'- dithiobis-2-nitrobenzoic acid (DTNB) (20 µl), and methanol (1.58 ml) and then they were incubated for 20 min in dark at 22°C. Absorbance was measured at 412 nm against blank (without adding supernatant). Total thiol concentration was calculated by using ε of 13,100 M^{-1} cm^{-1} and expressed as nmol/FWg.

The GSH level was determined according to Anderson (1985). Frozen seedlings were homogenized in 1.5 ml of 5% (w/v) sulfosalicylic acid. The homogenate was centrifuged at 12000 *g* for 20 min. Supernatant was used for subsequent analyses. 1 ml supernatant, 1 ml of 100 mM K-PO4 buffer (pH 7.0) including 0.5 mM Na_2EDTA and 100 µl of 3 mM DTNB were added and shaked thoroughly. Following 5 min, the absorbance was used at 412 nm. It was expressed as absorbance per FW.

In order to determine the level of non-protein thiol [Del longo et al., 1993], 1 ml of 100 mM phosphate buffer (pH 7.0) containing 0.5 mM Na_2EDTA and 1 ml of 3 mM DTNB were added on to 200 µl supernatant and shaked thoroughly. After 10 min, absorbance was taken at 412 nm. Non-protein thiol concentration was calculated by using an ε of 13,100 and expressed as nmol/FW g. Protein-bound thiol content was calculated by subtracting the content of non-protein thiols from total thiols.

3.2.8. Determination of Total Soluble Protein Content

Total soluble protein content was determined following to Bradford method [Bradford, 1976]. Weighed and frozen samples were extracted in mortars with 1 ml common suspension buffer (50 mM potassium phosphate (K-PO_4) buffer (pH 7.8) including 1 mM EDTA and 2% PVP). Extracts were centrifuged at 13,000 *g* for 30 min at 4°C. Supernatants were transferred into new tubes and kept on ice. Bradford solution (1X) was filtered before use. Bovine serum albumin (BSA) standards were prepared as concentrations of 0, 0.01, 0.02, 0.04, 0.06, 0.1, 0.16 mg/ml. Then, 5 ml Bradford were added to 0.5 ml of BSA standards and 0.5 ml of samples (25 times diluted). All samples and standards were prepared as 2 technical replicates. After vigorously shaking, the tubes were incubated at room temperature for 10 min in dark. Absorbances were read at 595 nm and standard curve was generated according to concentrations of BSA. The protein concentrations of samples were calculated by dividing of sample absorbances to slope of the standard curve and multiplying by the dilution factor. Total soluble protein contents were expressed as mg/ml/g FW and used for the enzyme activity experiments in order to load equivalent amounts of protein.

3.2.9. Determination of Antioxidant Enzyme Activities

CAT activity was determined according to the method of Chance and Maehly [1955]. Reaction medium included 50 mM K-PO_4 buffer (pH 7.0), 10 mM H_2O_2, and supernatant containing 100 µg protein. The reduction in absorbance was measured at 240 nm for 1.5 min (ε=39.4 $mM^{-1}cm^{-1}$). Activity of CAT was expressed as nmol/mg H_2O_2 destroyed per min.

APX activity was measured according to the method of Wang and colleagues [1991]. Reaction mixture contained 50 mM K-PO_4 buffer (pH 6.6), 0.25 mM ascorbate, 1 mM H_2O_2 and supernatant including 100 µg protein. The reduction in absorbance was measured at 290 nm for 1.5 min (ε=2.8 $mM^{-1}cm^{-1}$). Activity of APX was defined as nmol/mg oxidized ascorbate per min.

GR activity was determined according to the method of Sgherri and colleagues [1994]. The reaction medium consisted of 200 mM K-PO_4 buffer (pH 7.5) including 0.25 mM Na_2EDTA, 1.875 mM $MgCl_2$, 0.50 mM GSSG, 50 µM NADPH and supernatant containing 100 µg protein. NADPH oxidation was measured by recording the absorbance at 340 nm continuously for 1.5 min (ε=6.2 $mM^{-1}cm^{-1}$). Activity of GR was defined as nmol/mg oxidized NADPH per min.

Peroxidase (POX) activity was determined following the method of Quesada and colleagues [1990]. Reaction mixture contained 50 mM K-PO_4 buffer (pH 7.0), 0.02% o-dianizidine, 10 mM H_2O_2 and supernatant including 100 µg protein. The oxidation of reduced o-dianizidine was measured at 460 nm for 2.5 min (ε=2.8 $mM^{-1}cm^{-1}$). Activity of POX was defined as nmol/mg oxidized o-dianizidine per min.

GST activity was determined following the method of Habig and colleagues [1974]. Assay medium included 125 mM KPO_4 buffer (pH 7.8), 1 mM GSH, 1 mM 1-Chloro-2,4-dinitrobenzene (CDNB) and supernatant including 300 μg protein. Absorbance was recorded at 340 nm against the blank for 2.5 min. Activity of GST was calculated using the ε 9.6 $mM^{-1}cm^{-1}$, and expressed as nmol/mg product per min.

SOD activity was determined according to Gong et al. [2005]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8) including 100 mM Na_2EDTA, 1.3 mM L-methionine, 7.5 mM nitroblue tetrazolium (NBT), 0.4 mM riboflavin and enzyme extract containing 50 mg protein. The reaction was started by adding riboflavin solution and placing the tubes under white light for 15 min. A complete reaction mixture without enzyme, which gave the maximal color, served as a control. The reaction was stopped by switching off the light and putting the tubes in the dark. A non-irradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm and one unit of enzyme activity was considered as the amount of enzyme that reduced the absorbance reading to 50% in comparison with enzyme free tubes [Sairam and Srivastava, 2000].

3.3. Molecular Analyses in *Arabidopsis thaliana* Seedlings

3.3.1. Total RNA isolation

Total RNA was isolated by using TRIzol reagent (Ambion) according to Chomczynski and Sacchi [1987] from two-week-old *Arabidopsis thaliana* seedlings. Control and B-treated seedlings were ground in mortars with liquid nitrogen and approximately 0.2 g powder was transferred to pre-cooled eppendorf tubes. Subsequently, 1 ml of TRIzol reagent was added and vortexed for 15 min at room temperature and then the tubes were centrifuged for 5 min at maximum speed at room temperature. After 900 ml of the supernatant was transferred to a new tube and 180 ml chloroform was added, the tubes were vortexed and incubated at room temperature for 3 min. At the end of the incubation, it was centrifuged for 15 min at 4 °C at maximum speed. 450 ml from the upper phase was transferred into a new tube and 200 ml chloroform was added. The tubes were vortexed and then incubated at room temperature for 3 min. It was then spinned for 5 min at room temperature at maximum speed and pipetted 400 ml from the upper phase and transferred to a new tube. Equal volume of isopropanol was added and the tubes were inverted for several times and then incubated for 10 min at room temperature. Tubes were centrifuged for 10 min at room temperatures at maximum speed and supernatants were then removed. The pellets were washed with 1 ml 75% ethanol and left for 3 min and spinned down for 5 min at room temperature at maximum speed and then the supernatant was removed. In order to remove ethanol, it was centrifuged for an additional 15 s and air dried for 10 min. Finally, 50 ml DEPC-treated water was added and incubated for 15 min at 65 ^{o}C to dissolve pellet. Agarose gel electrophoresis was used to check the quality and integrity of the RNA. Then concentrations and quality of total RNAs were determined by NanoDrop (BioSpec-nano, Shimadzu Biotech, Japan). Total RNAs were kept at -80 ^{o}C until use.

3.3.2. Quantitative Real Time PCR (qRT-PCR)

Complementary DNAs (cDNAs) were obtained from total RNAs by using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) and were directly used for quantitative real time polymerase chain reaction (qRT-PCR).

qRT-PCR was conducted using Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific) on StepOnePlus™ Real-Time PCR System (Applied Biosystems) in order to measure and compare the expression levels of interested genes in *Arabidopsis thaliana* under control and toxic B conditions. For qRT-PCR analysis, 1 μl of cDNA product, 7 μl of 2X Master Mix, 0.3 μM final concentration of primers were supplied to 15 μl total volume with nuclease-free water. Primer names and sequences were given in Table 3.1. Primers were specifically designed according to coding sequence of *Arabidopsis thaliana* by the means of NCBI and Primer3 primer designing tool. The qRT-PCR conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 59 °C for 30 s and 72 °C for 30 s. The melting curve was adjusted as 95 °C for 15 s, 60 °C for 1 m and 95 °C for 15 s. The expression levels were normalized against to ACT2 and then relative fold change of expression for each comparison was calculated by $2^{-deltaCt}$.

Table 3.1: Primer list for *Arabidopsis thaliana* genes.

Primer name	Sequence 5’ to 3’
APX6_F	GGACGATCATTCTGGTGGCA
APX6_R	GGGCCGCCACATATTGAAAC
ATGR1_F	GGAGGAATTCGTGGCAGATG
ATGR1_R	ACCAAAAGCAGTGTTCGCAA
ATGR2_F	TCAGGACTTCCAGACCGAGT
ATGR2_R	GGTGAACACCCACTGTAGCA
ATGSH1_F	CAGTTCGAGCTTAGTGGTGC
ATGSH1_R	ATATCCTCCCGACGCCATTT
ATGSH2_F	CGTTTCCTGGCCTTAGTCGT
ATGSH2_R	CATGACTACCGCTCTTGGGT
ATGSTF2_F	AGTTTTCGGACACCCAGCTT
ATGSTF2_R	TGGTCAAGCCGTAGATGGA
ATGSTF6_SV1_F	GCTTGGGTTGCTGACATCAC
ATGSTF6_SV1_R	TTCAAATCAAACACTCGGCAGC
ATGSTF6_SV2_F	GCCGTTTGTTTTTGATCTCTCT
ATGSTF6_SV2_R	AAGGGGTTGCGAAGGATGAA
ATGSFT6_SV3_F	CTCAACTGGCAAGGACATGG
ATGSTF6_SV3_R	TCACCAAGCCTGTGTTCGTA
ATGSTF7_F	ATCTTCCGCAACCCTTTTGGTA
ATGSTF7_R	GGAGCCAAGGGAGACAAGT
ATGSTF8_F	GATCATCATGGCCAGTATCAAGG
ATGSTF8_R	GCTCTTGACTCGAAAAGCGTC
ATGSTU19_F	GGGATGAGGACAAGGATCGC
ATGSTU19_R	CCTCTGAGCATCATACAGCTTCT
ATGSTZ1_F	ACCCTGAGCCACCTTTGTTA
ATGSTZ2_R	TAACCCAGGCAGTCTTCTCC
ATPCS1_F	CTCCTCCGGCCATTGACTTT
ATPCS1_R	ACCTCCAAGGCCCTTTCCAT
CAT1_F	TGTCCTGCTCTTGTGGTTCC
CAT1_R	TCCCGATGAAGCACTTCTCAC
CSD1_F	TGCATGTCTACTGGTCCACA
CSD1_R	GAGGTCATCAGGGTCTGCAT
DHAR2_F	GGCGACTGTCCGTTTAGC
DHAR2_R	TCTCGGATCCGTCATTAGCG
MDAR2_F	CGCTGAACAAGCCGTAAAGG
MDAR2_R	TTTGGCGATTCCGGGTCATT
MSD1_F	AAATTCAACGGCGGAGGTCA
MSD1_R	TCAAGGGAGCCAAAGTGAGC
ACT2_F	CTTGACCTTGCTGGACGTGA
ACT2_R	AATTTCCCGCTCTGCTGTTG

3.3.3. Stem-Loop qRT-PCR

Expressions of microRNAs (miRNAs) were detected using stem-loop qRT-PCR method [Varkonyi-Gasic et al., 2007] with modification. It was carried out in a 12 µl total volume including 1 µg of total RNA, nuclease free water and 1µl stem-loop (SL) primer mix (2 µM). These mixtures were incubated at 65 °C for 5 min and subsequently, were taken to ice for 2 min. Then, 4 µL 5X Reaction Buffer, 2 µl of 10 mM dNTP, 1µl RiboLock RNase Inhibitor (20 U/µl) and RevertAid RT (200 U/µl) were added to each mixture (Thermo Scientific RevertAid First Strand cDNA Synthesis Kit). The reactions were incubated for 30 min at 16 °C followed by pulsed RT of 60 cycles at 30 °C for 30 s, 42 °C for 30 s and 50 °C for 1 s. The reaction was then terminated by incubating the tubes at 70 °C for 5 min. Negative control tubes included all components except RT enzyme or RNA template. The cDNAs obtained from stem-loop pulsed RT reaction were directly used for qRT-PCR as explained in section 3.3.2. The expression levels of miRNAs were normalized against to ACT2 and then, relative fold change of expression for each comparison was calculated by $2^{-deltaCt}$. miRNA sequences were obtained from miRBase and stem-loop RT and forward primers were specifically designed according to protocol of Varkonyi-Gasic and colleagues [2007] (Figure 3.1). Primer sequences for miRNAs were given in Table 3.2.

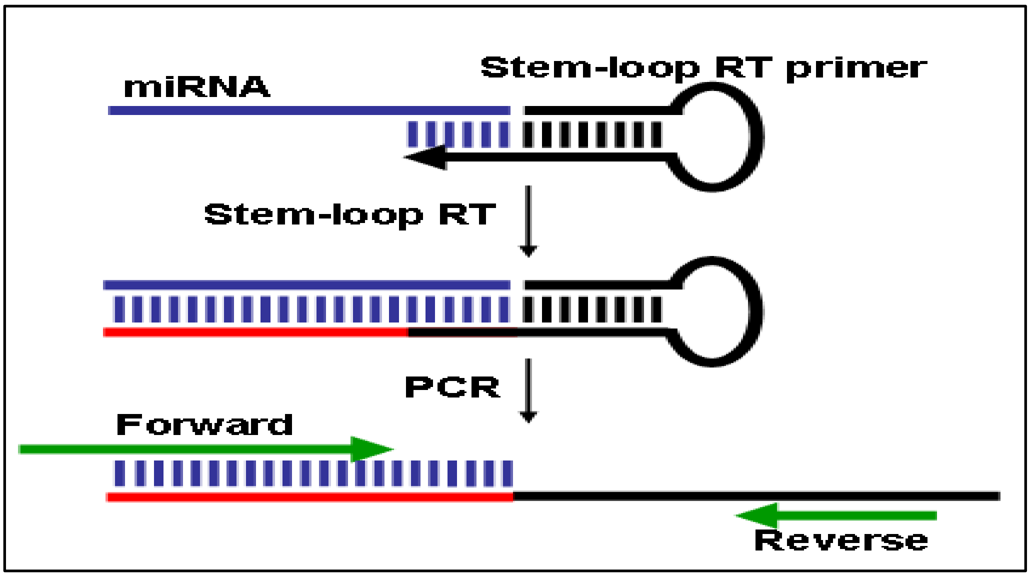

Figure 3.1: Schematic representation of stem-loop RT-PCR for miRNA assays.

Table 3.2: Primer list for *Arabidopsis thaliana* miRNAs.

Primer name	Sequence 5’ to 3’
miR398_SL RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGGGG
miR398_F	CGGCGGTGTGTTCTCAGGTCA
miR408_SL RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCCAGG
miR408_F	TGGCGGATGCACTGCCTCTTC
miR397_SL RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCATCAA
miR397_F	CGATATTCATTGAGTGCAGCG
miR156_SL RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGCTC
miR156_F	AGGCGGTGACAGAAGAGAGT
miR172_SL RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGCAG
miR172_F	CGGCGGAGAATCTTGATGATG
miR169_SL RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCGGCT
miR169_F	GATATGCAGCCAAGGATGACT
miR159_SL RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGAGC
miR159_F	CGGCGGTTTGGATTGAAGGGA
miR319_SL RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGGAG
miR319_F	CGGATATTGGACTGAAGGGAG
miR394_SL RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGAGGT
miR394_F	GGTAGTTTGGCATTCTGTCC
Universal_R	GTGCAGGGTCCGAGGT

3.4. Biochemical Analyses in Wheat Cultivars

Total soluble proteins were extracted from leaves of wheat cultivars by Bradford method [Bradford, 1976] as explained in section 3.2.8. Afterwards, GST activities of wheat cultivars were determined following the method of Habig and colleagues [1974] as explained in section 3.2.9.

3.5. Molecular Analyses in Wheat Cultivars

Total RNAs were isolated from 10-day-old seedlings of wheat cultivars as explained in 3.3.1. Leaf samples were ground in 2 ml-eppendorf tubes by Retsch MM400 grinder. Concentrations and quality of total RNAs were determined by NanoDrop (BioSpec-nano, Shimadzu Biotech). Total RNAs were kept at -80°C until use.

3.5.1. Computational Prediction of miRNA Targets and Primer Design

Computational target for miR172 was determined as “target of EAT1-B1 (TOE1-B1)” according to psRNATarget and named as Tae_TOE1. Computational target for miR319 was determined as Tae_MYB3 according to a study of Kumar and colleagues [2015]. Computational target for miR398 was determined as “Triticum aestivum cultivar HD 2009 cytosolic Cu/Zn superoxide dismutase mRNA” and named as Tae_CSD.

Primers for targets were specifically designed according to coding sequence of *Triticum aestivum* by means of NCBI and Primer3 primer designing tool. Primers for miRNAs designed according to sequences presented in miRBase. Sequences of primers were given in Table 3.3.

Table 3.3: Primer list of genes and miRNAs used for wheat cultivars.

Primer name	Sequence 5’ to 3’
Tae_miR398_SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGGGGG
Tae_miR398_F	CGGTGGTGTGTTCTCAGGTCG
Tae_miR172_SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGCAG
Tae_miR172_F	CGGCGGAGAATCTTGATGATG
Tae_miR319_SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGGAG
Tae_miR319_F	CGGATATTGGACTGAAGGGAG
Tae_GSTZ1_F	CCTCAGCATCCTCTCGTACC
Tae_GSTZ1_R	CTCAAGGCTCTCATCGGGG
Universal_R	GTGCAGGGTCCGAGGT
Tae_Actin_F	AGGTTCAGGTGCCCTGAGGT
Tae_Actin_R	CAACATCACACTTCATGATGGA
Tae_GAPDH_F	TTCAACATCATTCCAAGCAGCA
Tae_GAPDH_R	CGTAACCCAAAATGCCCTTG
Tae_U6_F	CCCTTCGGGGACATCCGATA
Tae_U6_R	CTCAAAAAAATTTGGACCATTTCT
Tae_MYB3_F	AAACTGCGGCTTCATGGATC
Tae_MYB3_R	TGAGAACTGATCGACCGAGG
Tae_TOE1_F	CCGGTGGCCAAGAAGAGC
Tae_TOE1_R	TTGCCGCAATCCCAGATATG
Tae_CSD_F	GCACCATCTTCTTCACCCAG
Tae_CSD_R	AGGGTTGAAGTGTGGTCCAG

3.5.2. Reverse Transcription-PCR (RT-PCR), qRT-PCR and Stem-Loop qRT-PCR

cDNAs for GSTZ1, target genes and internal controls (Actin, GAPDH and U6) were obtained from total RNAs by using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). In order to ensure for accurate cDNA synthesis and primer design, RT-PCR was carried out. 1.5 µl of cDNA product was amplified with 5x FIREPol® Master Mix (Solis BioDyne) with 12.5 mM $MgCl_2$, and specific primers (0.4 µM), together with in a 20 µl total volume of PCR reaction. The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 40 s, 55 °C for 30 s and 72 °C for 45 s, and final extension at 72 °C for 5 min. PCR products were run on 1% agarose gel and visualized by Image Lab.

qRT-PCR was conducted using Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific) on StepOnePlus™ Real-Time PCR System (Applied Biosystems). For qRT-PCR analysis, 1 µl of cDNA product, 7 µl of 2X Master Mix, 0.3 µM final concentration of primers were supplied to 15 µl total volume with nuclease-free water. The qRT-PCR conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 59 °C for 30 s and 72 °C for 30 s. The melting curve was adjusted as 95 °C for 15s, 60 °C for 1m and 95 °C for 15s. The expression levels were normalized against to Actin and then relative fold change of expression for each comparison was calculated by $2^{-deltaCt}$.

Expression of wheat miRNAs were detected using stem-loop qRT-PCR method of Varkonyi-Gasic et al. [2007] as explained in 3.3.3.

3.6. Statistical Analyses

All experiments were repeated four times and each replicate contained at least 15 seedlings. The obtained data were statistically analyzed by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test using SPSS statistical programme and presented as mean ± SEM. $p \leq 0.05$ was considered as significant. Data of qRT-PCR were analyzed by t-test and expressed as mean ± SEM ($p \leq 0.05$).

4. RESULTS

4.1. Physio-Biochemical Changes in *Arabidopsis thaliana* Exposed to B Toxicity

The growth of *Arabidopsis thaliana* seedlings was reduced under both B toxicity conditions and chlorosis was observed in their leaves (Figure 4.1).

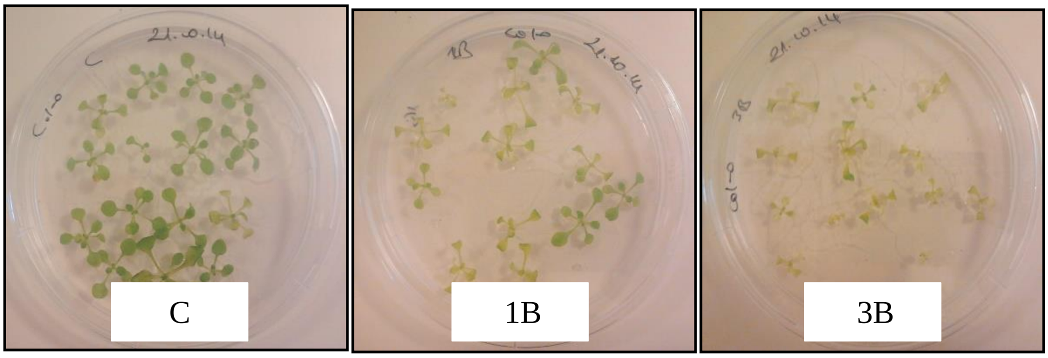

Figure 4.1: Phenotypic differences in 2 weeks old *Arabidopsis thaliana* seedlings exposed to B toxicity. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3.

A remarkable increase in H_2O_2 content was determined after 3mM B treatment (3B) but not after 1mM B treatment (1B) in comparison to controls (C) (Figure 4.2). A significant increase in lipid peroxidation level based on MDA content was observed in only 1B when compared to respective control plants (Figure 4.3). On the other hand, 1B did not lead to any significant change in free proline content, however, it increased two-fold under 3B compared to control condition (Figure 4.4). Similarly, sugar contents were not significantly changed in response to 1B, whereas 3B caused an increase in both total insoluble and soluble sugar contents (Figure 4.5-6).

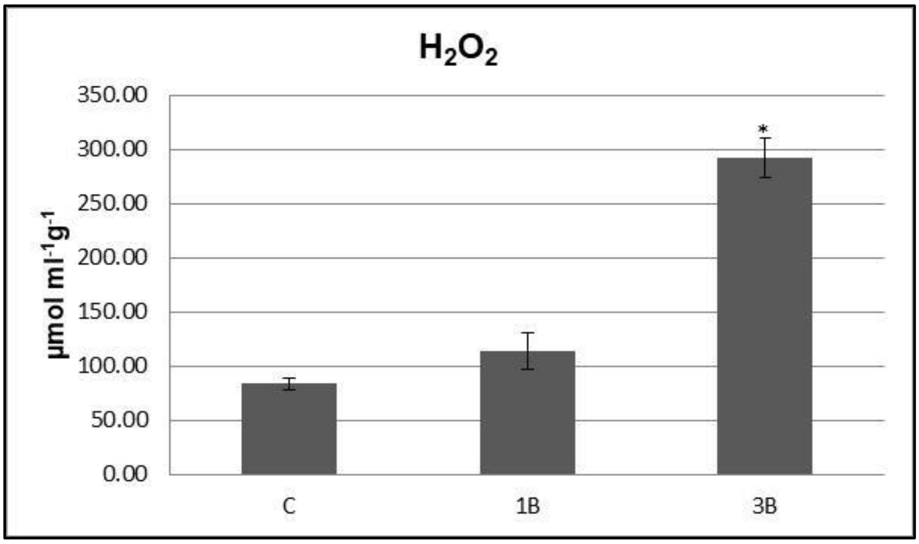

Figure 4.2: Changes in H_2O_2 contents of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

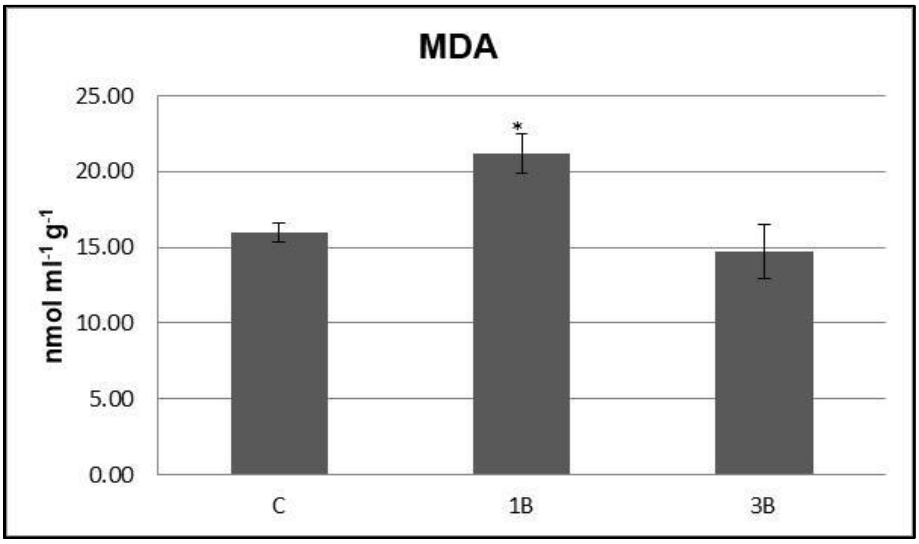

Figure 4.3: Changes in MDA contents of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

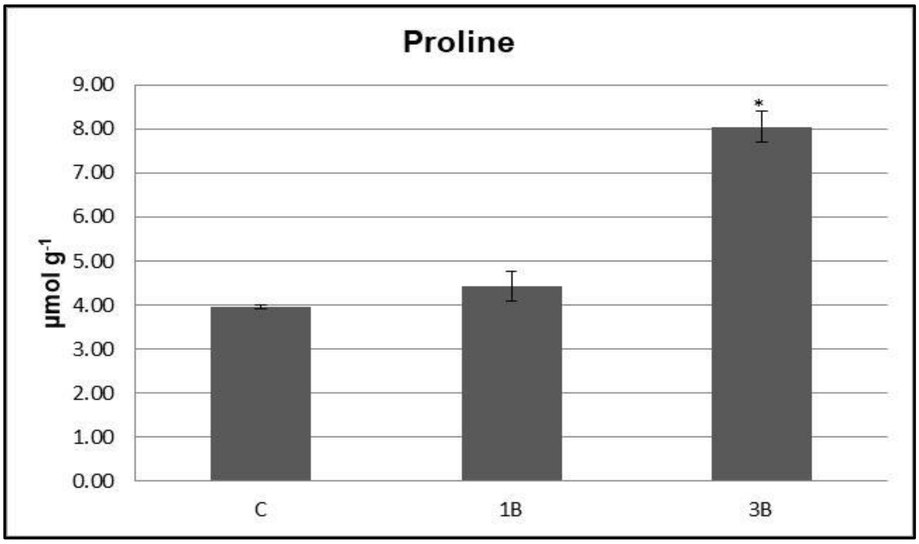

Figure 4.4: Changes in proline contents of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

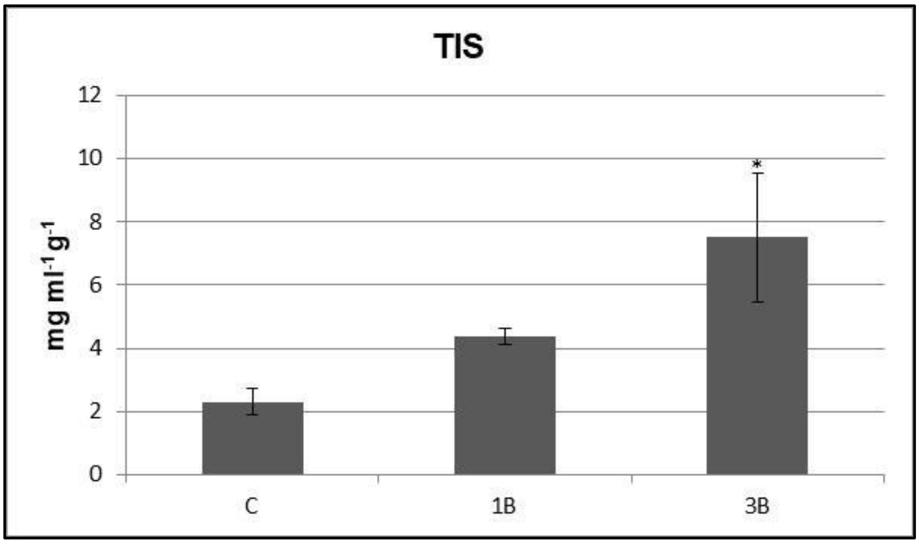

Figure 4.5: Changes in total insoluble sugar (TIS) contents of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

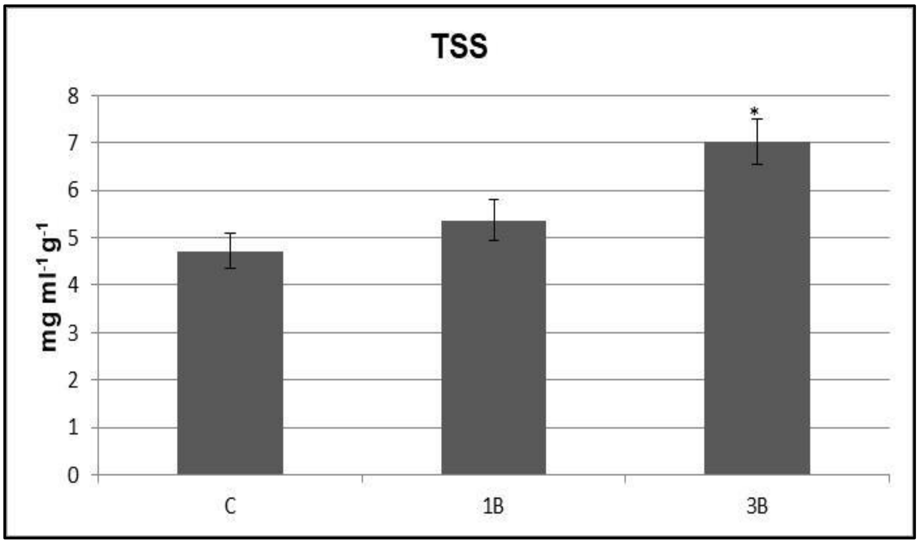

Figure 4.6: Changes in total soluble sugar (TSS) contents of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

Flavonoid contents in *Arabidopsis thaliana* seedlings increased significantly in 1B and continued to accumulate in 3B and there was no significant difference between 1B and 3B (Figure 4.7). Likewise, significant increases in anthocyanin contents were determined in both 1B and 3B conditions (Figure 4.8).

The contents of photosynthetic pigments reduced remarkably in *Arabidopsis thaliana* seedlings subjected to all B toxicity conditions (Figure 4.9-10-13). Namely, chlorophyll a (Chl a) level decreased under both 1B and 3B (Figure 13) and level of Chlorophyll b (Chl b) decreased under 1B and continued to decrease under 3B (Figure 4.10). Thus, Chl a+b level decreased significantly under both B toxicity conditions (Figure 4.11). In fact, more reduction was observed under 3B. However, Chl a/b ratio stayed stable after exposure to toxic B conditions when compared to control condition (Figure 4.14). Similar to other pigments, the content of carotenoid (Cx+c) pigment reduced dramatically under both B toxicity conditions (Figure 4.12).

The concentration of total soluble protein in *Arabidopsis thaliana* seedlings increased gradually with increasing level of B (Figure 4.15). Thus, the accumulation of this content was higher in 3B than in 1B condition.

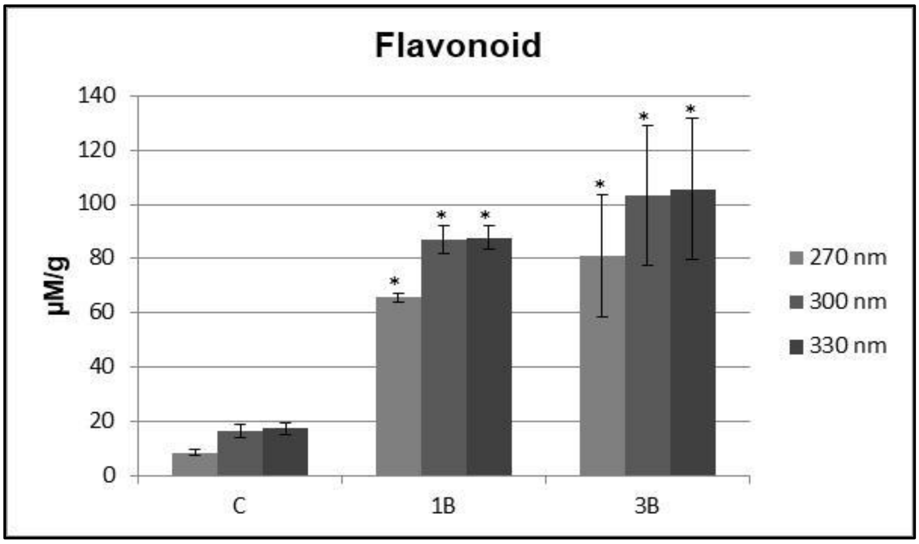

Figure 4.7: Changes in flavonoid contents of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

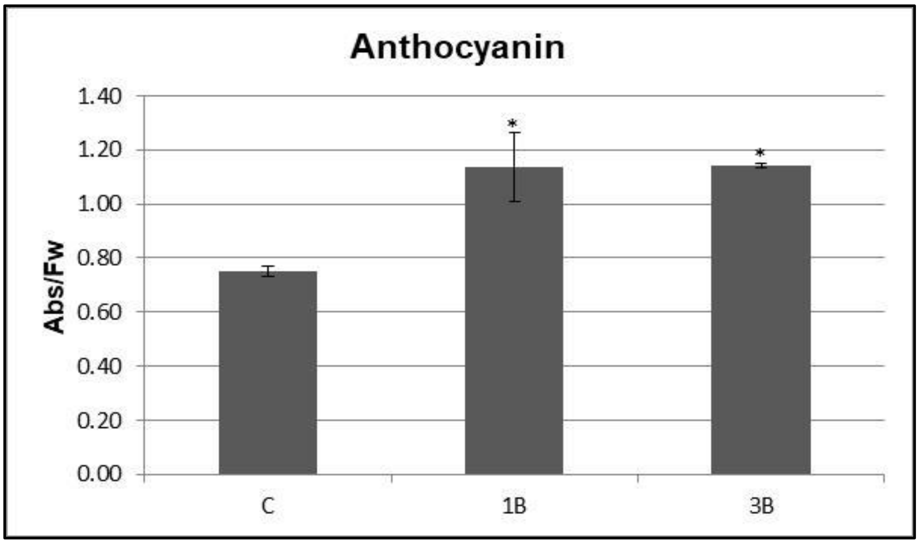

Figure 4.8: Changes in anthocyanin levels of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

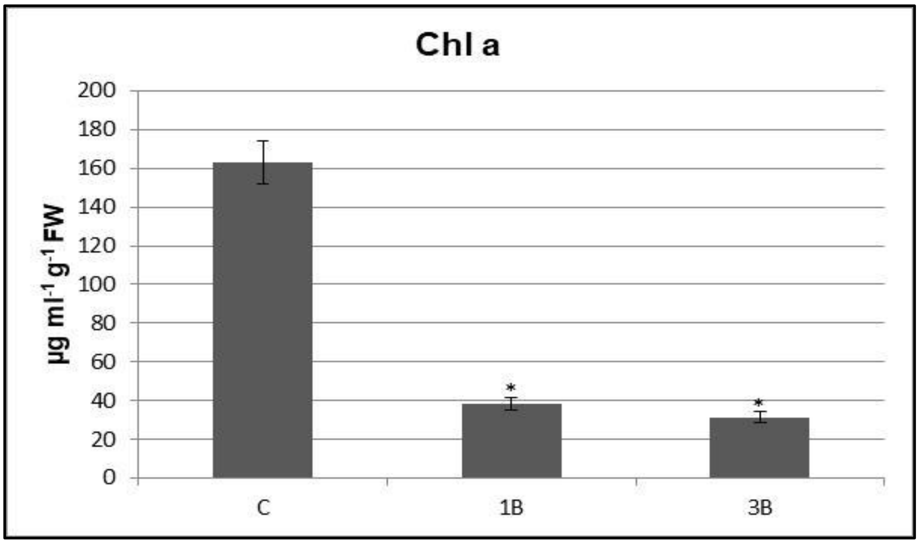

Figure 4.9: Changes in Chl a contents of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

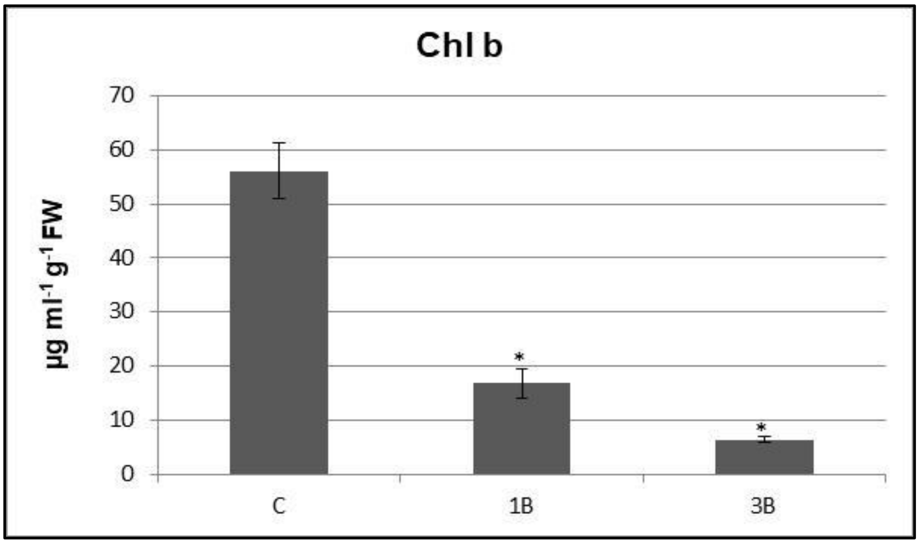

Figure 4.10: Changes in Chl b contents of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

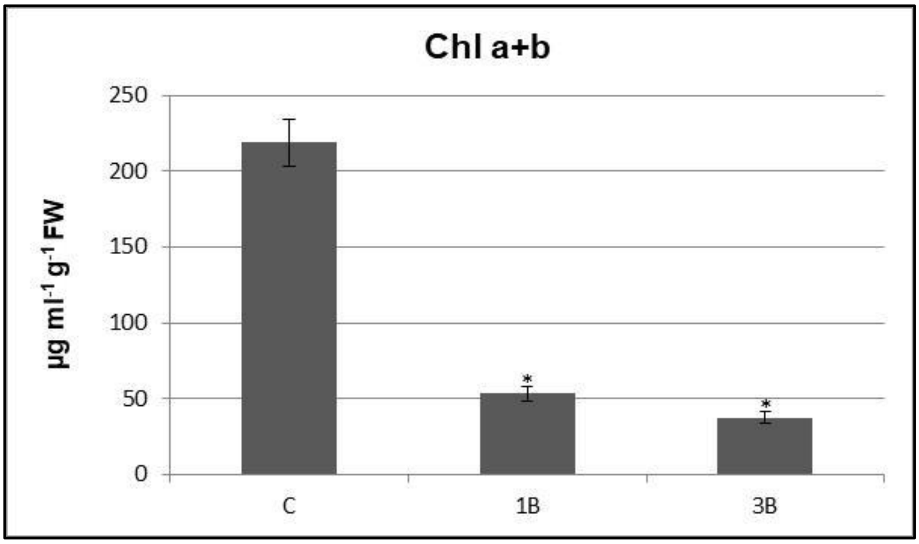

Figure 4.11: Changes in Chl a+b contents of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

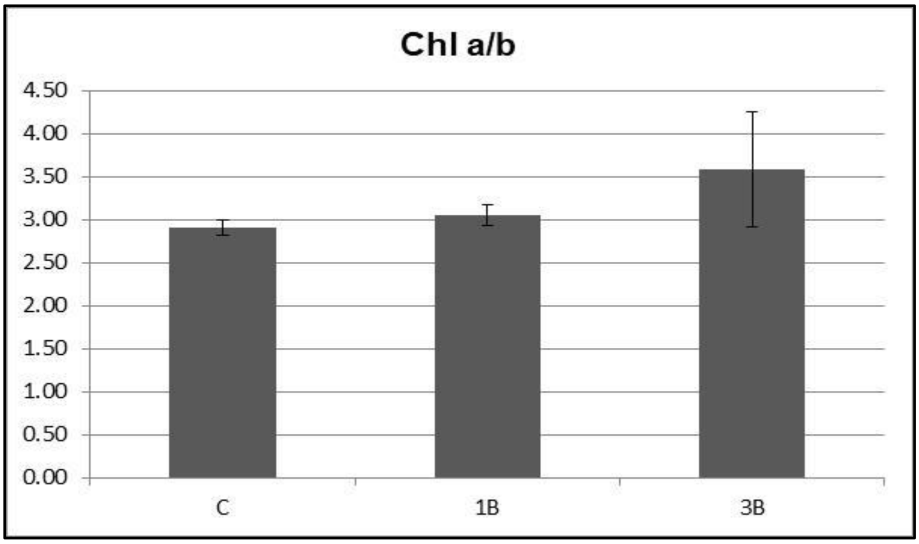

Figure 4.12: Changes in Chl a/b ratios of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

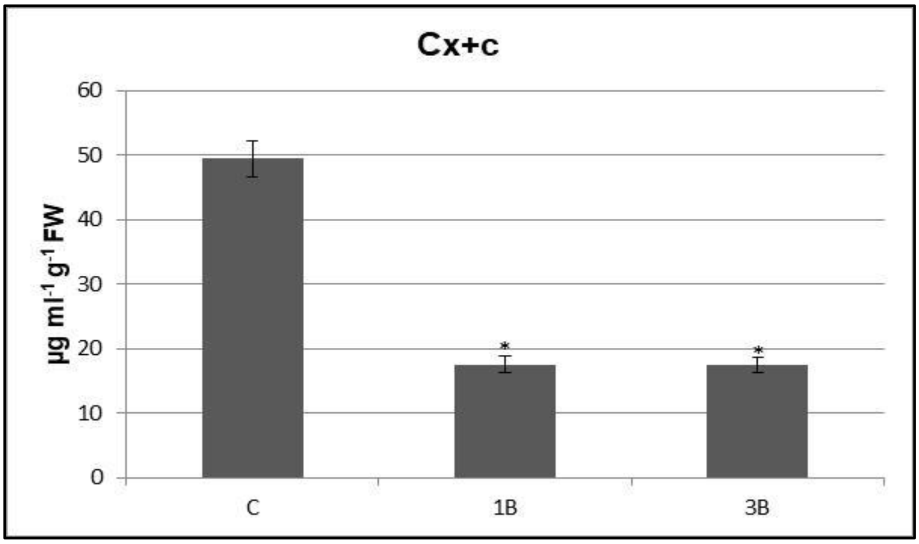

Figure 4.13: Changes in carotenoid contents of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

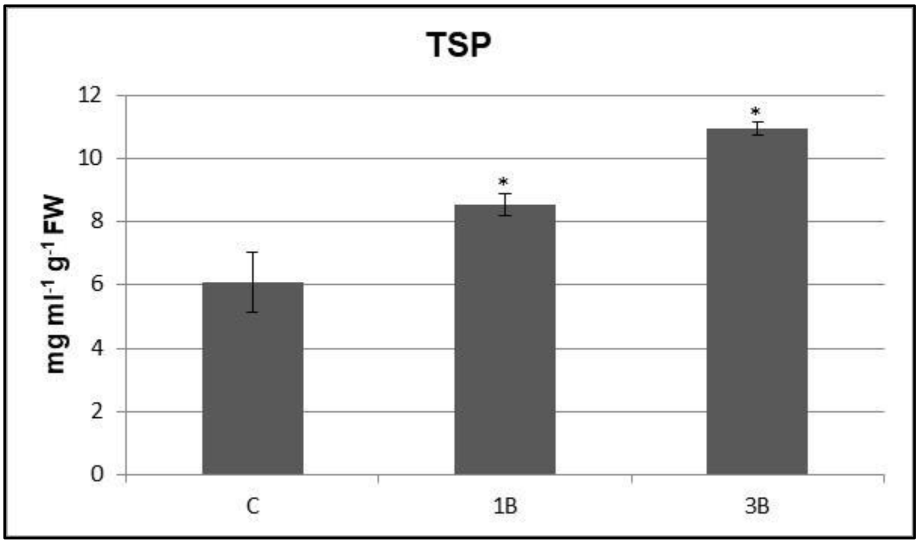

Figure 4.14: Changes in total soluble protein (TSP) contents of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

Antioxidant enzyme activities were generally induced under 1B condition, except for catalase (CAT) activity (Figure 4.15-16-17-18-19-20). CAT activity decreased under 1B and stayed stable under 3B (Figure 4.15). Also, 3B did not lead to any significant change in activity of glutathione reductase (GR) (Figure 4.16). On the contrary, the activities of ascorbate peroxidase (APX), glutathione *S*-transferase (GST) and peroxidase (POX) increased significantly in 3B as well as in 1B condition (Figure 4.17-18-19, respectively). Also, 3B treatment caused a remarkable induction of total superoxide dismutase (SOD) activity (Figure 4.20). As a conclusion, the most significant increases in activities of antioxidant enzymes were observed in SOD with almost ten-fold after 3B and in GST with four-fold after 1B (Figure 4.20-18, respectively).

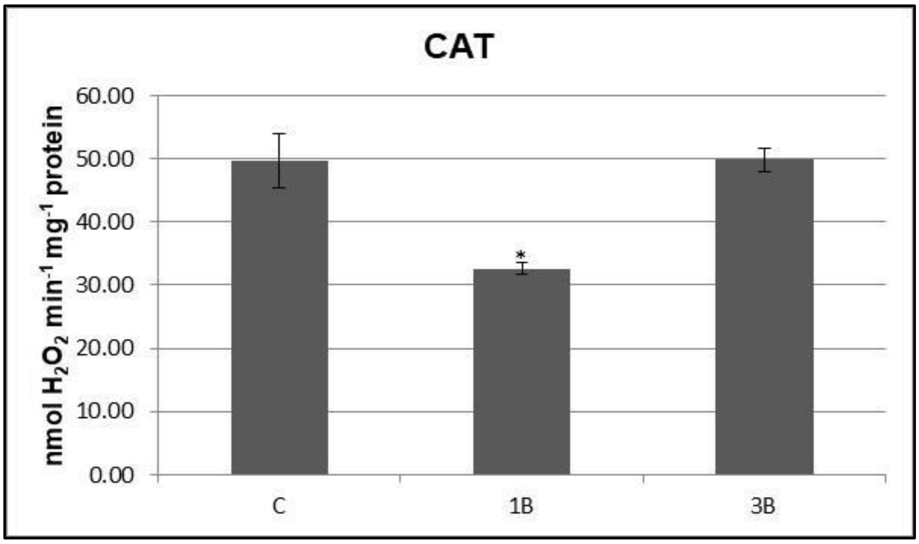

Figure 4.15: Changes in CAT activities of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

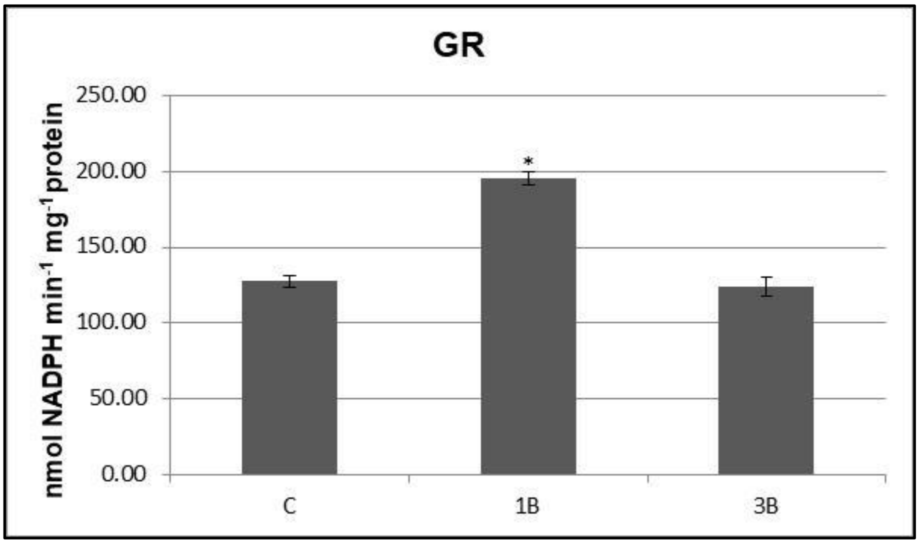

Figure 4.16: Changes in GR activities of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

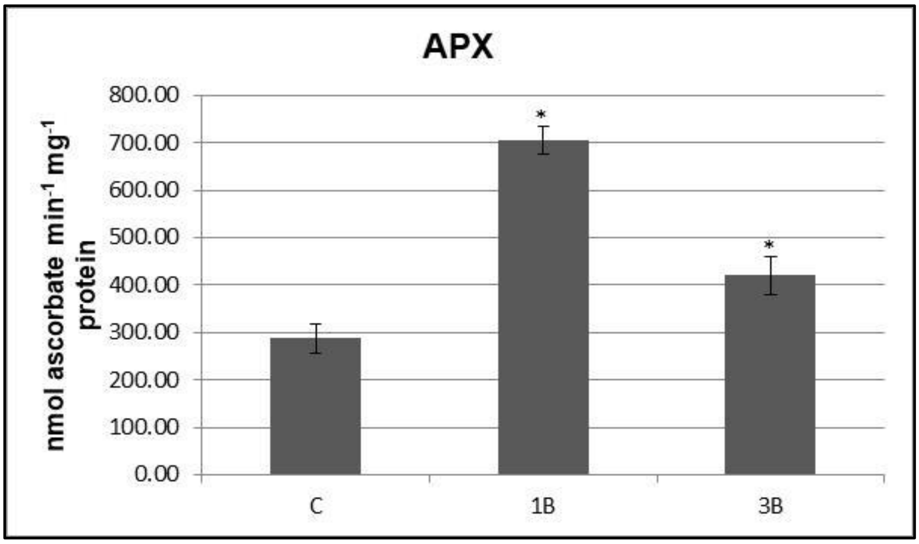

Figure 4.17: Changes in APX activities of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

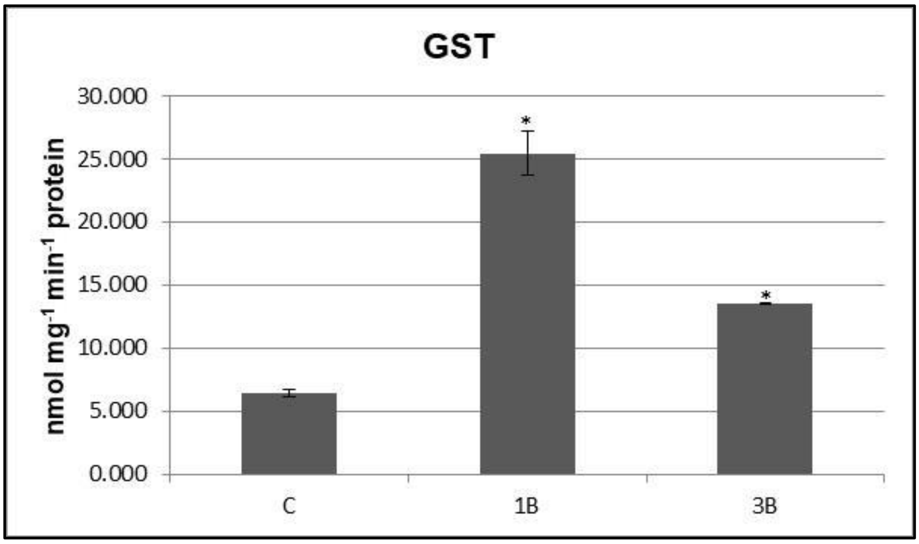

Figure 4.18: Changes in GST activities of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

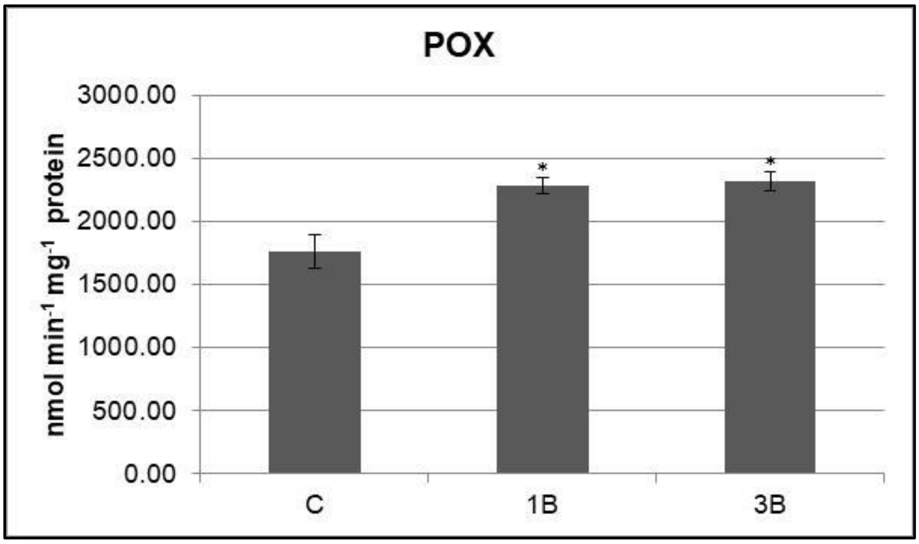

Figure 4.19: Changes in POX activities of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

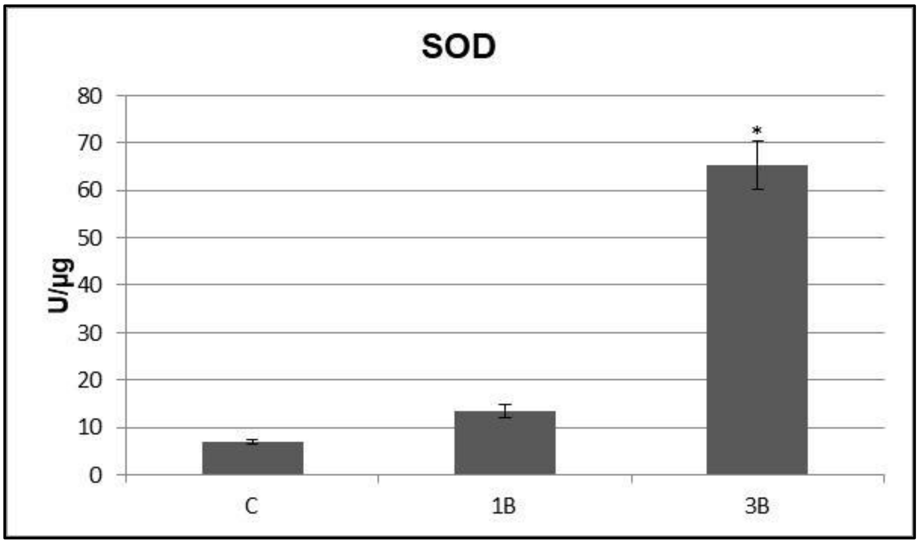

Figure 4.20: Changes in SOD activities of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

There was a significant increase in total thiol content in *Arabidopsis thaliana* seedlings under 3B condition, but not under 1B (Figure 4.21). Similarly, protein-bound thiols were increased only after 3B (Figure 4.22). However, the contents of non-protein thiols and reduced glutathione (GSH) did not significantly change following any of the toxic B conditions (Figure 4.23-24, respectively).

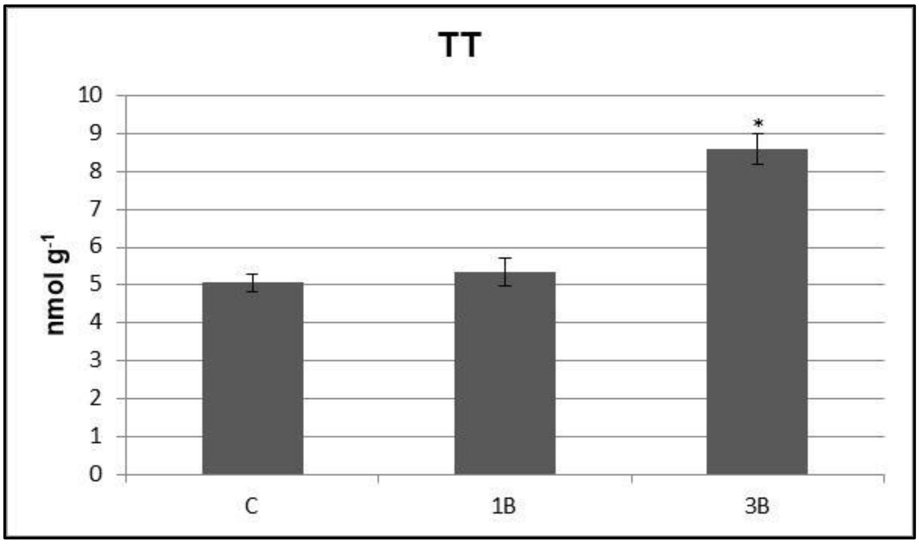

Figure 4.21: Changes in total thiol (TT) contents of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

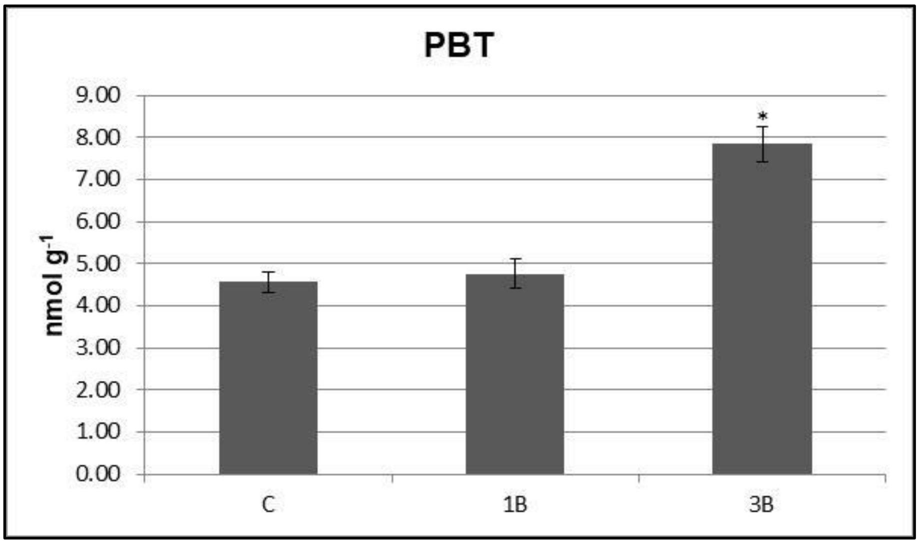

Figure 4.22: Changes in protein-bound thiol (PBT) contents of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

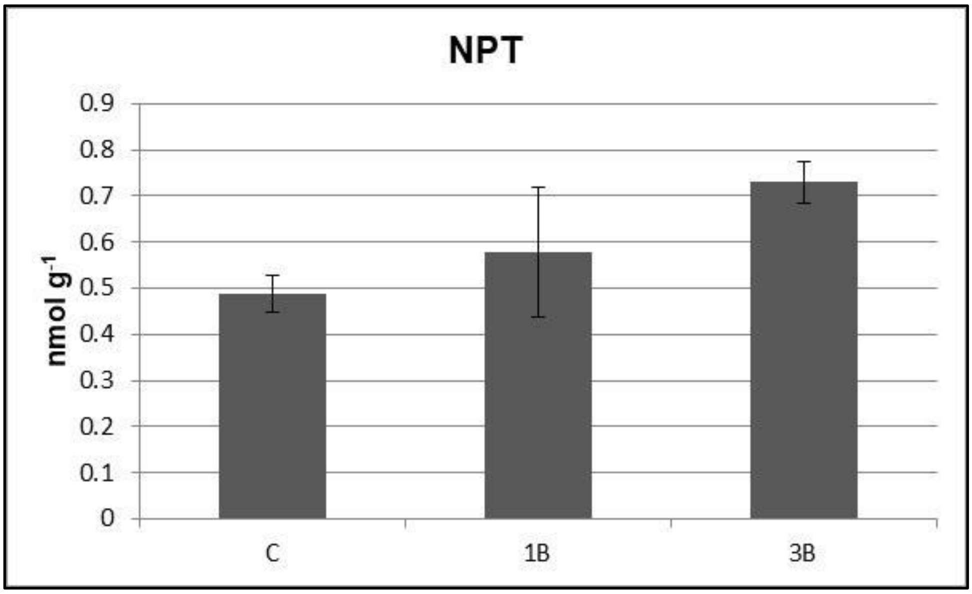

Figure 4.23: Changes in non-protein thiol (NPT) contents of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

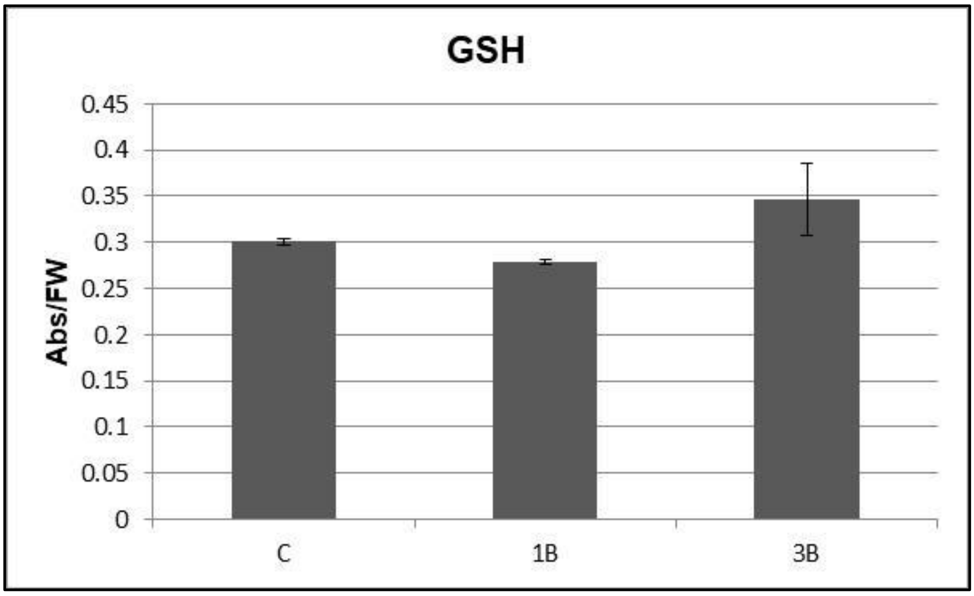

Figure 4.24: Changes in glutathione (GSH) contents of *Arabidopsis thaliana* seedlings exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

4.2. Molecular Regulations in *Arabidopsis thaliana* Exposed to B Toxicity

4.2.1. Determination of RNA Quality and Quantity

Quality and integrity of RNAs isolated from *Arabidopsis thaliana* leaves were visualized by agarose gel (Figure 4.25). Also, concentrations and 260/280 nm ratios of total RNAs were determined via NanoDrop (Table 4.1).

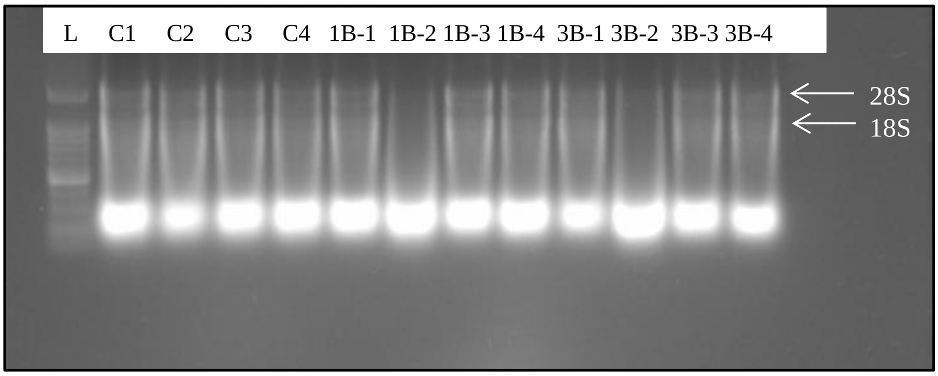

Figure 4.25: Visualization of total RNAs isolated from leaves of *Arabidopsis thaliana* on the agarose gel (L: 100 bp Sizer^{TM} (Intron), C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3). 28S and 18S rRNAs were indicated.

Table 4.1: Concentrations and ratios (260/280 nm) of isolated total RNAs from *Arabidopsis thaliana* leaves.

Sample	Concentration (ng/µl)	A260/A280
C-1	1274.3	2.06
C-2	928.7	2.05
C-3	832	2.06
C-4	1727.7	2.08
1B-1	2515.1	2.07
1B-2	1417.4	2.07
1B-3	1106.8	2.08
1B-4	2214.1	2.09
3B-1	1031.7	2.06
3B-2	1500.8	2.08
3B-3	1793.5	2.08
3B-4	1621.9	2.08

4.2.2. Transcriptional Regulations

Before real-time quantitative RT-PCR (qRT-PCR), RT-PCR was carried out for *Arabidopsis thaliana* genes in order to ensure that only one PCR amplicon was obtained and if it was at expected size. RT-PCR products of all genes were visualized on agarose gels which were given in Appendix B.

Both B toxicity treatments (1B and 3B) resulted in a significant increase in *CAT1* expression (Figure 4.26) whereas *APX6* expression was significantly increased after only 1B (Figure 4.27) comparing to control condition. The isoenzymes of SOD, Cu/Zn SOD (*CSD1*) and Mn SOD (*MSD1*), were upregulated by all toxic B conditions (Figure 4.28-29, respectively). In fact, 3B led to an almost four-fold increase in the expression level of *CSD1* (Figure 4.28).

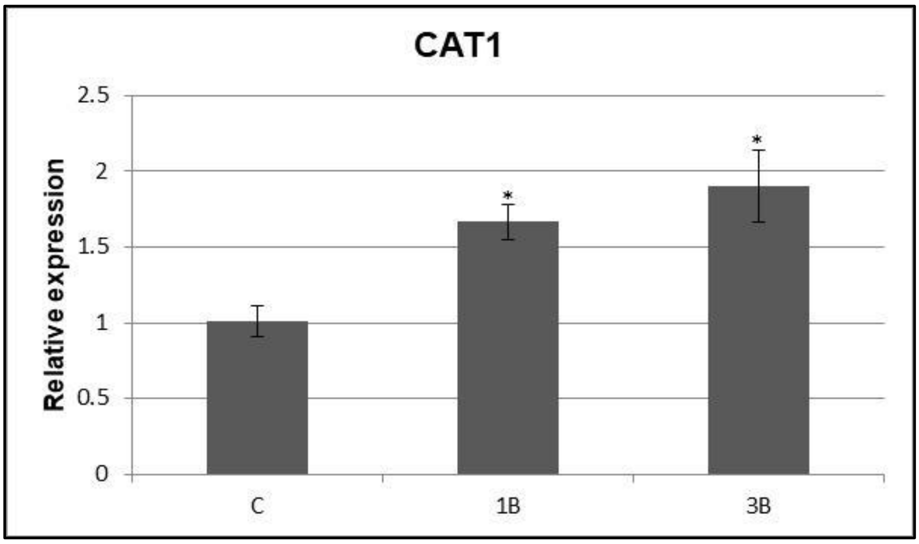

Figure 4.26: Relative expression level of *CAT1* gene in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

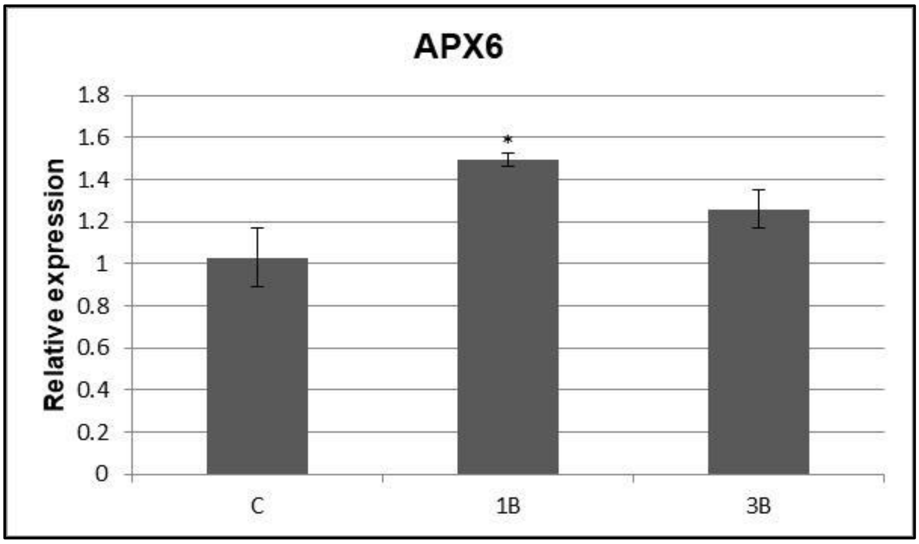

Figure 4.27: Relative expression level of *APX6* gene in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

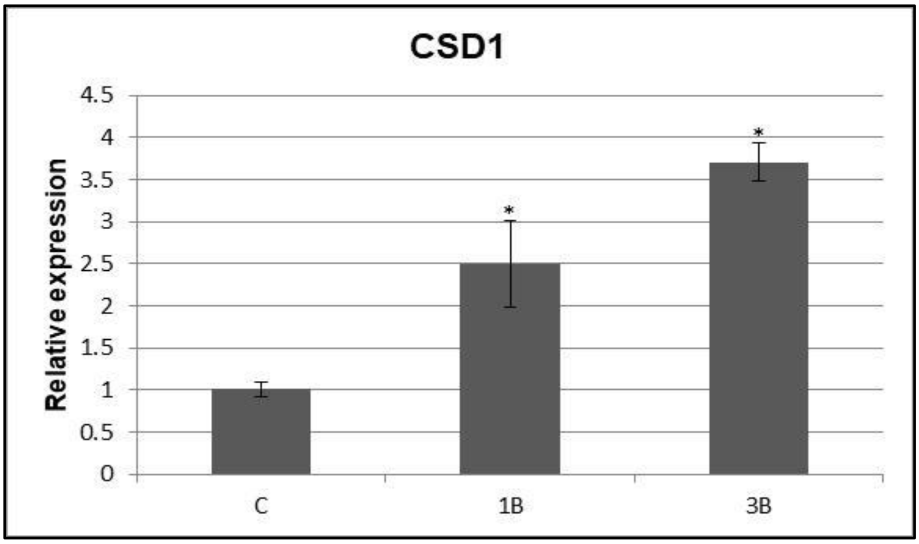

Figure 4.28: Relative expression level of *CSD1* gene in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

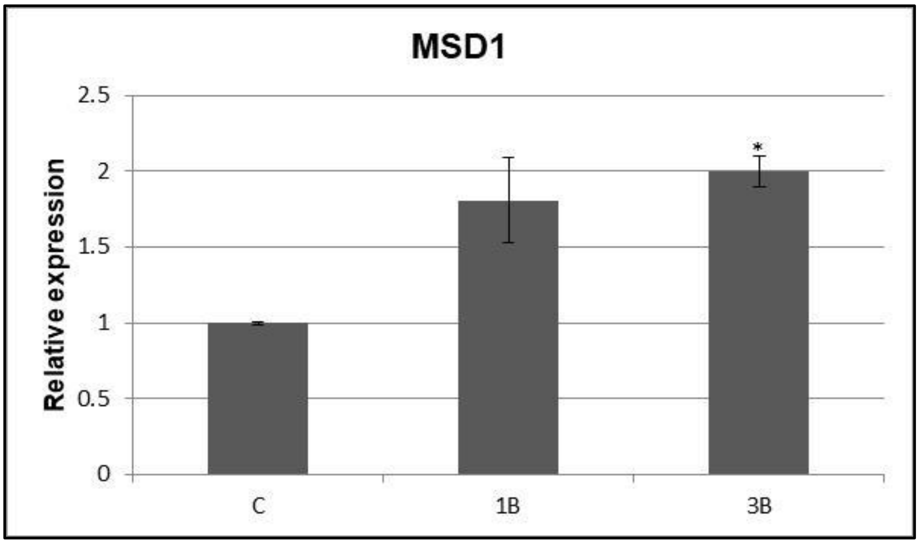

Figure 4.29: Relative expression level of *MSD1* gene in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

Increase in the expression level of glutamate cysteine ligase (*ATGSH1*) was observed under 3B (Figure 4.30). However, B toxicity did not cause any significant increase in glutathione synthetase (*ATGSH2*) (Figure 4.31). On the other hand, both *ATGR1* and *ATGR2* were not affected by 1B condition whereas they were significantly induced by 3B (Figure 4.32-33, respectively). These inductions were three- and two-folds when compared to control, respectively.

Although 1B condition led to an increase in phytochelatin synthase 1 (*PCS1*) expression, this was not significant (Figure 4.34). *PCS1* expression level was also stable after 3B (Figure 4.34). Monodehydroascorbate reductase 2 (*MDAR2*) expression level increased significantly after 1B but not in 3B (Figure 4.35). Moreover, both conditions of toxic B induced the expression of *DHAR2* (Figure 4.36). However, this induction was significant only in 3B condition as comparing to control.

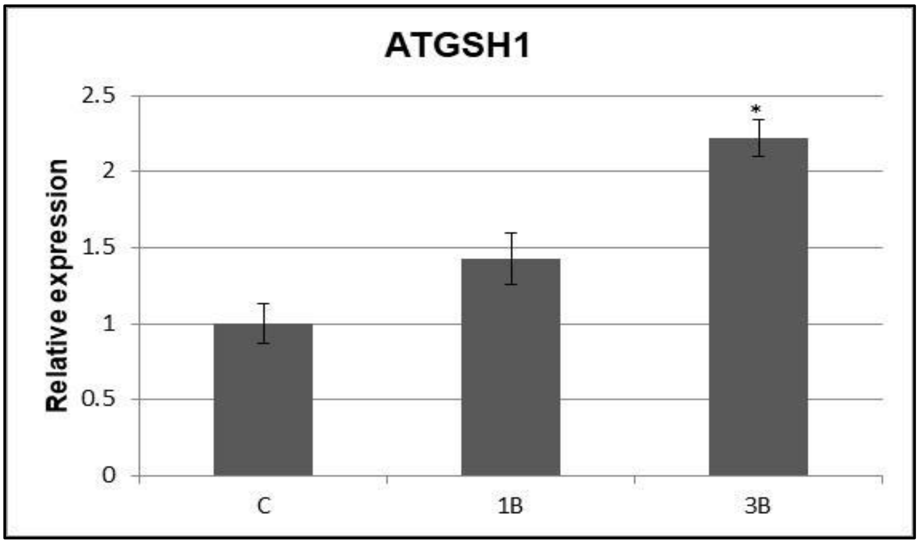

Figure 4.30: Relative expression level of *ATGSH1* gene in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

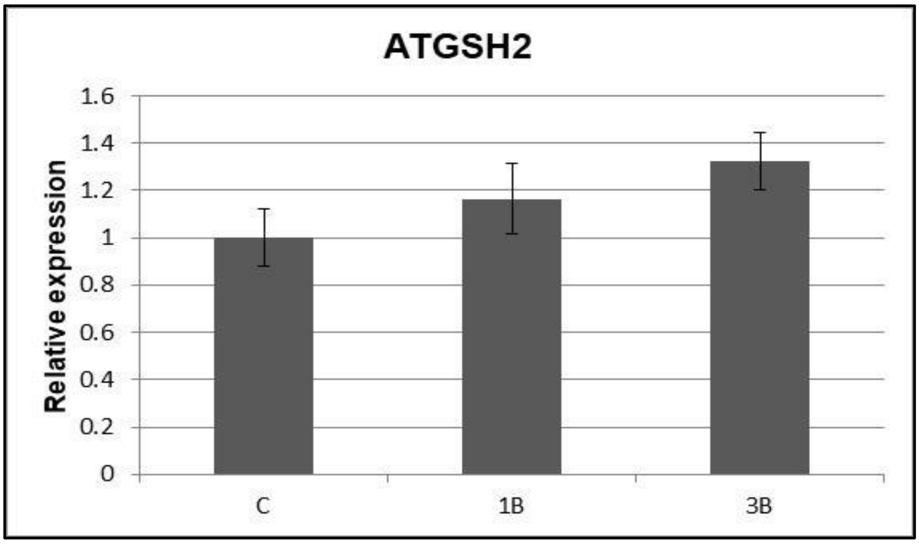

Figure 4.31: Relative expression level of *ATGSH2* gene in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

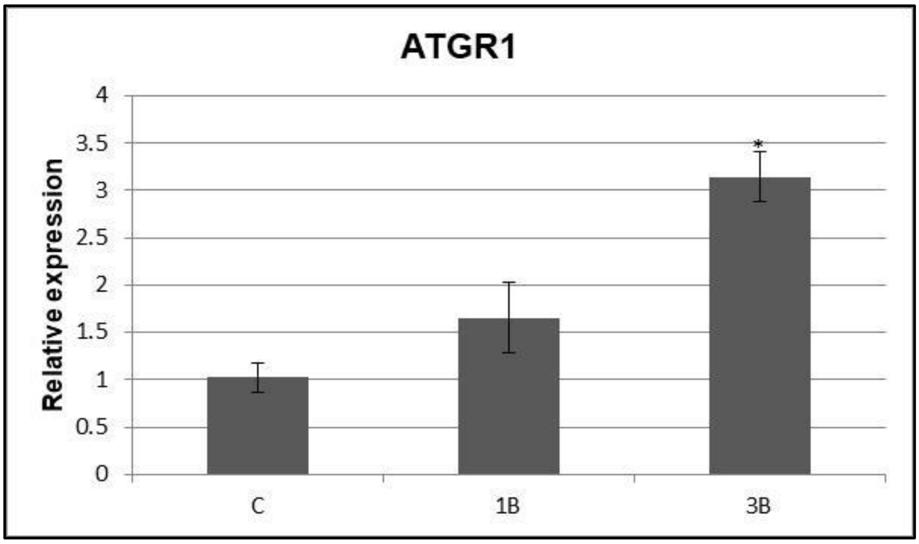

Figure 4.32: Relative expression level of *ATGR1* gene in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

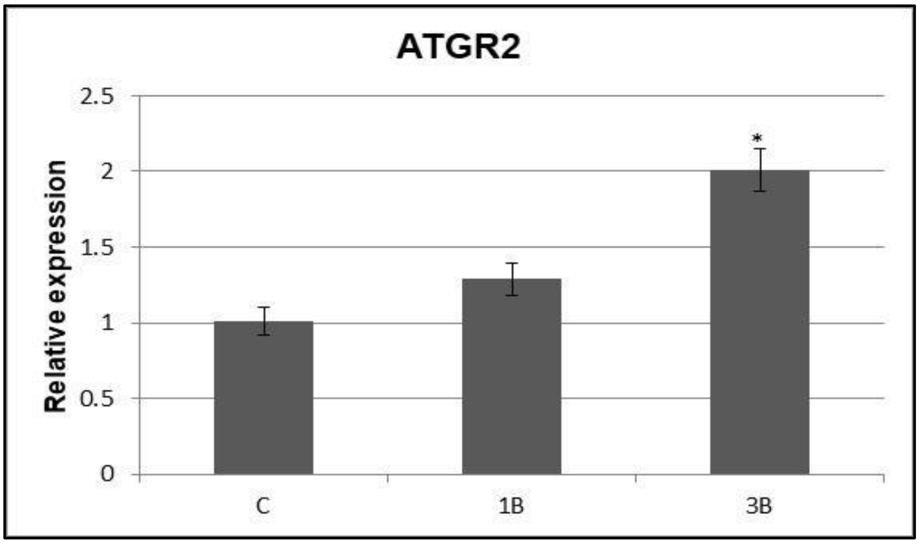

Figure 4.33: Relative expression level of *ATGR2* gene in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

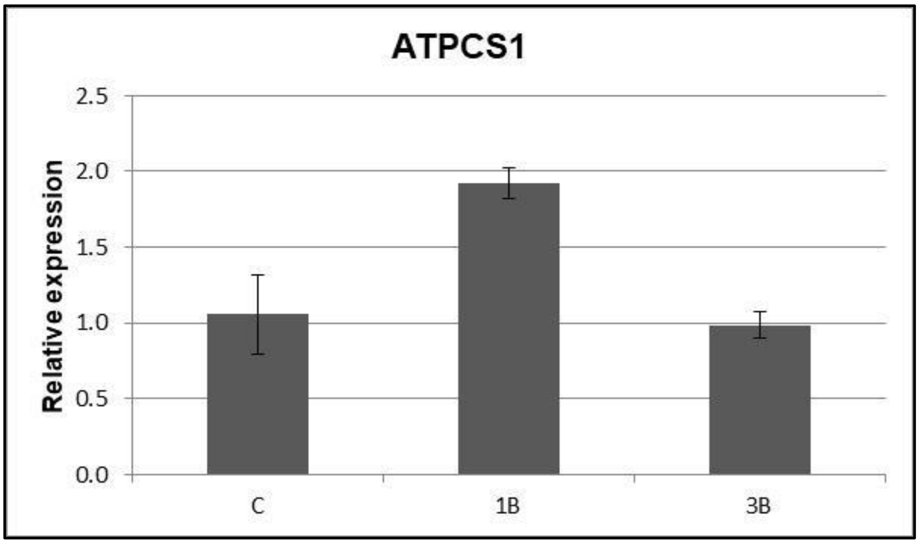

Figure 4.34: Relative expression level of *ATPCS1* gene in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

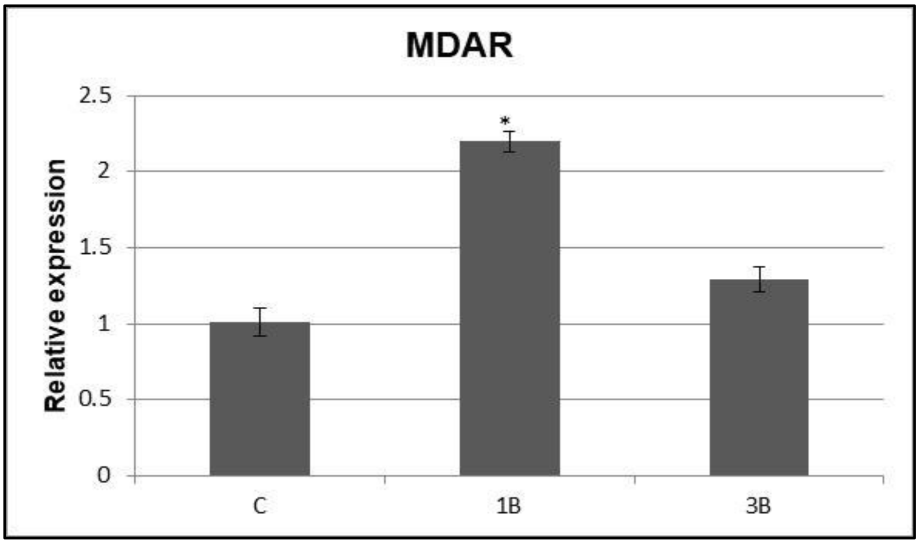

Figure 4.35: Relative expression level of *MDAR* gene in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

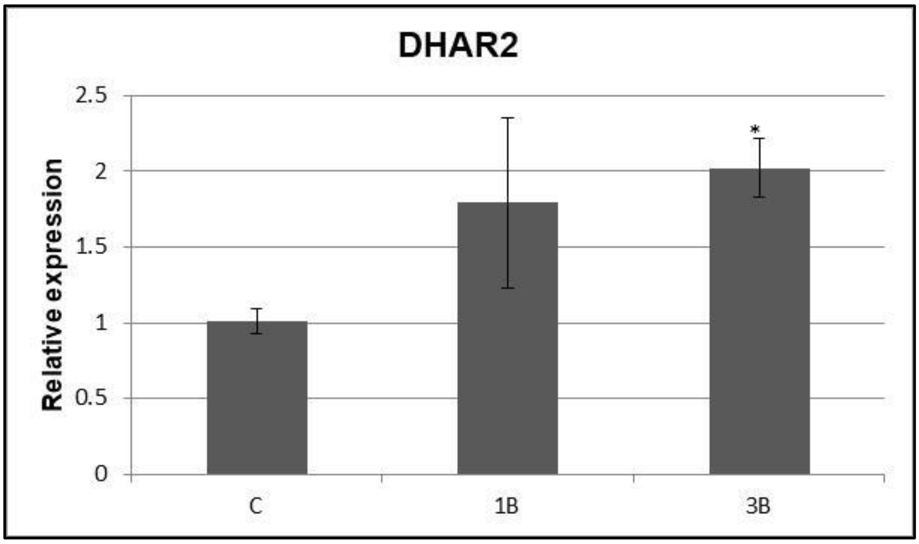

Figure 4.36: Relative expression level of *DHAR2* gene in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

1B condition did not cause any significant difference in the expression levels of phi classes of GST (Figure 4.37-38-39-40). Likewise, *ATGSTF2* and *ATGSTF8* expression levels were stable following 3B (Figure 4.37-40). However, 3B led to a significant decrease in the expression levels of both *ATGSTF6* and *ATGSTF7* (Figure 4.38-39). In fact, *ATGSTF7* was down-regulated to less than half after 3B when compared to control (Figure 4.39). In contrast to these members of phi class, expression levels of *ATGSTU19* and *ATGSTZ1* genes were induced remarkably after all toxic B treatments in comparison to control (Figure 4.41-42, respectively). These inductions were almost five- and four-folds for *ATGSTU19* after 1B and 3B, respectively (Figure 4.41), whereas *ATGSTZ1* was up-regulated almost three-fold by both B toxicity conditions (Figure 4.42).

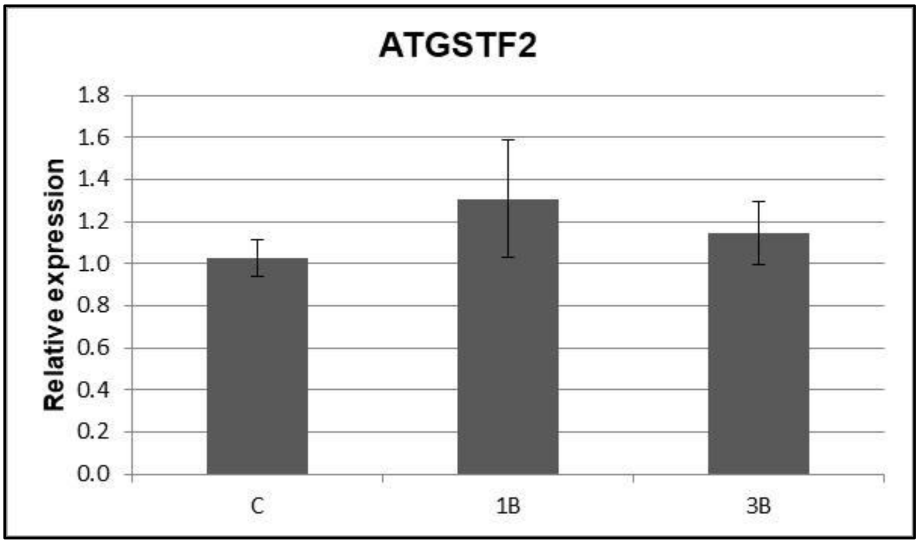

Figure 4.37: Relative expression level of *ATGSTF2* gene in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

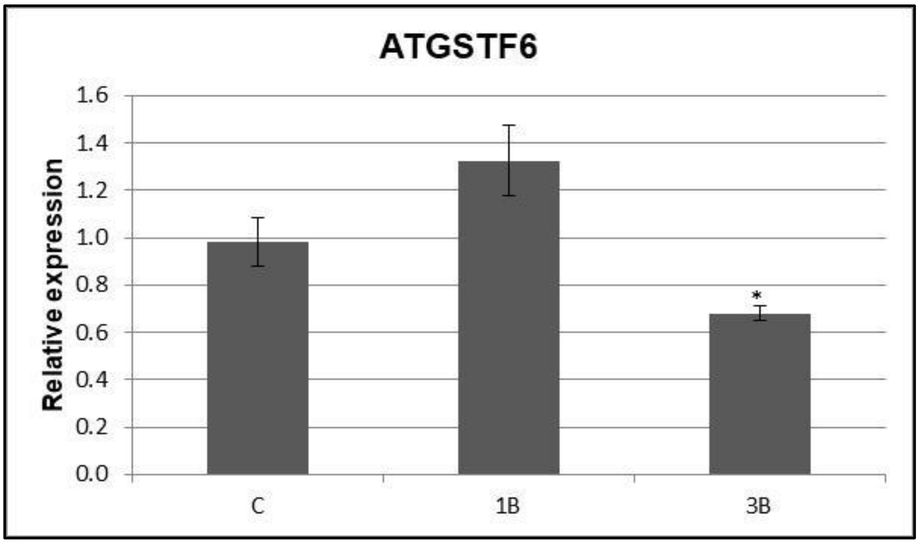

Figure 4.38: Relative expression level of *ATGSTF6* gene in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

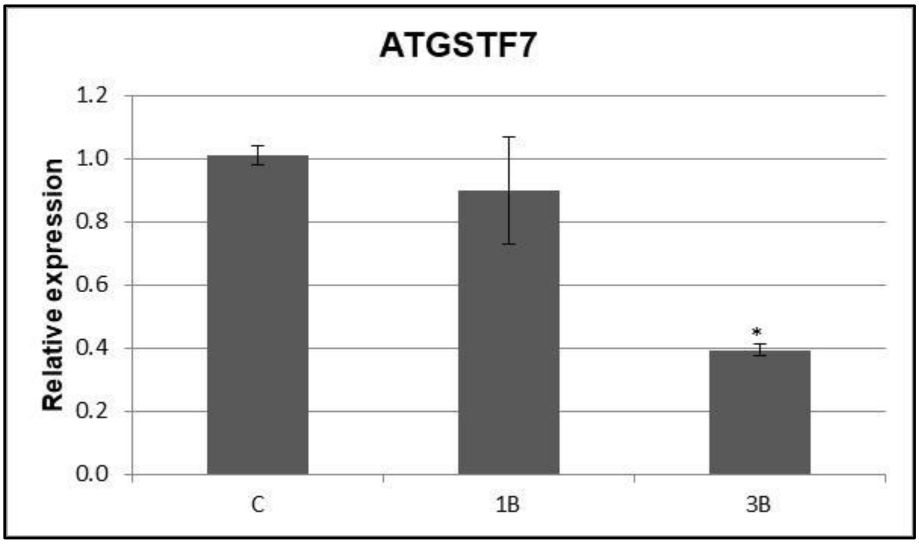

Figure 4.39: Relative expression level of *ATGSTF7* gene in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

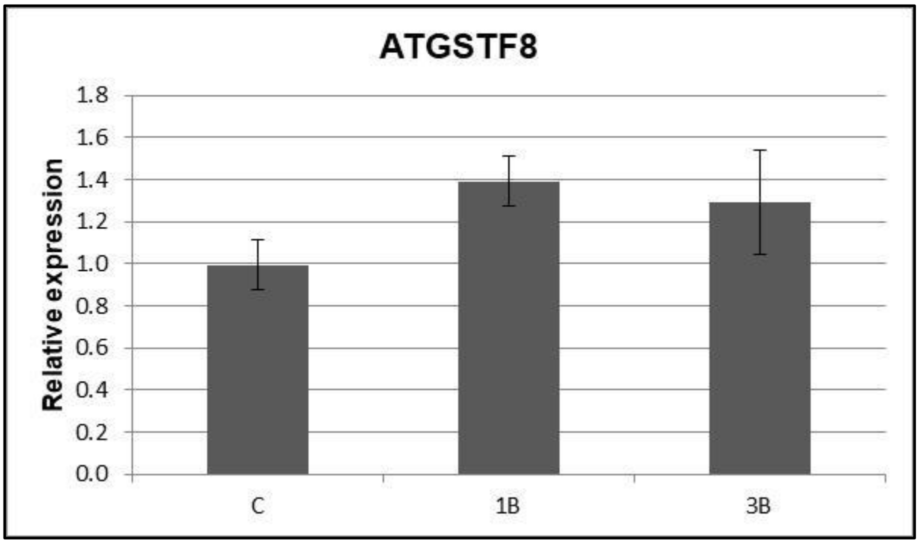

Figure 4.40: Relative expression level of *ATGSTF8* gene in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

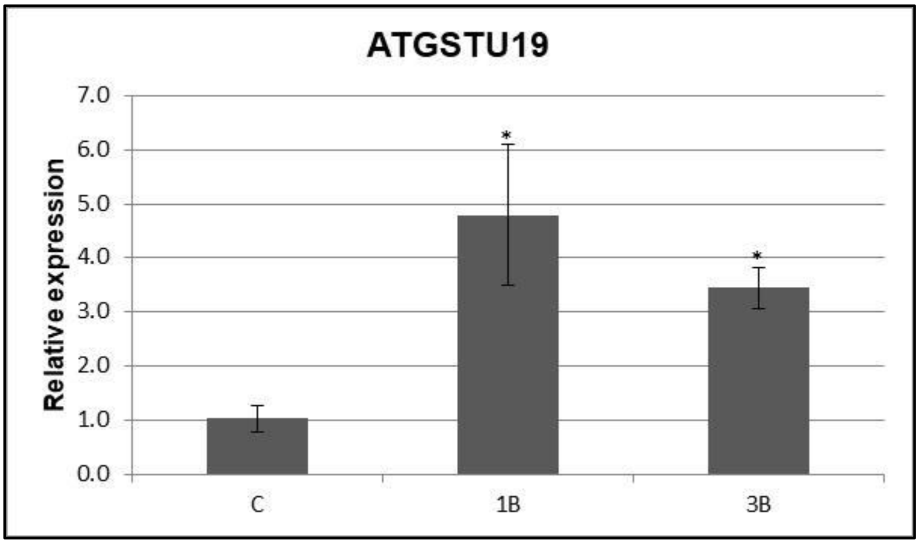

Figure 4.41: Relative expression level of *ATGSTU19* gene in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

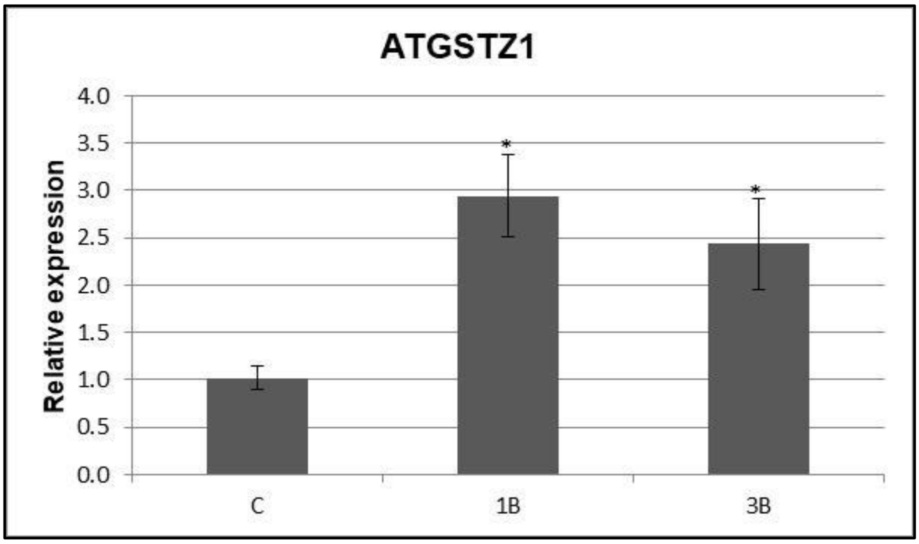

Figure 4.42: Relative expression level of *ATGSTZ1* gene in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

4.2.3. Post-Transcriptional Regulations

Based on the remarkable regulations of genes at transcriptional level in *Arabidopsis thaliana* under B toxicity, several miRNAs were chosen in order to investigate how these genes were regulated at post-transcriptional level under toxic B conditions. In this thesis, selected miRNAs and their targets in *Arabidopsis thaliana* were shown in Appendix C. One of the most significant increases was observed in *CSD1* expression. Therefore, expression levels of *miR398* were measured following toxic B treatments. The relative expression level of *miR398* increased significantly in 1B but stayed stable in 3B as compared to control (Figure 4.43).

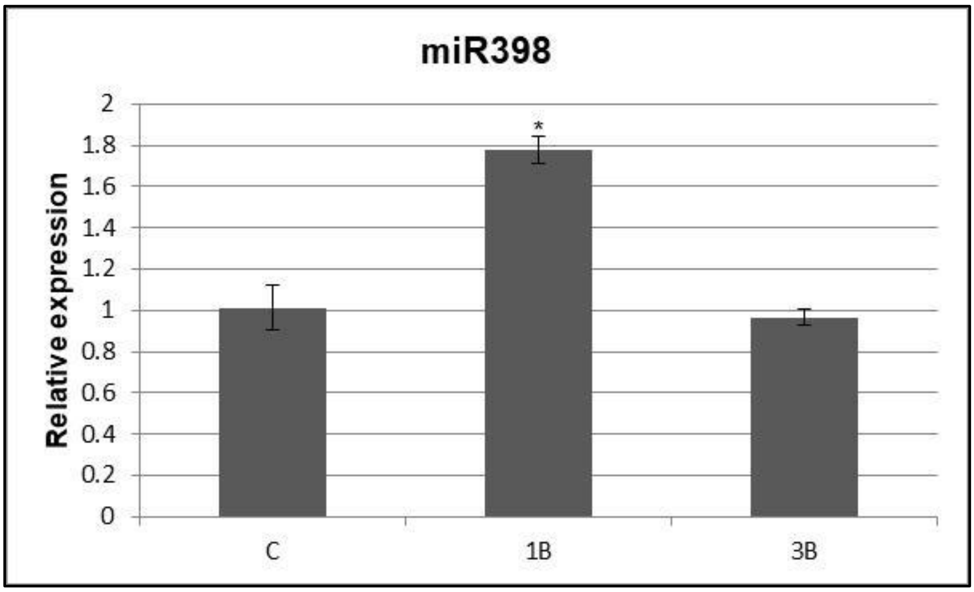

Figure 4.43: Relative expression level of *miR398* in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

We also evaluated comparative expression of *miR408* and *miR397* which target laccase mRNA. *miR408* expression level did not significantly change in response to both 1B and 3B conditions (Figure 4.44). On the other hand, 1B caused to a significant but negligible increase in *miR397* expression, while the level of *miR397* expression stayed stable following 3B (Figure 4.45).

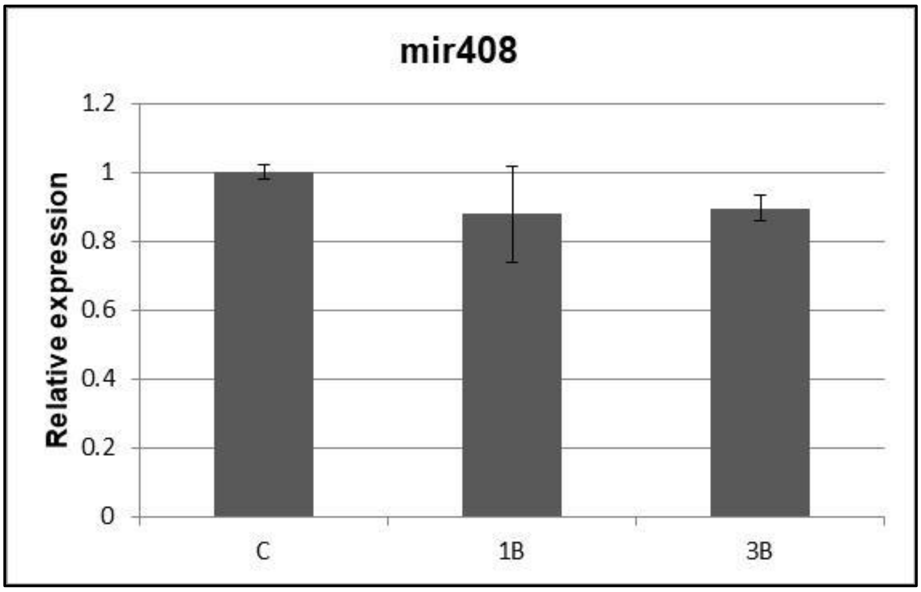

Figure 4.44: Relative expression level of *miR408* in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

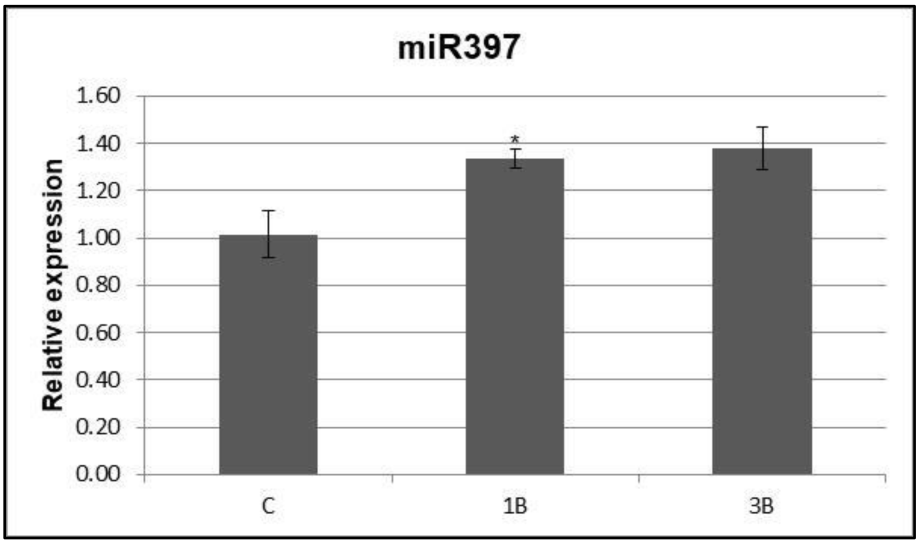

Figure 4.45: Relative expression level of *miR397* in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

On the other hand, with five-fold increase, the most significant up-regulation was determined in *ATGSTU19* expression. Thus, we searched the miRNAs targeting GST. However, we could not find any miRNA that directly targets a GST gene, except rsa-miR156 in *Raphanus sativus*, ssp-mir169 in sugarcane and non-conserved osa-mir1848 in rice. For this reason, the changes in expression levels of miR156 and miR169 were investigated under B toxicity in *Arabidopsis thaliana*. A slight, but significant, decrease in the expression level of *miR156* was observed under both B toxicity conditions (Figure 4.46). Both toxic B conditions induced the expression level of *miR169* (Figure 4.47). Especially, 1B treatment led to an almost four-fold increase in *miR169* expression.

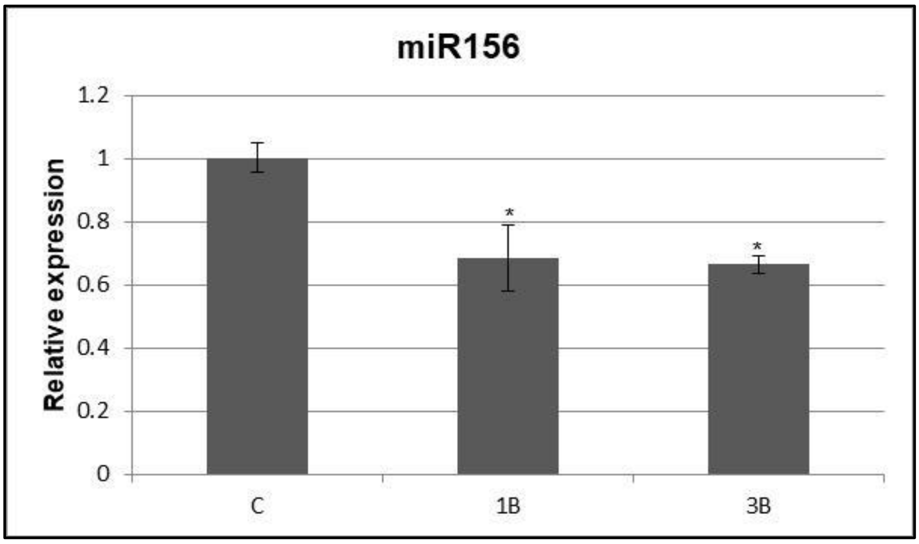

Figure 4.46: Relative expression level of *miR156* in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

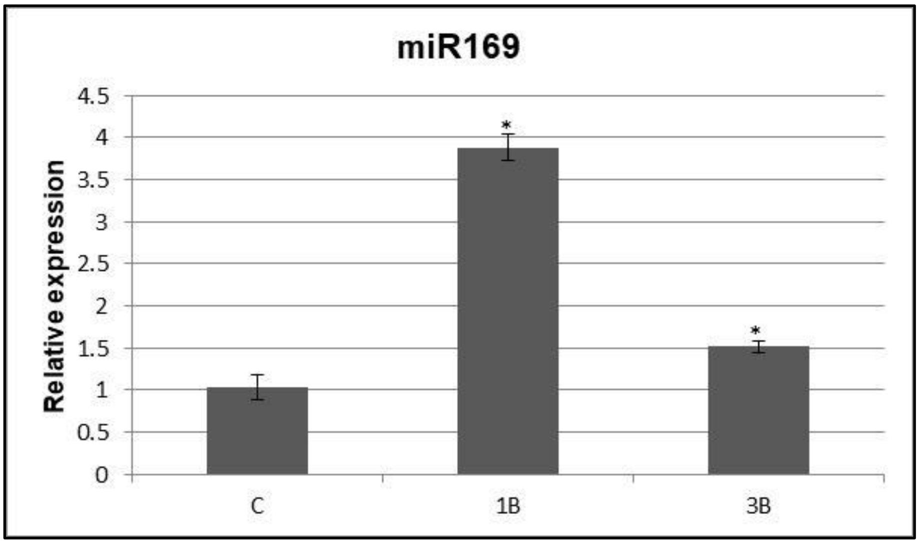

Figure 4.47: Relative expression level of *miR169* in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

Finally, we investigated regulations of jasmonate (JA)-related miRNAs including *miR159, miR319* and *miR394,* as well as ethylene-related *miR172*. The relative expression levels of *miR159, miR319* and *miR394* increased two-fold, three-fold and more than 2.5-fold by 1B treatment, respectively (Figure 4.48-49-50, respectively). On the contrary, they remained stable under 3B condition, except *miR319* with a very slight increase (Figure 4.49). Similarly, *miR172* expression was induced remarkably (almost four-fold) by 1B condition although it did not alter significantly after 3B supply (Figure 4.51).

Physiological, biochemical, transcriptional and post-transcriptional data from *Arabidopsis thaliana* were individually combined into a few tables and figures (Appendix D).

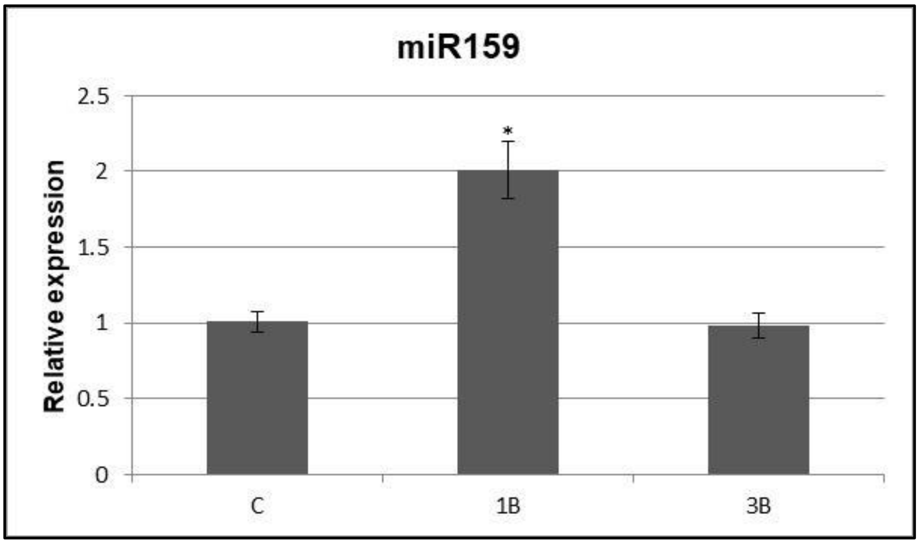

Figure 4.48: Relative expression level of *miR159* in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

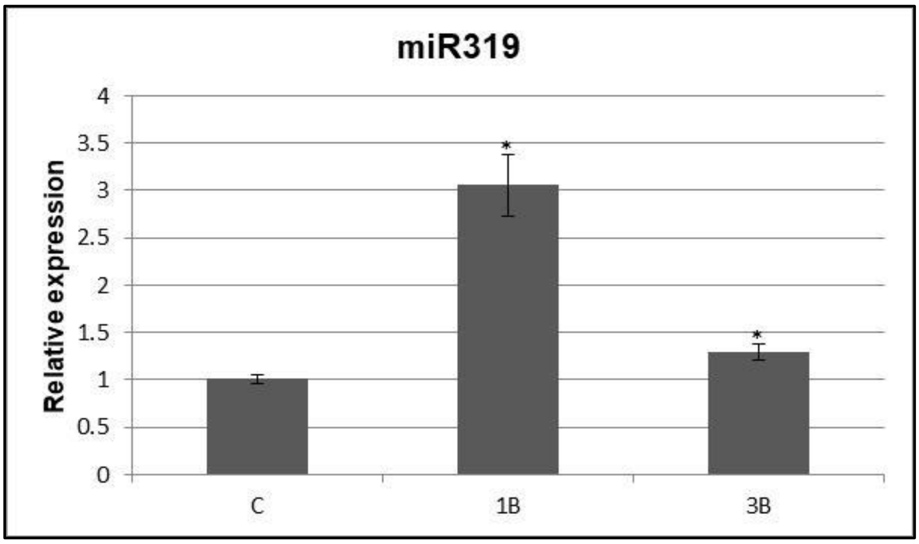

Figure 4.49: Relative expression level of *miR319* in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

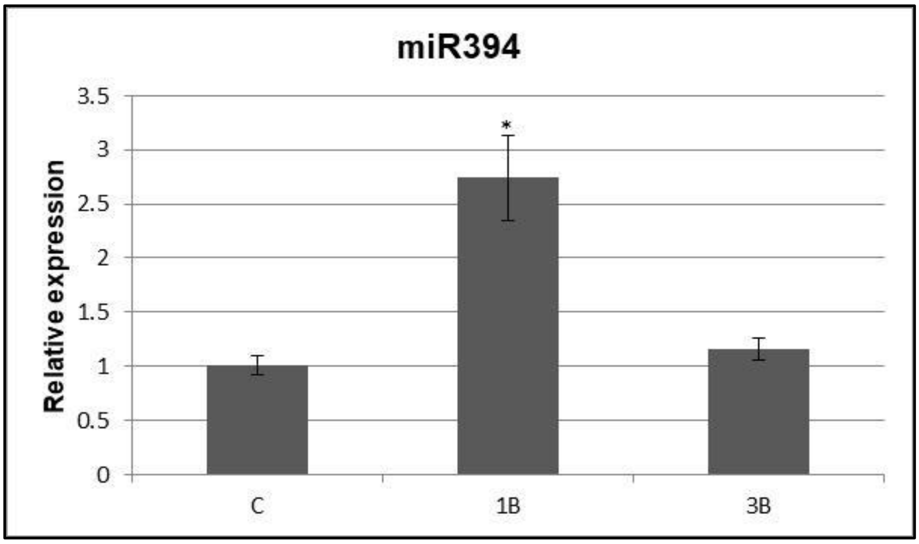

Figure 4.50: Relative expression level of *miR394* in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

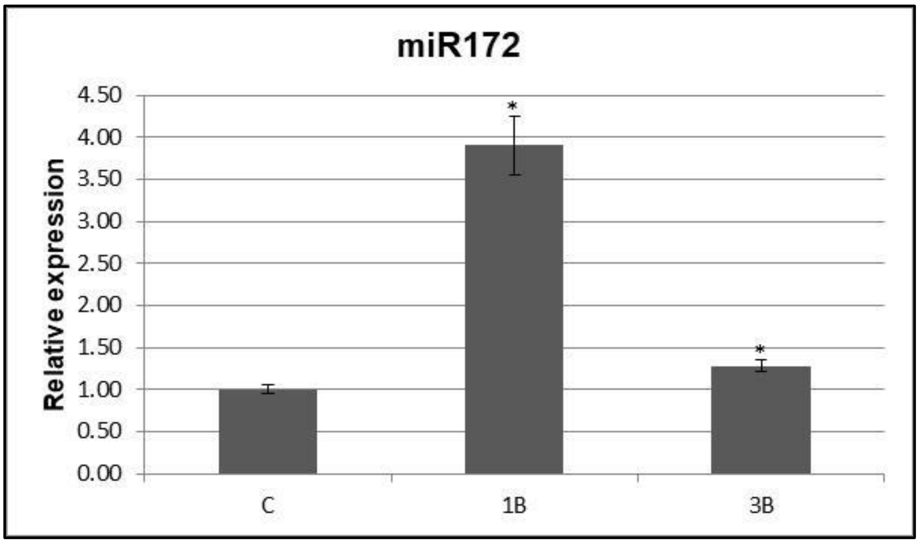

Figure 4.51: Relative expression level of *miR172* in *Arabidopsis thaliana*. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference.

4.3. Physio-Biochemical Regulations in Wheat Cultivars Exposed to B Toxicity

4.3.1. Phenotypic Differences Between Wheat Cultivars

At the end of 10 d, yellowing was observed in leaves of *Triticum aestivum* cv. Atay (B sensitive wheat cultivar), but not in *Triticum aestivum* cv. Bolal (B tolerant cultivar), under 1B condition (Figure 4.52-53, respectively). However, 3B caused a yellowing in leaves and necrosis in leaf apexes of both sensitive and tolerant cultivars. Moreover, 3B led to decreases in germination rate and shoot growth of both cultivars (Figure 4.52-53).

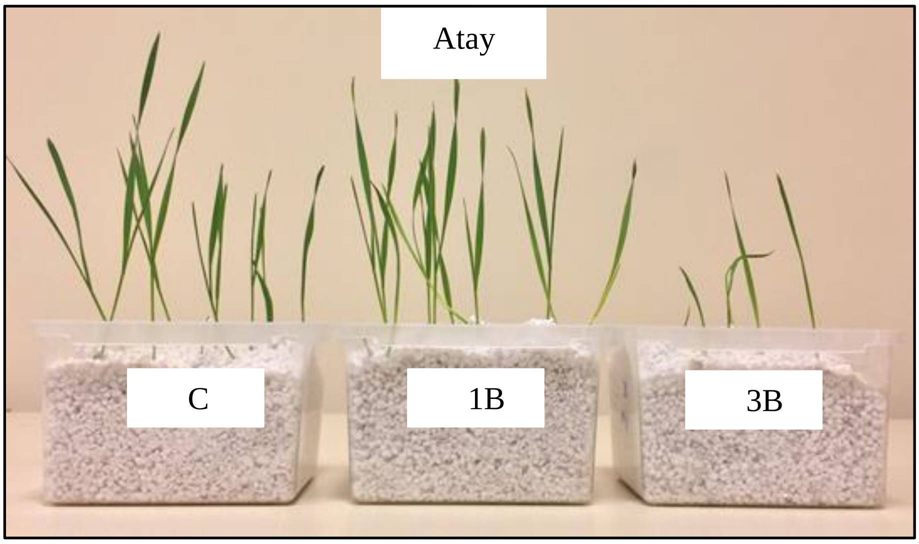

Figure 4.52: Phenotypic view of *Triticum aestivum* cv. Atay leaves after grown under control (C), 1 mM H_3BO_3 (1B) and 3 mM H_3BO_3 (3B) conditions for 10 days.

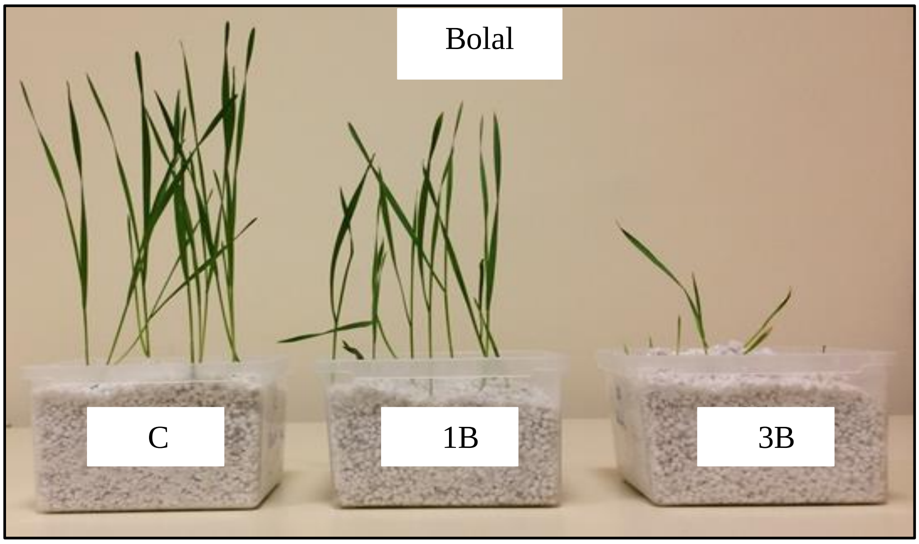

Figure 4.53: Phenotypic view of *Triticum aestivum* cv. Bolal leaves after grown under control (C), 1 mM H_3BO_3 (1B) and 3 mM H_3BO_3 (3B) conditions for 10 days.

4.3.2. Biochemical Analyses in Wheat Cultivars

GST activity was increased significantly in Atay under 1B condition (Figure 4.54). However, in contrast to Atay, 1B led to a significant decrease of GST activity in Bolal (Figure 4.55). On the other hand, 3B condition caused reduced activity of GST in both cultivars (Figure 4.54-55). When compared to control conditions, reduction rates of GST activities were 27% for Atay and almost 40% for Bolal at the end of 3B treatment. Moreover, Atay inherently had lower GST activity than Bolal in normal conditions (Figure 4.54-55).

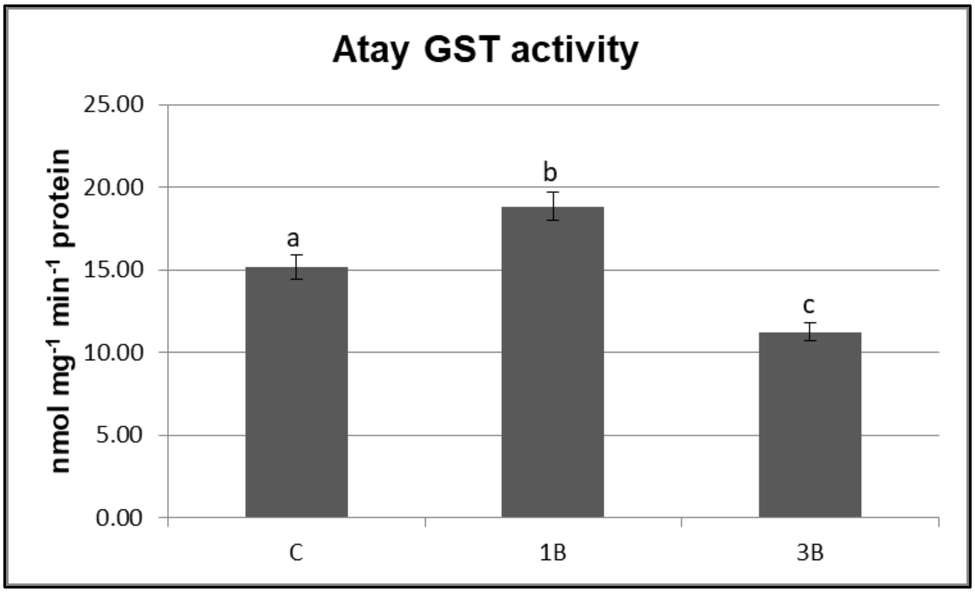

Figure 4.54: Changes in GST activities of Atay leaves exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

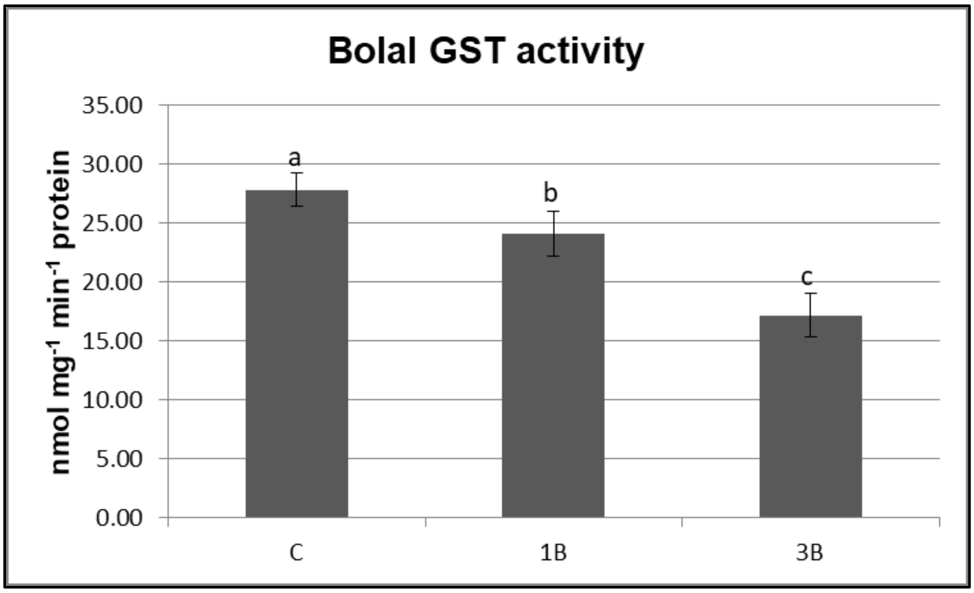

Figure 4.55: Changes in GST activities of Bolal leaves exposed to B stress. C: Control, 1B: 1 mM H_3BO_3, 3B: 3mM H_3BO_3. Asterisk indicates a significant difference.

4.3.3. Molecular Regulations in Wheat Cultivars

4.3.3.1. Determination of RNA Quality and Quantity

Quality and integrity of RNAs isolated from leaves of wheat cultivars exposed to toxic B were visualized by agarose gel (Figure 4.56-57). Also, concentrations and 260/280 nm ratios of total RNAs were determined via NanoDrop (Table 4.2).

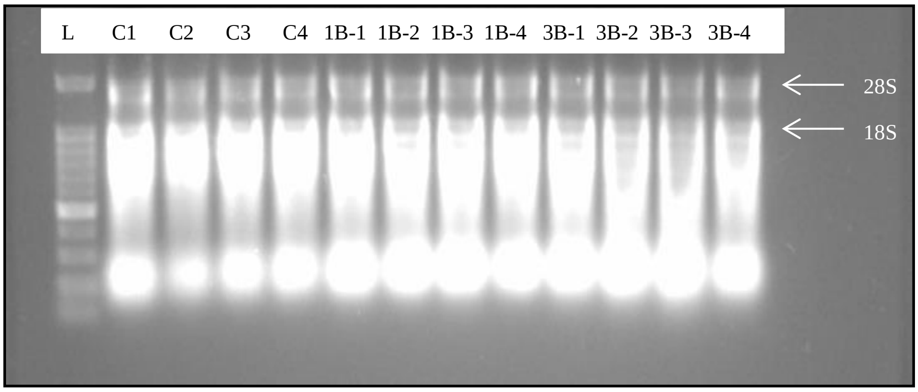

Figure 4.56: Visualization of total RNAs isolated from leaves of Atay on the agarose gel (L: 100 bp Sizer^{TM} (Intron), C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3). 28S and 18S rRNAs were indicated.

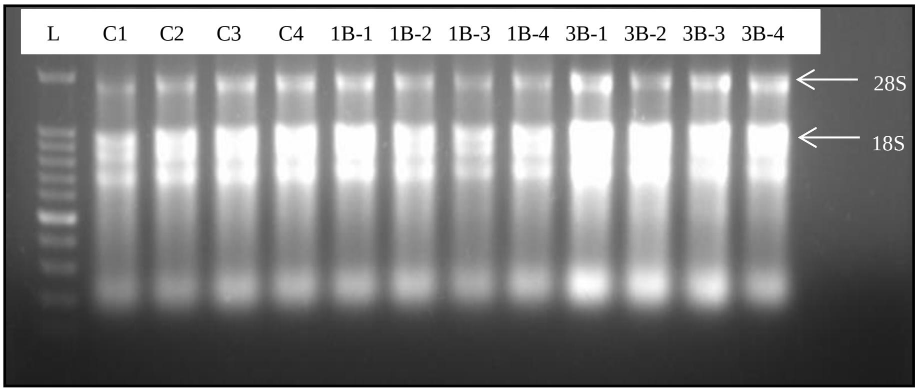

Figure 4.57: Visualization of total RNAs isolated from leaves of Bolal on the agarose gel (L: 100 bp Sizer^{TM} (Intron), C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3). 28S and 18S rRNAs were indicated.

Table 4.2: Concentrations and ratios (260/280 nm) of total RNAs isolated from leaves of wheat cultivars.

Sample	Atay		Bolal	
	Concentration (ng/µl)	A260/A280	Concentration (ng/µl)	A260/A280
C-1	398.5	2.0	309	2.0
C-2	293.7	2.0	331.3	2.0
C-3	345.7	2.0	375.5	2.0
C-4	348.5	2.0	371.6	2.0
1B-1	436.8	2.0	415.3	1.99
1B-2	448.9	2.05	324	2.04
1B-3	458.8	2.06	247.3	2.05
1B-4	446.9	2.05	314.5	2.0
3B-1	470.5	2.05	536.9	2.04
3B-2	463.3	2.0	452.8	2.04
3B-3	399.7	2.0	434.2	2.04
3B-4	368.8	2.0	374.1	2.03

4.3.3.2. Transcriptional and Post-Transcriptional Regulations

Before qRT-PCR analyses, RT-PCR was carried out for wheat genes in order to ensure that only one PCR amplicon is produced and its size is as estimated. All RT-PCR products were at expected sizes (Figure 4.58). However, unexpectedly, U6 primers gave product after amplification without reverse transcriptase (RT-) enzyme (Figure 4.59). For this reason, U6 primers were not used as internal control in qRT-PCR analyses. Moreover, GAPDH expression level was affected by B toxicity in qRT-PCR analysis. Therefore, this gene was not used as an internal control, as well.

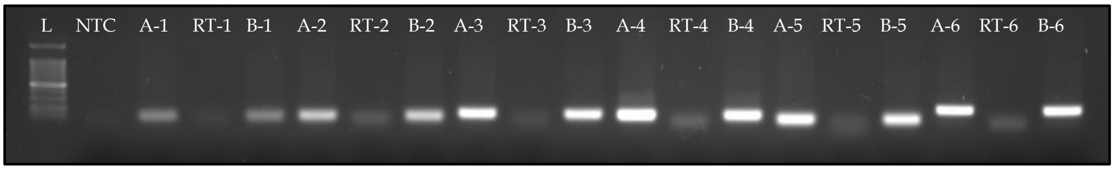

Figure 4.58: RT-PCR products on the agarose gel-1 (L: 100 bp Sizer^{TM} (Intron), NTC: no template control, A: Atay, B: Bolal, RT-: reverse transcriptase minus, 1: TOE1, 2: MYB3, 3: CSD, 4: GSTZ1, 5: Actin, 6: GAPDH).

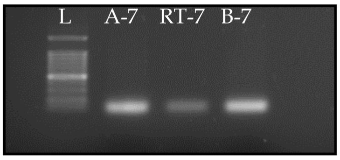

Figure 4.59: RT-PCR products on the agarose gel-2 (L: 100 bp Sizer^{TM} (Intron), A: Atay, B: Bolal, RT-: reverse transcriptase minus, 7: U6 sRNA)

Both 1B and 3B conditions led to remarkable increases in expression levels of *CSD* in both sensitive and tolerant cultivars (Figure 4.60). These increases were more remarkable in Atay with approximately ten and twelve-fold under 1B and 3B conditions, respectively, whereas five-fold increases were obtained in Bolal after these conditions (Figure 4.60). However, expression level of *miR398*, as CSD targeting miRNA, did not change significantly in Bolal under both toxic B conditions and in Atay after 3B (Figure 4.61). However, 1B caused a slight increase of *miR398* in Atay.

Similar to *CSD*, *GSTZ1* expressions were significantly up-regulated in both cultivars under all toxic B conditions (Figure 4.62). These inductions were greater in 3B condition than 1B. Especially, in Bolal there was an almost three-fold increase under 3B (Figure 4.62).

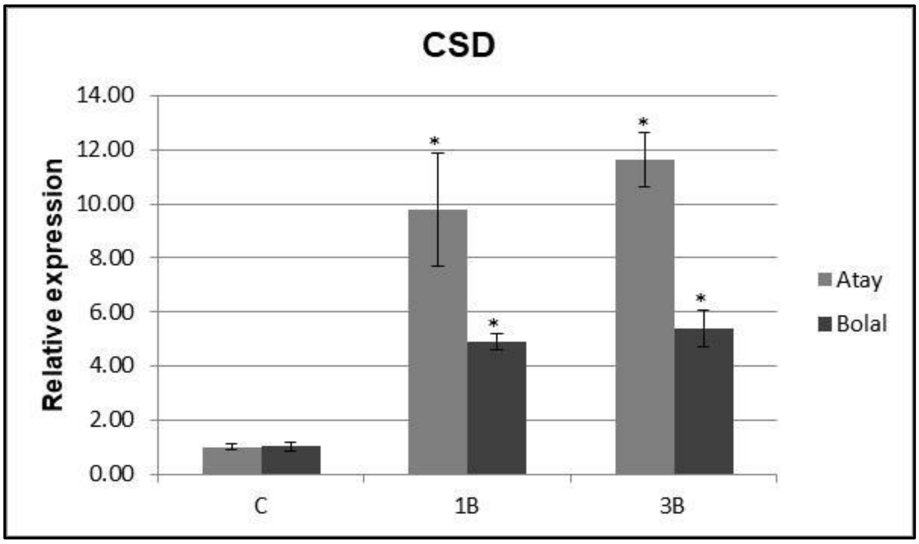

Figure 4.60: Relative expression level of *CSD* gene in wheat cultivars, Atay and Bolal. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference according to *t*-test.

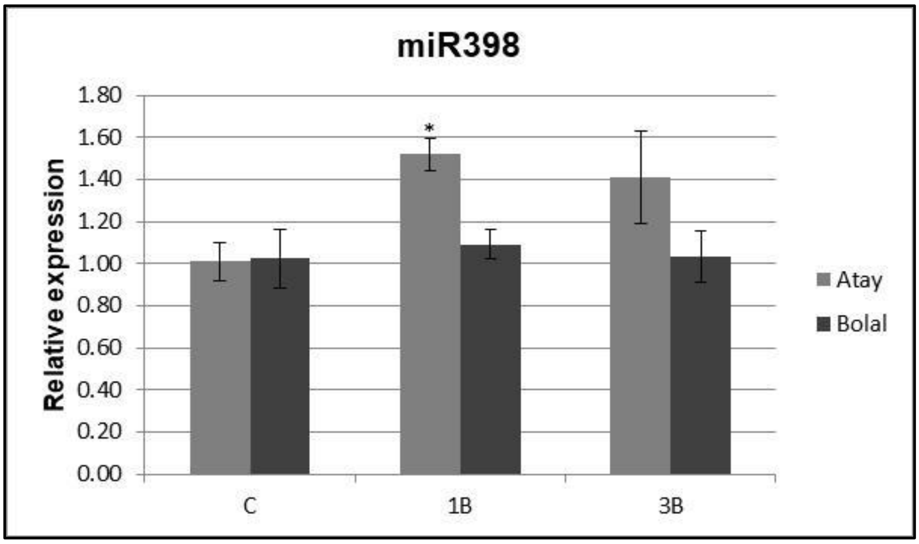

Figure 4.61: Relative expression level of *miR398* in wheat cultivars, Atay and Bolal. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference according to *t*-test.

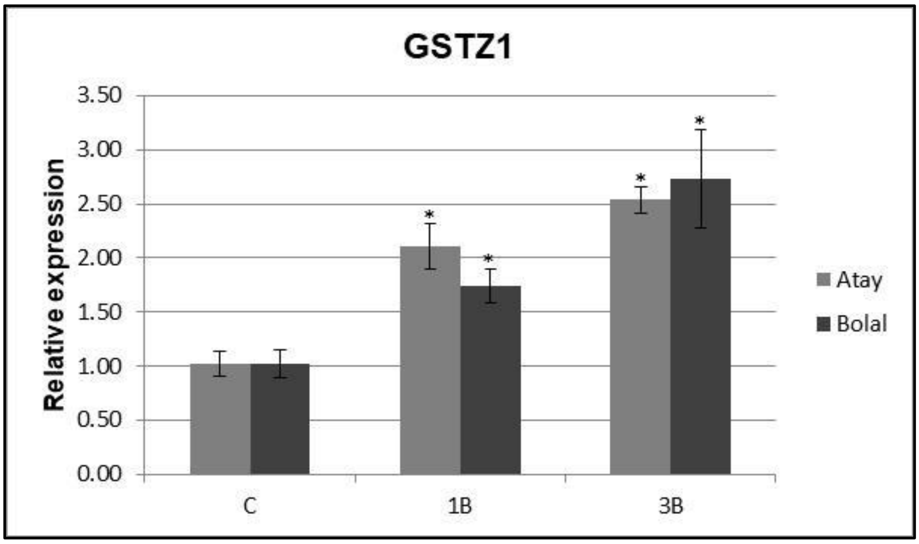

Figure 4.62: Relative expression level of *GSTZ1* in wheat cultivars, Atay and Bolal. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference according to *t*-test.

In Bolal, *miR319* expression was induced by both B toxicity conditions, but not significant (Figure 4.63). Likewise, 1B did not induce *miR319* in Atay (Figure 4.64). However, its expression level was increased slightly in 3B in this cultivar. In contrast to *miR319*, its target *MYB3* was induced significantly by B toxicity in both sensitive and tolerant cultivars (Figure 4.64). Indeed, *MYB3* expression level was increased more than four-fold by both B toxicity conditions in Atay. Furthermore, it was increased approximately six-fold by 1B and more than two-fold by 3B condition in Bolal (Figure 4.64).

Expression level of *miR172* was decreased significantly in Atay exposed to 1B condition whereas it stayed stable after 3B (Figure 4.65). On the other hand, 1B did not affect expression level of *miR172* in Bolal while 3B caused a non-significant increase in *miR172* expression (Figure 4.65). The target of *miR172,* TOE1, was induced by both 1B and 3B conditions in Atay, but only 1B induction was found statistically significant (Figure 4.66). In Bolal, *TOE1* expression level did not significantly change after both 1B and 3B treatments, however it decreased slightly after the latter (Figure 4.67).

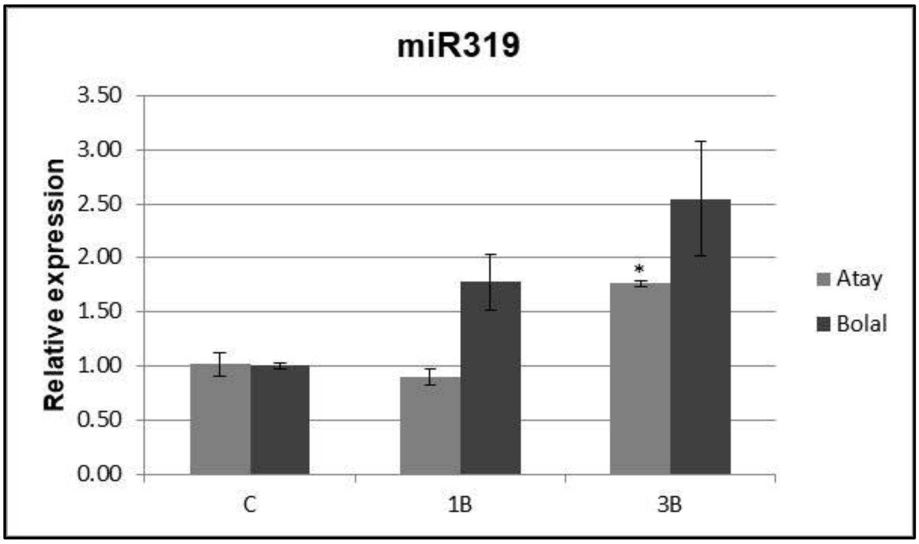

Figure 4.63: Relative expression level of *miR319* in wheat cultivars, Atay and Bolal. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference according to *t*-test.

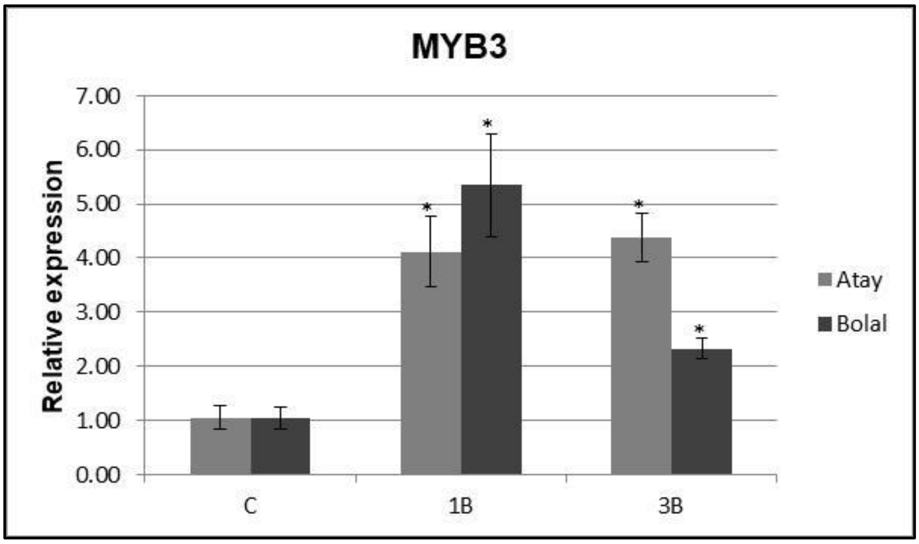

Figure 4.64: Relative expression level of *MYB3* in wheat cultivars, Atay and Bolal. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference according to *t*-test.

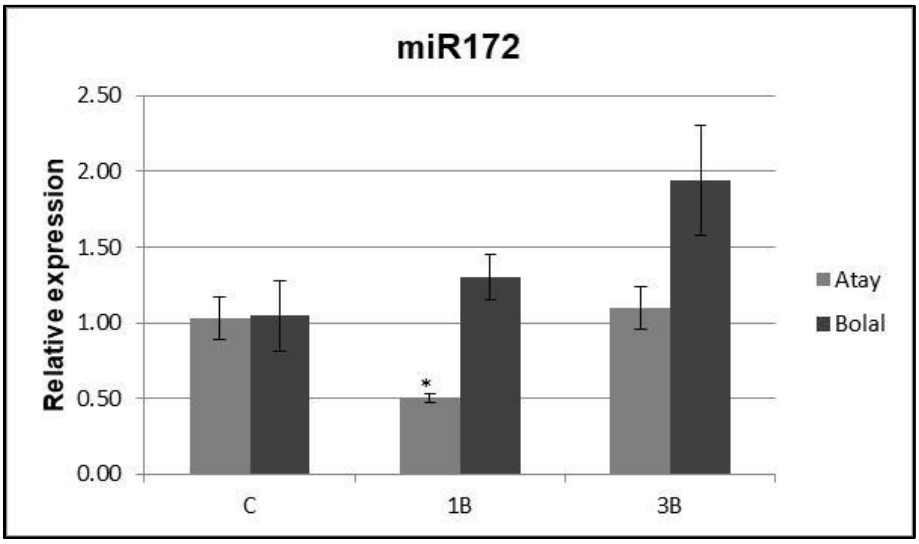

Figure 4.65: Relative expression level of *miR172* in wheat cultivars, Atay and Bolal. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference according to *t*-test.

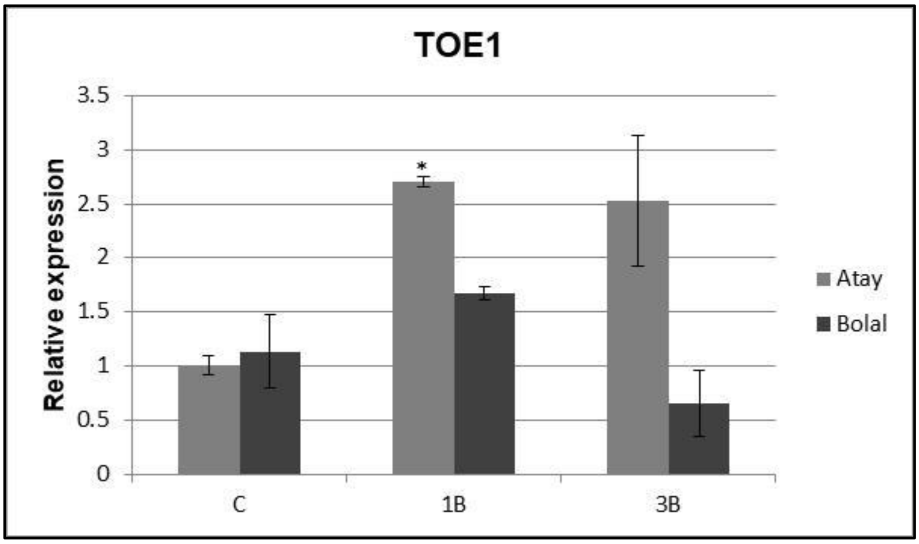

Figure 4.66: Relative expression level of *TOE1* in wheat cultivars, Atay and Bolal. C: control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Asterisk indicates a significant difference according to *t*-test.

5. DISCUSSION

Excess B in soils has been becoming a major limiting factor for cereal production in the world and thereby causing important yield loss, especially in arid areas [Nable et al., 1997; Emon et al., 2015]. Thus, it is crucial to understand the underlying mechanisms of B toxicity regulations in order to facilitate improving B tolerance of plants. A well-known mechanism of plants for avoiding from toxic effects of B is to export excess B by transporters [Miwa and Fujiwara, 2011] because tolerance to B toxicity is primarily associated with reduced B accumulation in tissues [Nable, 1988]. For instance, toxic B tolerance of a barley variety was attributed to active efflux of B from the roots [Hayes and Reid, 2004]. On the other hand, a subsequent work suggested that protection of intracellular processes was provided by exclusion of excess B from cytosol into the apoplast in both roots and shoots [Fitzpatrick and Reid, 2009]. However, the marker-assisted introgression of boron-efflux transporter genes in barley cultivars showed little to no improvement in yield in field trials conducted in B-contaminated soils [Emebiri et al., 2009; McDonald et al., 2010]. Meanwhile, the studies focused on cultivars differing in B tolerance clearly revealed that B content of plants exposed to extreme B was always higher, even if B-tolerant plants had lower B content than sensitive ones, and in each cultivar, oxidative damage could appear and antioxidative mechanisms could evoke [Cervilla et al., 2007; Ardıc et al., 2009]. In relation to that, B tolerant behaviour of *Citrus* was associated with re-regulation of B transport as well as activation of the antioxidant system [Martínez-Cuenca et al., 2015]. Thus, in addition to B transporters, other regulations such as enzymatic and non-enzymatic antioxidative mechanisms might have important roles for overcoming the B toxicity in plants. However, studies focusing on the antioxidative regulations under B stress do not display consistence in plants [Liu et al., 2005]. Therefore, the main goal of this thesis was to present an integrative view of B responsive regulations of antioxidative mechanisms including physiological, biochemical, transcriptional and post-transcriptional changes in *Arabidopsis thaliana*.

Germination of our *Arabidopsis thaliana* seeds was carried out in the medium with toxic B and as expected, leaf chlorosis, a typical visible symptom of excess B, was observed under B toxicity stress. Also, decrease in development and vigour were observed in leaf tissues of *Arabidopsis thaliana*. In fact, partial yellowing and growth inhibition were gradually increased with increasing toxic B level. Similarly, B treatment led to decreases in fresh and dry masses of *Arabidopsis thaliana* shoots as well as fresh masses of roots [Sürgün et al., 2016]. On the contrary, lengths, wet and dry weights did not significantly change following B toxicity stress in leaf and root tissues of wheat seedlings grown under high B for 5 d following growth in control condition for 8 d [Kayıhan et al., 2017]. This contradiction might be related to different seedling stages of different plants since plant developmental stage is one of the important characteristics for the specificity of the biological response to stresses [Gechev et al., 2006]. Contents of photosynthetic pigments in *Arabidopsis thaliana* leaves were dramatically decreased under high B stress. This reduction was gradual for Chl a and Chl b, in accordance with phenotypically viewed chlorosis of seedlings through increasing level of B toxicity. Inhibition of photosynthetic capacity by excess B was exhibited in many studies [reviewed by Landi et al., 2012]. Similar to our work, total chlorophyll contents decreased significantly in citrus [Han et al., 2009] and *Arabidopsis thaliana* after high B [Chen et al., 2014; Sürgün et al., 2016]. Moreover, excess B inhibits photosynthesis through alteration of electron transport rate via structural impairing of thylakoids [Pereira et al., 2000] or reduced contribution of NADPH and ATP because of impaired activity of some crucial enzymes involved in photosynthesis [Han et al., 2009]. In spite of decreased photosynthetic rate, total soluble (glucose, fructose, sucrose) and insoluble sugar (starch) contents were increased under B toxicity, especially 3B condition. Accumulation of these sugars might be associated with a dramatic inhibition of growth [Han et al., 2008] due to a limitation of their use by some metabolic processes involved in plant growth as under other types of stress. Meanwhile, other metabolic pathways that help to combat oxidative stress need to use these sugars in order to alleviate the toxicity symptoms of high B concentrations [Yokota and Konishi, 1990]. For instance, they are early precursors in AsA biosynthesis [Cervilla et al., 2007]. Supportively, Keles et al. (2004) suggested that high B caused an increase in the levels of AsA, glucose and fructose in orange plants.

Similar to sugar accumulation, dramatic increases in flavonoid and anthocyanin contents were observed during toxic B treatments. Likewise, the enhancements of flavonoid and anthocyanin contents were presented in tomato cultivars supplied with high B concentration [Cervilla et al., 2012]. Importantly, it has been recently suggested that anthocyanin plays photoprotective role in mesophyll cells when chloroplast functionality has been compromised by B toxicity and it has been shown that purple-leafed plants are more tolerant to B toxicity than green-leafed plants [Landi et al., 2014]. Moreover, a subsequent work exhibited that B tolerance of these purple-leafed plants arised from the higher phenolic content rather than reduced uptake or increased export of B [Pardossi et al., 2015]. The reduction of free B in tissues via complexation with anthocyanins and subsequent compartmentalization of B-anthocyanin complexes in vacuoles can provide a greater tolerance against high B [Landi et al., 2015].

In this thesis, we showed that 1B treatment caused an increase in MDA level in *Arabidopsis thaliana* indicating toxic B mediated oxidative damage because the level of MDA produced by peroxidation of membrane lipids was generally used as an indicator of oxidative damage [Eraslan et al., 2016]. Interestingly, the higher B concentration (3B) did not lead to lipid peroxidation in *Arabidopsis thaliana* due to stable MDA level. This result was in accordance with findings of Hamurcu and colleagues [2013] in soybean which suggested that level of lipid peroxidation was relatively decreased in response to increasing toxic B concentration. More clearly, MDA content increased under mild levels of B toxicity whereas it was lowered under moderate level, suggesting higher antioxidant activity of soybean [Hamurcu et al., 2013]. On the other hand, proline and H_2O_2 contents were significantly increased under only 3B treatment when compared to control and 1B conditions. Enhanced proline level accompanied with increased H_2O_2 content is a common response of plants against B toxicity [Karabal et al., 2003] and it is known that ROS may be detoxified by elevated levels of proline [Xiong and Zhu, 2002] which is inversely related with MDA concentration [Gunes et al., 2006; Molassiotis et al., 2006]. Martínez-Cuenca and colleagues [2015] supported this by showing increase in proline levels following high B treatment with a much lower increase in MDA.

As a major scavenger, SOD catalyzes the dismutation of $O_2{\cdot}^-$ to H_2O_2 and O_2 and this is an important protective step against biotic and abiotic stress-induced ROS production [Mittler, 2002]. In this thesis, SOD activity was markedly induced in *Arabidopsis thaliana* in 3B. This result can be verified with the expected increase in H_2O_2 content (3.5-fold) under 3B. Relatively lower increase in both SOD activity and H_2O_2 content was also observed following 1B treatment. Similarly, B toxicity-induced SOD activity, in parallel with raised amount of H_2O_2, was observed in maize [Esim et al., 2012], barley [Karabal et al., 2003], tomato [Cervilla et al., 2007], and chickpea exhibiting stringent contribution of SOD to B toxicity response [Ardıc et al., 2009].

Furthermore, the expression levels of SOD isoenzymes *MSD1* and *CSD1* were coincident with the total SOD activity. In fact, four-fold increments of *CSD1* gene expression can be mainly attributed to the increased activity of total SOD under 3B. In addition, we evaluated the expression level of *miR398* under 1B and 3B in order to gain better insight into transcriptional and post-transcriptional regulations of Cu/Zn SOD gene expression. Because Sunkar and colleagues [2006] demonstrated that Cu/Zn SOD isoenzymes were targets for miR398 in *Arabidopsis thaliana*. *miR398* expression was up-regulated by 1B although it remained unchanged under 3B comparing to control. Increased expression level of *miR398* probably suppressed its main target *CSD1* because the relatively low increases in total SOD activity and *CSD1* expression were observed under 1B. Furthermore, the stable level of *miR398* expression in 3B was coincident with the expected increase in total SOD activity and *CSD1* expression.

H_2O_2, produced with dismutation reaction of SOD, is still toxic and needs to be eliminated by conversion to H_2O in subsequent enzymatic reactions via CAT, APX, POX. In this study, activities of APX and POX changed with a slightly increase under 3B condition while activity of CAT remained stable which probably caused to accumulation of H_2O_2. This was verified by 3.5-fold increment of H_2O_2 content. On the other hand, both APX and POX activities were induced under 1B condition, while CAT activity was significantly decreased. The inverse modulation between APX and CAT was expectable since it was earlier shown that CAT-deficient mutant of barley compensated the reduction of CAT activity by enhancing activity of APX [Palatnik et al., 2002]. Similarly, induction of APX activity was observed in citrus while reduction of CAT activity was recorded after excess B exposure [Han et al., 2009]. Likewise, activities of CAT and APX were inversely regulated by drought stress in *Jatropha curcas* [Silva et al., 2012]. In agreement with our results, mild level of B toxicity caused induction of both APX and POX activities along with slight decrease of CAT activity in soybean [Hamurcu et al., 2013]. Inhibition of CAT activity was also observed in citrus [Keles et al., 2004] and in SC2121 cultivar of tomato [Oluk et al., 2012] under high B. Similarly, CAT activity decreased after 1.6 mM B in sensitive cultivar of chickpea but did not change after higher toxic level of B (as much as 6.4 mM) while APX activity increased and stayed stable respectively [Ardıc et al., 2009]. In our study, higher APX activity along with POX activity in 1B probably helped to maintain H_2O_2 content at normal level since ROS other than H_2O_2 could inactivate CAT [Escobar et al., 1996; Wang et al., 2004]. On the other hand, *APX6* expression was induced in 1B but did not alter in 3B in accordance with total APX activity. However, transcript level of *CAT1* (isozyme correspond to Class III catalase) showed a slightly increase following both toxic B conditions. This implied that changes of CAT activity did not absolutely correspond to the changes of *CAT1* expression under B toxicity conditions in *Arabidopsis thaliana*. Although *CAT1* has an important role in the decomposition of H_2O_2 generated under various abiotic stresses, *CAT2* and *CAT3* are the major catalases under normal conditions because in the cat1 RNAi lines, overall CAT activities were decreased by only 8% and the absence of *CAT1* did not affect cold-induced CAT activities in *Arabidopsis thaliana* [Due et al., 2008]. The expression of *CAT1* was activated by B toxicity as other environmental stresses [Due et al., 2008], implying that it might play a role in a feedback regulating of ROS signaling.

Similar to CAT, total GR activity and expression levels of *ATGR1* and *ATGR2* were not coordinately regulated in *Arabidopsis thaliana* under B toxicity stress. This might be related to possible post-transcriptional regulation of GR. Likewise, the *ZmGR1* is post-transcriptionally regulated in bundle sheath cells [Pastori et al., 2000]. On the contrary, Bashir and colleagues [2007] suggested that *GR1* and *GR2* genes did not have post-transcriptional regulation in rice, wheat and barley under stress conditions. Interestingly, Yannarelli et al. [2007] found that the changes in the GR isoform pattern did not occur as a result of an enhancement of GR mRNA and protein expression, indicating that post-translational modification might take place. Besides, unchanged GR activity might be related to inadequate supply of NADPH because the GSH-GSSG redox pair can effectively function only when there is an adequate NADPH [Potters et al., 2002]. Supportively, binding ability of B to the ribose moieties of NADPH [Reid et al., 2004] may reduce its function in toxic B condition. For this reason, further studies might be required to elucidate the molecular mechanisms of GR in plants.

In ascorbate-glutathione (AsA-GSH) cycle, following catalisation of ascorbic acid into monodehydroascorbate (MDHA) by APX, it rapidly converts to dehydroascorbate (DHA). Reduction back to AsA occurs by actions of both NAD(P)-dependent MDHAR and DHAR which needs GSH reduced by NADPH-dependent GR [Smirnoff, 2000]. In this study, expression levels of GR genes were coordinately regulated with *DHAR2* expression. Furthermore, the changes in expression level of *MDAR2* were in accordance with changes in *APX6* expression and total APX activity, implying that AsA-GSH cycle might contribute to trigger antioxidative responses in *Arabidopsis thaliana* in response to B toxicity.

Toxic 3B condition induced *ATGR1* and *ATGR2* expression but did not GR activity. This might be related to stable GSH level under 3B condition. GSH, as one of the most effective antioxidants of plant tissues, can protect the cells from various abiotic stresses via direct quenching of ROS; conjugation of toxic metals and other xenobiotics to GST; and acting as a precursor for the synthesis of PCs [reviewed by Hasanuzzaman et al., 2017]. GSH is biosynthesized in two sequential ATP-dependent reactions, catalyzed by glutamate cysteine ligase (GSH1; EC 6.3.2.2.) and glutathione synthetase (GSH2; EC 6.3.2.3). In this study, under 1B toxicity condition, GSH levels did not significantly change in *Arabidopsis thaliana*. Coordinately, expression levels of *ATGSH1* and *ATGSH2* stayed stable in 1B. On the other hand, *ATGSH1* expression was significantly increased in 3B condition, whereas GSH level and *ATGSH2* expression remained unchanged. This means that upregulation of *ATGSH1* expression might not directly induce GSH level in spite of rate-limiting step of GSH biosynthesis [Noctor et al., 2012] because translational activation of *GSH1* expression [Xiang and Bertrand, 2000] and a redox-based post-translational activation of GSH1 enzyme [Jez et al., 2004] have already suggested due to uncorrelated results between GSH1 expression and its activity in *Arabidopsis thaliana* [May et al., 1998].

Non-protein thiol content did not significantly change following 1B and 3B treatments. This might be related to GSH biosynthesis. As previously reported, an increase in GSH leads to an increase in non-protein thiol content, which promotes improvement of heavy metal tolerance in maize [Requejo and Tena, 2012]. PC content might be affected by stable GSH level because PCs are synthesized from GSH by action of phytochelatin synthase (PCS). Correlatively, transcript level of *PCS1* gene was not notably changed under both B toxicity conditions. On the other hand, protein-bound and total thiol contents were increased, through increasing level of B toxicity. The beginning of thiol metabolism is the uptake of sulfur and its successive assimilation [Wirtz and Hell, 2007]. Supportively, genes (ta.6012.1.s1_a_at and ta.3736.1.a1_x_at) encoding for sulfate transmembrane transporter were differentially regulated in leaf and root tissues of B-sensitive wheat cultivar under B toxicity condition [Kayıhan et al., 2017]. This means that some thiol containing compounds might have a role in B tolerance mechanism in plants.

The central position in GSH network is occupied by GST [Labrou et al., 2015]. GSTs have important roles in stress tolerance and other protective roles in plants [Nianiou-Obeidat et al., 2017]. In this thesis, four-fold increase in GST activity was observed in response to 1B treatment. Interestingly, the change in this activity was lower (more than two-fold) under 3B condition. This might be related to toxic B mediated oxidative stress because higher MDA level was observed in 1B treatment than 3B. Thus, it seems that total GST activity might dramatically increase in response to a sharp increase in MDA level under 1B. Correlatively, transgenic plants over-expressing one of the GST member suppress MDA concentration under stress conditions [Xu et al., 2016; Gao et al., 2016; Xu et al., 2017]. On the other hand, no correlation between GSH level and GST activity was found under B toxicity conditions. In other words, in spite of stable level of GSH, a dramatic increase in GST activity may indicate undisturbed usage and recycling of GSH. In fact, increased activities of GR along with GST probably may be a reason for stable level of GSH in 1B condition. Associatively, possible alternative pathways to convert GSSG back to GSH through class III peroxidases has been recently suggested due to uncorrelated results between GR and glutathione peroxidase in wheat [Liu et al., 2015].

Increased GST expression was shown to correlate with enhanced stress tolerance in tomato [Sun et al., 2010], wheat [Gallé et al., 2009] and barley [Rezaei et al., 2013]. Copper treatment increased expression levels of three phi class and one tau class members of GST superfamily genes in *Arabidopsis thaliana* [Smith et al., 2004]. Likewise, B toxicity led to differential regulations of some GST genes in barley [Öz et al., 2009] and wheat [Kayıhan et al., 2017]. In this study, both B toxicity conditions did not lead to a significant change in the expression levels of *ATGSTF2* and *ATGSTF8*, whereas 3B slightly reduced *ATGSTF6* and *ATGSTF7* expressions. It thus seems that phi class of GST might not involve in B tolerance or responsive mechanisms in *Arabidopsis thaliana* exposed to toxic B. On the other hand, *ATGSTU19* was markedly induced following B toxicity conditions. Recently, it was reported that overexpression of *ATGSTU19* caused an enhancement tolerance of salt, drought and methyl viologen stresses in *Arabidopsis thaliana* since the GST and other antioxidant enzymes activities and proline were increased and the expression of some late stress-response genes were activated even under normal growth conditions [Xu et al., 2016]. Likewise, ATGSTU19 can involve in tolerance mechanism against B toxicity in *Arabidopsis thaliana*. Furthermore, *ATGSTZ1* expression was dramatically increased following both B toxicity conditions. AtGSTZ1 is known for its role in tyrosine catabolism in *Arabidopsis thaliana* [Dixon et al., 2000]. It seems that tyrosine catabolism might be affected by B toxicity in plants. Likewise, Gao et al. [2016] found by using ThGSTZ1-overexpressing transgenic *Arabidopsis thaliana* that ThGSTZ1 regulated the activities and expression levels of protective enzymes and the ROS scavenging ability and thus it had a positive role in abscisic acid and methyl viologen tolerance. On the other hand, levels of *MDAR2* and *APX6* expressions and APX activity were in accordance with our findings related to expression levels of *ATGSTU19*, *ATGSTZ1* and total GST activity. In conclusion, we are sure that ATGSTU19 and ATGSTZ1 can have a role in a dramatic increase of total GST activity under B toxicity conditions and GST can have a special protective role in B toxicity tolerance in plants.

Recently, Yıldırım [2017] suggested a new internal B detoxification mechanism due to higher up-regulation of GST, HIPP and ABC transporters in poplar exposed to toxic B. On the other hand, compartmentalization of B-anthocyanin complexes in vacuoles has been recently suggested as one of the tolerance mechanisms against B-toxicity [Landi et al., 2015]. Accordingly, anthocyanin-GSH or -GST complexes can transiently bind to metal or metalloid ions and thus forming glutathionyl–anthocyanin–metal complexes and/or GST-anthocyanin-metal complexes can be sequestered into the vacuole and GST-anthocyanin-metal complexes can also be exported by ABC transporters. Supportively, we have already found that anthocyanin contents were significantly enhanced following B toxicity conditions. Moreover, ABC transporters are commonly and differentially up-regulated in two contrasting wheat cultivars under high B [Kayıhan et al., 2017].

To solve the underlying mechanism of a stress, the first step is to investigate stress responsive genes and their regulatory networks. In this thesis, in addition to expression of SOD isozymes and their respective activities, the most notable regulation was obtained in both expression and activity of GST in *Arabidopsis thaliana*. Therefore, in order to provide a more detailed insight to GST regulation, we tried to find miRNAs related to GST in the literature. However, we did not encounter any miRNA which directly targets a GST gene in *Arabidopsis thaliana*. Yet, miR156 and miR169 were suggested to direct the regulation of GST in radish [Xu et al., 2013] and in sugarcane [Gentile et al., 2015], respectively. Thus, we determined expression levels of these miRNAs in *Arabidopsis thaliana* under B toxicity. Both toxic B conditions led to significant decrease of *miR156* expression, indicating that it may be B toxicity responsive. Similarly, *miR156* was suggested as a B stress responsive miRNA in barley [Özhüner et al., 2013]. We found that centered site of *miR156* has imperfect complementary to 3' UTR region of *ATGSTU19* in *Arabidopsis thaliana*. Accordingly, repressed miR156 expression by toxic B conditions was correlated with up-regulation of ATGSTU19 expression. On the other hand, according to psRNATarget analysis, main target of miR156 in *Arabidopsis thaliana* is Squamosa promoter binding protein like transcription factor (SPL) which regulates developmental timing and phase transition from vegetative stage to reproductive stage in plants [Cardon et al., 1997]. Moreover, *SPL9* promotes transcription of *miR172b* in *Arabidopsis thaliana* [Wu et al., 2009]. In other words, miR156 indirectly contributes to the regulation of miR172. Supportively, in contrast to *miR156*, *miR172* was up-regulated by B toxicity. Especially, 1B led to four-fold increase in expression of *miR172* which regulates flowering time and floral organ identity in *Arabidopsis thaliana* by targeting APETALA2/ETHYLENE RESPONSE FACTORS (AP2/ERFs) [Zhao et al., 2007]. These transcription factors, as key components of ethylene signaling cascades, contribute to homeostasis by negative or positive regulations in response to biotic and abiotic stresses [Phukan et al., 2017]. In addition to ethylene responsive genes, jasmonate (JA) responsive genes are regulated by ERFs [Ou et al., 2011]. It is well known that GSTs are affected by JA and ethylene (Wagner et al., 2002; Moons, 2003; Shahrtash, 2013). Moreover, B toxicity was previously found to induce JA and ethylene related genes [Kayıhan et al., 2017; Öz et al., 2009]. Thus, we also determined the relative expression levels of JA related miRNAs including *miR159*, *miR319* and *miR394*. Interestingly, all JA/ethylene responsive miRNAs showed the same profile with GST under 1B. Moreover, *miR172* and *miR319* were significantly induced by both toxic B conditions but this induction was remarkable in 1B condition which indicates that these miRNAs might be B stress-responsive through regulating GST by means of ethylene and JA. Supportively, both JA and ethylene related genes were differentially regulated under B toxicity in barley [Öz et al., 2009] and wheat [Kayıhan et al., 2017]. This pattern was attributed to a possible cross-talk between B stress signaling and biotic stress [Öz et al., 2009] because JA controls responses to pathogen attack and wounding [Moons, 2003]. On the other hand, differential regulations of miR159, miR319, miR394 and miR156-miR172 may be a stress-adaptive response through modulation of morphological characteristics in plants [Khraiwesh et al., 2012].

According to psRNATarget analyses, *miR159*, *miR319* and *miR394* target *MYB* (Myeloblast), *TCP* (Teosinte branched 1, Cycloidea and Proliferating cell nuclear antigen factors) and F-*box* transcription factors in *Arabidopsis thaliana*, respectively. Likewise, *AP2* and *MYB* expressions were affected by B in wheat [Kayıhan et al., 2017]. Similar to *miR172* and *miR319*, *miR169* was also induced by both 1B and 3B, and this induction was remarkable after 1B. An essential role for *miR169* in stress-induced early flowering was suggested in *Arabidopsis thaliana* [Xu et al., 2013]. According to psRNATarget analysis, miR169 targets Nuclear factor Y (NF-Y) transcription factor in *Arabidopsis thaliana*. NF-YA regulates the expression of stress-responsive genes including GST, thus *ATGSTU24* and *ATGSTU25* were upregulated in NFY5-overexpressed transgenic *Arabidopsis thaliana* plants [Li et al., 2008]. However, we found that seed region of miR169 has perfect complementary to phi members of GST such as ATGSTF2, ATGSTF7, ATGSTF8. This might reflect reduced or stable levels of expression of these genes under B toxicity conditions.

It was earlier suggested that excess B could alter cell wall structure [Reid et al., 2004; Ghanati et al., 2005]. Supportively, Kayıhan and colleagues [2017] determined differential regulation of genes related to cell wall modification in wheat. Therefore, we investigated the regulations of *miR397* and *miR408* targeting laccase gene, *LAC*, which encodes a Cu-containing enzyme that involves in secondary cell wall integrity while plants are responding to an abiotic stress [Liang et al., 2006]. However, *miR397* expression level remained stable under both B toxicity conditions in this thesis. Contrarily, remarkable induction of *miR397* in B-sensitive *Citrus* cultivar in response to high B supply has been recently revealed [Huang et al., 2016]. Accordingly, *LAC* genes were repressed in that cultivar. On the other hand, *LAC* genes were also found as target of *miR408* in *Arabidopsis thaliana* [Thatcher et al., 2015]. In accordance with that, we could not determine any change in the expression of *miR408*, as *miR397*, in B toxicity conditions when compared to controls. Thus, we can suggest that B-toxicity might not affect cell wall modification at post-transcriptional level in *Arabidopsis thaliana*.

In order to confirm our most striking findings in cereals, we examined the regulation of GST not only at biochemical level, but also at molecular level in wheat cultivars differing in B tolerance. Furthermore, we investigated expressional regulations of two miRNAs, *miR319* and *miR172*, which were most significantly induced among miRNAs in *Arabidopsis thaliana* under B toxicity, and expressions of their targets, *MYB3* and *TOE1*, in wheat cultivars. We also checked transcriptional and post-transcriptional regulations of *CSD* in wheat.

In B-sensitive cultivar Atay, GST activity increased significantly after 1B condition. On the contrary, 1B led to decrease in GST activity of tolerant cultivar Bolal. On the other hand, GST activity was reduced in both cultivars by 3B. In other words, GST activity decreased through increasing level of B in wheat. Toxic symptoms were observed only in Atay under 1B condition. This might be related to increased GST activity. Moreover, non-induction of GST in Bolal under 1B may be due to inherent higher GST activity of this cultivar. However, B toxicity, as much as 3B, inhibited GST activity in both cultivars. Similarly, lower activity of GST in 3B than 1B was observed in *Arabidopsis thaliana*. On the contrary, *Tae-GSTZ1* expressions were significantly induced under both conditions in both cultivars. Similarly, both 1B and 3B caused up-regulation of *ATGSTZ1* whose mRNA has 71% identity with its orthologue in wheat, *Tae-GSTZ1*, according to BLAST analysis. In contrast to *Tae-GSTZ1*, *ATGSTZ1* expression was in accordance with GST activity. It seems likely that other members of GST might be responsible for reduction of GST activity or possible post-mechanisms are involved in regulation of GST in wheat under B toxicity. TaERF3 transcription factor activates transcription of GST6 by directly interacting with its promoter in wheat [Rong et al., 2014]. On the other hand, psRNATarget analysis shows that one of the targets of miR172 in wheat is TOE1 (Target of Early Activation Tagged 1) which belongs to putative AP2 like family. Thus, *miR172* might indirectly regulate GST expression in wheat. In agreement with that, *miR172* expression was decreased only in Atay after 1B, and GST activity was increased solely in this cultivar and in this condition. Unlike *Arabidopsis thaliana*, *miR172* stayed stable under 3B in Atay. However, *TOE1* expression was increased in both conditions in this cultivar. Both *miR172* and *TOE1* expressions were not altered significantly in Bolal, although a slight increase in level of *miR172* and a slight decrease in *TOE1* level were observed. miR172 seems likely to be regulated species specific and even, cultivar specific, under B stress. In fact, specific regulations of miRNA-mediated responses participate to varying tolerance capacity of species and also cultivars of same plant against abiotic stresses [Zhang, 2015].

Tae-miR319 expression did not change after 1B but increased significantly after 3B in Atay, whereas its target *MYB3* [Kumar et al., 2015] was remarkably induced in both conditions. On the other hand, increasing level of B led to higher induction of *miR319* and accordingly, lower induction of *MYB3* in Bolal. Contrarily, *miR319* expression was induced by 1B more than 3B in *Arabidopsis thaliana*. MYB transcription factors involve in many processes in plants, including stamen and petal development, embryogenesis, flavonoid and anthocyanin biosynthesis as well as biotic and abiotic stress responses or tolerance in plants via hormone signaling [reviewed by Ambawat et al., 2013]. Tae-MYB3 has a high similarity with AtMYB59 in *Arabidopsis thaliana* [Rahaie et al., 2013], whose expression is induced by JA and ethylene [Li et al., 2006]. Furthermore, higher miR319 expression accompanying low MYB3 in early stage of wheat was attributed to higher developmental rate [De Paola et al., 2016]. Thus, in our case remarkable induction of MYB3 may imply inhibition of development due to B stress.

The most remarkable induction of expression was observed in *CSD* with ten-fold and approximately twelve-fold increases in Atay in 1B and 3B conditions, respectively. Accordingly, increase of *miR398* expression was negligible under 1B and was stable under 3B in this B-sensitive cultivar. Similarly, less but still remarkable increase of *CSD* expression was determined in Bolal exposed to 1B (four-fold) and 3B (five-fold). Moreover, *miR398* expression remained same under both conditions in this cultivar. Stable level of *miR398* in both cultivars under B toxicity seems likely to contribute to induction of *CSD*. Furthermore, increased expression levels of *CSD* in both cultivars of wheat, as well as *Arabidopsis thaliana*, exhibited that induction of SOD may be a common response of plants against B toxicity.

To sum up, induced expression levels of GSTZ1, MYB3 and CSD were common responses for two wheat cultivars against B toxicity. On the contrary, between two cultivars, B toxicity conditions caused induction of TOE1 in only sensitive cultivar Atay. Thus, TOE1 regulation via miR172 may contribute to B tolerance in wheat.

In conclusion, this thesis shows the first demonstration of integrative view of toxic B-induced antioxidative regulations at physio-biochemical and molecular levels in *Arabidopsis thaliana*. It provided a better insight into biochemical and transcriptional regulations of enzymatic antioxidant machinery. When compared to 1B, higher B concentration (3B) did not provoke oxidative damage because it promoted accumulation of non-enzymatic antioxidants such as anthocyanins, flavonoids and proline, and strongly stimulated SOD expression and its respective activity in *Arabidopsis thaliana*. The remarkable induction of SOD isozyme CSD was also determined in wheat. On the other hand, AsA-GSH cycle was induced by toxic B at transcriptional level in *Arabidopsis thaliana*. However, non-correlation was observed between GR activity and expression levels of *ATGR1* and *ATGR2* genes. We found that expression level of *MDAR2* was coordinately regulated with *APX6* expression and total APX activity. These results were in accordance with expression levels of *ATGSTU19*, *ATGSTZ1* and total GST activity. Thus, unlike phi class GST members, ATGSTU19 and ATGSTZ1 can have an essential role in total GST activity in *Arabidopsis thaliana* under B toxicity conditions. Interestingly, increasing levels of B led to reduced activity of GST in wheat cultivars, despite the significant induction of *Tae-GSTZ1* by all toxic conditions in both cultivars.

Our findings supported a new hypothesis suggesting an internal B detoxification mechanism via GSH-GST conjugation in plants. However, further studies are required to better understand the possible role of GSH dependent pathways in the internal B detoxification mechanism in plants.

Obviously, B toxicity gave rise to induction of some JA and ethylene related miRNAs in *Arabidopsis thaliana*. Especially, miR156-miR172 and miR319 can have important roles in B toxicity response. And these post-transcriptional regulations were verified in wheat under excess B by demonstration of induced expression levels of *MYB3* and *TOE1*, for the first time in literature. Moreover, *TOE1* was differentially regulated in sensitive and tolerant cultivars, indicating miR172 and its target might be responsible for B tolerance in wheat. On the other hand, although B stress seems like to promote common responses with biotic stress, such as induction of JA and ethylene metabolism, these responses may occur as cell wall modification-independent manner because of the unchanged expression levels of *miR397* and *miR408* in *Arabidopsis thaliana* under high B conditions.

6. CONCLUSION

- This thesis was the first demonstration of integrative view of toxic B-induced antioxidative regulations at physio-biochemical and molecular levels in *Arabidopsis thaliana.*
- Chlorosis, a typical visible symptom of excess B, and reduced development and vigour were observed under high B conditions in leaves of *Arabidopsis thaliana*.
- Partial yellowing and growth inhibition were gradually increased with increasing toxic B level.
- Contents of photosynthetic pigments in *Arabidopsis thaliana* leaves were dramatically decreased under B toxicity. This reduction was in accordance with phenotypically viewed chlorosis of seedlings through increasing level of B toxicity.
- When compared to 1B, higher B concentration (3B) did not provoke oxidative damage because it promoted accumulation of non-enzymatic antioxidants such as anthocyanins, flavonoids and proline, and strongly stimulated SOD expression and its respective activity which led to over accumulation of H_2O_2.
- SOD activity was in accordance with transcriptional and post-transcriptional regulation of *CSD1*.
- Ascorbate-glutathione (AsA-GSH) cycle seems like to be induced by toxic B at transcriptional level.
- GR activity was not regulated coordinately with its expression level.
- Expression level of *MDAR2* was coordinately regulated with *APX6* expression and total APX activity. These results were in accordance with expression levels of *ATGSTU19, ATGSTZ1* and total GST activity.
- Phi class of GST might not involve in B tolerance or responsive mechanisms in *Arabidopsis thaliana* exposed to toxic B.
- ATGSTU19 and ATGSTZ1 can have a role in a dramatic increase of total GST activity under B toxicity conditions.
- Among the miRNAs, expression levels of miRNAs targeting transcription factors related to JA and ethylene metabolisms were induced remarkably in lower B toxicity condition but not in higher B toxicity condition. These

findings were in accordance with biochemical and transcriptional regulations of GST.

- Most remarkable regulations at post-transcriptional level were obtained in miR156-miR172 and miR319 in *Arabidopsis thaliana.*
- Expression levels of *miR408* and *miR397* remained stable under B toxicity, indicating lack of post-transcriptional regulation of genes related to cell-wall modification.
- Some striking results from *Arabidopsis thaliana* were screened at biochemical, transcriptional and post-transcriptional levels in B-sensitive and -tolerant wheat cultivars exposed to toxic B, for the first time in literature.
- 1B condition caused yellowing in leaves of sensitive cultivar while it did not cause any visible symptom in tolerant cultivar.
- GST activity was increased only in sensitive cultivar after 1B, however 3B inhibited activity of GST in both cultivars.
- Level of GST activity was always higher in B-tolerant wheat cultivar than the sensitive one.
- *Tae-GSTZ1* was induced significantly in both cultivars under all B stress conditions.
- GST can have a special protective role in B toxicity tolerance in plants.
- As in *Arabidopsis thaliana*, *CSD* was induced remarkably in wheat under B toxicity. However, its induction was much more remarkable in sensitive cultivar. Thus, SOD regulation seems likely a common response to B stress in plants.
- Remarkable increases in target of *miR319, MYB3*, in wheat under B toxicity indicated reduced development mediated by JA.
- There was no correlation for *miR172* expression between *Arabidopsis thaliana* and wheat cultivars, indicating this miRNA is specifically regulated in plants responding to excess B.
- Significant induction of *TOE1* in sensitive cultivar under B stress shows that *miR172* and its target (*TOE1*) can have a role in B tolerance mechanism in plants.

7. FUTURE PERSPECTIVES

In this thesis, investigation of B responsive antioxidative regulations at physio-biochemical, transcriptional and post-transcriptional levels, as an integrative approach, in a model plant *Arabidopsis thaliana* was aimed. Subsequently, confirmation of these findings in a cereal was aimed by using two contrasting wheat cultivars.

Although expressions of most antioxidant enzymes were in accordance with their respective activity, GR activity and expressions of *ATGR1* and *ATGR1* were not coordinately regulated. In the literature, some authors suggested post-transcriptional [Pastori et al., 2000] or post-translational regulation [Yannarelli et al., 2007] of GR whereas some authors suggested that post-transcriptional modification may not involve in GR regulation [Bashir et al., 2007]. Additionally, we are suggesting that limited availability of NADPH for GR function may be a reason for this consequence due to high B. Thus, more detailed studies should be carried out to ensure the underlying mechanism of GR regulation under B toxicity.

We determined a strong induction of GST activity in *Arabidopsis thaliana* in response to high levels of B. Further, we investigated transcriptional regulation of several GST genes from three different classes of this enzyme. We concluded that zeta and tau classes might involve in increasing activity of GST in response to B stress. However, there are 55 members within eight classes of this superfamily. Hence, examination of the expressional regulation of remaining GST members may provide more certain results in terms of B responsive GST regulation in plants. Moreover, we seriously wonder if there are any post-transcriptional regulations of GST in plants under B stress due to non-correlation between GST activity and *Tae-GSTZ1* expression in wheat.

We found that some JA and ethylene metabolism related miRNAs were differently regulated in *Arabidopsis thaliana* by B toxicity. Supportively, a possible cross-talk between biotic stress and B toxicity response were previously suggested. However, for a more reliable suggestion, we need to check the changes of JA and ethylene hormones at biochemical levels and furthermore, we also need to determine the expression levels of targets of these miRNAs in *Arabidopsis thaliana*.

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BIOGRAPHY

Doğa Selin KAYIHAN was born in 1987, Paris, FRANCE. She earned her B. Sc degree from Marmara University on Biology. She started Master’s program in Department of Molecular Biology and Genetics at Gebze Institute of Technology in 2009 and graduated in 2012. She started her PhD in 2012 at Gebze Technical University, Graduate School of Natural and Applied Sciences, Deparment of Molecular Biology and Genetics. Since 2010, she has worked in Department of Molecular Biology and Genetics at Gebze Technical University as a research assistant.

APPENDICES

Appendix A: The Published Article from This Thesis

Doğa Selin KAYIHAN, Ceyhun KAYIHAN, Yelda Özden Çiftçi, (2016), "Excess boron responsive regulations of antioxidative mechanism at physio-biochemical and molecular levels in *Arabidopsis thaliana*.", Plant Physiology and Biochemistry, 109: 337-345.

Appendix B: Visualization of RT-PCR Products of *Arabidopsis thaliana* Genes on Agarose Gels

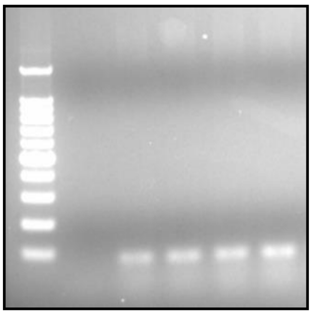

Figure B1.1: RT-PCR products of *Act2* from four biological replicates of *Arabidopsis thaliana* leaves on the agarose gel. L: 100 bp SizerTM (Intron); Expected band size: 93 bp.

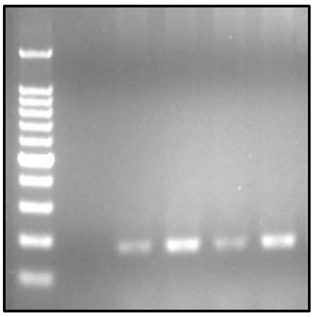

Figure B1.2: RT-PCR products of *APX6* from four biological replicates of *Arabidopsis thaliana* leaves on the agarose gel. L: 100 bp SizerTM (Intron); Expected band size: 189 bp.

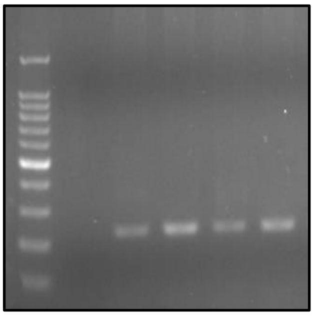

Figure B1.3: RT-PCR products of *ATGR1*from four biological replicates of *Arabidopsis thaliana* leaves on the agarose gel. L: 100 bp SizerTM (Intron); Expected band size: 235 bp.

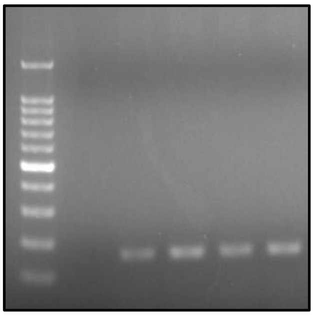

Figure B1.4: RT-PCR products of *ATGR2* from four biological replicates of *Arabidopsis thaliana* leaves on the agarose gel. L: 100 bp SizerTM (Intron); Expected band size: 172 bp.

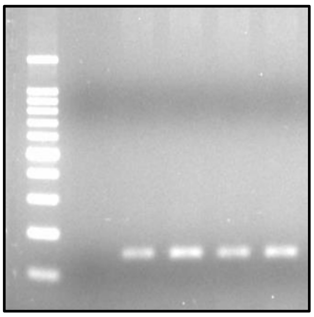

Figure B1.5: RT-PCR products of *ATGSH1*from four biological replicates of *Arabidopsis thaliana* leaves on the agarose gel. L: 100 bp SizerTM (Intron); Expected band size: 152 bp.

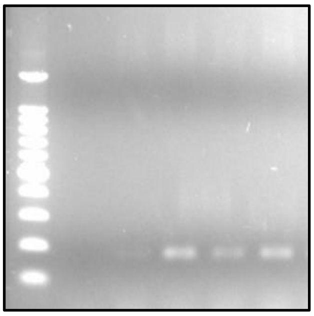

Figure B1.6: RT-PCR products of *ATGSH2* from four biological replicates of *Arabidopsis thaliana* leaves on the agarose gel. L: 100 bp SizerTM (Intron); Expected band size: 176 bp.

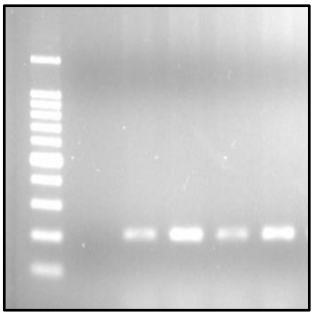

Figure B1.7: RT-PCR products of *ATPCS1* from four biological replicates of *Arabidopsis thaliana* leaves on the agarose gel. L: 100 bp SizerTM (Intron); Expected band size: 207 bp.

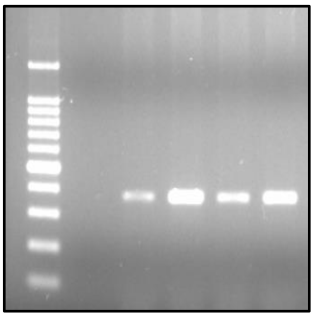

Figure B1.8: RT-PCR products of *ATGSTF2* from four biological replicates of *Arabidopsis thaliana* leaves on the agarose gel. L: 100 bp SizerTM (Intron); Expected band size: 380 bp.

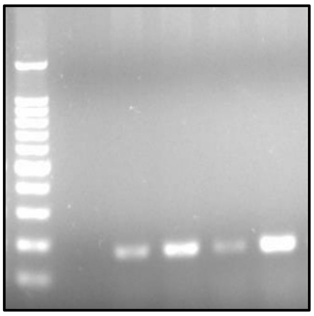

Figure B1.9: RT-PCR products of *ATGSTF6* from four biological replicates of *Arabidopsis thaliana* leaves on the agarose gel. L: 100 bp SizerTM (Intron); Expected band size: 182 bp.

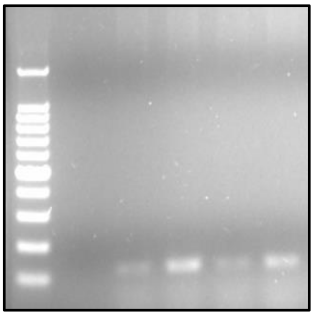

Figure B1.10: RT-PCR products of *ATGSTF7* from four biological replicates of *Arabidopsis thaliana* leaves on the agarose gel. L: 100 bp SizerTM (Intron); Expected band size: 135 bp.

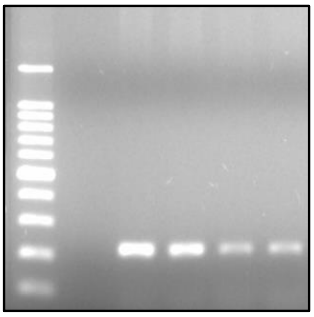

Figure B1.11: RT-PCR products of *ATGSTF8* from four biological replicates of *Arabidopsis thaliana* leaves on the agarose gel. L: 100 bp SizerTM (Intron); Expected band size: 216 bp.

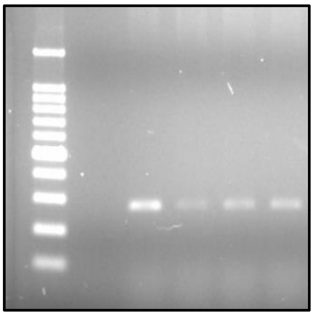

Figure B1.12: RT-PCR products of *ATGSTU19* from four biological replicates of *Arabidopsis thaliana* leaves on the agarose gel. L: 100 bp SizerTM (Intron); Expected band size: 288 bp.

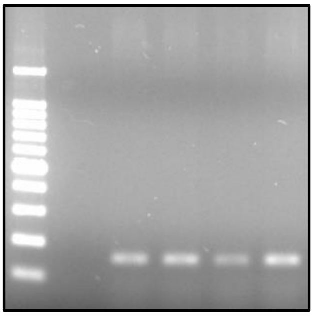

Figure B1.13: RT-PCR products of *ATGSTZ1* from four biological replicates of *Arabidopsis thaliana* leaves on the agarose gel. L: 100 bp SizerTM (Intron); Expected band size: 159 bp.

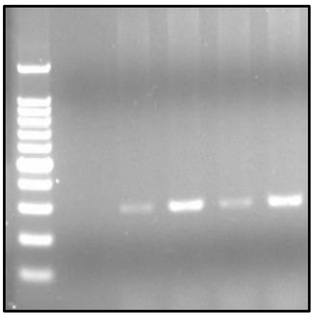

Figure B1.14: RT-PCR products of *CAT1* from four biological replicates of *Arabidopsis thaliana* leaves on the agarose gel. L: 100 bp SizerTM (Intron); Expected band size: 298 bp.

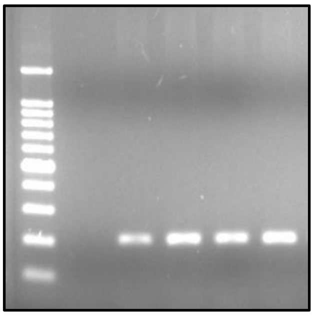

Figure B1.15: RT-PCR products of *CSD1* from four biological replicates of *Arabidopsis thaliana* leaves on the agarose gel. L: 100 bp SizerTM (Intron); Expected band size: 210 bp.

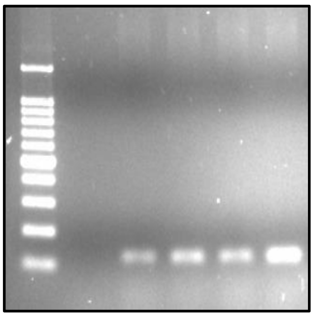

Figure B1.16: RT-PCR products of *MSD1* from four biological replicates of *Arabidopsis thaliana* leaves on the agarose gel. L: 100 bp SizerTM (Intron); Expected band size: 131 bp.

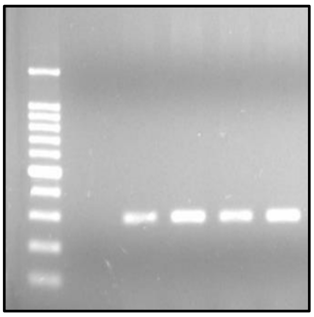

Figure B1.17: RT-PCR products of *DHAR2* from four biological replicates of *Arabidopsis thaliana* leaves on the agarose gel. L: 100 bp SizerTM (Intron); Expected band size: 310 bp.

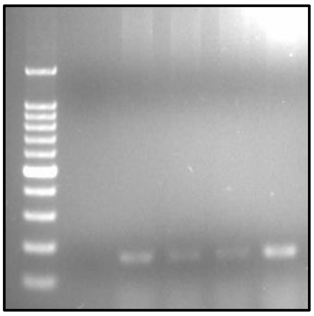

Figure B1.18: RT-PCR products of *MDAR* from four biological replicates of *Arabidopsis thaliana* leaves on the agarose gel. L: 100 bp SizerTM (Intron); Expected band size: 171 bp.

Appendix C: Selected miRNAs and Their Targets in *Arabidopsis thaliana*

Table C1.1: Selected miRNAs and Their Targets in *Arabidopsis thaliana*

miRNA	**Target**
miR398	Copper-zinc superoxide dismutase (CSD)
miR408	Laccase (LAC)
miR397	Laccase (LAC)
miR156	Squamosa promoter binding protein like transcription factor (SPL)
miR172	Apetala2/Ethylene Response Factor (AP2/ERF)
miR169	Nuclear factor Y transcription factor (NF-Y)
miR159	Myeloblast transcription factor (MYB)
miR319	Teosinte branched 1, Cycloidea and Proliferating cell nuclear antigen factors (TCP)
miR394	F-box transcription factor

Appendix D: Condensing Multiple Tables and Figures for Physio-biochemical and Molecular Data in *Arabidopsis thaliana*

Table D1.1: Changes in contents of total soluble sugars (TSS), total insoluble sugars (TIS), Chl a, Chl b, Cx + c, H_2O_2, proline (Pro), MDA, total soluble proteins (TSP), anthocyanins and flavonoids in seedlings of *Arabidopsis thaliana*.

Parameters	**C**	**1B**	**3B**
TSS (mg ml^{-1} g^{-1})	4.7±0.4 ^{a}	5.4±0.4 ^{a}	7±0.5 ^{b}
TIS (mg ml^{-1} g^{-1})	2.3±0.4 ^{a}	4.4±0.3 ^{a}	7.5±2 ^{b}
Chl a (µg ml^{-1} g^{-1})	162.9±10.9 ^{a}	38.1±3.2 ^{b}	31.3±2.8 ^{b}
Chl b (µg ml^{-1} g^{-1})	56.1±5.1 ^{a}	16.8±2.7 ^{b}	6.4±0.5 ^{b}
Cx+c (µg ml^{-1} g^{-1})	49.5±2.9 ^{a}	17.5±1.3 ^{b}	17.5±1.2 ^{b}
H_2O_2 (µmol ml^{-1}g^{-1})	83.9±5.3 ^{a}	114.4±16.7 ^{a}	292.1±18.6 ^{b}
Pro (µmol g^{-1})	4±0.04 ^{a}	4.4±0.3 ^{a}	8.1±0.4 ^{b}
MDA (nmol ml^{-1} g^{-1})	16±0.6 ^{a}	21.2±1.3 ^{b}	14.7±1.8 ^{a}
TSP (mg ml^{-1} g^{-1})	6.1±1 ^{a}	8.6±0.4 ^{b}	11±0.2 ^{c}
Anthocyanin (Abs g^{-1})	0.8±0.02 ^{a}	1.1±0.1 ^{b}	1.1±0.01 ^{b}
Flavonoid (µM g^{-1})	270 nm: 8.5±1.2 ^{a} 300 nm: 16.4±2.2 ^{a} 330 nm: 17.4±2.2 ^{a}	270 nm: 65.7±1.7 ^{b} 300 nm: 87±5.2 ^{b} 330 nm: 88±4.6 ^{b}	270 nm: 81±22.7 ^{b} 300 nm: 103.3±25.8 ^{b} 330 nm: 105.6±26.2 ^{b}

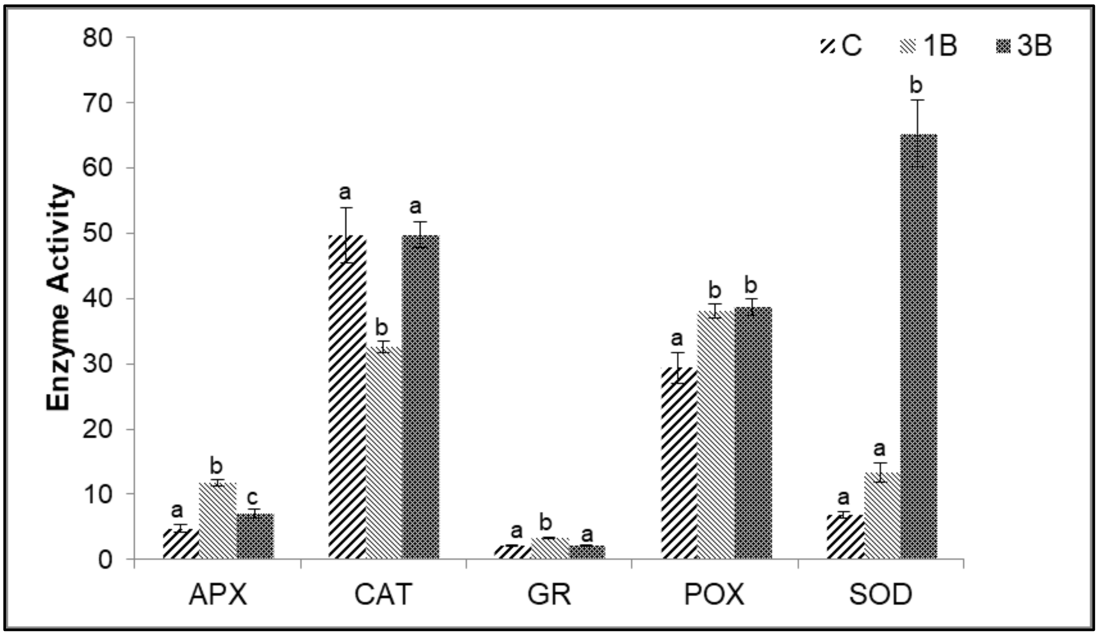

Figure D1.1: Activities of CAT (nmol min^{-1} mg^{-1}), APX (nmol s^{-1} mg^{-1}), GR (nmol s^{-1} mg^{-1}), POX (nmols^{-1} mg^{-1}) and SOD (U µg^{-1}) in seedlings of *Arabidopsis thaliana*. C: Control, 1B: 1 mM H_3BO_3 treatment, 3B: 3 mM H_3BO_3 treatment.

Table D1.2: Changes in the levels of total, non-protein and protein-bound thiols, GSH and total GST activity in seedlings of *Arabidopsis thaliana*. Values followed by different letters are significantly different at $P < 0.05$ level.

Parameters	**C**	**1B**	**3B**
Total thiol (nmol g^{-1})	5.1±0.2^{a}	5.3±0.4^{a}	8.6±0.4^{b}
Non-protein thiol (nmol g^{-1})	0.49±0.04^{a}	0.58±0.14^{a}	0.73±0.045^{a}
Protein-bound thiol (nmol g^{-1})	4.57±0.24^{a}	4.76±0.35^{a}	7.84±0.41^{b}
GSH (Abs/gFW)	0.30±0.003^{a}	0.28±0.003^{a}	0.35±0.039^{a}
GST (nmol min^{-1} mg^{-1})	6.43±0.32^{a}	25.43±3.02^{b}	13.55±0.05^{c}

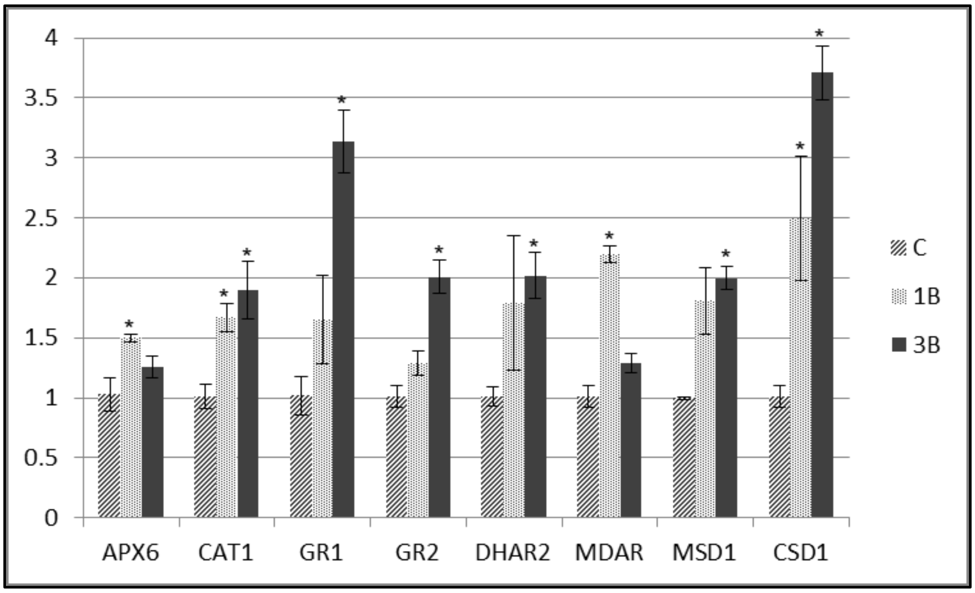

Figure D1.2: Relative expression level of APX6, CAT1, GR1, GR2, DHAR2, MDAR and CSD1 in *Arabidopsis thaliana* leaves in response to toxic B treatments. C: Control, 1B: 1 mM H_3BO_3 treatment, 3B: 3 mM H_3BO_3 treatment. The mean and SEM from four independent experiments are shown ($P<0.05$).

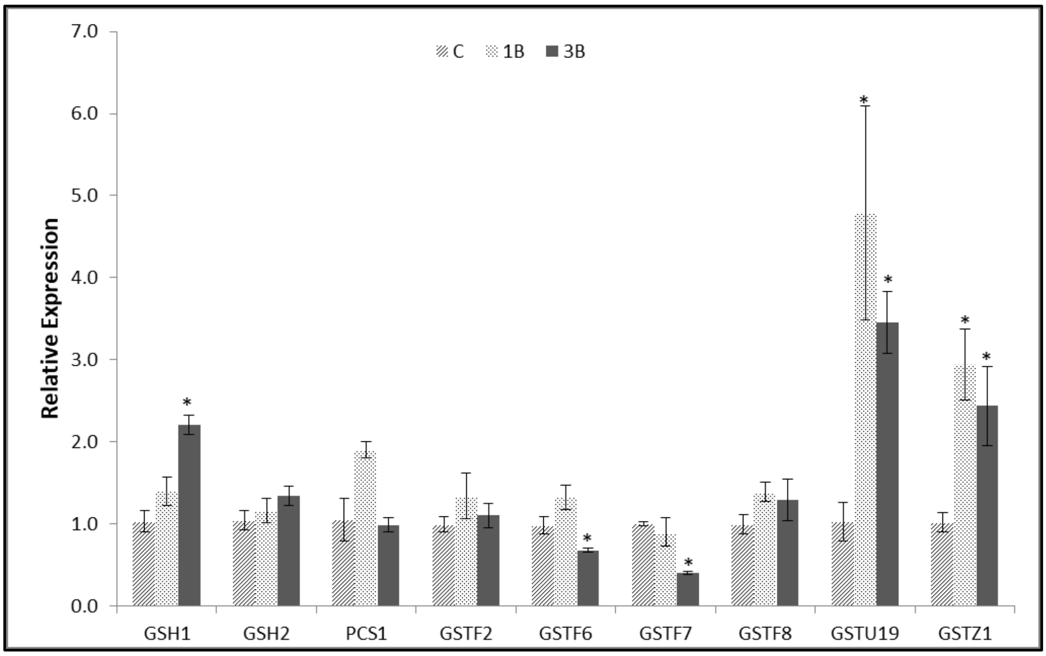

Figure D1.3: Relative expression level of GSH1, GSH2, PCS1, GSTF2, GSTF6, GSTF7, GSTF8, GSTU19 and GSTZ1 in *Arabidopsis thaliana* leaves in response to toxic B treatments. C: Control, 1B: 1 mM H_3BO_3 treatment, 3B: 3 mM H_3BO_3 treatment. The mean and SEM from four independent experiments are shown ($P<0.05$).